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Localization of Na^+/K^+-ATPase in the bovine corneal endothelium

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Abstract

A mouse monoclonal antibody has been used to localize Na^+/K^+-ATPase in the bovine corneal endothelium. The specificity of the antibody was demonstrated by reaction with a single protein of molecular mass 100 kDa on Western blots and immunoprecipitation of a complex consisting of 100 kDa and 50 kDa subunits. Treatment of the immunoprecipitated antigen with Peptide *N*-Glycanase F produced no change in the molecular mass of the 100 kDa protein, but resulted in a progressive decrease in the molecular mass of the 50 kDa subunit, to yield a core protein of molecular mass about 33 kDa. The pattern of deglycosylation suggested the presence of three *N*-linked glycans attached to the 33 kDa protein core. These results were consistent with the antibody being specific for the α subunit of the Na^+/K^+-ATPase. Immunocytochemical studies at the light and electron microscopic level demonstrated antibody binding to both the basal and lateral membranes of bovine corneal endothelial cells. This suggested a baso-lateral distribution of Na^+/K^+-ATPase in these cells, rather than the previously proposed lateral membrane-only distribution.

Key words: Corneal endothelium; ATPase, Na^+/K^+-; Monoclonal antibody; (Bovine)

1. Introduction

The Na^+/K^+-ATPase (EC 3.6.1.3.) is the membrane-bound enzyme responsible for the production of Na^+ and K^+ gradients across cell membranes. In the corneal endothelium, the activity of the Na^+/K^+-ATPase has been shown to be essential for the maintenance of corneal stromal hydration [1]. The Na^+ and K^+ gradients are believed to facilitate the active translocation of HCO_3^- ions across the endothelial cell layer, from the corneal stroma towards the aqueous humour. This bicarbonate ion 'pump' is thought to lower the osmotic potential in the stroma, thereby countering the innate tendency of the corneal stroma to imbibe water and swell [2–4]. Although a number of models of endothelial ion transport have been proposed, non have become generally accepted, and the nature of the coupling between the movement of HCO_3^- and other ions remains unresolved.

The Na^+/K^+-ATPase is composed of two distinct subunits (α and β), thought to exist as a tetramer ($\alpha_2\beta_2$) [5], although a number of other stoichiometries have been proposed [6]. The α subunit (molecular mass about 100 kDa) possesses the ouabain and ATP-binding sites, whilst the β subunit (molecular mass about 50 kDa) is believed to be involved in the correct insertion and stability of the $\alpha\beta$ complex in the plasma membrane [7,8].

Three isoforms of both the α (α1, α2 and α3) and β subunit (β1, β2 and β3) have been recognized [9,10], although the significance of the different isoforms has yet to be elucidated. The β2 isoform has recently been shown to be analogous to an adhesion molecule known as AMOG (adhesion molecule on glia) which can be found in the absence of an α subunit [11]. Monoclonal and polyclonal antibodies have been raised to all three Na^+/K^+-ATPase α subunits [9] and to two of the β subunits [12,13].

In the present study, a monoclonal antibody was characterized and used to assess the distribution of Na^+/K^+-ATPase in the bovine corneal endothelium. The localization of (Na + K)-ATPase is considered here

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as the first step in the formulation of models of endothelial ion transport.

This work has appeared in abstract form [14].

2. Materials and methods

2.1. Reagents and chemicals

Ouabain, diaminobenzidine tetrahydrochloride, nickel chloride, Tween 20, polyoxyethylene 10-lauryl ether, sodium dodecylsulfate, Triton X-100 and molecular weight markers were from Sigma (Poole, UK). EDTA was from Boehringer-Mannheim, Germany. Peptide *N*-glycanase F was from Calbiochem (Nottingham, UK). All other reagents were from BDH Chemicals, (Poole, UK) unless otherwise stated.

2.2. Source of antibody

A sample of ascites fluid containing an IgG1κ mouse monoclonal antibody raised against purified Na^+/K^+-ATPase from canine kidney, was generously donated by Dr. M. Caplan, Department of Cellular and Molecular Physiology, Yale University. This antibody has recently been described in the literature [15], where antibody specificity was demonstrated using an immunofluorescence staining technique.

2.3. Western blotting

Western blotting was performed against whole cell homogenates of bovine kidney or corneal endothelial cells, or against Na^+/K^+-ATPase samples purified by the method of Jorgensen [16]. Tissue was homogenized with ten strokes of a tight-fitting glass-teflon homogenizer and the supernatants solubilized for SDS-PAGE with 2% (w/v) SDS and 1% (w/v) dithiothreitol (DTT). Samples were heated for 30 min at 50°C (higher solubilization temperatures were avoided [17]), and run on 7.5% gels using the buffer system of Laemmli [18]. Gels were blotted onto nitrocellulose and blocked overnight in 'blocking solution', consisting of 1% (w/v) bovine serum albumin (BSA), 1% teleostean gelatin and 0.1% Tween 20 in PBS (20 mM phosphate-buffered saline, pH 7.4).

Blots were incubated overnight with 10 μg/ml monoclonal antibody in blocking solution, followed by a 2-h incubation with an anti-mouse IgG peroxidase conjugate (Sigma) and development with diaminobenzidine (DAB) plus $NiCl_2$ [19].

Attempts were made to separate the α and '$\alpha+$' isoforms of cerebral cortex Na^+/K^+-ATPase α subunits using low percentage SDS-gels, exactly as described by Sweadner [20]. The '$\alpha+$' form, which migrates more slowly than the 'α' form on these low percentage gels is thought to represent a mixture of the α2 and α3 isoforms, whereas the 'α' form is thought to contain solely the α1 (kidney) isoform. For this technique, microsomal membrane preparations from bovine and porcine kidney, and porcine brain were separated on 6% gels [20]. Blotting and immunostaining were repeated as above.

2.4. Immunoprecipitation

It was essential to solubilize Na^+/K^+-ATPase to allow efficient immunoprecipitation, however it was desirable to maintain the integrity of the $\alpha\beta$ complex throughout the solubilization and immunoprecipitation procedures. $\alpha\beta$ integrity was assessed using an enzyme assay [21] for Na^+/K^+-ATPase activity after solubilization with different types and concentrations of detergent. In the chosen procedure, microsomal samples (1 mg/ml final protein concentration, in PBS) were preincubated for 30 min at room temperature with SDS at 0.01% (w/v) final concentration. The samples were then diluted with an equal volume of polyoxyethylene 10-lauryl ether ($C_{12}E_{10}$) solution in PBS [22] and incubated at room temperature for 1 h. Non-solubilized material was pelleted by centrifugation at 100000 × *g* for 30 min at 4°C. At 0.2% (v/v) final concentration, $C_{12}E_{10}$ solubilized 70% of bovine microsomal membrane proteins, whilst retaining Na^+/K^+-ATPase enzyme activity (Figs. 1 and 2).

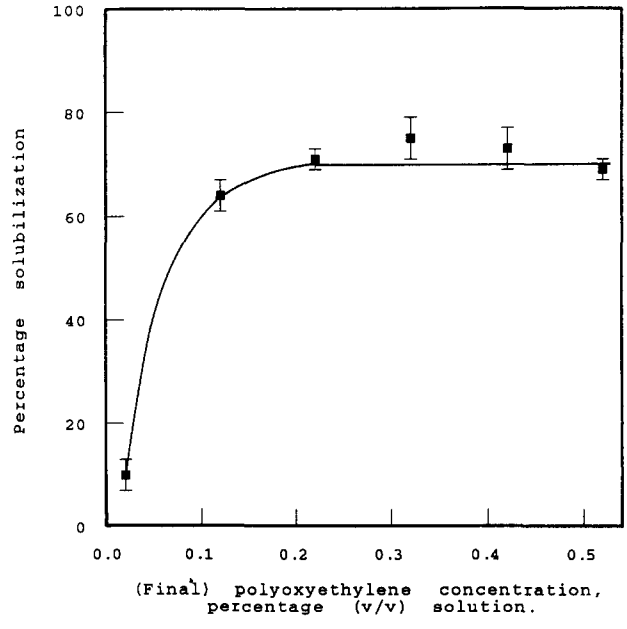

Fig. 1. Effect of polyoxyethylene ($C_{12}E_{10}$) concentration on the solubilization of microsomal membrane proteins. Cortical microsomes from bovine kidney (1 mg/ml) were incubated for 30 min with 0.01% (w/v) SDS in PBS and then diluted to 0.5 mg/ml with $C_{12}E_{10}$ solution and incubated for 60 min at room temperature. Soluble protein was recovered in the supernatant after centrifugation at 100000 × *g* for 30 min.

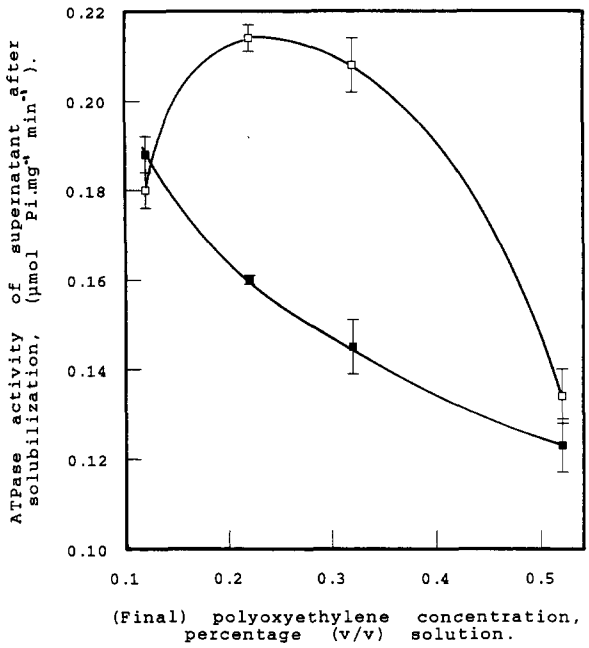

Fig. 2. Effect of polyoxyethylene ($C_{12}E_{10}$) concentration on the Na^+/K^+-ATPase activity of solubilized microsomal membrane proteins. Solubilized proteins were prepared as described in the legend of Fig. 1 and assayed for ATPase activity as described. Symbols; □, ouabain-sensitive ATPase activity; ■, total ATPase activity minus ouabain-sensitive ATPase activity.

The solubilized membrane proteins were biotinylated prior to immunoprecipitation to allow recognition during later analysis. A 10% solution of BSA was added to the solubilized microsomes (0.33–0.37 mg/ml protein) to a final concentration of 1 mg/ml, such that it became the most abundant protein present in the sample. The solution was then cooled on ice and protein amino groups were biotinylated with 0.5 mg/ml sulfo-NHS-biotin (Pierce) as described by Sargiacomo [23]. In preliminary experiments it was demonstrated that biotinylation occurred to apparently all proteins present in the sample and that biotinylation continued for at least 3 h under the conditions described above (data not shown).

Biotinylation was generally conducted for 60 min, and then stopped by the addition of 1 M Tris, 1 M glycine-HCl buffer (pH 7.5), to a final concentration of 20 mM Tris and glycine. The sample was then dialysed against three 1 h changes of 20 mM Tris, 20 mM glycine-HCl, pH 7.5 at room temperature. Polyclonal anti-mouse (IgG and IgM specific) antibodies (Sigma) were coupled to CNBr-activated Sepharose 4B (Fluka) by the method of Porath [24]. These antibody-beads were blocked for 1 h, in 'blocking solution' (see above) and then with a solution of solubilized microsomal membrane proteins (plus 1 mg/ml BSA) prior to the biotinylation step. After 3 h of this blocking incubation, the beads were gently pelleted and the supernatant containing the microsomes was removed (ready for biotinylation).

The biotinylated microsome/BSA sample was incubated (for 3 h) with an aliquot of anti-Na^+/K^+-ATPase monoclonal antibody at a final antibody concentration of 200 nM. Inappropriate (IgG and IgM) monoclonal antibodies were used at the same molar concentration in control incubations with biotinylated microsomes.

After the incubation, the antibody-antigen mixture was incubated with a sample of the anti-mouse beads for 3 h (0.75 ml packed bead volume plus 4 ml of antibody-antigen solution). The beads were washed with six 50 ml rinses of PBS and then eluted with 1 ml of 200 mM glycine-buffered saline (pH 2.5). The eluent was either analyzed by SDS-PAGE or used for deglycosylation experiments.

For SDS-PAGE analysis, samples were solubilized with a solution of 20% (w/v) SDS, 10% (w/v) dithiothreitol and 0.1 mg/ml Bromophenol blue in 500 mM Tris-HCl (pH 6.8), to give final concentrations of 2% SDS, 1% DTT and 50 mM Tris. The samples were heated at 50°C for 30 min and run on a 7.5% gel as described above. After Western blotting, the blot was blocked overnight in 'blocking solution' and then incubated for 2 h at room temperature with 2 μg/ml Extravidin peroxidase conjugate (Sigma). The blot was rinsed three times 30 min in Tris-buffered saline (pH 7.6) (TBS) and then developed with DAB/$NiCl_2$.

2.5. Deglycosylation

Peptide *N*-Glycanase F (PNGase F) was chosen for deglycosylation studies since it has been shown to fully deglycosylate the Na^+/K^+-ATPase β subunit [12,13]. The immunoprecipitated 'prospective αβ' protein complex was incubated with a serial dilution of the glycosidase in a medium having the final composition: Tris, 100 mM; EDTA, 37.5 mM; glycine, 100 mM; NaCl, 75 mM; SDS, 0.2% (w/v); Triton X-100, 0.2% (v/v); DTT, 0.2% (w/v) (pH 8.0).

The incubation was carried out for 16 h at 37°C, after which samples were solubilized and analyzed by Western blotting as described above. The blot was stained with Extravidin peroxidase (as above) to demonstrate the molecular weight of biotinylated proteins.

2.6. Enzyme assays

Na^+/K^+-ATPase enzyme activity was estimated using an NADH-coupled reaction based on that described by Barnett [21]. Assays were carried out at 25°C in the presence and absence of 0.1 mM ouabain. For $C_{12}E_{10}$-solubilized proteins, the enzyme activity was found to gradually decrease with time at 25°C. This

inactivation was found to be more rapid the higher the temperature [22,25].

2.7. Protein assays

Protein was estimated by the method of Bradford [26], although this method proved non-quantitative for insoluble membrane protein suspensions.

2.8. Immunocytochemical staining

Bovine eyes were obtained from a local abattoir and transported to the laboratory in a 'cool box'. The cornea and a 1–2 mm rim of sclera were dissected from an eye and rinsed gently in Hepes-buffered Ringer solution [27]. A sheet of endothelial cells was then removed by carefully scraping the posterior corneal surface with a plastic spatula. The intact cell sheet was shaken off the spatula into a 10 ml capacity glass beaker containing 2 ml of Ringer solution. A further nine eyes were treated similarly to obtain sufficient cell sheets for each immunolabelling experiment.

The cell sheets were fixed for 15 min at room temperature with the periodate-lysine-paraformaldehyde (PLP) fixative of McLean and Nakane [28] and then 'blocked' for 60 min with a solution of 1% (w/v) BSA in Ringer solution. After this blocking period, an aliquot of monoclonal antibody (20 μg protein) was added to the incubation solution and left to incubate for a further 60 min period, with gentle agitation. Control incubations were performed with inappropriate primary antibodies or following the omission of the primary antibody.

The cell sheets were rinsed six times with 10 ml of Ringer-1% BSA solution, by exchanging incubation solutions with a Pasteur pipette. Care was taken not to fragment the cell sheets during rinsing. The cells (in 4 ml Ringer solution) were then incubated for 1 h with a 1:200 dilution of anti-mouse IgG peroxidase antibodies (Sigma). After six further rinsing steps, bound peroxidase molecules were 'developed' using 0.5 mg/ml DAB and 0.003% (v/v) H_2O_2 in Ringer solution, for 5 min. The DAB solution was removed using five times 5 min rinses with 100 mM sodium phosphate buffer (pH 7.4). At this stage of processing, some cells were taken for examination under the light microscope, whilst the remaining cell sheets were post-fixed with 1% (w/v) osmium tetroxide (OsO_4) for 30 min (to increase the electron density of the DAB deposits). The cell sheets were rinsed again and then dehydrated with ethanol and embedded in 'LR White Resin' (London Resin, Basingstoke, UK) following established methods [29]. Sections of the embedded cells (100 nm thick) were examined without post-staining in a Jeol transmission electron microscope.

3. Results

3.1. Western blotting

The prospective anti-Na^+/K^+-ATPase monoclonal antibody detected a single immunoreactive band on Western blots of whole cell homogenates and samples of purified Na^+/K^+-ATPase. The antibody cross-reacted with proteins from dog, pig, ox, rabbit and rat kidney. In all cases the immunoreactive protein had an apparent molecular mass of about 100 kDa. Gels in which the α and $\alpha+$ isoforms from porcine cerebral cortex would have been expected to be separated produced only a single immunoreactive band and not a doublet. This immunoreactive band co-migrated with the α1 (kidney) protein, suggesting that the antibody detected only the α1 Na^+/K^+-ATPase α subunit isoform (Fig. 3). No antibody which could detect both α and $\alpha+$ isoforms was available and so a positive control could not be run to confirm that the $\alpha/\alpha+$ isoforms had indeed been separated.

3.2. Immunoprecipitation

Immunoprecipitation experiments provided the strongest evidence that the antibody was specific for the Na^+/K^+-ATPase. In this work a novel approach was taken, in that proteins were tagged with a biotin

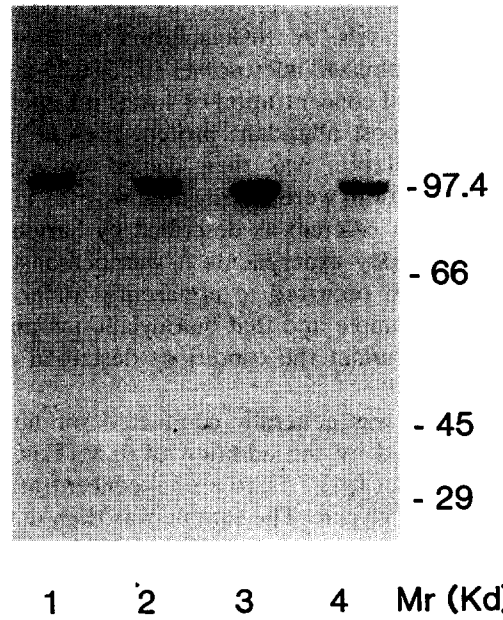

Fig. 3. Western blotting with the prospective anti-Na^+/K^+-ATPase monoclonal antibody. Microsomal preparations from porcine kidney (lane 1), porcine cerebral cortex (lanes 2and4) and bovine kidney (lane 3) were separated on a 6% gel under conditions designed to separate the α and $\alpha+$ isoforms, as described by Sweadner (20). Western blotted proteins were detected with the monoclonal antibody and labelled with a peroxidase-based detection system.

label, rather than a radiolabel prior to immunoprecipitation. Solubilization with the non-ionic detergent $C_{12}E_{10}$ (after opening sealed membrane vesicles with a low concentration SDS incubation) produced enzymatically active preparations in which 70% of the starting material had been solubilized (Figs. 1 and 2). Microsomal samples for these experiments were prepared from renal tissue of predominantly cortical origin: if solely medullary tissue was used then the Na^+/K^+-ATPase α subunit was found to be one of the most highly concentrated proteins present, thus potentially biasing future immunoprecipitation results.

The prospective anti-Na^+/K^+-ATPase antibody immunoprecipitated a dimeric protein complex consisting of subunits with apparent molecular masses 99.6 ± 1.7 kDa and 51.2 ± 0.5 kDa ($n = 8$, mean $\pm$ S.E.) (Fig. 4). Antibodies to proteins not present in the sample were themselves immunoprecipitated (as seen when Western blots were stained with general protein stains), but they did not co-precipitate any detectable (biotinylated) antigen. Similarly, omission of the primary antibody prevented the immunoprecipitation of any biotinylated proteins.

3.3. Deglycosylation

Deglycosylation of the anti-Na^+/K^+-ATPase antibody antigen with PNGase F produced a pattern characteristic of β subunit deglycosylation (not shown) whilst the molecular mass of the prospective α subunit remained constant. The PNGase F treatment reduced the molecular mass of the prospective β subunit from ≈ 50 kDa to 33 ± 1 kDa (mean $\pm$ S.E., $n = 3$) through the loss of three *N*-linked glycans (each oligosaccharide having a molecular mass of ≈ 7 kDa). These molecular masses and the degree of glycosylation are those that would be expected for the Na^+/K^+-ATPase β subunit [12,13,17].

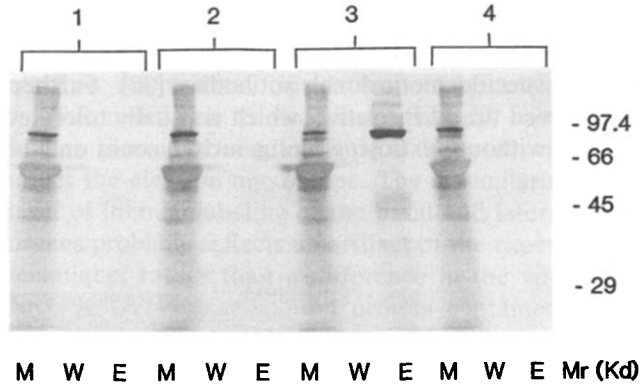

Fig. 4. Immunoprecipitation of biotinylated microsomal membrane proteins. Cortical microsomes from bovine kidney were solubilized and biotinylated as described under Materials and methods. Samples were then incubated for 3 h with primary antibodies as follows: lanes 1, no antibody; lanes 2, an anti-cytokeratin IgG, AE1/AE3 (Boehringer-Mannheim, Germany); lanes 3, the prospective anti-Na^+/K^+-ATPase IgG, and lanes 4, a partially characterized IgM, 'NAKA 61B3'. The samples were then immunoprecipitated using an anti-mouse affinity support. For each antibody tested, three samples (M, W and E) were analyzed (by SDS-PAGE, Western blotting and staining for biotin). Lanes M contained samples of the biotinylated microsomes, lanes W contained samples from the 'final rinse' solution (used just prior to low pH elution) and lanes E contained the immunoprecipitated protein (eluents). Note the immunoprecipitation of 100 kDa and 50 kDa proteins by the anti-Na^+/K^+-ATPase antibody.

3.4. Immunocytochemistry

Preliminary experiments on sections of (unfixed) renal tissue demonstrated a strong labelling of cortical and medullary tubules (not shown). The antibody failed

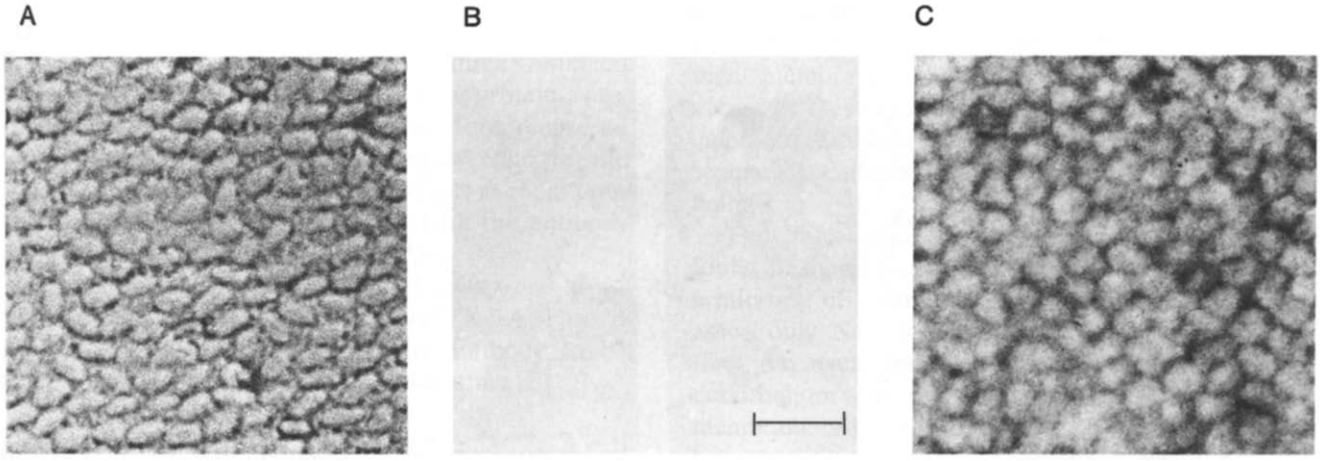

Fig. 5. Light microscopic immunocytochemical localization of anti-Na^+/K^+-ATPase antibody binding to bovine corneal endothelial cells. (A) Phase contrast image of non-immunostained bovine corneal endothelial cells, immediately after removal from Descemet's membrane. The 'hexagonal mosaic' appearance is evident when focusing on the apical surface of the cells. (B) Control for immunostaining. Bright field appearance of endothelial cells after omission of the anti-Na^+/K^+-ATPase antibody. Only diffuse background staining is apparent. (C) Bright field image of endothelial cells immunostained with the anti-Na^+/K^+-ATPase antibody, as described in Materials and methods. A high level of staining is evident along lateral membranes. (Magnification bar = 50 μm.)

to react with tissue which had been fixed with glutaraldehyde, a feature of most anti-Na^+/K^+-ATPase α subunit-specific monoclonal antibodies [30]. Furthermore, even the PLP fixative, which is usually tolerated [31–33] without destroying 'antigenicity', could only be used for up to about 1 h (at room temperature) without abolishing immunostaining. This prevented high-resolution antibody labelling of renal tissue (as a further indicator of antibody specificity) since antigenicity could only be maintained at the expense of tissue ultrastruc-

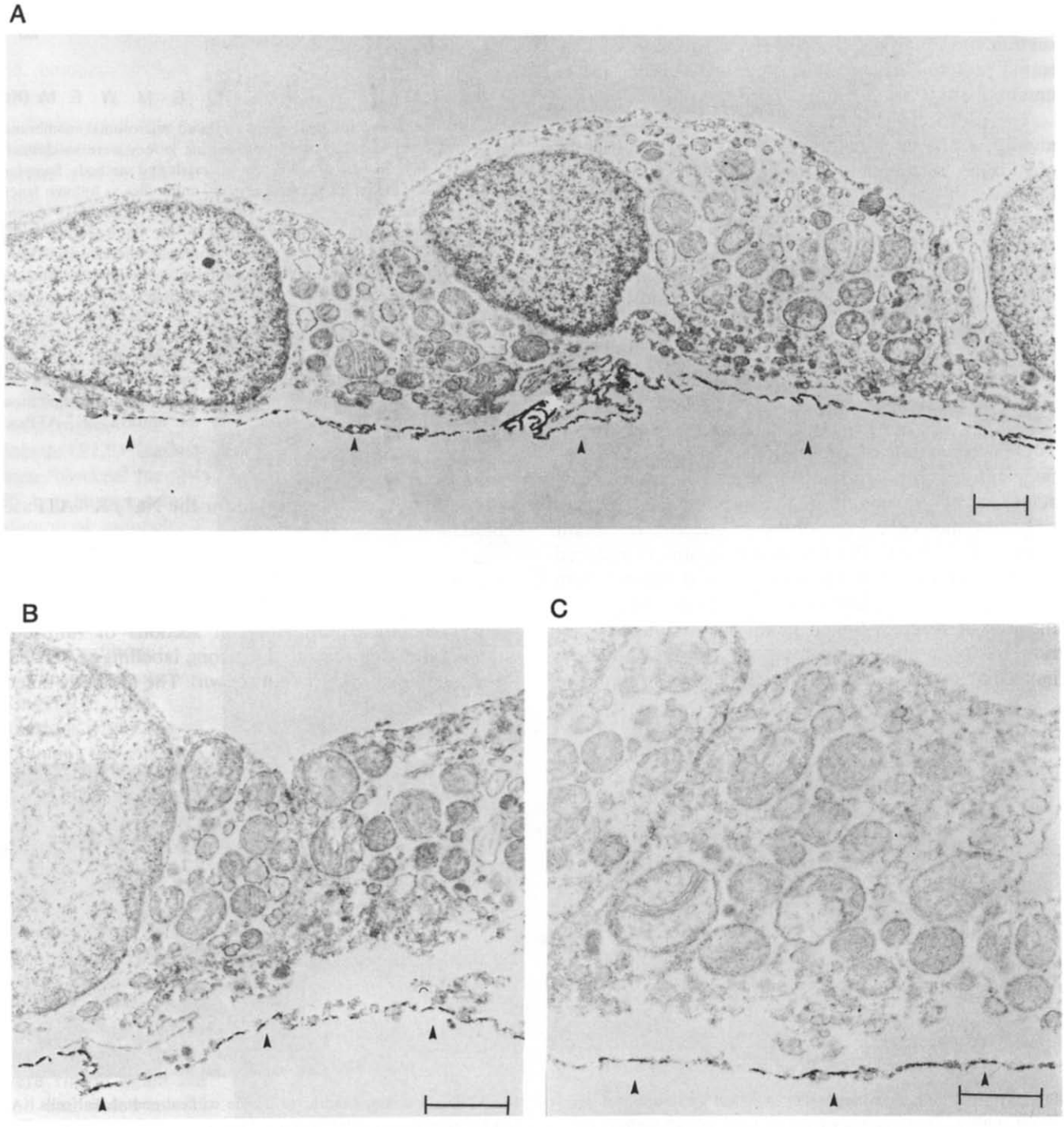

Fig. 6. Electron microscopic immunocytochemical localization of anti-Na^+/K^+-ATPase antibody binding to corneal endothelial cells. Cells labelled as described under Materials and methods. Cell sheets viewed in transverse-section (A, B and C) demonstrated electron dense reaction product deposition most strongly along basal membranes (arrowheads) and to a lesser degree along lateral membranes. (Magnification bars = 1 μm.)

ture. Attempts at post-embedding immunocytochemistry on plastic-embedded tissue also proved unsuccessful, even when tissue was prepared without fixation (by freeze-drying or freeze-substitution).

Fortunately, the antibody was shown to recognize an extracellular epitope, as demonstrated by the approach of Ovchinnikov [34] and hence this strategy was used in the labelling of corneal endothelial cell preparations.

Endothelial cells could be removed from their basement (Descemet's) membrane, allowing access for the antibodies to the basal as well as lateral and apical membranes. Cells immuno-labelled with the antibody in this manner demonstrated strong labelling of the lateral cell membranes, when the cells were viewed with the light microscope (Fig. 5). Cells viewed in transverse-section with the electron microscope showed that immuno-labelling of the basal membrane was also present (Fig. 6).

4. Discussion

4.1. Characterization of the monoclonal antibody

The antibody used in this study reacted with a single protein of molecular mass approx. 100 kDa on Western blots of whole cell homogenates. In blots of Na^+/K^+-ATPase purified by the method of Jorgensen [16], a protein co-migrating with the α subunit was immunostained. This suggested that the antibody was specific for the Na^+/K^+-ATPase α subunit.

The antigen complex bound by the antibody was then analyzed to provide further evidence of specificity. The antigen was found to exist as a dimeric protein complex, the 100 kDa protein and a 50 kDa subsidiary. Deglycosylation with PNGase F suggested that the 100 kDa protein was not *N*-glycosylated, but that the 50 kDa protein contained three *N*-linked oligosaccharides attached to a 33 kDa protein core. Although active Na^+/K^+-ATPase was not recovered by the immunoprecipitation procedure (due to the $C_{12}E_{10}$ solubilization and the incubations at room temperature), these data are consistent with the antibody being specific for the Na^+/K^+-ATPase.

A previous study in which COS-1 cells were transfected with either $\alpha 1$, $\alpha 2$ or $\alpha 3$ Na^+/K^+-ATPase α subunit cDNA [15] also found this antibody to be specific for cells expressing the $\alpha 1$ isoform.

4.2. Na^+/K^+-ATPase localization in corneal endothelial cells

When corneal endothelial cells were immunolabeled with the anti-Na^+/K^+-ATPase antibody and then viewed under the light microscope, strong levels of staining were seen along lateral cell membranes. When the cells were viewed in transverse section under the electron microscope, this lateral membrane staining was often difficult to discern. Conversely, basal membranes showed strong levels of staining when viewed under the electron microscope. The dissimilarity in the level of immunolabeling of the basal and lateral membranes probably reflects an artifact of the experimental technique, rather than a difference in the amount of Na^+/K^+-ATPase α subunit protein contained in the two membrane domains. Although antibodies would have had free access to the apical and basal membranes of endothelial cells during the primary and secondary antibody incubations, the access of antibodies to the lateral membranes would have been sterically hindered. This would have led to (the observed) lower levels of staining on lateral membranes compared to basal membranes.

Previous attempts to localize Na^+/K^+-ATPase in the corneal endothelium have used histochemical methods [35–37]. In these techniques Na^+/K^+-ATPase is localized by virtue of its *p*-nitrophenyl phosphatase (*p*-NPPase) activity. In these earlier studies, histochemical incubations were performed on relatively thick sections of fresh or fixed/frozen tissue, without first removing the endothelial cell layer from Descemet's membrane. Reaction product was confined to the lateral membrane only, the apical and basal membranes appearing to possess no Na^+/K^+-ATPase enzyme activity.

In view of the baso-lateral localization of Na^+/K^+-ATPase immunoreactivity established during the present study, it seems possible that the apparent lack of basal membrane staining encountered in earlier studies was due to reduced access of reagents to enzyme sites located on the basal cell membrane, due to the presence of Descemet's membrane. It is possible that the contact between the endothelial cells and their basement membrane provided a barrier to the diffusional access of these reagents, but that when access is provided (to antibodies) by removing the cells from Descemet's membrane, then the pump sites may be labelled.

The baso-lateral distribution of Na^+/K^+-ATPase found in the present study is unexceptional. Although a minority of polarized epithelia appear to possess an apical-only Na^+/K^+-ATPase distribution [30], rather than the usual baso-lateral distribution, the corneal endothelium was the only tissue in which a 'lateral membrane-only' distribution of Na^+/K^+-ATPase had been proposed.

When taken in conjunction with the evidence that no net movement of sodium occurs across the corneal endothelium under short-circuit conditions [38], this work suggests that all sodium-coupled transport takes place across the baso-lateral membrane.

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