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Proteinases inhibit H^+-ATPase and Na^+/H^+ exchange but not water transport in apical and endosomal membranes from rat proximal tubule

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A marked increase in water permeability can be induced in *Xenopus* oocytes by injection of mRNA from tissues that express water channels, suggesting that the water channel is a protein. In view of this and previous reports which showed that proteinases may interfere with mercurial inhibition of water transport in red blood cells (RBC), we examined the influence of trypsin, chymotrypsin, papain, pronase, subtilisin and thermolysin on water permeability as well as on ATPase activity, H^+-pump, passive H^+ conductance, and Na^+/H^+ exchange in apical brush-border vesicles (BBMV) and endosomal (EV) vesicles from rat renal cortex. H^+ transport was measured by Acridine orange fluorescence quenching and water transport by stopped-flow light scattering. As measured by potential-driven H^+ accumulation in BBMV and EV, proteinase treatment had little effect on vesicle integrity. In BBMV, ecto-ATPase activity was inhibited by 15–30%, Na^+/H^+ exchange by 20–55%, and H^+ conductance was unchanged. Osmotic water permeability (P_f) was 570 $\mu m/s$ and was inhibited 85–90% by 0.6 mM $HgCl_2$; proteinase treatment did not effect P_f or the $HgCl_2$ inhibition. In EV, NEM-sensitive H^+ accumulation and ATPase activity were inhibited by >95%. P_f (140 $\mu m/s$) and $HgCl_2$ inhibition (75–85%) were not influenced by proteinase treatment. SDS-PAGE showed selective digestion of multiple polypeptides by proteinases. These results confirm the presence of water channels in BBMV and EV and demonstrate selective inhibition of ATPase function and Na^+/H^+ exchange by proteinase digestion. The lack of effect of proteinases on water transport contrasts with previous data in RBC which showed that proteinases prevent the inhibition of water transport by mercurials. We conclude that the water channel may be a small integral membrane protein which, unlike the H^+-ATPase and Na^+/H^+ exchanger, has no functionally important membrane domains that are sensitive to proteolysis.

Introduction

Functional water channels are responsible for the high transepithelial water permeability of the mammalian proximal tubule, thin descending limb, and vasopressin-stimulated collecting duct (for reviews see Refs. 1 and 2). The presence of water channels that exhibit a high osmotic water permeability coefficient (P_f), low energy of activation (E_a), and sensitivity to mercurial sulfhydryl reagents, has been demonstrated in luminal and contraluminal membranes isolated from mammalian proximal tubule [3–7]. Similar water channels have been found in endosomal vesicles (EV) from kidney cortex [8] and papilla [9], and in clathrin-coated vesicles from kidney cortex [10]. Recent data showed that a population of EV (labeled by endocytosis of 6-carboxyfluorescein) with very high osmotic water permeability is induced in renal papilla following vasopressin treatment of Brattleboro rats [9]. Labeled vesicles with high water permeability were not present in the papilla of vasopressin-deficient rats. The vasopressin-sensitive pool of EV that shuttles water channels to and from the luminal membrane of the collecting duct principal cells is probably responsible for the

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regulation of water permeability by vasopressin in the kidney collecting duct. Although EV from proximal tubule apical membrane also contain functional water channels [8], it is not known whether water permeability in proximal tubule is subject to physiological regulation.

Although it has been proposed that water transporters represent proteins or special protein-lipid or lipid units [2], recent data that functional, $HgCl_2$-sensitive water channels are expressed in *Xenopus* oocytes microinjected with mRNA from kidney and reticulocytes suggest that water channels are proteins [11]. In red blood cell (RBC) membranes, which also contain water channels, radiation and proteolysis did not affect water transport, but decreased its sensitivity to mercurial inhibition [12–14]. These results suggest the presence of a protein subunit, sensitive to mercurials and proteinases, that regulates membrane water permeability. Based on these observations we have tested the effect of a series of proteinases on control and $HgCl_2$-inhibited osmotic water transport in rat renal cortical brush-border vesicles (BBMV) and EV. These vesicles have either their extracellular (BBMV) or cytoplasmic (EV) membrane surface exposed. Whereas several transport (Na^+/H^+ exchange, H^+-pump) and enzyme (H^+-ATPase, ecto-ATPase) activities in these vesicles were partially or completely inhibited by some proteinases, the $HgCl_2$-sensitive water permeability remained unaffected.

Material and Methods

Preparation of renal cortical vesicles

BBMV and EV were isolated from cortical homogenates of Sprague-Dawley rat kidney by the Mg-aggregation method of Biber et al. [15] and the differential and Percoll density gradient centrifugation method of Sabolić and Burckhardt [16], respectively. BBMV were enriched in leucine arylamidase activity, a marker for luminal membranes, 16.9 ± 0.2 times, and in the basolateral membrane marker, $(Na^+ + K^+)$-ATPase, 1.4 ± 0.2 times ($n = 4$). In EV, the enrichment factors for the NEM-sensitive H^+-pump and leucine arylamidase activity were 35.5 ± 0.6 and 2.7 ± 0.1 ($n = 4$), respectively.

Vesicles were washed twice with KCl-buffer containing 300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)/tris(hydroxymethyl)aminomethane (Tris), pH 7.4, and were diluted with the same buffer to a protein concentration of 10 mg/ml.

Proteinase treatment

Vesicles were treated with the following proteinases: trypsin, chymotrypsin, papain, pronase, subtilisin, and thermolysin (Boehringer-Mannheim, Indianapolis, IN). An aliquot of vesicles (100 μl, 1 mg protein) was mixed with a stock proteinase solution to yield a final proteinase concentration of 1 mg/ml. The mixture was incubated for 60 min at 37°C. The reaction was stopped by addition of 1 mM (final concentration) phenylmethyl-sulfonylfluoride (PMSF) and cooling the samples on ice. Control vesicles were treated identically but without proteinase.

ATPase activity

ATPase activity was measured in the absence and presence of *N*-ethylmaleimide (NEM). As demonstrated previously [17,18], the NEM-insensitive ATPase in BBMV represents an ecto-ATPase, whereas the NEM-sensitive ATPase in BBMV and EV represents the vacuolar type H^+-ATPase. Vesicles were diluted with ice-cold KCl-buffer to a protein concentration of 0.5 mg/ml (BBMV) or 1 mg/ml (EV) (not including the protein contributed by proteinase). The reaction mixture for the ATPase assay in a total volume of 0.2 ml comprised: 150 mM KCl, 5 mM $MgSO_4$, 50 mM Hepes-Tris (pH 7.4), 1 mM levamisole, 2 mM ouabain, 5 μg/ml oligomycin, 0.5 mM vanadate, with or without 1 mM NEM, and 10 μg (BBMV) or 20 μg (EV) vesicle protein. The inhibitors were present to abolish the activities of alkaline phosphatase, $(Na^+ + K^+)$-ATPase, mitochondrial H^+-ATPase, Ca^{2+}-ATPase, and the vacuolar type H^+-ATPase, respectively [17,18]. After preincubation of the mixture at 37°C for 10 min, the reaction was started by adding a stock solution of Tris-ATP (final concentration, 5 mM). The reaction was terminated after 15 min by addition of 1 ml of an ice-cold stop solution (2.9% ascorbic acid, 0.45% ammonium molybdate and 2.86% sodium dodecyl sulphate (SDS) in 0.48 H HCl). Further processing of the samples and colorimetric measurement of liberated P_i was performed as described by Dean et al. [19].

Fluorometric measurements

The formation and dissipation of transmembrane proton gradients in control and proteinase-treated vesicles were measured from the fluorescence of Acridine orange (AO), a pH-gradient sensitive dye that accumulates in acidic compartments [20,21].

To measure the effect of proteinases on vesicle integrity (vesiculation), 20 μl BBMV or EV loaded with KCl-buffer (0.2 mg vesicle protein, not including the proteinase content) was added to 2 ml of K^+-free buffer (TMACl-buffer: 300 mM mannitol, 100 mM tetramethylammonium chloride, 5 mM $MgSO_4$, 5 mM Hepes-Tris (pH 7.4)) containing 6 μM AO, 2.5 μM valinomycin, and 2.5 μM carbonyl cyanide *p*-chloromethoxyphenylhydrazone (CCCP). Under these conditions, i.e. in the presence of a high K^+ and H^+ conductances, the outward K^+ gradient induced the development of an inside-negative K^+-diffusion poten-

tial. This potential was a driving force for the CCCP-mediated intravesicular H^+ uptake measurable by the quenching of AO fluorescence. The quenching signal required the presence of sealed vesicles [20]. In some experiments, vesicles were washed two times with proteinase-free, KCl-buffer and the protein concentration was adjusted to 10 mg/ml before fluorescence measurements in order to remove proteinases.

H^+ conductance and Na^+/H^+ exchange in BBMV were measured as described previously [20,22]. Briefly, proteinase-treated vesicles were washed twice with proteinase-free, KCl-buffer and the protein concentration was adjusted to 10 mg/ml. An aliquot of vesicles (20 μl, 0.2 mg protein) was added to 2 ml TMACl-buffer containing 6 μM AO and 2.5 μM valinomycin. The rate of intravesicular acidification driven by the induced K^+-diffusion potential, observed as the time-dependent quenching of AO fluorescence, provides a measure of the intrinsic H^+ conductance of the vesicle membrane [20]. The H^+ conductance was estimated from the initial linear rate of fluorescence decrease. The rate of the fluorescence decrease in the presence of the outward K^+ gradient was subtracted for the rate observed in its absence (the reaction measured in KCl-buffer), and was expressed as fluorescence change per min ($\Delta F/min$). After the intravesicular acidification was fully developed, 25 μl stock solution of TMACl or NaCl (final cation concentration 12.2 mM) was added, and the rate of ΔpH dissipation (fluorescence recovery) was recorded. The initial rate of fluorescence recovery in the presence of Na^+, corrected for the recovery observed with TMACl, represented Na^+/H^+ antiport [22]. Initial rates were estimated from the fluorescence during the first 3 s following addition of a cation, and were expressed as $\Delta F/min$. In some experiments, amiloride was added from an aqueous stock (final concentration 0.5 mM) before addition of NaCl or TMACl.

H^+-pump activity in control and proteinase-treated EV was measured by ATP-driven, Cl^--stimulated quenching of AO fluorescence [21]. An aliquot of EV (10 μl, 0.1 mg protein) was added to 2 ml KCl-buffer which contained 6 μM AO, 2.5 μM valinomycin, and 1.5 mM Tris-ATP. When required, CCCP was added from ethanol stock (final concentration in the assay, 2.5 μM) to dissipate the transmembrane H^+ gradient.

Fluorescence was monitored at 37°C over 1 s intervals in an SLM-Aminco 8000 fluorometer (Urbana, IL) interfaced to an IBM/PC computer. Fluorescence was excited at 485 nm, and emission was filtered by two Schott glass OG515 cut-on filters in series. Samples were stirred continuously.

Osmotic water permeability

Osmotic water permeability in BBMV and EV was measured by stopped-flow light scattering [7]. Control and proteinase-treated vesicles were washed twice in a low-osmolality buffer which comprised: 100 mM mannitol, 5 mM Hepes-Tris (pH 7.4). Stopped-flow experiments were performed at 23°C on a Hi-Tech SF-51 apparatus (Wiltshire, UK) that has a 1-ms dead time. An aliquot of vesicles was mixed with an aliquot of hyperosmolar solution of sucrose to give a 100 mM inwardly directed sucrose gradient. The time course of 90° light scattering at 500 nm was recorded by a MINC/23 computer (Digital Equipment, Maynard, MA) for subsequent analysis. No significant change in the intensity of scattered light was observed when the vesicles were mixed with isoosmotic buffers. The calculation of osmotic water permeability (P_f) from the time course of scattered light intensity and vesicle surface-to-volume ratio was described previously [7]. Vesicle geometry was evaluated by electron microscopy (see below).

Electron microscopy of isolated vesicles

Ultrathin frozen sections of isolated vesicles were stained with uranyl acetate according to the method of Tokuyasu [23]. Briefly, vesicles were fixed with 0.5% glutaraldehyde, washed with PBS, and embedded in 3% agar. The small solid agar blocks were infiltrated with 2.3 M sucrose overnight. The blocks were frozen in liquid nitrogen and ultrathin sections (60 nm) were cut and mounted on carbon/Parlodion-coated copper grids. The specimen-containing grids were washed with PBS, further fixed with 1% glutaraldehyde, washed with water, and stained with 2% Uranyl acetate for 5 min. Thereafter, the grids were destained with and embedded in 2% methyl cellulose, dried, and viewed in a Philips CM10 electron microscope.

Morphometric analyses were made from the electronmicrographic images of isolated vesicles by using a calibrated eye-piece. In round vesicles, the diameter was estimated by measuring the greatest distance between two opposite points at the external surface of the vesicle membrane. In oval vesicles, diameters along short and long axes of the vesicle were measured, averaged, and the result was used as a reference diameter in calculations of vesicle surface and volume.

SDS-polyacrylamide electrophoresis

Control or proteinase-treated vesicles were washed twice with proteinase-free buffer. Membrane proteins were solubilized by boiling for 5 min in sample buffer [1% SDS, 30 mM Tris/HCl, pH 6.8, 12% (vol/vol) glycerol]. Aliquots of 150 mg protein were loaded onto 13% Laemmli SDS-polyacrylamide gels (SDS-PAGE) and electrophoresed at 15 mA for 12 h. The gel was stained with 0.2% Coomassie blue in acetic acid/methanol/water (10:50:40, v.v), and destained with dye-free solution. The molecular weight markers (Bethesda Research Laboratories, MD, USA) were:

lysozyme (14300), β-lactoglobulin (18400), carbonic anhydrase (29000), ovalbumin (43000), bovine serum albumin (68000), phosphorylase B (97400), and myosin (H-chain) (200000).

Statistics

The recordings, electronmicrographs, and SDS-PAGE gels are representative of two or more experiments performed on different vesicle preparations. Other data are expressed as mean ± S.E. Statistical differences between defined groups of the data were evaluated by the Student's *t*-test.

Results

Electron microscopy of isolated vesicles

Electron micrographs of negatively-stained apical and endosomal membrane vesicles isolated from rat renal cortex are shown in Fig. 1. The membrane bilayers in both types of vesicles are clearly visible. BBMV (A) are larger in size and exhibit greater variation in size and shape than EV (B). Morphometric analysis revealed a broader size distribution of BBMV diameters then EV diameters (Fig. 2). The average diameter of BBMV was 274 ± 6 nm. The calculated surface, volume, and surface-to-volume ratio of BBMV were $236 \cdot 10^{-3}$ μm^2, $10.7 \cdot 10^{-3}$ μm^3, and $2.2 \cdot 10^5$ cm^{-1}, respectively. The average diameter of EV was 175 ± 4 nm, surface $96 \cdot 10^{-3}$ μm^2, volume $2.8 \cdot 10^{-3}$ μm^3, and surface-to-volume ratio $3.4 \cdot 10^5$ cm^{-1}.

Before testing of the effect of proteinases on osmotic water transport in isolated vesicles, a series of experiments was performed to demonstrate the positive action of proteinases in the membranes. In BBMV we tested the effect of proteinases on (a) ecto-ATPase activity, an enzyme with a catalytic site of broad nucleotide specificity at the external domain [18,24], (b) membrane H^+ conductance, and (c) Na^+/H^+ antiporter activity. H^+ conductance and Na^+/H^+ antiport are intrinsic properties of the luminal membrane [20]. In EV, we measured the activity of a vacuolar-type H^+-pump and the corresponding NEM-sensitive ATPase activity (H^+-ATPase). The effect of proteinases on vesicle integrity (vesiculation) and the protein pattern by SDS-PAGE was also examined.

Proteinase effect on brush-border membrane vesicles

The integrity of proteinase-treated BBMV was tested by monitoring K^+ diffusion potential-driven, CCCP-mediated intravesicular acidification (Figs. 3A, B, and 4). We first tested whether AO fluorescence quenching is sensitive to the number of sealed BBMV. Vesicles were diluted with KCl-buffer, incubated at 37°C for 60 min, and the quenching amplitude was measured in response to addition of different amounts of vesicle protein. Fig 3A shows that increasing amounts of BBMV (0–0.2 mg protein) gave a progressively larger amplitude of fluorescence quenching curves. No quenching was observed in the absence of vesicles in the buffer (record 0) or in the presence of BBMV and 0.1% Triton X-100 (not shown). The relative quenching amplitudes in Fig. 3 B indicate that a 50% decrease in BBMV (from 0.2 to 0.1 mg protein) gave about 30% decrease in quenching amplitude. With fewer vesicles (< 0.1 mg vesicle protein), a steep and nearly linear decrease in quenching amplitude was obtained.

Compared with control vesicles (Fig. 4A, recording 1), the vesicles treated with proteinases (recordings 2–7, see legend) exhibited a slightly smaller fluorescence quenching, suggesting that proteinase treatment had a small effect on BBMV integrity. However, the majority of vesicles remained sealed.

Ecto-ATPase activity, measured in these vesicles, is shown in Table I. As shown previously [25], BBMV isolated by aggregation with divalent cations are oriented right-side-out. We have recently demonstrated that these membranes possess an ecto-ATPase activity that is insensitive to NEM [18,24]. In the present studies, in the absence of NEM, BBMV had an ATPase activity of 734 ± 30 nmol P_i/min mg protein (n = 6). In the presence of 1 mM NEM, ATPase activity decreased to 654 ± 13 nmol P_i/min mg protein (Table I, Control). The small NEM-sensitive component of ATPase activity is due to the vacuolar-type ATPase present in contaminating intracellular and/or unsealed brush-border membranes [18,21]. As shown in Table I, all proteinase-treated vesicles exhibit a decrease in ecto-ATPase activity compared to control BBMV. The decrease ranged from 10% (chymotrypsin) to 30% (pronase).

A limited effect of proteinases on vesiculation, observed in Fig. 4A, may effect the measurement of H^+ conductance and Na^+/H^+ exchange. To eliminate this possibility, BBMV were washed with proteinase-free buffers following incubation with proteinases. In washed vesicles, the extent of acidification in control and proteinase-treated BBMV was similar (Fig. 4B), possibly due to removal by washing of the nonvesiculated membranes.

The representative set of recordings demonstrate the development of a K^+ diffusion potential-driven intravesicular uptake of H^+ due to intrinsic H^+ conductance of the membrane, followed by dissipation of the ΔpH via Na^+/H^+ exchange (Fig. 5). In accord with previous observations [26], 0.5 mM amiloride added after development of the pH gradient caused a limited additional fluorescence quenching due to nonspecific interaction of AO fluorescence with amiloride. Subsequent addition of Na^+ caused no fluorescence recovery, indicating the presence of amiloride-sensitive Na^+/H^+ antiport.

As shown in Table I, the H^+ conductance in proteinase-treated BBMV did not differ significantly from control. Na^+/H^+ antiport was not inhibited by trypsin and chymotrypsin. Papain and subtilisin inhibited Na^+/H^+ antiport by 20%, whereas thermolysin, and pronase inhibited by 30%, and 55%, respectively.

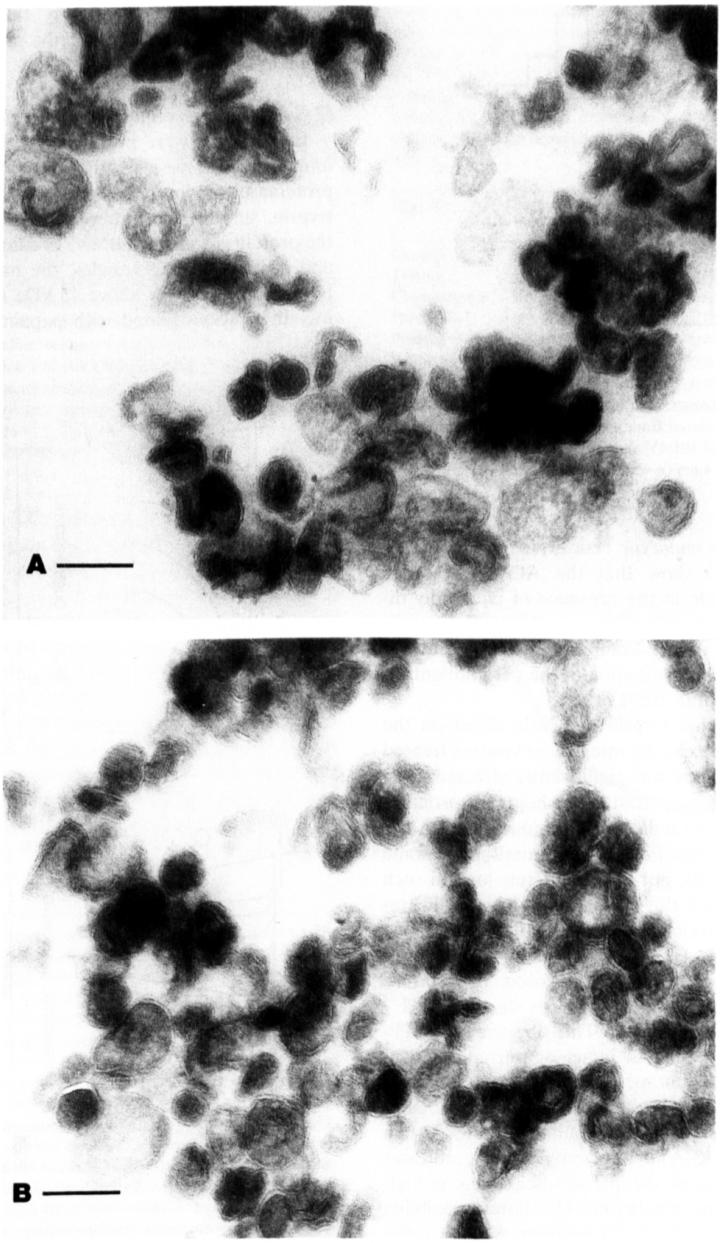

Fig. 1. Electron micrographs of ultrathin (60 nm) frozen sections of isolated rat renal cortical brush-border (A) and endosomal membrane vesicles (B) negatively stained with uranyl acetate. The vesicles are sharply delineated by a double membrane. Brush-border membrane vesicles are larger and more heterogeneous in size and shape than endosomal vesicles. Bar = 0.25 μm.

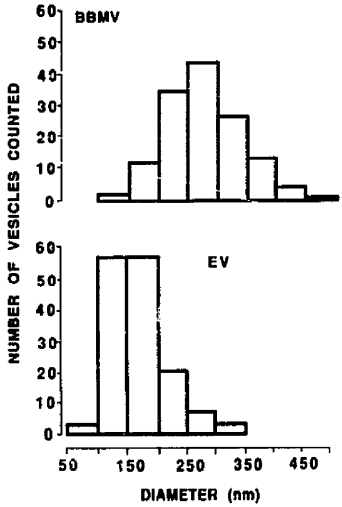

Fig. 2. Histograms of diameters of brush-border (BBMV) and endocytic vesicles (EV) calculated from analysis of electron micrographs. The average diameter of BBMV and EV is 274 ± 6 nm (n = 140) and 175 ± 4 nm (n = 150), respectively.

Proteinase effect on endocytic vesicles

Fig. 3C and D show that the AO fluorescence quenching amplitude in the presence of outwardly directed K^+ gradients, valinomycin, and CCCP, is sensitive to the amount of EV. Comparing Figs. 3A and C it is noticeable that the dissipation of the pH gradients in EV was slower than in BBMV.

Fig. 6A shows that trypsin had little effect on the integrity of EV whereas the integrity of vesicles treated with other proteinases was significantly affected compared to control, the greatest effect being on pronase-treated EV. The data indicate that, unlike BBMV, the integrity of EV is significantly compromised by some proteinase treatments, but not completely lost. In such vesicles we measured the H^+-pump activity, an intrinsic marker for endosomal membrane [16,21]. As shown in Fig. 6B in control vesicles there was a significant ATP-driven intravesicular accumulation of protons. However, EV treated with proteinases showed no H^+-pump-mediated acidification. The absence of ATP-driven H^+ accumulation in intact or partially vesiculated endosomal membranes may mean that the proteinases increased H^+ conductance of the vesicle membrane and thus dissipated the pump-mediated ΔpH, and/or inhibited H^+-ATPase activity. Fig. 6A indicates that the dissipation of the pH gradients was not affected by proteinase treatment. The latter possibility was tested by measuring NEM-sensitive ATPase activity in control and proteinase-treated EV (Table II). In the absence of NEM, the isolated EV contained an ATPase activity of 147 ± 8 nmol P_i/min mg protein (n = 6). About 45% of this activity was sensitive to NEM (Table II, Control), and represented intrinsic vacuolar-type H^+-ATPase [17]. As shown in Table II, NEM-sensitive ATPase activity was abolished in proteinase-treated EV. Therefore, the removal of H^+-ATPase activity accounts for the absence of ATP-dependent acidification.

SDS-PAGE of proteinase-treated vesicles

Experiments were performed with control and proteinase-treated vesicles that had been washed with proteinase-free buffers. As shown in Fig. 7, chymotrypsin, subtilisin and thermolysin had little effect on the protein pattern in BBMV. In contrast, in trypsinized and pronase-treated vesicles, the majority of the protein bands with MW above 43 kDa decreased in staining. In vesicles treated with papain, no clear protein

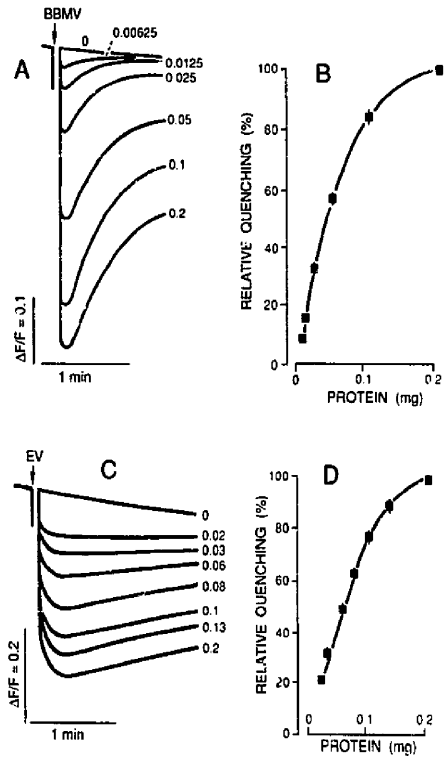

Fig. 3. Dependence of Acridine orange quenching amplitude on amount of BBMV and EV. Vesicles were preincubated at 37°C for 60 min. Aliquots (20 μl) of either brush-border (BBMV) or endosomal vesicles (EV), preloaded with KCl-buffer and containing indicated amounts of vesicle protein (0–0.2 mg), were diluted in TMACl-buffer containing Acridine orange, valinomycin and CCCP (see Methods). The quenching amplitudes were recorded (A and C) and plotted as a relative amplitude (B and D). Each point represents the mean ± S.E. of three to four measurements in vesicles pooled from three different preparations.

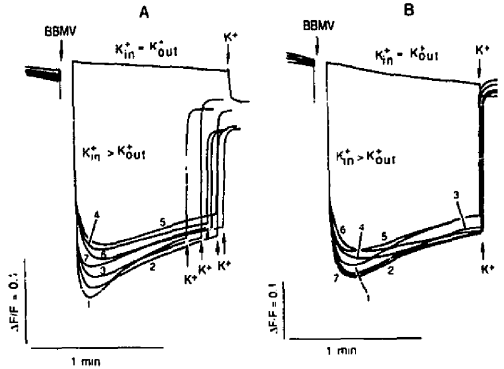

Fig. 4. Effect of proteinase treatment on integrity of BBMV before (A) and after washing with proteinase-free buffers (B). Vesicles were added to KCl-buffer ($K^+_{in} = K^+_{out}$) or TMACl-buffer ($K^+_{in} > K^+_{out}$) which contained Acridine orange, valinomycin and CCCP. At the indicated time (arrow), a 1 M stock solution of KCl was injected into outside buffer (final concentration of K^+, 25 mM) to dissipate the ΔpH. In this and subsequent figures the proteinases were: 1, control (no proteinase); 2, trypsin; 3, chymotrypsin; 4, papain; 5, pronase; 6, subtilisin and 7, thermolysin.

band was stained. The effect of papain was not due to interaction of papain with Coomassie blue staining because no bands were observed also by silver staining (not shown). *

In EV, trypsin decreased the intensity of several bands of apparent MW above and below 43 kDa (Fig. 7, EV). As in BBMV, papain treatment resulted in loss of the protein band pattern. The effect of other proteinases in EV was limited to a few protein bands.

The majority of the preceding experiments clearly show that the proteinases had an effect on transport and enzyme systems present in BBMV and EV and proved therefore that proteinases are able to act from

* The preceding experiments showed that the papain-treatment of BBMV and EV had only minimal effect on vesiculation. The activities of ecto-ATPase, an enzyme of the MW about 100 kDa [27], and of Na^+/H^+ exchanger were only partially inhibited. These findings clearly contrast with the complete disappearance of protein bands on SDS-PAGE of papain-treated vesicles. In a separate experiment (results not shown) we examined the mechanism of papain action by incubating BBMV either (a) as above, in the presence of papain at 37°C for 60 min, followed by the addition of PMSF and washing of the vesicles with papain-free buffer, or (b) by addition of papain after incubation of the vesicles in the papain-free buffer, followed by the addition of PMSF and washing, or (c) by addition of PMSF after incubation of BBMV in a papain-free medium, followed by the addition of papain and washing. All three conditions yielded a similar protein pattern of the vesicles on SDS-PAGE, with loss of the protein bands. Therefore the main action of papain occurred after the incubation of vesicles with papain was completed, probably during the preparation and boiling of the membranes in sample buffer before SDS-PAGE.

TABLE I

Ecto-ATPase activity, H^+ conductance and Na^+/H^+ exchange in proteinase-treated brush-border membrane vesicles

Shown are means ± S.E. obtained with four to six vesicle preparations. The ecto-ATPase activity was measured by the P_i liberation assay in unwashed vesicles and represents the residual ATPase activity in the presence of 1 mM NEM. H^+ conductance and Na^+/H^+ exchange were measured by the quenching method of Acridine orange fluorescence in vesicles following removal of proteinases by washing as described in the Materials and Method section. Statistical significance vs. Control: * $P < 0.05$, and ** $P < 0.01$

Enzyme	Ecto-ATPase [a]	H^+ Conductance [b]	Na^+/H^+ Exchange [b]
Control	654 ± 13	0.139 ± 0.015	0.115 ± 0.013
Trypsin	569 ± 16 **	0.156 ± 0.010	0.110 ± 0.027
Chymotrypsin	589 ± 13 **	0.159 ± 0.014	0.130 ± 0.012
Papain	560 ± 7 **	0.148 ± 0.018	0.093 ± 0.016 *
Pronase	465 ± 7 **	0.121 ± 0.015	0.053 ± 0.004 **
Subtilisin	574 ± 8 **	0.130 ± 0.010	0.089 ± 0.019 *
Thermolysin	572 ± 7 **	0.139 ± 0.014	0.080 ± 0.009 **

[a] nmol P_i/min mg protein.
[b] ΔF/min.

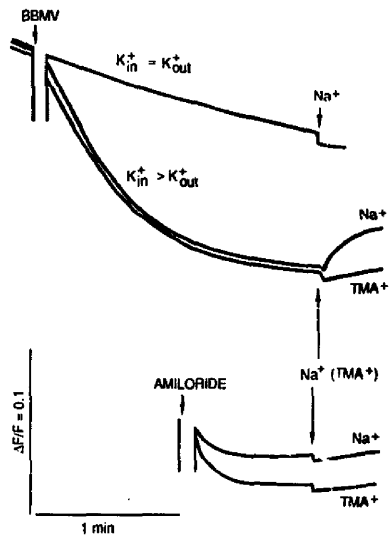

Fig. 5. H^+ conductance and Na^+/H^+ exchange in brush-border membrane vesicles. BBMV, preloaded with KCl-buffer, were added to same ($K^+_{in} = K^+_{out}$) or TMACl-buffer ($K^+_{in} > K^+_{out}$) which contained AO and valinomycin. The rate of fluorescence quenching in the presence of outward K^+ gradient is a measure of a H^+ conductance. At the indicated time (arrow), concentrated chloride salts of either Na^+ or TMA^+ were added into outside buffer to induce a dissipation of the ΔpH via Na^+/H^+ exchange. Final concentrations of added cations was 12.2 mM. Amiloride (final concentration 0.5 mM) was added about 1 min before cations in order to block the Na^+/H^+ antiporter activity. Transport rates are summarized in Table I.

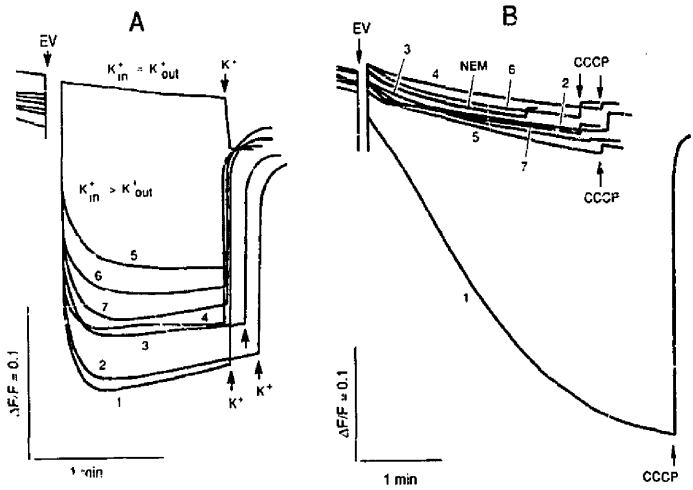

Fig. 6. Vesiculation (A) and H^+-pump activity (B) in control and proteinase-treated endocytic vesicles. (A) Vesicles were added to KCl-buffer ($K^+_{in} = K^+_{out}$) or TMACl-buffer ($K^+_{in} > K^+_{out}$) which contained Acridine orange (AO), valinomycin and CCCP. At the arrow, a 1 M stock solution of KCl was injected into outside buffer (final concentration of K^+, 25 mM) to dissipate the ΔpH. (B) Vesicles were added to KCl-buffer which contained AO, valinomycin and ATP. At the arrow, CCCP was added to dissipate the ΔpH. NEM = the H^+-pump activity in control vesicles preincubated with 1 mM NEM at room temperature for 5 min, and measured in a buffer containing the same concentration of the inhibitor. The recordings are labeled as in Fig. 4.

both the external and cytoplasmic sides of the vesicle membrane.

Effect of proteinases on osmotic water transport

Fig. 8 shows the time course of light scattering in response to a 100 mM inwardly directed sucrose gradient in control BBMV (A) and EV (B) in the presence and absence of 0.6 mM $HgCl_2$. $HgCl_2$ strongly inhibited water transport as reported previously [3–8]. In the absence of $HgCl_2$, the calculated osmotic water permeability in control BBMV and EV was 571 ± 83 and 141 ± 14 μm/s, respectively. As shown in Table

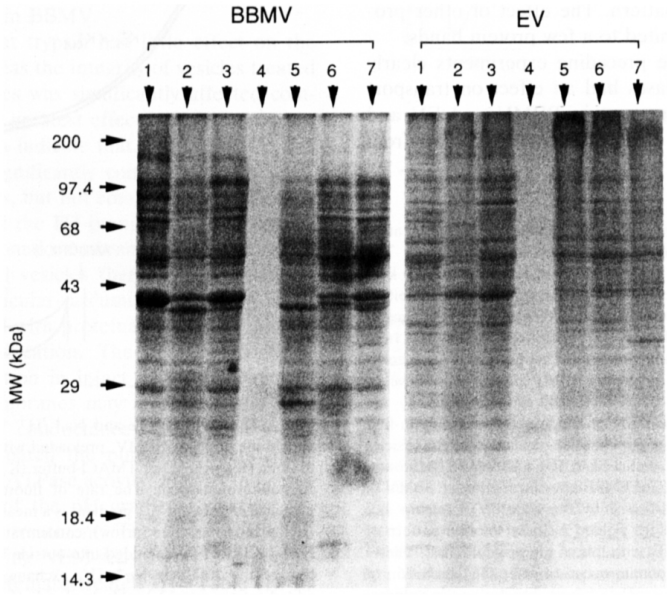

Fig. 7. SDS-polyacrylamide gel electrophoresis of control and proteinase-treated brush-border (BBMV) and endosomal vesicles (EV). The labeling of the lanes corresponds to the recordings in Fig. 4. The gels are typical of two to three separate studies.

TABLE II

Effect of proteinases on NEM-sensitive ATPase activity in rat renal cortical endocytic vesicles

ATPase activity was measured by the P_i liberation assay in unwashed vesicles as described in the Materials and Methods section. The NEM-sensitive ATPase is the difference between the ATPase activity in the absence and presence of 1 mM NEM. Shown are means ± S.E. of the data obtained with four to six vesicle preparations. Statistical significance vs. Control: * $P < 0.01$

Enzyme	NEM-sensitive ATPase (nmol P_i/min mg protein)
Control	63.5 ± 6.56
Trypsin	0.1 ± 0.05 *
Chymotrypsin	0.7 ± 0.52 *
Papain	2.9 ± 0.28 *
Pronase	2.0 ± 0.85 *
Subtilisin	2.7 ± 0.92 *
Thermolysin	5.0 ± 1.72 *

III, in the absence of $HgCl_2$, the water permeability following proteinase treatment in BBMV and EV remained unchanged. $HgCl_2$ inhibited water permeability in BBMV and EV by 90% and 80%, respectively. There was no significant effect of proteinase treatment on control and $HgCl_2$-inhibited water permeability in BBMV and EV.

Our data in isolated vesicles conflicts with a previous report of Benga et al., who found that the mercurial inhibition of water transport in human RBC can be

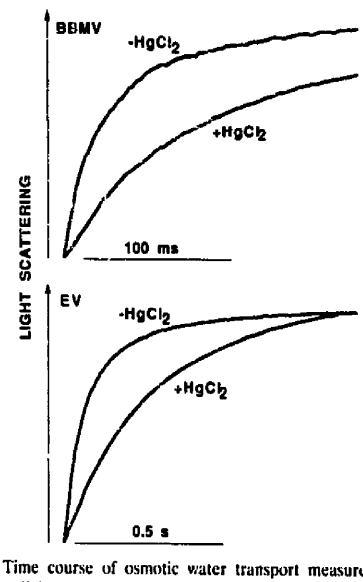

Fig. 8. Time course of osmotic water transport measured by the stop-flow light scattering in BBMV and EV. Each time course corresponds to a 100 mM inwardly directed sucrose gradient in the absence (−$HgCl_2$) or presence of 0.6 mM $HgCl_2$. P_f values are summarized in Table III.

TABLE III

Osmotic water permeability (P_f) in rat renal cortical brush-border (BBMV) and endosomal vesicles (EV) treated with various proteinases

P_f (mean ± S.E.) was measured in three to four vesicle preparations in the absence or presence of 0.6 mM $HgCl_2$ and is expressed in $\mu m/s$

Enzyme	BBMV		EV	
	−$HgCl_2$	+$HgCl_2$	−$HgCl_2$	+$HgCl_2$
Control	571 ± 83	63 ± 18	141 ± 15	29 ± 3
Trypsin	596 ± 78	74 ± 26	130 ± 9	28 ± 1
Chymotrypsin	652 ± 93	90 ± 36	150 ± 19	27 ± 4
Papain	623 ± 42	70 ± 18	153 ± 9	36 ± 4
Pronase	548 ± 55	67 ± 18	130 ± 2	29 ± 3
Subtilisin	527 ± 61	67 ± 19	150 ± 3	24 ± 2
Thermolysin	57[illegible]	85 ± 23	136 ± 4	35 ± 3

inactivated by papain [12]. We therefore reexamined the effect of papain on osmotic water permeability in human RBC and on the inhibition of water permeability by *p*-chloromercurybenzene sulfonate (pCMBS). For this purpose, human RBC were obtained by veinupuncture, washed twice in phosphate-buffered saline, and diluted to 0.2 vol% for incubation with papain and stopped flow measurements. When present, pCMBS was added in a final concentration of 1 mM 30 min prior to stopped-flow studies. In three sets of experiments performed as reported previously [28], P_f at 23°C was 0.021 ± 0.001 cm/s in the absence, and 0.0024 ± 0.0003 cm/s in the presence of pCMBS. Preincubation of the cells with papain (1 mg/ml) for 1 h had no significant effect on control water transport (0.023 ± 0.003 cm/s), and did not prevent the inhibition of water transport by pCMBS (0.0026 ± 0.0003 cm/s).

Discussion

Our data show that multiple proteinases caused marked inhibition of the NEM-sensitive ATPase (H^+-pump) in EV, and a significant inhibition of the Na^+/H^+ exchanger and ecto-ATPase in BBMV. The vacuolar H^+-ATPase in EV has 9–10 different subunits [29]. A subunit with MW of 70–73 kDa has a nucleotide binding site and has been assumed to possess ATPase catalytic activity. Strong inhibition of NEM-sensitive ATPase and H^+-pump activity by multiple proteinases confirms that the 70–73 kDa subunit is a peripheral membrane protein exposed at the outside membrane surface [30]. The effect of proteinases on ecto-ATPase activity was limited (10–30%). Similar limited effects of proteinases (trypsin, chymotrypsin, papain) on ecto-ATPase activity were also reported in hepatocytes in primary culture [31]; therefore the catalytic domain of this ATPase is less susceptible to proteolysis than that of the vacuolar-type H^+-ATPase.

Na^+/H^+ exchange was inhibited by several proteinases but notably not by trypsin. Weinman et al. [32] found that trypsin at low concentrations (< 50 μg/ml) and brief incubation times (< 10 min) stimulated Na^+/H^+ antiport in solubilized and reconstituted BBMV, whereas at higher concentrations (> 100 μg/ml) and after prolonged incubation (> 20 min), trypsin strongly inhibited activity of the exchanger. Taken together, these findings suggest that in solubilized membranes the antiporter is exposed to trypsin from the cytoplasmic surface, whereas in our closed right-side-out BBMV, the antiporter has no domain sensitive to trypsin. H^+ conductance was not affected by proteinase treatment, supporting the conclusion that passive (non-ion coupled) H^+ transport in these membranes is mediated by lipidic and non-specific protein-lipid pathways [33].

In accordance with previous studies [3–8], we confirmed the presence of mercurial-sensitive water transporters in renal cortical brush-border and endosomal membranes. The presence of water channels in proximal tubule endocytic (Ref. 8, and this paper) and clathrin-coated vesicles [10] suggests that the water channels that facilitate the transcellular reabsorbtion of water driven by small osmotic gradients are inserted into and retrieved from apical membranes. However, it is not known whether the number of water channels present in the proximal tubule apical membrane is subject to physiological regulation. Also, the physical nature of the water transporting pathway remains uncertain. Whereas ion channels and membrane transporters such as the Na^+/H^+ exchanger and H^+-ATPase are clearly proteins (as demonstrated also in this paper by their sensitivity to proteolysis), several lines of evidence have raised the possibility that the water transporting pathway may be composed, in part, of non-protein elements such as specialized membrane lipids. In human red cells, osmotic water permeability was not inhibited by proteinases [22] or by high energy radiation up to 4 M_{rad} [13], whereas inhibition by mercurials was prevented by these treatments. In apical membrane vesicles from rabbit proximal tubule, osmotic water permeability was not inhibited by radiation [14]. The radiation inactivation findings are consistent with the water channel being a small protein, an oligomeric assembly of proteins, or a nonprotein pathway. Recent data showed that mRNA from kidney, toad bladder and reticulocytes resulted in the expression of functional water channels when injected into *Xenopus* oocytes [11]. These data suggest that mRNA directs the translation of protein(s) which may comprise the water channel itself, or which direct the synthesis of other components, such as lipids, that might form the water channel.

In view of the data that suggest the proteinaceous nature of the water channel [11], we measured proteinase effects on brush-border membrane and endosomal water transport to examine whether the water transporter has functional peptide domains that are accessible to proteinase digestion. Surprisingly, high concentrations of proteinases, which caused significant protein digestion on gels and inhibition of other protein-mediated processes, did not affect osmotic water permeability or the ability of $HgCl_2$ to inhibit water permeability. Because the orientation of the water transporter in BBMV is probably right-side-out and in EV is cytoplasmic-side-out, it is concluded that neither the external nor the cytoplasmic surface of the water transporter is sensitive to proteinase treatment.

In contrast to a study by Benga et al. [12], we did not find an effect of papain on osmotic water transport or on the pCMBS inhibition of water transport in human erythrocytes. The experimental procedures were the same, except that Benga et al. measured diffusional water permeability by nuclear magnetic resonance whereas we measured osmotic water permeability. Because of the high ratio of osmotic-to-diffusional water permeability in RBC, the measurement of osmotic water transport has significantly better sensitivity to resolve inhibitory effects. RBC osmotic water permeability is > 90% inhibited by pCMBS whereas diffusional water permeability is inhibited by 50% [34]. In addition, the measurement of proton relaxation times by nuclear magnetic resonance requires very concentrated cell suspensions (50% v/v in the study of Benga et al. [12]) and can be seriously confounded by small changes in cell volume and the permeation of extracellular paramagnetic quenchers.

In summary, in our hands, proteinases had a significant inhibitory effect on several transport and enzyme systems in right-side-out BBMV and cytoplasmic-side-out EV. However, the water transport itself, and the mercurial inhibition of water transport in these vesicles remained unaffected by proteinases. We thus conclude that the water channel in renal cortical membranes may be a small integral membrane protein with no functionally important membrane domains that are sensitive to proteolysis from either side of the cell membrane. Our data, however, do not exclude the possibility that the water channel is a lipoprotein or a special lipid arangement in the water permeable membranes.

References

1 Brown, D. (1989) Am. J. Physiol. 256, F1–F12.
2 Verkman, A.S. (1989) Am. J. Physiol. 257, C837–C850.
3 Meyer, M.S. and Verkman, A.S. (1987) J. Membr. Biol. 96, 107–119.
4 Pratz, J., Ripoche, P. and Corman, B. (1986) Biochim. Biophys. Acta 856, 259–266.
5 Van der Goot, F.G., Podevin, R.A. and Corman, B.J. (1989) Biochim. Biophys. Acta 986, 332–340.

6 Van Heeswijk, M.P.E. and Van Os, C.H. (1986) J. Membrane Biol. 92, 183–193.
7 Verkman, A.S., Dix, J.A. and Seifter, J.L. (1985) Am. J. Physiol. 248, F650–F655.
8 Ye, R., Shi, L.-B., Lencer, W.I. and Verkman, A.S. (1989) J. Gen. Physiol. 93, 885–902.
9 Verkman, A.S., Lencer, W.I., Brown, D. and Ausiello, D.A. (1988) Nature 333, 268–269.
10 Verkman, A.S., Weyer, P., Brown, D. and Ausiello, D.A. (1989) J. Biol. Chem. 264, 20608–20613.
11 Zhang, R., Logee, K.A. and Verkman, A.S. (1990) J. Biol. Chem. 265, 15375–15378.
12 Benga, G., Popescu, O. and Pop, V.I. (1983) Cell Biol. Int. Rep. 7, 807–818.
13 Dix, J.A., Ausiello, D.A., Jung, C.Y. and Verkman, A.S. (1985) Biochim. Biophys. Acta 821, 243–252.
14 Verkman, A.S., Dix, J.A., Seifter, J.L., Skorecki, K.L., Jung, C.Y. and Ausiello, D.A. (1985) Am. J. Physiol. 249, F806–F812.
15 Biber, J., Stieger, B., Haase, W. and Murer, H. (1981) Biochim. Biophys. Acta 647, 169–176.
16 Sabolić, I. and Burckhardt, G. (1990) Methods Enzymol. 191 (Biomembranes, Part V), 505–529.
17 Sabolić, I. and Burckhardt, G. (1988) Biochim. Biophys. Acta 937, 398–410.
18 Turrini, F., Sabolić, I., Zimolo, Z., Moewes, B. and Burckhardt, G. (1989) J. Membrane Biol. 107, 1–12.
19 Dean, G.E., Fishkes, H., Nelson, P. and Rudnick, G. (1984) J. Biol. Chem. 259, 9569–9574.
20 Sabolić, I. and Burckhardt, G. (1983) Biochim. Biophys. Acta 734, 210–220.
21 Sabolić, I., Haase, W. and Burckhardt, G. (1985) Am. J. Physiol. 248, F835–F844.
22 Mačković, M., Zimolo, Z., Burckhardt, G. and Sabolić, I. (1986) Biochim. Biophys. Acta 862, 141–152.
23 Tokuyasu, K.T. (1980) Histochem. J. 12, 381–403.
24 Čulić, O., Sabolić, I. and Žanić-Grubišić, T. (1990) Biochim. Biophys. Acta 1030, 143–151.
25 Evers, C., Haase, W., Murer, H. and Kinne, R. (1978) Membrane Biochem. 1, 203–219.
26 Sabolić, I. and Burckhardt, G. (1984) Biochim. Biophys. Acta 772, 140–148.
27 Lin, S.-H. (1989) J. Biol. Chem. 264, 14403–14407.
28 Tsai, S.T., Zhang, R. and Verkman, A.S. (1991) Biochemistry 30, 2087–2092.
29 Forgac, M. (1989) Physiol. Review 69, 765–796.
30 Brown, D., Gluck, S. and Hartwig, J. (1987) J. Cell Biol. 105, 1637–1648.
31 Lin, S.-H. and Russell, W.E. (1988) J. Biol. Chem. 263, 12253–12258.
32 Weinman, E.J., Dubinsky, W.P., Diah, G., Steplock, D. and Shenolikar, S. (1989) J. Membr. Biol. 109, 233–241.
33 Verkman, A.S. (1987) J. Bioenerg. Biomembr. 19, 481–493.
34 Macey, R.I. (1984) Am. J. Physiol. 246, C195–C203.