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PROTON PATHWAYS IN RAT RENAL BRUSH-BORDER AND BASOLATERAL MEMBRANES

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The quenching of acridine orange fluorescence was used to monitor the formation and dissipation of pH gradients in brush-border and basolateral membrane vesicles isolated from rat kidney cortex. The fluorescence changes of acridine orange were shown to be sensitive exclusively to transmembrane ΔpH and not to membrane potential difference. In brush-border membrane vesicles, an Na^+ (Li^+)-H^+ exchange was confirmed. At physiological Na^+ concentrations, 40–70% of Na^+-H^+ exchange was mediated by the electroneutral Na^+-H^+ antiporter; the remainder consisted of Na^+ and H^+ movements through parallel conductive pathways. Both modes of Na^+-H^+ exchange were saturable, with half-maximal rates at about 13 and 24 mM Na^+, respectively. Besides a Na^+ gradient, a K^+ gradient was also able to produce an intravesicular acidification, demonstrating conductance pathways for H^+ and K^+ in brush-border membranes. Experiments with Cl^- or SO_4^{2-} gradients failed to demonstrate measurable Cl^--OH^- or SO_4^{2-}-OH^- exchange by an electroneutral antiporter in brush-border membrane vesicles; only Cl^- conductance was found. In basolateral membrane vesicles, neither Na^+(Li^+)-H^+ exchange nor Na^+ or K^+ conductances were found. However, in the presence of valinomycin-induced K^+ diffusion potential, H^+ conductance of basolateral membranes was demonstrated, which was unaffected by ethoxzolamide and 4,4′-diisothiocyanostilbene-2,2-disulfonic acid. A Cl^- conductance of the membranes was also found, but antiporter-mediated electroneutral Cl^--OH^- or SO_4^{2-}-OH^- exchange could not be detected by the dye method. The restriction of the electroneutral Na^+-H^+ exchanger to the luminal membrane can explain net secretion of protons in the mammalian proximal tubule which leads to the reabsorption of bicarbonate.

Introduction

The function of the proximal tubule in regulation of acid-base balance of body fluids is to secrete H^+ and reabsorb HCO_3^- (for reviews see Refs. 1, 2). Secreted protons serve to titrate filtered HCO_3^- and to form carbonic acid which diffuses out of the lumen, probably as CO_2 and water. In the proximal tubule cell, carbonic acid is formed by carbonic anhydrase-catalysed hydration of CO_2 and is subsequently split into H^+ and HCO_3^-. Protons are secreted across the luminal membrane and HCO_3^- leaves the cell across the basolateral membrane. Therefore, a functional asymmetry between brush-border and basolateral membrane with respect to transport mechanisms for H^+(OH^-) and HCO_3^- has to be assumed.

An Na^+-H^+ exchanger, which drives uphill

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid; Mes, *N*-morpholinoethanesulfonic acid; TMA^+, tetramethylammonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TCS, tetrachlorosalicylanilide; DiS-$C_2(5)$, 3,3-diethylthiadicarbocyanine; DIDS, 4,4-diisothiocyanostilbene-2,2-disulfonic acid (disodium salt); SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

secretion of H^+ coupled to the downhill movements of Na^+, is supposed to be the major mechanism responsible for H^+ secretion in the proximal tubule. Sodium-coupled H^+ transport has been demonstrated in microperfusion experiments in vivo [3], in isolated tubules [4,5], and in brush-border membrane vesicles derived from proximal tubules [6–11]. Furthermore, an ATP-driven proton pump, located in the luminal membrane, has been proposed to play a role in acidification of the proximal tubular fluid [4,5,12–15]. Direct evidence for an ATP-driven H^+ extrusion in isolated renal brush-border membrane vesicles has been provided by Kinne-Saffran et al. [16]. An electroneutral Cl^--OH^- exchange mechanism, demonstrated in rabbit renal brush-border membrane vesicles [17], may not contribute significantly to H^+ or OH^- movements across the luminal membrane, as transcellular Cl^- transport was shown to be negligible [18]. The physiological signficance of H^+ conductance found in isolated brush-border membrane vesicles [9,10] is unknown.

So far, very little is known about H^+ pathways in the contraluminal membrane of proximal tubular epithelial cells. Experiments with isolated basolateral membranes have not been performed. Electrophysiological studies [19,20] have suggested conductance of the basolateral membrane for OH^- (or H^+ in opposite direction) rather than for HCO_3^-. This conductance was inhibited by acetazolamide, suggesting membrane-bound carbonic anhydrase being involved, and also by SITS [20]. The inhibition of glycodiazine (HCO_3^-) reabsorption by peritubularly applied SITS in microperfusion experiments on proximal tubules [15] is in agreement with the electrophysiological data.

In this work we have used the ΔpH-dependent fluorescence quenching of acridine orange to study H^+ transport in isolated brush-border and basolateral membrane vesicles from rat kidney cortex. With this technique we were able to demonstrate directly that the Na^+-H^+ exchange is restricted to the luminal membrane. This finding, which is in agreement with preliminary data of Ives et al. [21], can explain the net secretion of H^+ in the proximal tubule.

Material and Methods

Membrane preparations

Rat renal brush-border membrane vesicles were prepared by precipitation with 10 mM $CaCl_2$ [22]. In the final membrane preparations, the specific activity of the marker enzyme for luminal membranes, leucine arylamidase (EC 3.4.1.2) was enriched 14.7 ± 1.21 times (mean $\pm$S.E., $n = 14$) with respect to the starting homogenate. The enrichment factor for the specific activity of the basolateral membrane marker enzyme, $(Na^+ + K^+)$-ATPase (EC 3.6.1.3), was 0.66 ± 0.15. Basolateral membrane vesicles were separated from other membranes on a Percoll gradient [23]. The enrichment factor of the $(Na^+ + K^+)$-ATPase activity 29.2 ± 3.55 ($n = 12$), whereas that for leucine arylamidase was 1.81 ± 0.17 with respect to the starting homogenate.

The vesicles were preloaded with a buffer of desired ionic composition by two washings followed by an incubation in the same buffer at room temperature for 2 h. The composition of the preloading buffers are described in the legends of the figures. The protein concentration of the final vesicle preparations was adjusted to 16 mg/ml and was measured with the Bio-Rad protein assay kit using bovine serum albumin as a standard.

Measurement of ΔpH

The changes in transmembrane ΔpH were visualized by using the fluorescence quenching of acridine orange [9–11], a method originally described by Schuldiner et al. [24]. The measurement was started by dilution of 10 μl of vesicles into 2 ml buffer containing 6 μM acridine orange. The fluorescence was continuously recorded at 25°C in a Shimadzu RF 510 spectrofluorophotometer (excitation, 493 nm; emission, 525 nm). During the measurement, the samples were continuously stirred. If not stated otherwise, ionophores (final concentrations: gramicidin, 1 μg/ml; valinomycin, 2.5 μM; FCCP, 5 μM; TCS, 1 μM) were added from ethanol stocks before addition of vesicles. Control samples contained an equivalent amount of ethanol, which was less than 0.5% of the total buffer content.

Fluorescence quenching was expressed relatively (in %) to the initial fluorescence observed in

the absence of transmembrane pH and ion gradients.

Measurements of the electrical potential difference

Changes in membrane potential difference were monitored by recording continuously the fluorescence changes of the potential-sensitive dye, DiS-$C_2(5)$. 20 μl of vesicles (10 mg protein/ml) were added to 2 ml buffer containing 3 μM DiS-$C_2(5)$. Valinomycin (final concentrations 2.5 μM) was added 2 min later to create a K^+ diffusion potential. Fluorescence quenching was expressed relative to the steady fluorescence level recorded after the addition of vesicles.

Materials

Acridine orange, TCS and DiS-$C_2(5)$ were obtained from Eastman Kodak (Rochester, NY, U.S.A.), Valinomycin and FCCP from Boehringer (Mannheim, F.R.G.), DIDS from Pierce Chem. Co. (Rockford, IL, U.S.A.); Ethoxzolamide was from Upjohn Ltd. (Kalamazoo, MI, U.S.A.) and Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were reagent grade. TMA^+ gluconate was made by titrating TMA^+ hydroxide pentahydrate with gluconic acid (50% solution in water).

Results

The ΔpH-dependence of acridine orange fluorescence quenching

In order to calibrate the dye response, various pH differences were applied across the vesicle membrane. When vesicles with internal pH 6.0 were diluted into acridine-orange-containing buffers, which were alkaline with respect to intravesicular pH, and immediate drop in fluorescence was observed indicating an intravesicular uptake of the dye and quenching of its fluorescence. This drop, measured 6 s after addition of vesicles, was linearly related to the imposed transmembrane pH difference between 0.8 and 2.8 units in both brush-border and basolateral membrane vesicles (Fig. 1). At constant ΔpH, the initial drop was proportional to the concentration of vesicle protein (up to 0.2 mg/sample, not shown). This experiment shows that acridine orange can be used as an indicator of ΔpH in brush-border as well as basolateral membrane vesicles.

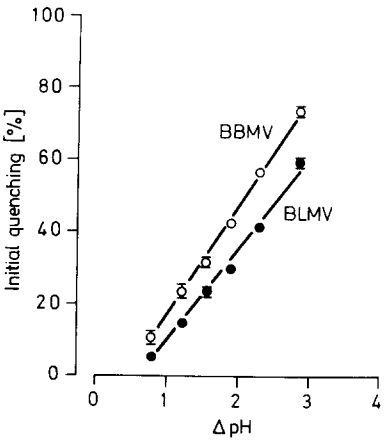

Fig. 1. The ΔpH-dependent intial quenching of acridine orange fluorescence in brush-border (BBMV) and basolateral (BLMV) membrane vesicles. Vesicles were preloaded with 300 mM mannitol/150 mM choline chloride/5 mM Hepes/5 mM Mes-Tris (pH 6.0) and diluted into buffers of various pH which contained the same additions as above plus 6 μM acridine orange. The recordings of fluorescence were 6 s after addition of the vesicles. Each point represents the mean $\pm$S.E. of 3–4 determinations.

Na^+ gradient and Li^+ gradient-dependent formation of ΔpH

When Na^+-loaded brush-border membrane vesicles were diluted into buffer containing acridine orange and the same concentration of Na^+ and H^+, a negligible drop of fluorescence was observed (Fig. 2, $Na^+_{in=out}$). In contrast, the dilution of Na^+-loaded brush-border membrane vesicles into Na^+-free buffer resulted in intravesicular acidification due to Na^+-H^+ exchange, as shown by a time-dependent decrease in the fluorescence (C). In several preparations, the maximum varied between 27% and 35%. According to Fig. 1, this corresponds to a transmembrane ΔpH between 1.2 and 1.5 units. The transmembrane ΔpH was completely abolished after addition of Na^+ to the external buffer (arrows). The rate and the degree of H^+ gradient formation due to a Na^+ gradient were only slightly affected by the protonophore tetrachlorosalicylanilide (TCS), indicating that H^+ conductance is not limiting for Na^+-driven proton movements in brush-border membranes. However, in the presence of TCS, the H^+ gradient formed dissipated more quickly. Addition of gramicidin (Gr) caused a strong intravesicular acidification

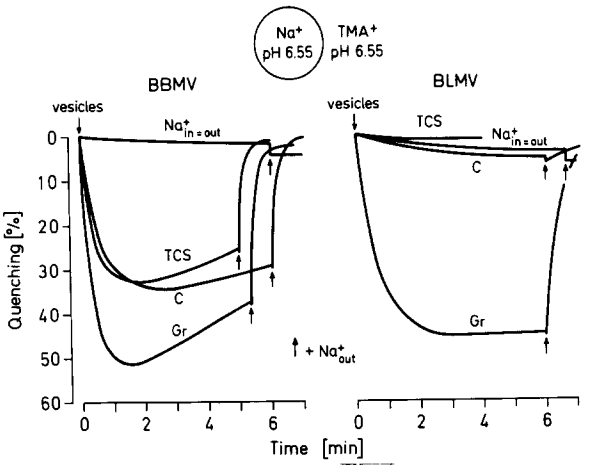

Fig. 2. Na^+-gradient-dependent formation of ΔpH in brush-border (BBMV) and basolateral (BLMV) membrane vesicles. Vesicles were preloaded with 300 mM mannitol/150 mM Na^+ gluconate/10 mM Hepes-Tris (pH 6.55) and diluted into buffer comprising 6 μM acridine orange/300 mM mannitol/150 mM TMA^+ gluconate (150 mM Na^+ gluconate for $Na^+_{in=out}$)/10 mM Hepes-Tris (pH 6.55). Additions were: TCS, gramicidin (Gr) or an equivalent amount of ethanol in controls (C). The arrows indicate an injection of 50 μl 3 M NaCl into external buffer to collapse the pH gradient.

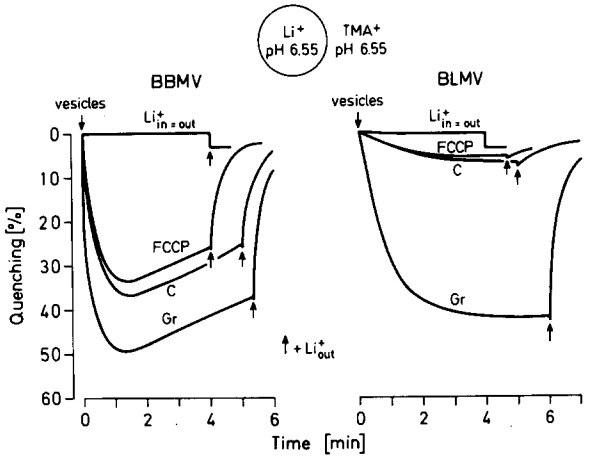

Fig. 3. Li^+-gradient-dependent formation of ΔpH in brush-border (BBMV) and basolateral (BLMV) membrane vesicles. Vesicles were preloaded with 300 mM mannitol/150 mM lithium gluconate/10 mM Hepes-Tris (pH 6.55) and diluted into buffer comprising 6 μM acridine orange/300 mM mannitol/150 mM TMA^+ gluconate (150 mM lithium gluconate for $Li^+_{in=out}$)/10 mM Hepes-Tris (pH 6.55). Additions were: FCCP, gramicidin (Gr) or an equivalent amount of ethanol in controls (C). The arrows indicate an injection of 50 μl 3 M LiCl into external buffer to collapse the pH gradient.

due to artificial Na^+-H^+ exchange [25].

In contrast to brush-border membrane vesicles, the intravesicular acidification due to a Na^+ gradient was negligible in basolateral membrane vesicles, indicating the absence of Na^+-H^+ exchange in these membranes (C). As TCS did not increase the signal, the absence of Na^+ conductance in basolateral membranes must be assumed as well. The gramicidin-mediated Na^+-H^+ exchange led to the same quenching as in brush-border membrane vesicles, but the dissipation of the signal was slower.

Li^+-loaded brush-border and basolateral membrane vesicles gave the same signals as the Na^+-loaded vesicles (Fig. 3), confirming previous findings that Li^+ can substitute for Na^+ on the Na^+-H^+ exchanger [7,10,26]. Here, FCCP was used as protonophore instead of TCS, with similar results.

Electroneutral and electrically driven Na^+-H^+ exchange

Na^+-gradient-dependent intravesicular acidification can be due to the action of an electroneutral Na^+-H^+ exchanger and/or electrically driven Na^+ and H^+ movements through parallel conductance pathways. We tested the contribution of both mechanisms to Na^+-H^+ exchange by addition of valinomycin in the presence of 100 mM K^+ from both sides of the vesicle membrane. In this case, the Na^+ diffusion potential as a possible driving force for H^+ uptake is minimized, and only an electroneutral Na^+-H^+ exchange should be observed.

The broken line in Fig. 4 shows the Na^+ gradient-dependent formation of ΔpH in brush-border membrane vesicles in the absence of K^+. In the presence of K^+, a slightly smaller acidification and a faster dissipation rate of the formed pH gradient was achieved (C). By addition of valinomycin the final degree of acidification was strongly reduced (Val), the quenching being only 60% (40–60% in three preparations) of that without K^+, and the pH gradient dissipated more quickly. Thus, in this experiment, 60% of Na^+-H^+ exchange is mediated by electroneutral antiporter and 40% is due to Na^+ and H^+ movements through parallel conductance pathways. When present, both valinomycin and FCCP completely abolished intravesicular acidification because of the simultaneously

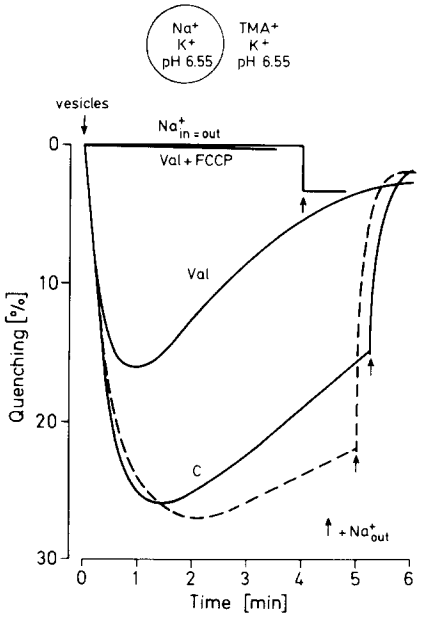

Fig. 4. Electroneutral and electrically driven Na^+-H^+ exchange in brush-border membrane vesicles. — — —, Vesicles were preloaded with 100 mM mannitol/100 mM TMA^+ gluconate/150 mM sodium gluconate/10 mM Hepes-Tris (pH 6.55) and diluted into buffer comprising 6 μM acridine orange/100 mM mannitol/250 mM TMA^+ gluconate (100 mM TMA^+ gluconate and 150 mM sodium gluconate for Na^+_{in-out}/10 mM Hepes-Tris (pH 6.55). ———, Vesicles were preloaded with 100 mM mannitol/100 mM potassium gluconate/150 mM sodium gluconate/10 mM Hepes-Tris (pH 6.55), and diluted into buffer comprising 6 μM acridine orange/100 mM mannitol/100 mM K^+ gluconate/150 mM TMA^+ gluconate/10 mM Hepes-Tris (pH 6.55). Additions were: valinomycin (Val), FCCP or an equivalent amount of ethanol in control (C). The arrows indicate an injection of 50 μl 3 M NaCl into external buffer to collapse the pH gradient.

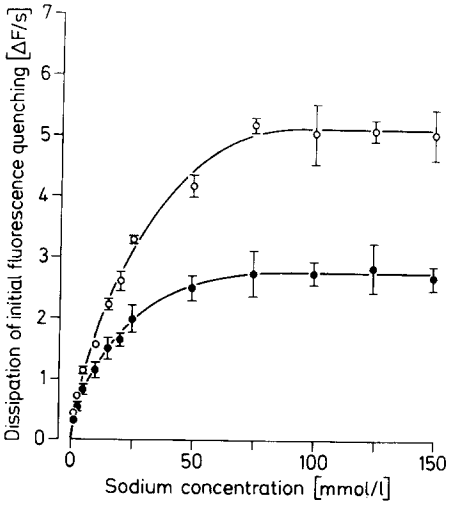

Fig. 5. Kinetics of Na^+-H^+ exchange in brush-border membrane vesicles in the presence (○) and absence (●) of a Na^+ diffusion potential. ○, Vesicles were preloaded with 290 mM mannitol/150 mM TMA^+ gluconate/20 mM Mes-Tris (pH 5.8) and diluted into buffer containing 6 μM acridine orange/290 mM mannitol/150 mM TMA^+ gluconate/20 mM Hepes-Tris (pH 8.3). ●, Vesicles were preloaded with 290 mM mannitol/100 mM potassium gluconate/50 mM TMA^+ gluconate/20 mM Mes-Tris (ph 5.8), and diluted into buffer containing 6 μM acridine orange/2.5 μM valinomycin/290 mM mannitol/100 mM potassium gluconate/50 mM TMA^+ gluconate/20 mM Hepes-Tris (pH 8.3). 10 s after addition of vesicles, solutions of TMA^+ gluconate (controls) or sodium gluconate were injected during continuous fluorescence recording. The resulting initial rates of fluorescence recovery ($\Delta F/s$) for Na^+-containing samples were determined and corrected for those observed in controls. Each datum represents the mean ± S.E. of four separate experiments.

increased K^+ and H^+ conductances of the membranes.

Kinetics of Na^+-H^+ exchange

The effect of various Na^+ concentrations on Na^+-H^+ exchange was tested in pH-jump experiments ($pH_{in} = 5.8$; $pH_{out} = 8.3$). The rate of dissipation of the present pH gradient increases with extravesicular Na^+ concentration and is a measure of Na^+-H^+ exchange. Again, electroneutral and electrically driven Na^+-H^+ exchange were distinguished by the use of valinomycin and equimolar K^+ concentrations on both sides of the membrane.

As shown in Fig. 5 (closed circles), the Na^+-H^+ exchange mediated by the electroneutral antiporter was saturable with half-maximal rate at about 13 mM Na^+. In the absence of K^+ and valinomycin (open circles), an additional component was found which most probably represents Na^+-H^+ exchange through conductance pathways. This component (difference between open and closed circles) also showed saturability, with a half-maximal rate at about 24 mM Na^+. Na^+-H^+ exchange by the antiporter and by conductance pathways did not obey simple Michaelis-Menten kinetics, but rather showed two processes with low and high affinity for Na^+ (not shown). These results require further detailed examination.

At 150 mM Na^+, electrically driven Na^+-H^+ exchange accounted for 30–60% of the total Na^+-

H^+ exchange signal (four experiments). This finding again indicates conductive pathways for Na^+ and H^+ in brush-border membranes and is thus in agreement with data shown in Fig. 4.

K^+-gradient-dependent formation of ΔpH

These experiments were performed to test directly for the existence of H^+ conductance in brush-border and basolateral membranes. When K^+-loaded brush-border membrane vesicles were diluted into K^+-free buffer, a small signal of acidiciation occurred (Fig. 6,C), suggesting small intrinsic K^+ and H^+ conductances in these membranes. FCCP slightly stimulated intravesicular acidification, additionally proving the presence of an intrinsic K^+ conductance. A strong intravesicular acidification signal was found after addition of valinomycin (Val). This quenching was due to an increased uptake of H^+ into the vesicles by a conductance pathway in consequence to an inside-negative K^+ diffusion potential. FCCP further stimulated the H^+ flux into the vesicles, as shown by a large fluorescence quenching (Val + FCCP).

In basolateral membrane vesicles, no quenching

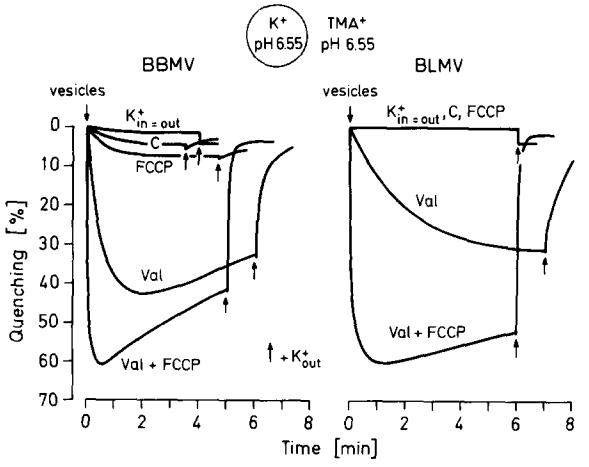

Fig. 6. K^+-gradient-dependent formation of ΔpH in brush-border (BBMV) and basolateral (BLMV) membrane vesicles. Vesicles were preloaded with 300 mM mannitol/150 mM potassium gluconate/10 mM Hepes-Tris (pH 6.55) and diluted into buffer comprising 6 μM acridine orange/300 mM mannitol/150 mM TMA^+ gluconate (150 mM K^+ gluconate for K^+_{in-out})/10 mM Hepes-Tris (pH 6.55). Additions were: valinomycin (Val), FCCP or an equivalent amount of ethanol in controls (C). The arrows indicate an injection of 50 μl 3 M KCl into external buffer to collapse the pH gradient.

was observed with a K^+ gradient in control (C) and FCCP-containing vesicles, demonstrating the absence of measurable intrinsic K^+ conductance in these membranes. In contrast, valinomycin induced a time-dependent drop in fluorescence (Val), indicating the presence of an H^+ conductance in basolateral membranes. The rate of acidification was smaller as compared to that in brush-border membrane vesicles. In the presence of both ionophores, the maximal quenching was similar to that in brush-border membrane vesicles, but the dissipation of the pH gradient was slower.

In order to prove that the fluorescence quenching in the previous experiment was really due to an intravesicular acidification and not to a ΔpH-independent, electrophoretic movement of acridine orange, the following two experiments were designed.

K^+-gradient-dependent formation of ΔpH; effect of low and high intravesicular buffer capacity

K^+-gradient-dependent formation of ΔpH in membrane vesicles should be smaller with high intravesicular buffer capacity.

For this purpose, membrane vesicles of the same preparation, but preloaded with buffers of low or high capacity, were compared. The experimental procedure was the same as in Fig. 6. It is obvious that brush-border membrane vesicles with high intravesicular buffer capacity (Fig. 7B) failed to develop significant quenching of acridine orange fluorescence under all conditions. The same results were obtained with basolateral membrane vesicles (data not shown). This suggests a transmembrane ΔpH as a driving force for acridine orange uptake.

Effect of ΔpH and membrane potential on acridine orange fluorescence quenching

According to Jacobs and Stewart [27], ammonium buffer should dissipate any pH gradient across the membrane by nonionic diffusion of NH_3. This allows us to test the effect of membrane potential on acridine orange fluorescence in the absence of a generated ΔpH.

The formation of ΔpH was visualized with acridine orange (Fig. 8A) and membrane potential with DiS-C_2(5) (Fig. 8B). In the absence of a K^+ gradient, no signal of intravesicular acidification and membrane potential was observed (Fig. 8A

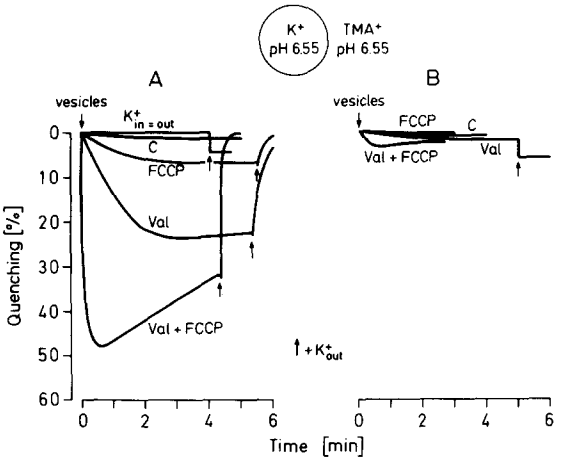

Fig. 7. Effect of low (A) and high (B) intravesicular buffer capacity on the K^+-gradient-dependent formation of ΔpH in brush-border membrane vesicles. (A) Vesicles were preloaded with 299 mM mannitol/150 mM K^+ gluconate/10 mM Hepes/1 mM Mes-Tris (pH 6.55) and diluted into buffer containing 6 μM acridine orange/299 mM mannitol/150 mM TMA^+ gluconate (150 mM potassium gluconate for $K^+_{in=out}$)/ 10 mM Hepes/1 mM Mes-Tris (pH 6.55). (B) Vesicles were preloaded with 100 mM mannitol/150 mM potassium gluconate/10 mM Hepes/200 mM Mes-Tris (pH 6.55) and diluted into buffer containing 6 μM acridine orange/100 mM mannitol/150 mM TMA^+ gluconate/10 mM Hepes/200 mM Mes-Tris (pH 6.55). Additions were: valinomycin (Val), FCCP or an equivalent amount of ethanol in controls (C). The arrows indicate an injection of 50 μl 3 M KCl into external buffer to dissipate the pH gradient.

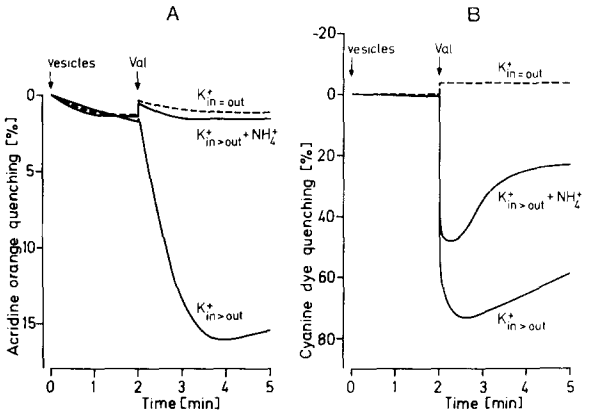

Fig. 8. A different response of acridine orange (A) and potential-sensitive dye (B) to the K^+-driven ΔpH and K^+ diffusion potential in brush-border membrane vesicles: discrimination with NH_4Cl. ———, Vesicles were preloaded with 150 mM KCl/10 mM Hepes-Tris (pH 6.55) and diluted into buffer containing either 6 μM acridine orange (A) or 3 μM DiS-C_2(5) (B), 150 mM TMA^+ chloride (150 mM KCl for $K^+_{in=out}$) and 10 mM Hepes-Tris (pH 6.55). - - - - - -, The same as above, except that 30 mM NH_4Cl was present in the external buffer to dissipate pH gradients. At the indicated time (Val), valinomycin was added to induce a K^+ diffusion potential.

and B, respectively, $K^+_{in=out}$). A K^+ gradient in the presence of valinomycin resulted in intravesicular acidification (Fig. 8A, $K^+_{in>out}$). At the same time, the development of a membrane potential was recorded (Fig. 8B, $K^+_{in>out}$). In the presence of NH_4Cl membrane potential was decreased, but still present, yet no quenching of acridine orange fluorescence developed (Fig. 8B and A, respectively, $K^+_{in>out} + NH^+_4$). This experiment proved acridine orange's being sensitive exclusively for ΔpH and not for membrane potential.

Effect of ethoxzolamide and DIDS on proton conductance

The effect of the carbonic anhydrase inhibitor, ethoxzolamide, on proton conductance in basolateral membrane vesicles was tested (experiments not shown). K^+-loaded vesicles were diluted into K^+-free buffers containing valinomycin and ethoxzolamide. In comparison with controls (without ethoxzolamide) the same conductance for H^+ was observed in the presence of this inhibitor.

Disulfonic stilbenes are known to inhibit transport of anions and concomitant transport of H^+ at the basolateral side of the renal proximal tubule [15,28–30]. We have tested the effect of DIDS on the H^+ conductance in basolateral membrane vesicles. In preliminary experiments, we found that SITS and DIDS strongly quenched acridine orange fluorescence. Therefore, the vesicles were preincubated into DIDS-containing buffers (25 or 50 μM DIDS) at pH 8.4 for 30 min and unbound DIDS was removed by washing. Control vesicles passed through the same procedure but without DIDS. By this experimental approach, DIDS was found to inhibit irreversibly SO_4^{2-} transport in basolateral membrane vesicles (Friedrich, T. and Burckhardt, G., unpublished data). When K^+-loaded vesicles were diluted into valinomycin-containing K^+-free buffer, no difference in H^+ conductance between untreated and DIDS-treated basolateral membrane vesicles was observed (data not shown).

Cl^--gradient-dependent formation of ΔpH

When Cl^--free brush-border membrane vesicles were diluted into Cl^--containing buffer, an intravesicular acidification occurred (Fig. 9, C), which could suggest the presence of an electroneutral Cl^--OH^- exchange and/or Cl^- and H^+ conductances. The signal was small, about 10% of that found with a Na^+ gradient. The rate of acidification was slightly accelerated by FCCP. However, in the absence of membrane potential (K^+ + Val) no signal of acidification was recorded. Therefore, an electroneutral Cl^--OH^- exchange was not detectable. The observed intravesicular acidification was due to H^+ movements driven by a Cl^- diffusion potential.

Similar signals of the Cl^- gradient-dependent intravesicular acidifications were obtained in basolateral membrane vesicles (Fig. 9). In both kinds of membrane vesicle, ethoxzolamide had no effect on Cl^- gradient-dependent acidification signals (data not shown).

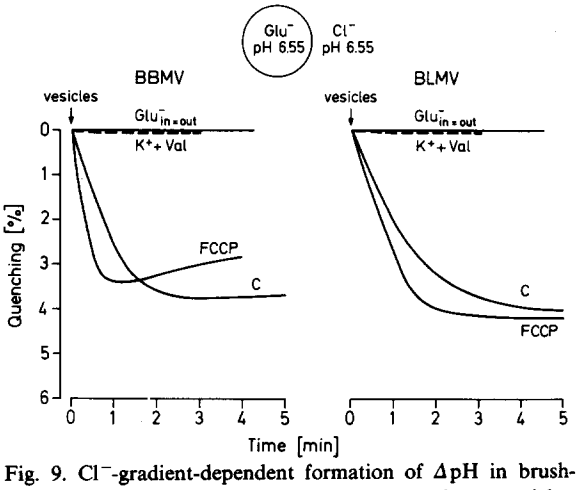

Fig. 9. Cl^--gradient-dependent formation of ΔpH in brush-border (BBMV) and basolateral (BLMV) membrane vesicles. ———, Vesicles were preloaded with 300 mM mannitol/150 mM TMA^+ gluconate/10 mM Hepes-Tris (pH 6.55) and diluted into buffer comprising 6 μM acridine orange/300 mM mannitol/150 mM TMA^+ chloride (TMA^+ gluconate for $Glu^-_{in=out}$)/10 mM Hepes-Tris (pH 6.55). ------, Vesicles were preloaded with 200 mM mannitol/50 mM potassium gluconate/150 mM TMA^+ gluconate/10 mM Hepes-Tris (pH 6.55) and diluted into buffer comprising 6 μM acridine orange/2.5 μM valinomycin/200 mM mannitol/50 mM potassium gluconate/150 mM TMA^+ chloride/10 mM Hepes-Tris (pH 6.55). Additions were: valinomycin (Val), FCCP or an equivalent amount of ethanol in controls (C). Note a 10-fold smaller ordinate scale in comparison with those of previous figures.

The effect of other anion gradients on acridine orange fluorescence

In experiments with SO_4^{2-} gradients (75 mM, out > in) we found no signal of intravesicular acification in brush-border and basolateral membrane vesicles (experiments not shown). The results are consistent with the absence of measureable SO_4^{2-}-OH^- exchange and SO_4^{2-} conductance in each kinds of membrane. With regard to basolateral membrane vesicles, this is in contrast to findings in tracer experiments which clearly revealed the existence of SO_4^{2-}-OH^- exchange in these membranes (Löw, I., Friedrich, T. and Burckhardt, G., unpublished data). Also, we observed no change in intravesicular pH in basolateral membrane vesicles by using gradients (20 mM, out > in) of L-glutamate, citrate, phosphate, *p*-aminohippurate or thiosulfate (75 mM, out > in).

Discussion

Proton movements in rat renal brush-border and basolateral membrane vesicles were investigated by using the ΔpH-sensitive fluorescence dye, acridine orange. Whereas proton conductance pathways were found in both brush-border and basolateral vesicles, the Na^+-H^+ exchange was restricted to the brush-border membrane.

Several lines of evidence suggested that acridine orange is a useful tool for study of transmembrane pH differences not only in brush-border [9–11] but also in basolateral membrane vesicles (present studies): (a) in both types of vesicle, acridine orange fluorescence decreased with increasing preset transmembrane ΔpH (inside acidic) and showed the same sensitivity, allowing the detection of pH differences of more than 0.5 units; (b) pH gradients generated by ionophore-mediated Na^+-H^+ and K^+-H^+ exchange led to a similar fluorescence decrease in brush-border and basolateral membrane vesicles; (c) the influence of the intravesicular buffer capacity on the acridine orange signals as well as the different responses of acridine orange and of potential-sensitive dye proved that acridine orange is sensitive exclusively to ΔpH and not to a transmembrane potential difference.

Proton conductance pathways in brush-border and basolateral membrane vesicles were directly demonstrated by proton uptake driven by an in-

side-negative K^+ diffusion potential. With respect to data from rat luminal vesicles, presented in this paper, similar results have been obtained with rabbit renal brush-border membrane vesicles [9,10,32]. Concerning the proton conductance in basolateral membrane vesicles, no comparable results have been published as yet, but our findings would be in agreement with electrophysiological data obtained in vivo [19,20]. However, in contrast to those findings, we could not show any inhibition of H^+ conductance by ethoxzolamide and DIDS. The lack of effect of DIDS in our experiments is also at odds with the observations of Ullrich et al. [15], who found an inhibition of glycodiazine reabsorption (proton secretion) by peritubularly applied SITS.

In contrast to proton conductance pathways which are present in both kinds of membrane vesicle, Na^+ conductance was found only in brush-border membrane vesicles. Considerable Na^+ gradient-driven proton uptake into brush-border membrane vesicles in addition to antiporter-mediated Na^+-H^+ exchange indicated a relatively high Na^+ conductance of these membranes. This exchange by conductance pathways was saturable with respect to Na^+, indicating possibly a limited number of conductive sites within the membrane. The presence of Na^+ conductance in isolated brush-border membrane vesicles has also been shown previously in experiments using acridine orange [9,10], potential-sensitive probes [31,32], and tracer techniques [33]. In agreement to our data, $^{22}Na^+$ uptake studies with isolated rabbit renal luminal membrane vesicles [34] showed that, at physiological Na^+ concentrations, diffusion (probably by conductance pathways) accounted for about 50% of the total Na^+ transport. In contrast to the large Na^+ conductance in brush-border membrane vesicles, electrophysiological experiments on rat proximal tubular cells in vivo demonstrated a very small conductance of the luminal membrane for Na^+ [35]. The absence of Na^+ conductance in basolateral membrane vesicles, however, would be in agreement with electrophysiological studies in intact cells in vivo [35] and in vitro [36].

Small K^+ gradient-driven intravesicular acidifications in the absence of valinomycin indicated a small intrinsic K^+ conductance of luminal membrane vesicles. In basolateral membrane vesicles, no K^+ conductance could be detected. These results are in contradiction to electrophysiological measurements in rat [35], rabbit [36] and *Necturus* [37] proximal tubule, all of which indicated a high K^+ conductance of luminal and/or antiluminal membranes.

Our experiments revealed the presence of Cl^- conductances, but failed to show a measurable antiporter-mediated electroneutral Cl^--OH^- exchange in both brush-border and basolateral membranes. Possibly, no slow antiporter-mediated Cl^--OH^- exchange was detected because of low sensitivity of acridine orange method below ΔpH 0.5. By tracer techniques, Warnock and Yee [17] demonstrated the existance of an electroneutral Cl^--OH^- exchange and a Cl^- conductance in rabbit renal luminal membrane vesicles. However, similar experiments performed recently confirmed the presence only of Cl^- conductance in these vesicles [38] and are thus in agreement with our data. Comparable experiments with isolated basolateral membrane vesicles have not previously been performed. Electrophysiological studies showed very small or undetectable Cl^- conductance of luminal and antiluminal membranes of mammalian proximal tubule cells [35,36]. Likewise, Cl^- conductance was found to be small in cold-blooded animals [37,39–41]. Our experiments also indicated only a small Cl^- conductance in isolated rat renal brush-border and basolateral membrane vesicles.

Hence, the conductance pathways for H^+, Na^+, K^+, Cl^-, as found in our experiments, are in agreement with previously published data also obtained with isolated membrane vesicles, but considerable differences exist with respect to results of electrophysiological studies in intact cells. Moreover, the physiological significance of high Na^+ and H^+ conductances of the luminal membrane is doubtful. A high Na^+ conductance would tend to dissipate transmembrane Na^+ gradients, and all Na^+-dependent transport processes, including Na^+-H^+ exchange, would be diminished. A high H^+ conductance would dissipate transmembrane H^+ gradients and thus counteract net H^+ secretion. In addition, the missing or small K^+ conductance in the vesicles cannot account for the electrical potential difference of -74 mV (negative inter-

ior) found in vivo [35]. We have, therefore, to assume that the permeability pattern in isolated membrane vesicles does not reflect the characteristics of the membranes in vivo. It is possible that the membranes are altered during the preparation procedure. If this is the case, even ion permeabilities of brush-border and basolateral membranes cannot be compared, as both kinds of membrane are prepared by different methods (Ca^{2+} precipitation versus density gradient separation). An alternative explanation may be that during isolation of membrane vesicles cellular factors which control the permeability status of the membranes are lost.

Despite the results concerning ion permeabilities of vesicles have to be considered with caution, isolated membrane vesicles provide an excellent tool to study molecularly coupled transport processes. Our data unequivocally demonstrated that electroneutral Na^+-H^+ (Li^+-H^+) exchange is restricted to the luminal membrane. These results agree with preliminary findings of Ives et al. [21], who reported a copurification of Na-H^+ exchange with marker enzymes for brush-border membranes along a Percoll density gradient. In contrast, Boron and Boulpaep [42] claimed the presence of a Na^+-H^+ exchange in the luminal as well as the contraluminal membrane of salamander proximal tubule.

According to our kinetic experiments, Na^+-H^+ exchange by the antiporter is saturated at physiological Na^+ concentrations with a half-maximal rate at about 13 mM Na^+. This rate is close to [10,11], or 2–3-times higher [7,26,34] than, the apparent K_m values found with rabbit renal brush-border membrane vesicles. The V_{max} of the electroneutral exchanger was only 40–70% of the total Na^+-H^+ exchange. Because of artificially increased Na^+ and H^+ conductances in isolated brush-border membrane vesicles, the contribution of the electroneutral to the total Na^+-H^+ exchange in probably underestimated.

The asymmetric distribution of the Na^+-H^+ exchange (and possibly of a proton-ATPase [16], which was not investigated in this study) can explain a net H^+ secretion in the mammalian proximal tubule. Electroneutral anion exchanges (Cl^--OH^-, SO_4^{2-}-OH^- and others) seem not to be involved significantly in proton transport across the luminal and antiluminal membrane. The pathways for H^+ and HCO_3^- (OH^-) in the contraluminal membrane, as indicated by electrophysiological studies in vivo, have to be characterized further.

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