Biochimica et Biophysica Acta, 772 (1984) 140–148
Elsevier

BBA 72062

EFFECT OF THE PREPARATION METHOD ON Na^+-H^+ EXCHANGE AND ION PERMEABILITIES IN RAT RENAL BRUSH-BORDER MEMBRANES

IVAN SABOLIĆ * and GERHARD BURCKHARDT **

Max-Planck-Institut für Biophysik, Kennedyallee 70, 6000 Frankfurt/Main (F.R.G.)

(Received November 2nd, 1983)

Key words: Na^+-H^+ exchange; Ion transport; Acridine orange; Brush-border membrane; (Rat kidney cortex)

The ΔpH-dependent quenching of Acridine orange was used to characterize Na^+-H^+ exchange and K^+ and H^+ conductances in brush-border membrane vesicles isolated by precipitation with either $CaCl_2$ or $MgCl_2$ from rat kidney cortex. A transmembrane pH difference of 2.5 units (inside acidic) was imposed and the initial rate of its dissipation was followed after injecting a puls of tetramethylammonium gluconate (control) or sodium or potassium gluconate. In membranes isolated by $CaCl_2$, the Na^+-H^+ exchange was partially electroneutral (45% to 77% of the total exchange) and the rest was due to electrically coupled Na^+ and H^+ movements through conductive pathways in the membranes. In membranes prepared by $MgCl_2$, the rate of total Na^+-H^+ exchange was about twice as high as that in membranes obtained by $CaCl_2$ precipitation. However, total and electroneutral exchanges were equal indicating negligible electrically coupled Na^+ and H^+ movements in these membranes. $K_{0.5}$ for Na^+ in all preparations was in the same range, being in average 30 mM. Amiloride was a competitive inhibitor of Na^+-H^+ exchange in membranes obtained with both preparations; K_i values ranged between 0.1 and 0.58 mM. The rates of ΔpH-dissipation with K^+ gradients (± valinomycin) were by 50% to 150% higher in membranes prepared with $CaCl_2$ than in membranes isolated with $MgCl_2$ indicating much higher H^+ and K^+ conductances in membranes obtained with $CaCl_2$. Therefore, the rate of Na^+-H^+ exchange as well as the conductances for various ions in the isolated brush-border membranes depend on membrane preparation.

Introduction

Available evidence from proximal tubular cells [1–3] and brush-border membrane vesicles derived from proximal tubules [4–10] clearly suggests electroneutral Na^+-H^+ exchange as the main mechanism for H^+ secretion and Na^+ absorption in the proximal tubule of mammalian kidney. An Na^+-H^+ exchanger is present exclusively in the luminal, but not in contraluminal membrane [10,11], and is specifically inhibited by amiloride [2,5,6,8,9]. However, the amiloride effects on the exchanger, as observed in Acridine orange studies [8,9], were recently questioned by finding of a nonspecific interaction of amiloride and Acridine orange [12,13].

Conductances for H^+, Na^+, K^+, and Cl^- were also found in isolated brush-border membrane vesicles [7,8,10]. The pattern of conductances, as observed in isolated membranes, does not agree with that in intact cells, so that the physiological significance of all these conductances is doubtful

* Permanent address: Department of Physiology, Faculty of Medicine, University of Zagreb, Zagreb, Yugoslavia.
** To whom correspondence should be addressed.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; Mes, *N*-morpholinoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TMA^+, tetramethylammonium.

[10]. It is possible that the membranes are altered during the preparation procedure or, alternatively, that the cellular factors which control the permeability status of the membranes are lost in isolated membranes [10].

In the present report, we have tested two widely used techniques for preparation of luminal membrane vesicles, precipitation with $CaCl_2$ [14] and $MgCl_2$ [15], on the rate of Na^+-H^+ exchange and the conductances for Na^+, H^+ and K^+. Great differences between the two preparations were found which indicate that the permeability pattern as well as the activity of the electroneutral Na^+-H^+ exchanger can be affected by the membrane preparation. In addition, we show an approach by which the specific inhibition of Na^+-H^+ exchange by amiloride can be demonstrated also in Acridine orange studies.

Material and Methods

Membrane preparation

Brush-border membrane vesicles were prepared from rat kidney cortex by precipitation with either 10 mM $CaCl_2$ [14] (further on: Ca-preparation) or 12 mM $MgCl_2$ [15] (Mg-preparation). In addition to the divalent cations, the preparation buffers contained 10 mM mannitol, 2 mM Tris-HCl (pH 7.1) (further on: buffer A) or 300 mM mannitol, 5 mM EGTA, 12 mM Tris-HCl (pH 7.4) (buffer B). In both preparations the homogenization of the cortical tissue was performed by an Ultra-Turrax (two times 60 s, instrument setting at 180 V) (Janke and Kunkel, Staufen, F.R.G.). All other steps in the preparations were the same as described previously [14,15]. With respect to the starting homogenate, the final membrane preparations were enriched 10–15-times in the specific activity of the marker enzyme for luminal membranes, leucine arylamidase (EC 3.4.1.2). The enrichment factor for the $(Na^+ + K^+)$-ATPase (EC 3.6.1.3), a marker for basolateral membranes, was always less than 1.0.

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 preloading buffers contained either 290 mM mannitol, 150 mM TMA^+ gluconate, 20 mM Mes/Tris, pH 5.8 (further on: TMA buffer, pH 5.8) or 290 mM mannitol, 100 mM K^+ gluconate, 50 mM TMA^+ gluconate, 20 mM Mes/Tris, pH 5.8 (K buffer, pH 5.8). The protein concentration of the isolated membrane vesicles was adjusted to 16 mg/ml and was measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

Measurement of ΔpH

The changes in transmembrane pH-gradients were vizualized by using the ΔpH-dependent quenching of Acridine orange fluorescence [7–10,16]. 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. Under all experimental conditions, the fluorescence of Acridine orange before addition of vesicles was adjusted to the same level (100%) by changing the instrument gain.

A preset ΔpH ($pH_{in} = 5.8$; $pH_{out} = 8.3$) was imposed in all experiments [7,9,10], and the rate of dissipation of the pH gradient was followed after injecting a puls of a salt into the outside buffer. The composition of the outside buffer was either 290 mM mannitol, 150 mM TMA^+ gluconate, 20 mM Hepes/Tris, pH 8.3 (further on: TMA buffer, pH 8.3) or 290 mM mannitol, 100 mM K^+ gluconate, 50 mM TMA^+ gluconate, 20 mM Hepes/Tris, pH 8.3 (K buffer, pH 8.3).

1.74 ml buffer (pH 8.3) was placed into the cuvette. The stock solutions of Acridine orange and either valinomycin dissolved in ethanol or an equivalent amount of ethanol were added (in a total volume of 10 μl) to give final concentrations of 6 μM, 2.5 μM and 0.25%, respectively. Vesicles (10 μl, $pH_{in} = 5.8$) were added and the dissipation of the initial quenching was recorded. Exactly 10 s after addition of vesicles, 0.25 ml of TMA^+ gluconate (control) or Na^+ or K^+ gluconate solutions were injected during continuous fluorescence recording and the resulting initial rates of fluorescence recovery were determined. To prevent osmotic and pH changes in the outside buffer, all stock solutions of cations were made isotonic by appropriate additions of TMA^+ gluconate and were buffered to pH 8.3.

The initial rates of the reactions were determined by drawing the tangents on the record-

ings obtained in the first 2.5 s following the addition of salts. The initial rates for Na^+- or K^+-containing samples were corrected for those observed in controls and were expressed as the difference in fluorescence change per 2.5 s ($\Delta F/2.5$ s). The measurements were made at least in triplicate with each preparation.

When present, amiloride dissolved in water was added prior to vesicles addition in the final concentration indicated in the figure and table legends. Statistical analyses were made by means of Student's *t*-test.

Acridine orange was obtained from Eastman Kodak (Rochester, NY, U.S.A.), valinomycin from Boehringer (Mannheim, F.R.G.), amiloride was a gift from Merck, Sharp and Dohme Research Laboratories (West Point, PA, U.S.A.); PMSF was from Sigma (St. Louis, MO, U.S.A.), trifluoperazine-HCl from Röhm Pharma (Darmstadt, F.R.G.), and mepacrine from ICN Pharmaceuticals Inc. (New York, NY, U.S.A.). All other chemicals were reagent grade. TMA^+ gluconate was prepared by titrating tetramethylammonium hydroxide pentahydrate with gluconic acid (50% solution in water).

Results

Kinetics of Na^+-H^+ exchange

The effect of various Na^+ concentrations on the dissipation of a transmembrane ΔpH was tested in brush-border membrane vesicles isolated by Ca- or Mg-preparation. The rate of dissipation of the preset pH difference increased with extravesicular Na^+ concentration (Fig. 1). Na^+-gradient-dependent rates of ΔpH-dissipation can be due to the action of an electroneutral Na^+-H^+ exchanger and electrically driven Na^+ and H^+ movements through parallel conductive pathways in isolated membranes [10]. The contribution of these two mechanisms to the total rate of Na^+-H^+ exchange can be determined by the use of valinomycin and equimolar K^+ concentrations on both sides of the vesicle membrane. By this approach, an Na^+ diffusion potential as a possible driving force for increased H^+ extrusion is minimized, and only electroneutral Na^+-H^+ exchange is recorded [10].

As shown in Fig. 1 (open circles), at each Na^+ concentration, the rate of total Na^+-H^+ exchange (absence of K^+ and valinomycin) in the membranes obtained by Ca-preparation was about half of that in the membranes isolated by Mg-preparation. However, when K^+ and valinomycin were present (closed circles), the rate of Na^+-H^+ exchange in membranes obtained by Ca-preparation was about 35% smaller, but remained unchanged in membrane vesicles isolated by Mg-preparation. The results indicate the presence of electroneutral and electrically coupled Na^+-H^+ exchange in the membranes prepared by Ca-preparation, the latter being probably due to simultaneously present Na^+ and H^+ conductances in these membranes. In membranes obtained by Mg-preparation, electrically coupled Na^+ and H^+ movements through conductance pathways are virtually absent; only electroneutral Na^+-H^+ exchange was recorded, which was 3–4-times bigger than in membranes isolated by Ca-preparation.

In the measured range, the rates of Na^+-H^+ exchange in all preparations obeyed Michaelis-Menten kinetics (insets in Fig. 1). In membranes obtained by Ca-preparation, the values of $K_{0.5}$ (Na^+) and V_{max} for electroneutral Na^+-H^+ ex-

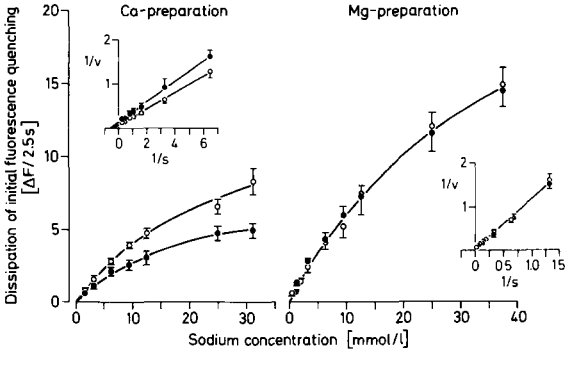

Fig. 1. Kinetics of Na^+-H^+ exchange in brush-border membrane vesicles isolated by Ca- or Mg-preparation. (○) Total Na^+-H^+ exchange: vesicles were preloaded with TMA buffer, pH 5.8, and diluted into TMA buffer, pH 8.3, which contained Acridine orange and ethanol. (●) Electroneutral Na^+-H^+ exchange: vesicles were preloaded with K buffer, pH 5.8, and diluted into K buffer, pH 8.3, which contained Acridine orange and valinomycin. The initial rates of fluorescence recovery for samples containing Na^+ were corrected for controls containing TMA^+ and are shown as means ±S.E. of four membrane preparations. The insets show the Lineweaver-Burk plots of the same data.

TABLE I

KINETIC CHARACTERISTICS OF Na^+-H^+ EXCHANGE IN RAT RENAL BRUSH-BORDER MEMBRANE VESICLES ISOLATED BY EITHER Ca- OR Mg-PREPARATION; INHIBITION BY AMILORIDE

Measurements were performed as described in the legends of Figs. 1 and 3. The values for $K_{0.5}(Na^+)$ and V_{max} were obtained from Lineweaver-Burk plots by calculation, and those for K_i(amiloride), by calculation from Dixon plots. Shown are the means ± S.E. for the number of preparations indicated in brackets. n.s., not significant. n.d., not determined.

Preparation	Na^+-H^+ exchange	$K_{0.5}(Na^+)$ (mM)	V_{max} (ΔF/2.5 s)	K_i(amiloride) (mM)
Ca^{2+}	Total	34.9 ± 8.31(4)	16.7 ± 2.57	0.23 ± 0.025(3)
	Electroneutral	16.0 ± 1.55(4)	7.2 ± 1.31	0.36 ± 0.053(3)
	p	< 0.05	< 0.01	n.s.
Mg^{2+}	Total	34.5 ± 4.22(4)	28.0 ± 4.58	0.26 ± 0.084(5)
	Electroneutral	31.7 ± 7.89(4)	27.9 ± 4.21	n.d.
	p	n.s.	n.s.	

change were half of those for total exchange (Table I). However, a significant difference in $K_{0.5}$ in these preparations is compromised by a considerable scatter of the data from experiment to experiment; the $K_{0.5}$ values ranged between 12.2 and 53.5 mM Na^+. In membranes prepared with $MgCl_2$, no difference in $K_{0.5}$ (Na^+) and V_{max} was observed between the total and electroneutral exchange; $K_{0.5}$ was about 33 mM and V_{max} about twice as high as the total exchange in the membranes prepared with $CaCl_2$.

Inhibition of Na^+-H^+ exchange by amiloride

Recently published papers [12,13] pointed out an interference of amiloride with the determination of proton gradients by Acridine orange. The fact that amiloride quenches Acridine orange fluorescence but does not dissipate transmembrane pH gradients [12] was used here to demonstrate a specific inhibition of Na^+-H^+ exchange in brush-border membrane vesicles isolated by Ca- and Mg-preparation.

The initial rates of dissipation of the preset pH gradients in control experiments (150 mM $TMA^+_{out} > TMA^+_{in}$) were not influenced by up to 1.5 mM amiloride (Table II) proving again that amiloride does not act as a permeant base [12]. By changing the instrument gain after the addition of amiloride always to the same level (100%), the recorded curves following the addition of vesicles were only shifted up, in direction of the baseline (for illustration see Fig. 2). As also shown in Table II, the initial rates of dissipation of transmembrane ΔpH were increased in the presence of a K^+ gradient (12.4 mM K^+_{out}, 0 K^+_{in}) and valinomycin by 1.3 fluorescence units/2.5 s over the control (TMA^+). These initial rates were not affected by amiloride concentrations up to 1.5 mM excluding effects of amiloride on K^+ and H^+ conductances.

Amiloride, however, inhibited the initial rates of fluorescence recovery dependent on Na^+ gradi-

TABLE II

EFFECT OF AMILORIDE ON THE RATE OF FLUORESCENCE RECOVERY IN THE PRESENCE OF TMA^+ OR K^+ GRADIENTS

Vesicles were preloaded with TMA buffer, pH 5.8, and diluted into TMA buffer, pH 8.3, which contained Acridine orange, valinomycin and the indicated final concentrations of amiloride. The stock solutions of TMA^+ or K^+ gluconate were injected 10 s after the addition of vesicles and the rate of fluorescence recovery was recorded (for illustration, see Fig. 2). The final concentration of the injected salts in the cuvette was 150 mM; the concentration of K^+ was 12.4 mM. The initial rates of fluorescence recovery of samples with K^+ were corrected for controls (TMA^+). Each datum is a mean ± S.E. for the number of determinations indicated in the brackets obtained from a representative single preparation.

Amiloride concn. (mM)	Rate of fluorescence recovery (ΔF/2.5 s)	
	TMA^+	K^+
0	1.23 ± 0.05(6)	1.30 ± 0.06(4)
0.5	1.17 ± 0.07(3)	1.30 ± 0.07(4)
1.0	1.26 ± 0.02(5)	1.30 ± 0.07(4)
1.5	1.17 ± 0.09(3)	1.30 ± 0.08(5)

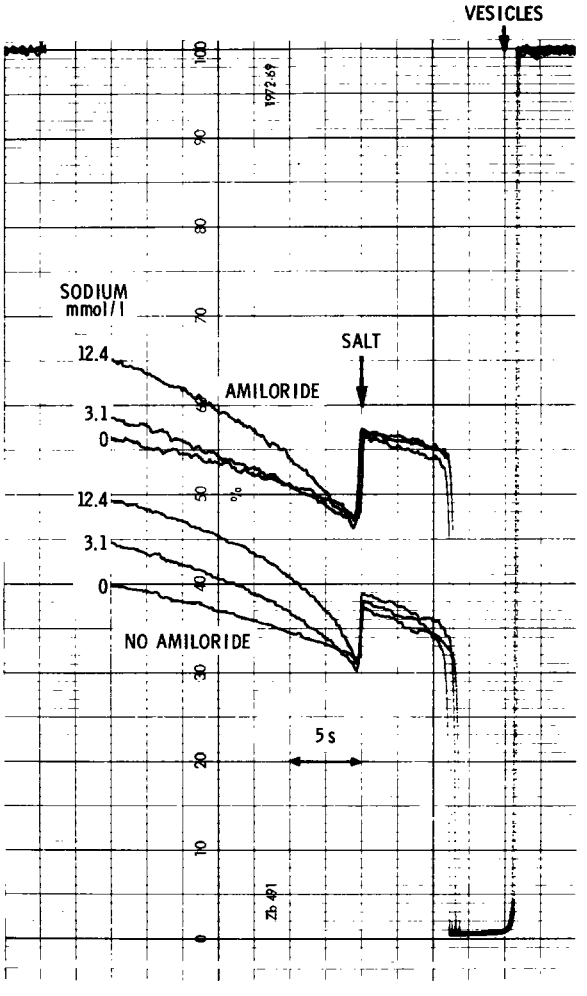

Fig. 2. The inhibition by amiloride of the initial rates of fluorescence recovery dependent on Na^+ gradients. Vesicles were prepared by Mg-preparation, preloaded with TMA-buffer, pH 5.8, and diluted into TMA buffer, pH 8.3, which contained Acridine orange and ethanol, without or with 1 mM (final concentration) amiloride. The stock solutions of TMA^+ or Na^+ were injected 10 s after the addition of vesicles (arrow) and the subsequent recovery of the fluorescence was followed. The final Na^+ concentration in the cuvette was either 3.1 or 12.4 mM. The direction of the recordings is from right to left.

ents as illustrated in Fig. 2. The inhibition was stronger at lower Na^+ concentration indicating a competition between amiloride and Na^+. As shown in Fig. 3 for membranes isolated by Mg-preparation, the initial rates of Na^+-H^+ exchange were measured at two Na^+ concentrations, 3.1 mM and 12.4 mM, in the presence of the indicated amiloride

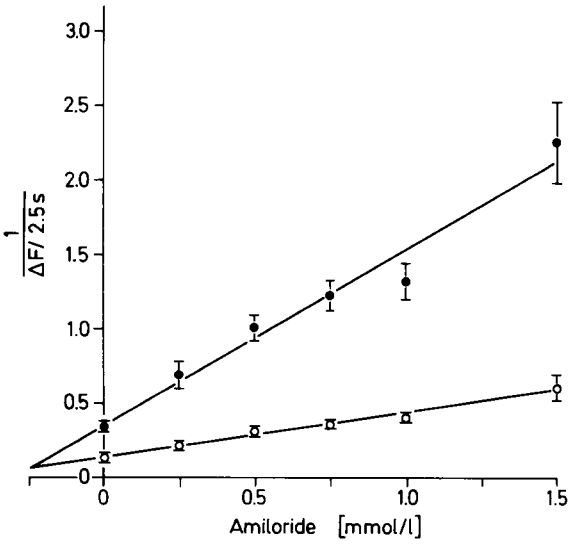

Fig. 3. The inhibition by amiloride of the initial rates of Na^+-H^+ exchange in brush-border membrane vesicles isolated by Mg-preparation (Dixon plot). The measurements were performed as described in the legend of Fig. 2, except that the amiloride concentration varied as indicated. The initial rates of the fluorescence recovery for samples containing Na^+ were corrected for controls. The final concentrations of Na^+ were 3.1 mM (●) or 12.4 mM (○). Each point is a mean ± S.E. of data from five preparations.

concentrations. The Dixon plot of the data revealed a competitive inhibition of the Na^+-H^+ exchange by amiloride. The K_i for amiloride ranged between 0.1 and 0.58 mM, averaging 0.28 ± 0.084 mM (Table I). The values of K_i for total and electroneutral exchange in membranes obtained by Ca-preparation were in the same range.

The effect of various preparative conditions on maximal rate of total and electroneutral Na^+-H^+ exchange

Preceding experiments showed the presence of electroneutral and electrically coupled portions of total Na^+-H^+ exchange in membranes prepared with $CaCl_2$. In membranes obtained by Mg-preparation, electrically coupled Na^+-H^+ exchange was virtually absent. In order to investigate the cause of formation of the electrically coupled Na^+-H^+ exchange, we compared the rates of the total and electroneutral Na^+-H^+ exchange at a Na^+ gradient (150 mM Na^+_{out}, 0 Na^+_{in}) under various preparative conditions (Table III). At this

Na^+ concentration, the recorded rate of Na^+-H^+ exchange was maximal.

In vesicles obtained by Ca-preparation (buffer A), the maximal rate of electroneutral Na^+-H^+ exchange was always smaller by 23% to 55% than total exchange indicating that a quarter to one half of total exchange corresponds to electrically coupled movements of Na^+ and H^+ through conductive pathways existing in these membranes. In membranes isolated by Mg-preparation (buffer B), the maximal rate of total exchange was about twice as high as that in Ca-preparation, and no influence of K^+ and valinomycin was observed, suggesting again negligible electrically coupled movements of Na^+ and H^+ through conductive pathways in these membranes.

When 12 mM $MgCl_2$ was present instead of 10 mM $CaCl_2$ in a buffer normally used in Ca-preparation (buffer A), the total Na^+-H^+ exchange was smaller than in membranes prepared with $MgCl_2$ in buffer B, but again no indication for significant electrically coupled Na^+-H^+ exchange was found. A smaller total Na^+-H^+ exchange in these experiments indicates that possibly the osmolarity and/or the pH of the homogenization buffer can also affect the rate of the exchanger. On the contrary, by preparing membrane vesicles with $CaCl_2$ instead of $MgCl_2$ in buffer B (EGTA omitted), the total exchange in the vesicles was also smaller than in Mg-preparation (buffer B), but electrically coupled movements of Na^+ and H^+ were observed.

Vesicles prepared by Ca-preparation in the presence of a protease inhibitor, PMSF, or a phospholipase-A_2 inhibitor, mepacrine [17,18], as well as trifluoperazine-HCl, an inhibitor of calmodulin-mediated processes [19], retained the pattern of the total and electroneutral Na^+-H^+ exchange as compared with preparation without inhibitors; the total exchange was smaller than in membranes prepared with $MgCl_2$ and a significant electrically coupled part of total exchange was always visible (Table III). When membrane vesicles prepared by Mg^{2+} (buffer B) were incubated for 2 h in the presence of 10 mM Ca^{2+}, no difference between total and electroneutral Na^+-H^+ exchange was found (24.25 ± 0.21 and 24.95 ± 0.35 ($n = 3$) ΔF/2.5 s, respectively) indicating that conductances for Na^+ and H^+ were formed during the membrane preparation in Ca-buffers.

The effect of various preparative conditions on H^+ and K^+ conductance

The above-mentioned experiments clearly demonstrated that the membranes contain significant conductive pathways for Na^+ and H^+ only when

TABLE III

THE INFLUENCE OF DIFFERENT PREPARATIONS ON MAXIMAL RATES OF TOTAL AND ELECTRONEUTRAL Na^+-H^+ EXCHANGE

Vesicles were preloaded with either TMA buffer, pH 5.8, and diluted into TMA buffer, pH 8.3, which contained Acridine orange and ethanol (total Na^+-H^+ exchange) or with K buffer, pH 5.8, and diluted into K buffer, pH 8.3, which contained Acridine orange and valinomycin (electroneutral Na^+-H^+ exchange). If indicated, 1 mM PMSF, 0.1 mM mepacrine, or 0.01 mM trifluoperazine-HCl (TFP) were present in the preparation buffers. Initial rates of fluorescence recovery for samples containing Na^+ (150 mM Na^+_{out}, 0 Na^+_{in}) were corrected for controls (TMA^+). Each datum is the mean ± S.E. of the number of preparations indicated by n. The composition of the buffers A and B is described under Material and Methods. Statistically significant differences ($p < 0.05$) between total and electroneutral exchange are indicated with asterisks.

Precipitating cation	Preparation buffer	n	Na^+-dependent fluorescence recovery (ΔF/2.5 s)	
			Ethanol	$K^+_{i=0}$ + valinomycin
10 mM Ca^{2+}	A	7	13.22 ± 0.90	8.56 ± 1.01 *
12 mM Mg^{2+}	B	6	23.41 ± 1.43	22.61 ± 1.26
12 mM Mg^{2+}	A	4	14.51 ± 0.25	14.67 ± 0.51
10 mM Ca^{2+}	B (− EGTA)	5	18.48 ± 1.00	14.41 ± 1.41 *
10 mM Ca^{2+}	A (+ PMSF)	3	15.75 ± 0.35	11.52 ± 0.81 *
10 mM Ca^{2+}	A (+ mepacrine)	3	15.61 ± 1.29	11.10 ± 0.34 *
10 mM Ca^{2+}	A (+ TFP)	4	16.64 ± 0.69	12.68 ± 1.00 *

TABLE IV

THE INFLUENCE OF DIFFERENT PREPARATIONS ON K^+ AND H^+ CONDUCTANCE

Vesicles were preloaded with TMA buffer, pH 5.8, and diluted into TMA buffer, pH 8.3, which contained Acridine orange and either ethanol (K^+ conductance) or valinomycin (H^+ conductance). Shown are the initial rates of fluorescence recovery for samples containing K^+ (150 mM K^+_{out}, 0 K^+_{in}) and corrected for controls. The data are means ± S.E. of the number of preparations indicated by n. Statistically significant differences ($p < 0.05$) in comparison to the corresponding Mg-preparation (buffer B) are indicated by asterisks. The composition of the buffers A and B is described in Material and Methods. The concentrations of PMSF, mepacrine, and trifluoperazine-HCl (TFP) in the preparation buffers were 1 mM, 0.1 mM, and 0.01 mM, respectively.

Precipitating cation	Preparation buffer	n	K^+-dependent fluorescence recovery (ΔF/2.5 s)	
			Ethanol	Valinomycin
10 mM Ca^{2+}	A	7	1.11 ± 0.17 *	5.90 ± 0.26 *
12 mM Mg^{2+}	B	6	0.73 ± 0.09	3.78 ± 0.30
12 mM Mg^{2+}	A	4	0.76 ± 0.18	3.90 ± 0.11
10 mM Ca^{2+}	B (− EGTA)	5	1.34 ± 0.10 *	6.20 ± 0.10 *
10 mM Ca^{2+}	A (+ PMSF)	3	1.40 ± 0.12 *	6.02 ± 0.37 *
10 mM Ca^{2+}	A (+ mepacrine)	3	1.59 ± 0.05 *	6.80 ± 0.47 *
10 mM Ca^{2+}	A (+ TFP)	4	1.47 ± 0.10 *	6.55 ± 0.38 *

prepared with $CaCl_2$. A lack of the electrically coupled Na^+-H^+ exchange in the membranes prepared by $MgCl_2$ can be explained by the absence of Na^+ and/or H^+ conductances in these membranes. With the present method it is not possible to visualize Na^+ conductance independently of the conductance for H^+. However, an H^+ conductance of the membranes can be demonstrated separately by imposing a K^+ gradient (150 mM K^+_{out}, 0 K^+_{in}) in the presence of valinomycin. By this way, a K^+ diffusion potential (inside positive) acts as a driving force for increased H^+ efflux through conductive pathways for H^+, and is thus a qualitative measure of H^+ conductances in the membranes.

As indicated by the increased rates of ΔpH dissipation in the presence of a K^+ gradient and valinomycin (Table IV), an H^+ conductance is present in the membranes isolated with both preparations. These rates were by 50% to 75% higher in membranes isolated with $CaCl_2$ compared to membranes obtained by Mg-preparation, indicating a higher H^+ conductance in membranes prepared with $CaCl_2$. PMSF, mepacrine or trifluoperazine-HCl, had no effect on the calcium-induced high H^+ conductance. Considerable electrically coupled Na^+-H^+ exchange in membranes prepared by $CaCl_2$ indicates also a high Na^+ conductance in these membranes. As electrically coupled Na^+-H^+ exchange was absent in Mg-prepared membranes, one has to assume a very small Na^+ conductance in these membranes.

A relative measure of the K^+ conductance of the membranes was obtained from the rate of ΔpH dissipation in the presence of a K^+ gradient (150 mM K^+_{out}, 0 K^+_{in}), but without valinomycin. In this case, K^+ moved through intrinsic conductive pathways in the membranes, and an inside-positive diffusion potential, thus formed, was a driving force for extrusion of H^+. These signals were much smaller than those in the presence of valinomycin. As shown in Table IV, ΔpH dissipation driven by a K^+ gradient was between 50% and 120% higher in membranes prepared with $CaCl_2$ than in membranes prepared by $MgCl_2$. This pattern was not changed when the vesicles were prepared with $CaCl_2$ in the presence of PMSF, mepacrine or trifluoperazine-HCl.

An addition of 10 mM $CaCl_2$ to the vesicles isolated by Mg-preparation as well as an addition of 12 mM $MgCl_2$ to vesicles obtained by Ca-preparation, 2 h or immediately before the measurement, did not change the conductance pattern for H^+ and K^+ (data not shown). This suggests that the observed conductive pathways were formed in the membranes during the preparation.

Discussion

Using the ΔpH-sensitive dye Acridine orange we showed that brush-border membrane vesicles

isolated from rat kidney cortex by precipitation with either $CaCl_2$ or $MgCl_2$ differ significantly in the rate of Na^+-H^+ exchange, and in Na^+, H^+ and K^+ conductances.

Electronetural Na^+-H^+ exchange saturated at physiological Na^+ concentrations (Ref. 10, and this study). Kinetic experiments revealed the same affinity of the Na^+-H^+ exchanger for Na^+ in Ca- and Mg-preparations with half-maximal rate at about 30 mM Na^+. The observed apparent K_m for Na^+ is 2–6-times higher than that in rabbit renal brush-border membrane vesicles [5,8,9,20–23]. In contrast to K_m, Ca- and Mg-preparations differed markedly in the maximal rate of the electroneutral Na^+-H^+ exchanger being about 3–4-times higher in membranes prepared by Mg-precipitation.

The Na^+-H^+ exchange was competitively inhibited by amiloride. As shown in this paper, amiloride does not interact with H^+ and K^+ conductances, and in agreement with our previous findings [12] and those from Cassano et al. [24], does not act as a permeant buffer dissipating preset pH differences. Therefore, in pH-jump experiments amiloride can be used with Acridine orange after proper correction for the amiloride-dependent fluorescence quenching. The K_i for amiloride was in the same range for Ca- and Mg-preparations, between 0.1 and 0.58 mM. The affinity of the exchanger for amiloride is about one order of magnitude smaller in rat kidney than in rabbit kidney [6,22,23]. Our findings agree with data from perfused rat proximal tubules and in situ experiments. Chan and Giebisch [25] observed an incomplete inhibition of bicarbonate reabsorption by 1 mM amiloride at a luminal Na^+ concentration of 5 mM. At physiological Na^+ concentrations, Ullrich and Rumrich found an inhibition of glycodiazine reabsorption (H^+ secretion) in situ by only 14% in the presence of 1 mM amiloride (unpublished observations). In similar studies by Howlin et al. [26], 1 mM amiloride inhibited tubular acidification in the rat kidney by 25–36% at physiological Na^+ concentrations. Using the average values for $K_{0.5}$ (Na^+) and K_i (amiloride) from our work (30 mM and 0.28 mM, respectively), one can calculate that at physiological Na^+ concentrations Na^+-H^+ exchange is inhibited by about 40%.

Brush-border membrane vesicles prepared by Ca-precipitation showed a considerable Na^+-H^+ exchange via conductance pathways for Na^+ and H^+. In vesicles prepared by Mg-precipitation, electrically driven Na^+-H^+ exchange was not detected (Ref. 24, and this study) indicating that Ca- and Mg-prepared vesicles differ with respect to their H^+ and/or Na^+ conductance. An H^+ conductance is present in membrane vesicles obtained by either preparation as visualized by K^+ diffusion potential-driven H^+ efflux. The absence of electrically coupled Na^+-H^+ exchange in Mg-prepared vesicles must therefore be due to a negligible Na^+ conductance. In vesicles prepared by Ca-precipitation, a greater K^+-driven H^+ efflux and electrically coupled Na^+-H^+ exchange indicate higher H^+ and Na^+ conductances in accordance to previous experiments with Acridine orange [8,10], potential-sensitive probes [27,28] and tracer technique [20,28].

Differences between Ca- and Mg-prepared brush-border membrane vesicles were previously indicated by transport experiments and determination of the fatty acid composition of the membranes. Biber et al. [15] found higher rates of Na^+-dependent D-glucose and phosphate transport in membrane vesicles isolated by Mg^{2+}/EGTA precipitation. The data presented in this paper provide an explanation for the observed differences: membrane vesicles prepared with $CaCl_2$ are more leaky to Na^+, thus allowing the faster dissipation of an Na^+ gradient through conductive pathways. The content of free fatty acids and lysophosphatides in brush-border membranes prepared by Ca-preparation was exceptionally high in comparison to Mg-preparation [29]. The reason for this difference was suggested to be an activation of membrane-bound phospholipase A by Ca^{2+}. When EGTA was present in the preparation buffers, the content of lysophospholipids decreased significantly. A similar mechanism has been also proposed for the increased H^+ and K^+ conductances in rat gastric membranes prepared in the absence of EGTA [30].

The differences between Ca- and Mg-prepared membrane vesicles described in this study may also be due to an action of Ca^{2+}-activated phospholipases. However, the pattern of Na^+-H^+ exchange and ion conductances in membranes prepared with $CaCl_2$ was not changed in the presence

of mepacrine, an inhibitor of phospholipase A_2 [17,18], and trifluoperazine, a specific inhibitor of Ca^{2+}-calmodulin-mediated processes. Ca^{2+}-calmodulin has been found to increase the activity of phospholipase A_2 [19]. As PMSF did not alter the properties of Ca-prepared vesicles, an intrinsic proteolytic activity (which is present in plasma membranes of proximal tubule cells [31]) is either insensitive to PMSF or does not affect membrane conductances. A direct influence of Ca^{2+} on Na^+-H^+ exchange and permeability pattern can be excluded because preloading of Mg-prepared vesicles with 10 mM Ca^{2+} had no effect. At present, we are not able to explain the cause of the differences in the permeability pattern for various ions and the rate of Na^+-H^+ exchange in membranes prepared with $CaCl_2$ and $MgCl_2$. Nevertheless, our data suggest that results concerning ion permeabilities of vesicles have to be considered with caution as they depend on membrane preparation and may not represent the in vivo properties of the membrane.

Acknowledgement

We thank Dr. K.J. Ullrich for reading of the manuscript and for valuable comments.

References

1 Bichara, M., Paillard, M., Leviel, F. and Gardin, J.P. (1980) Am. J. Physiol. 238, F445–F451
2 Schwartz, G.J. (1981) Am. J. Physiol. 241, F380–F385
3 Bichara, M., Paillard, M., Leviel, F., Prigent, A. and Gardin, J.P. (1983) Am. J. Physiol. 244, F165–F171
4 Murer, H., Hopfer, U. and Kinne, R. (1976) Biochem. J. 154, 597–604
5 Kinsella, J.L. and Aronson, P.S. (1980) Am. J. Physiol. 238, F461–F469
6 Kinsella, J.L. and Aronson, P.S. (1981) Am. J. Physiol. 241, F374–F379
7 Reenstra, W.W., Warnock, D.G., Yee, V.J. and Forte, J.G. (1981) J. Biol. Chem. 256, 11663–11666
8 Burnham, C., Munzesheimer, C., Rabon, E. and Sachs, G. (1982) Biochim. Biophys. Acta 685, 260–272
9 Warnock, D.G., Reenstra, W.W. and Yee, V.J. (1982) Am. J. Physiol. 242, F733–F739
10 Sabolić, I. and Burckhardt, G. (1983) Biochim. Biophys. Acta 734, 210–220
11 Ives, H.E., Yee, V.L. and Warnock, D.G. (1983) J. Biol. Chem. 258, 13513–13516
12 Sabolić, I. and Burckhardt, G. (1983) Biochim. Biophys. Acta 731, 354–360
13 Dubinsky, W.R. and Frizzel, R.A. (1983) Am. J. Physiol. 245, C157–C159
14 Evers, C., Haase, W., Murer, H. and Kinne, R. (1978) Membrane Biochem. 1, 203–219
15 Biber, J., Stieger, B., Haase, W. and Murer, H. (1981) Biochim. Biophys. Acta 647, 169–176
16 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70
17 Vargaftig, B.B. and Hai, N.D. (1972) J. Pharm. Pharmac. 24, 159–161
18 Flower, R.J. and Blackwell, G.J. (1976) Biochem. Pharmacol. 25, 285–291
19 Cheung, W.Y. (1980) Science 207, 19–27
20 Nord, E.P., Wright, E.M. and Fine, L.G. (1983) Kidney Int. 23, 264 (Abstract)
21 Kinsella, J.L. and Aronson, P.S. (1981) Am. J. Physiol. 241, C220–C226
22 Aronson, P.S., Suhm, M.A. and Nee, J. (1983) J. Biol. Chem. 258, 6767–6771
23 Ives, H.E., Yee, V.J. and Warnock, D.G. (1983) J. Biol. Chem. 258, 9710–9716
24 Cassano, G., Stieger, B. and Murer, H. (1984) Pflügers Arch., in the press
25 Chan, Y.L. and Giebisch, G. (1981) Am. J. Physiol. 240, F222–F230
26 Howlin, K.J., Alpern, R.J. and Rector, F.C., Jr. (1983) Proc. 29th Congress Int. Union Physiol. Sci., Sydney, p. 422 (Abstr.)
27 Wright, S.H., Krasne, S., Kippen, I. and Wright, E.M. (1981) Biochim. Biophys. Acta 640, 767–778
28 Wright, E.M., Schell, R.R. and Krasne, S. (1983) Kidney Int. 23, 241 (Abstract)
29 Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577
30 Im, W.B. and Blakeman, D.P. (1982) Biochem. Biophys. Res. Commun. 108, 635–639
31 Rankin, B.B., McIntyre, T.M. and Curthoys, N.P. (1980) Biochem. Biophys. Res. Commun. 96, 991–996