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Mechanism of coupling between Cl^- and OH^- transport in renal brush-border membranes

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The coupling mechanism for Cl^- and H^+/OH^- transport in renal brush-border vesicles was examined from intravesicular pH changes following imposed H^+ and Cl^- gradients. Vesicles were loaded with 6-carboxyfluorescein and exposed to H^+ gradients and Cl^-, gluconate, or sulfate gradients, each with and without a K^+/valinomycin voltage clamp. Parallel experiments were performed with vesicles equilibrated with 10 mM HCO_3^- or 5 mM formate. Rate of H^+/OH^- transport was determined from the initial rate of change in 6-carboxyfluorescein fluorescence, vesicle buffer capacity and the relationship between fluorescence and vesicle pH. In contrast to gluconate or sulfate, Cl^- caused enhanced H^+/OH^- transport under all conditions. This difference was eliminated with voltage clamping in the presence of gluconate, SO_4^{2-}, or HCO_3^-, but not in the presence of formate. These findings were not affected by the method of preparation of the vesicles. Electrically coupled Cl^-/OH^- transport was not inhibited by 100 μM DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) or 100 μM DBDS (4,4′-dibenzamidostilbene-2,2′-disulfonate). SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) was found to be a protonophore at concentrations > 500 μM. As a control for the method, we demonstrated amiloride inhibitable, electroneutral Na^+-H^+ exchange (H^+ flux = 107 ± 9 nmol/s per mg, 100 mM Na^+) and electroneutral, DBDS inhibitable Cl^--HCO_3^- exchange in sealed human red blood cell ghosts. Therefore, electroneutral Cl^--OH^- or HCO_3^- exchange does not measurably contribute to Cl^- transport in the proximal tubule brush border. Cl^--formate exchange with formic acid recyling appears to be the only electroneutral coupling mechanism between Cl^- and OH^- transport demonstrable in renal brush-border membrane vesicles.

Introduction

Although NaCl is the major salt reabsorbed from the renal proximal tubule, the mechanisms responsible for NaCl transport across this epithelium are poorly understood. In late proximal tubule fluid ($[HCO_3^-] < 7$ and $[Cl^-] = 125$ mM), where the reabsorbed salt is almost entirely NaCl, approx. 50% of the Cl^- is transported passively across the paracellular pathway while the remaining 50% of the reabsorbed Cl^- is transported through the proximal tubular cells [1–4]. It has been proposed [5,6] that a Cl^-/OH^- exchanger could work in parallel with a Na^+/H^+ exchanger to effect net transport of NaCl. A Na^+/H^+ ex-

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate.

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changer has been well described in this tissue and Cl^-/OH^- or Cl^-/HCO_3^- exchangers have been found in many other membranes [7–9]. However, definitive evidence for Cl^--OH^- or Cl^--HCO_3^- exchange in the renal proximal tubule has been diffucIt to obtain.

Three different groups have studied the pH dependence of $^{36}Cl^-$ uptake into brush border vesicles. Two have found evidence for electroneutral exchange [6,10] while the third finds no such evidence [11]. A fourth group also failed to find evidence for electroneutral Cl^--OH^- exchange in renal brush-border vesicles using the acridine orange method to study H^+ or OH^- transport [12]. None of these groups fully addressed the possibility that the putative anion exchanger might require HCO_3^-. More importantly, it has never been determined whether the imposed pH gradients persist for the entire period of $^{36}Cl^-$ uptake.

Work from several other laboratories has established that renal brush-border membrane vesicles prepared by a variety of techniques have a high H^+/OH^- permeability [13–18]. In voltage-clamped vesicles with exogenously added buffers of from 10 to 50 mM, pH gradients collapse with an exponential time constant of 10–15 s [13–16, 18]. Thus, earlier isotopic Cl^- uptake studies were probably performed under the condition of a rapidly changing pH gradient, complicating their interpretation.

In order to overcome this experimental difficulty, we studied the effect of Cl^- and other anions on H^+/OH^- transport in renal brush-border membrane vesicles. So that Cl^--OH^- exchange and Na^+-H^+ exchange could be studied under similar conditions, we developed a method to study the collapse of both inwardly ($pH_{in} > pH_{out}$) and outwardly ($pH_{out} > pH_{in}$) directed H^+ gradients with a time resolution of < 1 s [17]. Using this method, it was possible to detect high activity levels of electroneutral Na^+-H^+ exchange and modest levels of electrically coupled Cl^--OH^- or Cl^--HCO_3^- exchange. However, the ability of Cl^- to stimulate H^+/OH^- transport was eradicated by voltage clamping. Thus, Cl^- driven H^+/OH^- transport appears to result from diffusion potentials formed by the Cl^- chemical gradient and a Cl^- conductance pathway.

Materials and Methods

Materials

Unless otherwise specified, all chemicals were of reagent grade and were obtained from Sigma (St. Louis, MO). 6-Carboxyfluorescein was obtained from Molecular Probes (Junction City, OR), and SITS from ICN (Chicago, IL). DBDS was synthesized by the method of Kotaki et al. [19].

Vesicle preparation

Renal brush-border membrane vesicles were prepared as previously described [13,20]. Briefly, the renal cortex was dissected from the kidneys of 1–2 kg female New Zealand white rabbits and homogenized at 4°C in 50 mM sucrose, 50 mM Hepes-Tris, 5 mM EGTA (pH 6) with a Sorvall Omni mixer. After exposure of the homogenate to 12 mM $MgCl_2$ for 30 min at 4°C, brush-border membranes were isolated by differential centrifugation [13,20]. Where specified, $MgCl_2$ was replaced by equimolar $CaCl_2$. Maltase and ($Na^+ + K^+$)-ATPase activities exhibited enrichments of 12- and 0.3-fold over the crude homogenate, respectively. Vesicles were either assayed immediately after preparation or frozen for up to 1 month at −70°C. Freezing did not alter any of the enzymes or transport processes being studied. Protein concentration, maltase, and ($Na^+ + K^+$)-ATPase activities were asayed as described previously [21]. In several experiments, brush-border vesicles were prepared by sucrose density gradients as previously described [21]. Briefly, crude membranes were prepared from homogenized renal cortex by differential centrifugation and loaded on to 35–48% sucrose gradients at pH 7. After centrifugation to equilibrium (16 h), fractions were collected and assayed for maltase and ($Na^+ + K^+$)-ATPase activity which were typically 8- and 0.6-fold enriched, respectively. In the indicated experiments, protease inhibitors (0.05 mg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, and 0.1 mg/ml pepstatin) were included throughout the preparation.

Sealed human red blood cell ghosts were prepared as previously described [22].

H^+/OH^- transport measurements

Vesicles (20 mg protein/ml) were incubated for

12 h with 100 μM 6-carboxyfluorescein. In some cases (see Results), vesicles were incubated for 30 min with 6-carboxyfluorescein diacetate rather than overnight with the unesterified compound. Dye remaining in solution was removed by three washes in 50 vol. of buffer not containing the dye followed by centrifugation at 300000 × *g* for 10 min. Vesicles containing entrapped 6-carboxyfluorescein were maintained as a packed pellet at 4°C until the time of the experiment to minimize dye leakage [18].

10 μl of the 6-carboxyfluorescein-loaded, packed brush-border vesicles (40 mg of protein per ml) were added to buffer (50 mM Mes-Tris (pH 6 or 7), 250 mM sucrose, 150 mM K^+ gluconate (termed 'standard buffer') or 75 mM K_2SO_4) and baseline 6-carboxyfluorescein fluorescence was determined using an SLM 8225 fluorometer (Urbana, IL). Small volumes of acid (gluconic or H_2SO_4) were added to cause a specific change in solution pH. Within 2 s after addition of acid, 100 mosM *N*-methylglucamine chloride, *N*-methylglucamine sulfate, or *N*-methylglucamine gluconate was added. The time-course of fluorescence intensity (excitation 486 nm, emission 512 nm) was followed for 100 s and fitted to a single exponential with an IBM PC XT computer. The intial slope of the exponential was used to determine the intial rate of change in intravesicular pH using the pH calibration of 6-carboxyfluorescein as previously described [18]. This value was then converted directly to H^+ flux (nmol/s per mg of protein) using the intravesicular buffer capacity [18].

To measure the rate of Na^+-H^+ exchange using 6-carboxyfluorescein, pH 6 vesicles loaded with dye were diluted into pH 6 assay buffer (50 mM Mes-KOH, 250 mM sucrose, 150 mM potassium gluconate) and small volumes of *N*-methylglucamine were added to cause a rapid increase in extravesicular pH to 7.0. 100 mosM sodium gluconate or potassium gluconate was then rapidly added.

In all cases where voltage clamping was required, vesicles were incubated with 150 mM K^+ on both sides of the membrane and 50 μg valinomycin was added per mg of protein. While this does not necessarily insure a membrane potential of 0 mV, rheogenic H^+ flux is not limited by the formation of diffusion potentials under these conditions [13].

Anion exchange in sealed red blood cell ghosts

Ghosts were incubated for 12 h in 250 mM sucrose, 40 mM tripotassium citrate, 50 mM Hepes-Tris, 100 μM 6-carboxyfluorescein. Dye remaining in solution was removed by washing ghosts three times with dye-free buffer prior to assay. To measure the rate of H^+/OH^- transport, 10 μl of packed ghosts were diluted into 2 ml of 250 mM sucrose, 40 mM potassium citrate, 50 mM Hepes-Tris (pH 7.0). Citric acid was added to decrease the solution pH to 5.0 and 6-carboxyfluorescein fluorescence was monitored as described above. 10 mM *N*-methylglucamine chloride or iso-osmotic *N*-methylglucamine citrate was added within 2 s to examine the effect of Cl^- on collapse of the pH gradient.

Extravesicular pH measurements

To measure the rate of H^+/OH^- flux by changes in extravesicular pH, brush-border vesicles were washed twice with unbuffered 250 mM sucrose, 150 mM KCl. 400 μl of packed brush-border vesicles (40 mg of protein per ml) were incubated in 2 ml of the same solution for 15 min in a cuvette which was continuously stirred and bubbled with 100% N_2. Extravesicular pH was monitored after addition of 5–10 μl of 0.05 M H_2SO_4 or 0.1 M KOH using a Corning pH electrode and a Corning model 125 pH meter interfaced to a Digital Equipment Corp. MINC/23 computer. Extravesicular buffer capacity was 0.2 mequiv./l per pH unit under these conditions.

Statistics

All data are presented as mean ± S.D. for *N* observations. Where comparisons are made *P* values were obtained using Student's *t*-test. All significant differences are denoted by *.

Results

Rate of collapse of pH gradients in brush-border membranes

Reenstra et al. [16] first reported rapid collapse of outwardly directed pH gradients ($pH_{in} = 6$, $pH_{out} = 7.5$) in renal brush-border vesicles using

acridine orange fluorescence quenching. When fitted to a single exponential, the collapse of pH gradients had a time constant of 220 s in the absence of voltage clamping and 20 s in the presence of a 150 mM K^+ and valinomycin voltage clamp. We have confirmed this finding in two recent studies of the H^+ permeability of brush-border membranes [13,14]. In addition, we showed that the endogenous buffer capacity of renal brush-border membranes is 125 mequiv./l per pH unit, making the time constant for collapse of pH gradients relatively independent of exogenously added buffers [13]. In the past, pH gradient-stimulated Cl^- uptake in voltage-clamped vesicles has been studied on time scales longer than 20 s [6,10,11]. Because a rapidly collapsing pH gradient could alter the interpretation of these isotopic studies, we re-examined the time-course of pH gradient collapse using two additional independent methods.

Vesicles loaded with the pH-sensitive fluorescent dye 6-carboxyfluorescein were voltage clamped and exposed to a 0.5 unit inwardly directed pH gradient (Fig. 1A). Intravesicular pH declined exponentially with a time constant of 12 s, slightly faster than the value found by Reenstra et al. [16] for outwardly directed gradients using the acridine orange method.

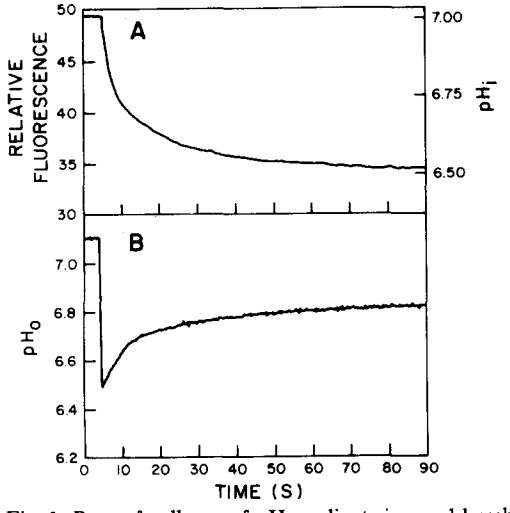

Fig. 1. Rate of collapse of pH gradients in renal brush-border membrane vesicles. In (A) vesicles (400 μg of protein) were equilibrated in pH 7 assay buffer (see Methods) with 50 μg of valinomycin/mg of protein and gluconic acid was added to rapidly change extravesicular pH to 6.5. Time constant (τ) for the slow portion of the curve was 12.4 ± 0.4 s ($n = 13$, typical experiment shown). In (B) vesicles (8 mg of protein) were suspended in 2 ml of buffer and 10 μl of 0.05 M H_2SO_4 was rapidly added. Extravesicular pH was followed with a pH electrode. τ for the recovery phase of pH equilibration was 10.1 ± 0.5 s ($n = 4$, typical experiment shown).

To confirm this finding using an independent method, a similar experiment was carried out with vesicles in a buffer-free assay solution. By excluding buffer, it was possible to detect changes in extravesicular pH with standard pH electrode during H^+/OH^- transport across the brush-border membrane (Fig. 1b). After addition of small volumes of H_2SO_4, pH fell rapidly (< 1 s) due to titration of the extravesicular solution and then returned slowly (time constant 10 s) due to equilibration of the intravesicular and extravesicular spaces. This time constant, which measures the rate of H^+/OH^- transport across the membrane, is in agreement with the data obtained using intravesicular 6-carboxyfluorescein. Since pH gradients in renal brush-border membrane vesicles collapse with a time constant of 10–20 s as confirmed by three independent techniques, it is necessary that initial ion flux rates be determined within 10–20 s after the pH gradient is established. Since fluorescence measurements of intravesicular pH can be made within 500 ms (conventional fluorometry) or 2 ms (stopped flow fluorometry), we used intravesicular pH measurements to examine the coupling mechanism for Cl^- and H^+/OH^- transport in renal brush-border membrane vesicles.

Influence of Cl^- on collapse of pH gradients

Coupling between Cl^- transport and pH gradients in proximal tubule brush-border membrane vesicles has been amply demonstrated in the past [6,10,11]. In order to determine whether or not this coupling is electroneutral, the effect of voltage clamping on Cl^--stimulated H^+/OH^- transport was compared with its effect on SO_4^{2-} or gluconate-stimulated H^+/OH^- transport. After establishment of a 2 unit pH gradient ($pH_{in} = 7.0$, $pH_{out} = 5.0$), 200 mosM *N*-methylglucamine chloride or *N*-methylglucamine gluconate was added to vesicles (Fig. 2). Cl^- enhanced the rate of collapse of the pH gradient by approx. 2-fold (Table I), indicating cotransport of H^+/Cl^- or

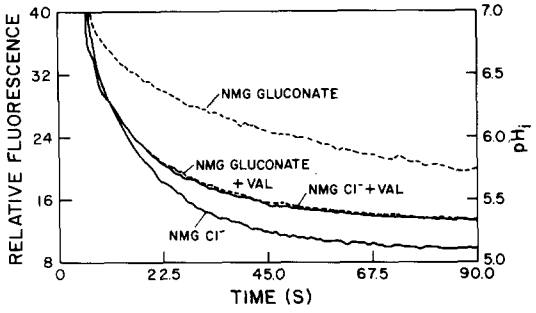

Fig. 2. Effect of anions on collapse of pH grdients in renal brush-border membrane vesicles. Vesicles (400 μg of protein) were incubated in pH 7 buffer and the extravesicular space was rapidly titrated to pH 6 with gluconic acid as in Fig. 1. 2 s later, 200 mosM *N*-methylglucamine chloride (NMG Cl^-), or *N*-methylglucamine gluconate (NMG gluc) was rapidly added either with (+Val) or without (−Val) 50 μg valinomycin/mg of protein. τ for the fitted exponentials were 33.3 ± 2.1 (NMG gluc), 13.6 ± 0.3 (NMG Cl^-), 14.9 ± 0.4 (NMG Cl^- + Val), and 15.2 ± 0.9 (NMG gluc + Val).

exchange of Cl^-/OH^-. However, upon addition of valinomycin, the difference in the rate of H^+/OH^- transport induced by Cl^- and gluconate was eliminated. Similar results were obtained with a 1 unit pH gradient and with SO_4^{2-} as the anion (Table I). Lastly, similar results were obtained when the assays were performed with buffer containing 1 mM Ca^{2+} or Mg^{2+} in the assay solution.

To test for the presence of electroneutral Cl^--HCO_3^- exchange, the experiment shown in Fig. 2 was repeated with vesicles that were pre-incubated with 10 mM HCO_3^- buffered to pH 7 with KOH. After imposition of a 2 unit pH gradient (pH_{out} = 5.0, pH_{in} = 7.0), addition of anions resulted in H^+/OH^- transport at the rate of 19.7 ± 0.6 nmol/s per mg of protein (Cl^-) or 7.9 ± 0.4 nmol/s per mg (gluconate). When valinomycin was added, the rate of H^+/OH^- transport after addition of the two anions was equalized (17.7 ± 0.9 nmol/s per mg (Cl^-) or 16.9 ± 1.3 nmol/s per mg (gluconate). These results were similar to those observed without HCO_3^- and suggest that electroneutral Cl^--HCO_3^- exchange is absent in renal brush-border membrane vesicles.

Since it has recently been proposed [23] that Cl^- may be transported across the renal brush border in exchange for small organic anions like formate, we examined the effect of formate on Cl^--stimulated collapse of pH gradients (Table II). Vesicles at pH 7 were loaded with 5 mM

TABLE I

RATE OF COLLAPSE OF H^+ GRADIENTS IN PRESENCE OF ANION GRADIENTS

Brush-border vesicles (400 μg of protein) at pH 7 were loaded with 6-carboxyfluorescein, washed three times and added to 2 ml of the standard buffer (pH 7). 2 s after addition of vesicles, sufficient gluconic acid was added to reduce solution pH to 6.0 or 5.0 as indicated. Within 2 s, 200 mosM *N*-methylglucamine chloride, *N*-methylglucamine gluconate or *N*-methylglucamine sulfate was added. Rate of collapse of the pH gradient was determined and converted to H^+ flux as described in Methods. Identical results were obtained when vesicles were loaded with 6-carboxyfluorescein diacetate for 30 min instead of 6-CF overnight. (N = 4 for all points.)

		H^+/OH^- flux (nmol H^+/s per mg protein)	
	pH(in)/pH(out):	7.0/6.0	7.0/5.0
−Val	Cl^-	7.8 ± 0.3	20.9 ± 0.7
	gluconate	4.5 ± 0.1 *	8.7 ± 0.3 **
	SO_4^{2-}	–	8.4 ± 0.5
+Val	Cl^-	5.8 ± 0.1	18.8 ± 0.5
	gluconate	5.9 ± 0.2	18.8 ± 0.6
	SO_4^{2-}	–	18.4 ± 1.5

* $P < 0.001$.
** $P < 0.0001$.

TABLE II

EFFECT OF FORMATE ON RATE OF COLLAPSE OF H^+ GRADIENTS IN PRESENCE OF ANION GRADIENTS

In the − formate condition, brush-border vesicles (400 μg of protein, pH 7) were assayed as in Table I. In the + formate condition, vesicles (400 μg of protein) were incubated overnight with 6-carboxyfluorescein and 5 mM formic acid, washed three times and added to 2 ml of the standard buffer (pH 7) containing 0.5 mM formic acid. 2 s after addition of vesicles, sufficient gluconic acid was added to reduce solution pH to 6. Within 2 s, 200 mosM *N*-methylglucamine chloride, or *N*-methylglucamine gluconate. Rate of collapse of the pH gradient was determined and converted to H^+ flux as described in Methods. (N = 3 for all points.)

		H^+/OH^- flux (nmol H^+/s per mg protein)	
		− formate	+ formate
−Val	Cl^-	8.8 ± 0.5	14.5 ± 1.6
	gluconate	5.5 ± 0.2 *	7.5 ± 1.0 *
+Val	Cl^-	7.8 ± 0.9	15.0 ± 0.7
	gluconate	6.9 ± 1.2	9.6 ± 0.6

* $P < 0.001$.

formate and assayed at pH 6 in a solution containing 0.5 mM formate so that formic acid would be in equilibrium across the membrane and the results would not be confounded by spontaneous formic acid diffusion. As in Table I, the rate of collapse of this pH gradient was measured in the presence of gluconate or chloride both with and without a K^+ and valinomycin voltage clamp (Table II). In contrast to the results obtained with HCO_3^- or OH^- alone, Cl^- exhibited a significant effect on H^+/OH^- transport which was not eliminated with voltage clamping.

Ability of Cl^- to generate pH gradients in brush-border membrane vesicles

To examine the process of anion-dependent H^+/OH^- transport further, we studied the ability of inwardly directed anion gradients to form outwardly directed H^+ gradients. Addition of 100 mM *N*-methylglucamine chloride to vesicles equilibrated in 150 mM potassium gluconate (pH 7) decreased intravesicular pH with an H^+/OH^- flux of 1.0 ± 0.1 nmol H^+/s per mg of protein (Fig. 3). However, in the presence of 50 μg valinomycin/mg of protein this was reduced to 0.0 ± 0.1 nmol H^+/s per mg. *N*-Methylglucamine gluconate did not cause formation of a pH gradient in the presence or absence of valinomycin (data not shown). Thus, electrically coupled Cl^-/H^+ or OH^- transport is detectable in the absence of a preformed pH gradient, but electroneutral coupling cannot be demonstrated under these conditions.

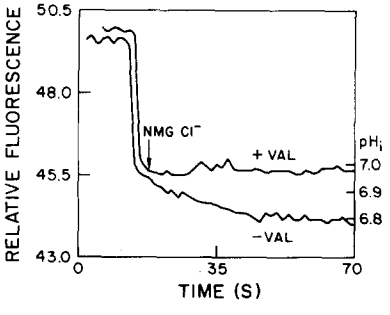

Fig. 3. Formation of pH gradients by Cl^- gradients in renal brush-border membrane vesicles. At the arrow, 200 mosM *N*-methylglucamine chloride (NMG Cl) was added to vesicles (400 μg of protein) in the standard buffer either with (+Val) or without (−Val) 50 μg of valinomycin per mg of protein). See text for rates of H^+ transport.

Effect of the method of preparation on Cl^-/OH^- exchange

Preparation method has been reported to alter ionic conductances and transport processes in renal brush-border membrane vesicles [24]. In addition, it is possible that an electroneutral Cl^--OH^- exchange system may be damaged during membrane preparation by proteases released from lysosomes of the proximal tubule. To rule out this possibility, Cl^--OH^- exchange was studied in vesicles prepared by a variety of techniques (Table III). Neither Ca^{2+} aggregation nor sucrose density gradient preparations altered the electrogenic coupling of Cl^- to H^+/OH^- transport observed with vesicles prepared by Mg^{2+} aggregation. Transport in vesicles prepared by Mg^{2+} aggregation in the presence of three different protease inhibitors also was indistinguishable from transport in control vesicles.

Na^+-H^+ exchange in brush-border vesicles

Electroneutral Na^+-H^+ exchange has been studied in brush-border vesicles by a number of workers [21,25,26]. In order to show that this process could be detected by the 6-carboxyfluorescein method, vesicles were loaded with dye and equilibrated at pH 6. After rapidly raising the extravesicular pH to 7.0, 100 mM sodium gluco-

TABLE III

EFFECT OF METHOD OF PREPARATION ON ANION-INDUCED COLLAPSE OF pH GRADIENTS

Vesicles were prepared by each method as described in Methods. In each case, 400 μg of vesicle protein (equilibrated at pH 7) was added to 2 ml of the standard (pH 7) assay buffer. Sufficient gluconic acid was added to drop the solution pH to 5 and 200 mosM *N*-methylglucamine gluconate or *N*-methylglucamine chloride was rapidly added. Electrically coupled Cl^--OH^- exchange is the difference between the initial rates of transport in the presence of Cl^- vs. gluconate. Electroneutral is the same value obtained in the presence of valinomycin ($N = 3$ for all points.)

Preparation method	Electrically coupled	Electroneutral
Mg^{2+} aggregation	12.2 ± 0.5	0.7 ± 0.6
Ca^{2+} aggregation	11.9 ± 0.7	1.2 ± 0.9
Sucrose gradient	12.2 ± 0.8	0.4 ± 1.0
Protease inhibitors	12.7 ± 0.6	0.3 ± 0.7

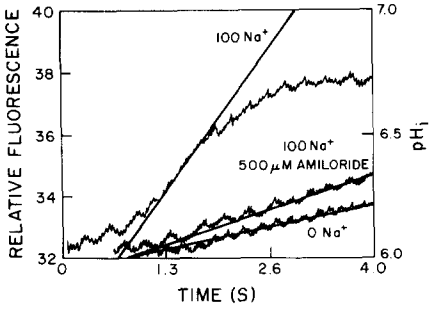

Fig. 4. Detection of Na^+-H^+ exchange by 6-carboxyfluorescein method. Vesicles were washed and suspended in standard assay buffer at pH 6. *N*-Methylglucamine was added to raise the solution pH to 7; 2 s later, 100 mM sodium gluconate (Na^+) was added either with (+amiloride) or without 500 μM amiloride.

nate was added (Fig. 4). The initial rate of collapse of the pH gradient was highly dependent on Na^+ concentration (data not shown) and was inhibited 95% by 500 μM amiloride. The results were unchanged by voltage clamping. Na^+-H^+ exchange could also be detected in the absence of a pH gradient. Vesicles were equilibrated at pH 6 and 100 mM sodium gluconate was added (Fig. 5). Na^+ caused the formation of a pH gradient at the rate of 0.8 ± 0.1 nmol H^+/s per mg of protein which was inhibited > 95% by 500 μM amiloride. Similar vesicles incubated with *N*-methylglucamine gluconate did not develop pH gradients (data not shown). Thus, both in the presence and absence of pre-formed pH gradients, electroneutral Na^+-H^+ exchange was readily detected by the 6-carboxyfluorescein method.

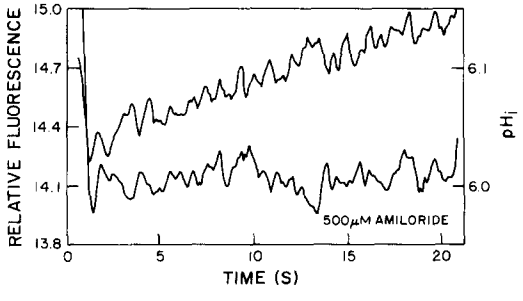

Fig. 5. Formation of pH gradients by Na^+-H^+ exchange in renal brush-border membrane vesicles. Vesicles (400 μg of protein) were assayed in the standard buffer at pH 6 with 50 μg valinomycin/mg of protein. 100 mM sodium gluconate was quickly added with or without 500 μM amiloride.

Cl^--base exchange in sealed red blood cell ghosts detected by 6-carboxyfluorescein method

To demonstrate that Cl-base exchange could be detected by the 6-carboxyfluorescein method in a system where it is known to exist, pH changes were measured in human red blood cell ghosts in response to Cl^- or citrate (Fig. 6). In voltage-clamped red cell ghosts, Cl^- caused collapse of a 2.0 unit pH gradient 7 times more rapidly than citrate. This effect was inhibited 90% by 100 μM DBDS. To show that 6-carboxyfluorescein itself does not inhibit the red cell anion exchanger, we studied the effect of 6-carboxyfluorescein on Cl^-/SO_4^{2-} countertransport in intact red cells. Cl^--SO_4^{2-} exchange is accompanied by net transport of osmoles to maintain electroneutrality. This results in changes in cell size detectable by light scattering. When 155 mM NaCl was added to 20 μl of packed red blood cells in a final volume of 2 ml (130 mM Na_2SO_4, 5 mM phosphate buffer (pH 7.4)), cells swell at a rate of 10 ± 1 arbitrary light scattering (600 nm) units/min. This was reduced to 3 ± 1 units/min by 50 μM DBDS. In the presence of 150 μM 6-carboxyfluorescein, basal swelling was 9 ± 1 units/min and 3 ± 1 units/min in the presence of 50 μM DBDS. Thus 6-carboxyfluorescein does not itself inhibit anion exchange.

Effect of stilbene derivatives on brush-border anion transport

Anion exchange systems are inhibited by the stilbene disulfonates and their derivatives [6–11].

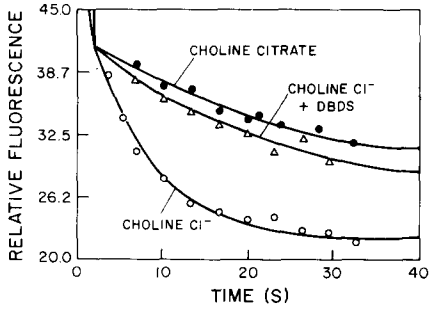

Fig. 6. Demonstration of Cl^--base exchange in human red blood cell ghosts. Effect of Cl^- or citrate on collapse of a 2 pH unit gradient was studied in voltage clamped (100 mM K^+, 50 μg of valinomycin/mg of protein) ghosts. Fitted time constants were 4.7 ± 0.8 s (Cl^-), 35.3 ± 4.3 s (Cl^- + DBDS), and 54.3 ± 4.5 s (citrate).

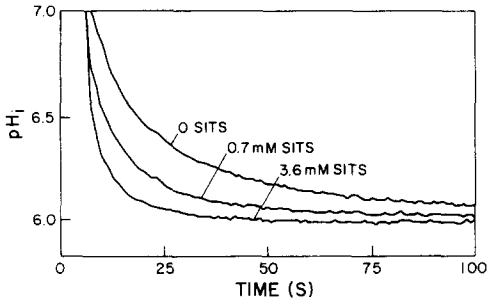

Fig. 7. Protonophoric effect of SITS. Vesicles were washed and incubated in standard pH 7 assay buffer. 2 unit pH gradient (pH_o) was imposed in presence of indicated concentrations of SITS. Fitted time constants were 13.1 ± 0.8 s (control), 9.3 s (0.7 mM SITS), and 4.7 s (3.6 mM SITS) in a representative sample of three identical experiments.

Although specific coupling between Cl^- and OH^- or HCO_3^- transport was not detected in the experiments described above, we examined the effects of stilbene disulfonates on anion-OH^- exchange to substantiate this finding. To test for the possibility of stilbene-inhibitable Cl^--OH^- exchange, the anion stimulated collapse of preformed H^+ gradients was studied in the presence of DIDS, DBDS and SITS. At concentrations of SITS which have been used by others (1–5 mM, Ref. 10), SITS was found to enhance the rate of collapse of the pH gradient (Fig. 7, Table IV). This was not due to interference of SITS with 6-carboxyfluorescein fluorescence, because preincubation with SITS for 10 min did not alter the baseline intravesicular fluorescence. Because SITS acts as a protonphore at these high concentrations, interpretation of results obtained with this compound becomes difficult. DIDS and DBDS (which both have much higher affinity for anion exchangers than SITS) did not spontaneously collapse the pH gradient at concentrations from 50 to 200 μM. Neither DIDS nor DBDS altered the rate of H^+/OH^- transport induced by Cl^- (Table IV), further supporting the conclusion that there is no stilbene-inhibitable anion-OH^- exchange system on the rabbit proximal tubule brush border.

TABLE IV

EFFECT OF ANION TRASPORT INHIBITORS ON CL^-/OH^- TRANSPORT IN RENAL BRUSH-BORDER VESICLES

Brush-border vesicles (400 μg of protein) were assayed for Cl^--OH^- exchange as described in the legend to Fig. 1. DIDS, DBDS, or SITS at the indicated concentrations were added to the assay buffer prior to addition of vesicles. ($N = 3$ for all points.)

Addition	Collapse of pH gradient
None	5.2 ± 0.6
DIDS (50 8gmM)	5.3 ± 0.8
DIDS (100 μM)	5.2 ± 0.4
DBDS (100 μM)	5.1 ± 0.5
SITS (500 μM)	6.8 ± 1.0
SITS (2 mM)	10.7 ± 0.9 *
SITS (5 mM)	15.4 ± 0.7 *

* $P < 0.001$.

Discussion

The molecular mechanisms responsible for Cl^- transport in the late renal proximal tubule have not been defined. It has been proposed that Cl^--OH^- exchange, stimulated by luminal acidity in the late proximal tubule, operates in parallel with the Na^+/H^+ exchanger to cause net transport of NaCl. Evidence both supporting and contradicting this idea has come from studies of pH gradient-stimulated uptake of Cl^- in brush-border vesicles prepared from the renal cortex [6,10–22].

Work in this laboratory [13,14,18] and by others [6,10,11,15,16] has established that (in the presence of typically used buffer concentrations) H^+ gradients decay more rapidly than Cl^- gradients in renal brush-border vesicles (10–20 s for H^+ gradients [11,13–16] vs. 1–2 min for Cl^- gradients [6,10]). In the setting of these rapidly changing pH gradients, studies of isotopic Cl^- uptake are subject to significant artifact. Since optical measurement of pH gradients can be made with better time resolution (< 1 s) than isotopic measurements, we chose to reexamine the issue of Cl^--OH^- exchange in renal proximal tubule brush-border vesicles by studying the effect of Cl^- on H^+/OH^- transport rather than the effect of pH on Cl^- transport.

The experiments presented in this paper provide evidence that, under the conditions of an adequate voltage clamp, Cl^- has no effect on HCO_3^- or OH^- transport over and above that seen with impermeant anions (gluconate or SO_4^{2-}, Fig. 2). In the absence of a voltage clamp, Cl^- gradients do cause significant H^+ transport which

can result in the formation of detectable pH gradients. Thus, it has been deduced [6,15] that renal brush-border vesicles must have a significant Cl^- conductance. The physiological relevance, if any, of this conductance has not been determined.

Because the 6-carboxyfluorescein method fails to show electroneutral Cl^-/HCO_3^- transport in renal brush-border vesicles, it was important to show that this method could detect such transport in a system where it is known to exist. Therefore, we examined Cl^--base exchange in human red blood cell ghosts using 6-carboxyfluorescein. Electroneutral exchange in red cells was 7-fold greater than electrically coupled exchange and was inhibited by low concentrations of stilbene disulfonates. Thus, the 6-carboxyfluorescein method can detect Cl^--HCO_3^- exchange when it is present.

The methods outlined in this paper can be used to detect electroneutral Na^+-H^+ exchange in brush-border membranes. Using a 1 pH unit, outwardly directed H^+ gradient, the H^+ flux induced by 100 mM Na^+ is approx. 100 nequiv. H^+/s per mg of protein. Given the errors inherent in the methods we use, electroneutral Cl^--OH^- or HCO_3^- exchange could be at most 1 nequiv. H^+/s per mg of protein under an equivalent driving force (2 pH unit inwardly directed gradient, $Cl^-_{out} = 100$ mM, $Cl^-_{in} = 0$). Therefore, electroneutral Cl^--OH^- exchange is at most 1% as active as Na^+-H^+ exchange when assayed under equivalent chemical driving forces. Thus, it seems unlikely that Na^+/H^+ and Cl^-/OH^- transporters could be acting in concert to produce net transport of NaCl in the proximal tubule. This is especially true in the early proximal tubule where the driving forces for Na^+-H^+ exchange are similar to those for Cl^--OH^- exchange. Of interest, Liu and Cogan [27] have recently demonstrated that Cl^- transport is quite brisk (200 pmol/mm per min) in the early proximal tubule and relatively constant along the length of the tubule. Thus, the early proximal tubule appears to be the site of significant Cl^- transport.

Two previous isotopic studies suggest that electroneutral Cl^--OH^- exchange is present in rabbit renal brush-border membrane vesicles [6,10]. In both of these studies, Cl^- uptake was stimulated by inwardly directed pH gradients in the presence of a voltage clamp. In both of these studies the enhanced rate of Cl^- uptake persisted for at least 2 min. We demonstrate that, under the condition of an adequate voltage clamp and 50 mM exogenous buffer (Mes-Tris), pH gradients in renal brush-border vesicles collapse > 90% in < 30 s. This raises the possibility that voltage clamping was not complete in the isotopic studies. Indeed, critical experiments in both papers were done with $K^+_{in} = K^+_{out} = 4$ mM. We have previously shown [18] that 100 mM K^+ is necessary to fully voltage clamp brush-border membrane vesicles in the presence of a 2 unit pH gradient. Shiuan and Weinstein [10] showed that pH gradient-stimulated Cl^- uptake is inhibited by 5 mM SITS. However, the initial rate of Cl^- uptake was only slightly inhibited by SITS. The major effect of SITS was to decrease the maximal level of intravesicular Cl^- achieved. This observation is consistent with the idea that SITS behaves as a protonophore at these concentrations (see Fig. 7). Thus, the ability of 5 mM SITS to inhibit Cl^--OH^- exchange derives simply from its ability to collapse the pH gradient.

A third study of isotopic Cl^- uptake in the presence of a pH gradient [11] failed to show electroneutral Cl^--OH^- exchange, in agreement with our findings. However, this study examined isotopic Cl^- transport from 30 s to 5 min, neglecting the possibility that pH gradients might cause enhanced Cl^- transport at much earlier time points, prior to collapse of the pH gradient.

By examining the formation and collapse of pH gradients in the presence of Cl^- and other anions, we have been able to establish an upper limit for Cl^--OH^- exchange activity in comparison to Na^+-H^+ exchange activity in renal brush-border membrane vesicles. The results indicate that electroneutral Cl^--OH^- exchange probably does not play a significant role in the absorption of Cl^- by the proximal tubule epithelial cell. In addition, by showing that formate enhances the Cl^--stimulated collapse of pH gradients in renal brush border membrane vesicles, we have added support to the notion that brush-border membranes exhibit Cl^--formate exchange with formic acid recycling [23]. While the quantitative contribution of this system to proximal tubule Cl^- transport is not yet known, it is possible that Cl^--formate exchange, and not

Cl^--OH^- or Cl^--HCO_3^- exchange, is responsible for Cl^- transport in the renal proximal tubule.

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