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ION PATHWAYS IN RENAL BRUSH BORDER MEMBRANES

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The absorbance change of the weak base dye probe, Acridine orange, was used to monitor alterations of pH gradients across renal brush border membrane vesicles. The presence of Na^+/H^+ or Li^+/H^+ exchange was demonstrated by diluting Na_2SO_4 or Li_2SO_4 loaded vesicles into Na^+- or Li^+-free solutions, which caused dye uptake. About 20% of the uptake was abolished by lipid permeable cations such as valinomycin-K^+ or tetraphenylphosphonium, indicating perhaps the presence of a finite Na^+ conductance smaller than electroneutral Na^+/H^+ exchange. The protonophore tetrachlorosalicylanilide raised the rate of dye uptake under these conditions, hence the presence of an Na^+ conductance greater than the H^+ conductance was suggested. K^+ gradients also induced changes of pH, at about 10% of the Na^+ or Li^+ rate. Partial inhibition (21%) was seen with 0.1 mM amiloride indicating that K^+ was a low affinity substrate for the Na^+/H^+ exchange. Acceleration both by tetrachlorosalicylanilide (2-fold) and valinomycin (4-fold) suggested the presence of 2 classes of vesicles, those with high and those with low K^+ conductance. The larger magnitude of the valinomycin dependent signal suggested that 75% of the vesicles had a low K^+ conductance. Inward Cl^- gradients also induced acidification, partially inhibited by the presence of tetraphenylphosphonium, and accelerated by tetrachlorosalicylanilide. Thus both a Cl^- conductance greater than the H^+ conductance and a Cl^-/OH^- exchange were present. The rate of Na^+/H^+ exchange was amiloride sensitive with a pH optimum of 6.5 and an apparent K_m for Na^+ or Li^+ of about 10 mM and an E_A of 14.3 kcal per mol. A 61-fold Na_2SO_4 gradient resulted in a pH gradient of 1.64 units which increased to 1.8 with gramicidin. An equivalent NaCl gradient gave a much lower ΔpH even in the presence of gramicidin showing that the H^+ and Cl^- pathways could alter the effects of the Na^+/H^+ exchange.

Introduction

Various proton pathways across biomembranes are known to exist. Primary active H^+ pumps include the ATPases of mitochondria [1], chloroplasts [2], *Neurospora* [3] and gastric mucosa [4]. Bacteriorhodopsin is a light driven H^+ pump [5]. An alternative form of proton transport is provided by chemically coupled porters such as the Na^+/H^+ antiporter of certain bacteria [7]. Proton conductance is also present in the F_0 fragment of mitochondrial complex V allowing electrical coupling between H^+ gradients and fluxes of other conducting species [8]. H^+ permeability of the phospholipid component of bilayers is relatively low, the observed rates being largely due to flux of neutral species such as HCl [9].

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Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DCCD, *N*,*N'*-dicyclohexylcarbodiimide; TCS, tetrachlorosalicylanilide.

Of major significance in eukaryotic cell plasma membranes is a coupled Na^+/H^+ exchange process. This has been studied extensively in renal and intestinal brush border vesicles. For example, medium acidification resulting from inward Na^+ gradients has been demonstrated using pH electrodes [10], and Na^+ gradient dependent alkalinization of the vesicle interior was demonstrated by trapping of the weak acid dimethyloxazolidine [11]. Earlier data suggested that perhaps this was the sole pathway available for H^+ in brush borders, but recently, intestinal brush borders have been shown to possess a Cl^-/OH^- exchange activity [12]. A combination of Na^+/H^+ and Cl^-/OH^- exchange would result in indirectly coupled NaCl cotransport and such a system has been postulated to be involved in volume regulation in red cells [13]. Na^+/Li^+ countertransport has been studied in red cells [14] and the ability of the Na^+/H^+ antiporter of brush border membrane vesicles to utilize Li^+ suggests that the cation exchange found in red cells may be due to the Na^+/H^+ antiporter.

For further studies of the Na^+/H^+ exchange, the use of a dye probe sensitive to pH gradients such as Acridine orange has many advantages including rapidity of response, sensitivity, and use of small quantities of material [15]. In this study the H^+ gradient dependent accumulation of Acridine orange by renal brush border membranes has been used to characterise further some of the ion pathways across renal brush border membranes, as well as additional properties of the Na^+/H^+ antiporter. These data have been presented in preliminary form [16] and are similar in many respects to those of Reenstra et al. [17] using the same dye probe.

Materials and Methods

Preparation

Rabbit renal brush border membrane vesicles were prepared by a modification of the Ca^{2+}-precipitation method of Crane et al. [18]. Albino Norwood rabbits were killed by decapitation and the kidneys immediately excised. The cortices were dissected from the medulla and homogenized in 2 volumes (v/w), of ice cold 60 mM sucrose and 4 mM Tris-HCl, pH 7.4, for three strokes at 1000 rev./min in a teflon/glass homogenizer. The homogenate was then diluted 1:10 (v/v) in the same buffer. This diluted homogenate was further homogenized with a Brinkman Polytron at intermediate speed for three 20-s intervals with a minimum of 20 s cooling time between each. A 1 M $CaCl_2$ solution was then added to the homogenate, bringing the final $CaCl_2$ concentration to 10 mM. This mixture was stirred for 15 min at 4°C and then centrifuged at $1500 \times g$ for 20 min. The pellet was discarded and the supernate centrifuged 20 min at $27000 \times g$. This pellet, which contained the brush border membranes, was resuspended in 30 ml of 300 mM sucrose, 2 mM Pipes/Tris, pH 6.5, and centrifuged again for 20 min at $27000 \times g$. This step was repeated once. Protein concentration was determined using a Biorad protein assay kit and was adjusted to 10 mg/ml in the same buffer. Enrichment of the brush border markers was 9-fold [18].

Proton uptake experiments were performed in an Aminco DW-2 spectrophotometer in the dual beam mode, using Acridine orange [15]. The absorbance was monitored at 492 nm using 546 nm as the reference wavelength, and both rate and magnitude of the pH gradient could be monitored.

Calibration of the dye response

The principle of the calibration was to apply pH gradients of varying magnitude across the membrane and measure the dye response. This was done by modifying the preparative method so that the final two washes were carried out at pH 5.5. The vesicles were then incubated at 4°C overnight in a solution containing 95 mM K_2SO_4, 123 mM sucrose, 20 μM Acridine orange and 5.4 mM Mes/Tris at pH 5.5 at a protein concentration of 2 mg/ml. 45 μl of this suspension was then injected into 2.7 ml stirring dilution medium which contained 95 mM K_2SO_4, 110 mM sucrose, 20 μM dye, and 10 mM buffer at various pH values. At pH 6.3 and below the buffers were 10 mM Mes/Tris. Above pH 6.3 the buffers were 10 mM Pipes/Tris. The maximal change of absorbance difference between 492 nm and 546 nm was used. In these high ionic strength solutions, pH gradient independent binding of Acridine orange was minimal, and the use of polystyrene cuvettes prevented the binding of the dye to the surface of the

cuvette which in glass cuvettes produced a slow decline in the optical signal. For measurement of the pH gradient produced due to an outward Na^+ gradient, vesicles from the same preparation were loaded at pH 6.5 with 95 mM Na_2SO_4 instead of K_2SO_4, and diluted as above with a medium of identical pH. The change in absorbance thus provided a direct measure of the pH gradient.

Use of H^+ loaded vesicles

For some experiments, a pH gradient was preformed as above by loading vesicles at pH 5.5 and then diluting into a medium at pH 7.5. After the signal had stabilised, ions such as Na^+, Li^+, K^+ or Cl^- could be added to the external solution in the absence or presence of ionophores to determine properties of hydrogen and other ion flux pathways across the brush border membrane vesicles.

Sodium/proton exchange assay

Brush border membrane vesicles in 300 mM sucrose and 2 mM Pipes/Tris, pH 6.5, at a protein concentration of 10 mg/ml were diluted 5-fold into a preloading solution containing 119 mM Na_2SO_4, 78 mM sucrose, and 6.25 mM Pipes/Tris, pH 6.5. After an overnight incubation at 4°C, the vesicles were assumed to contain 95 mM Na_2SO_4, 5.4 mM Pipes/Tris, pH 6.5, and 123 mM sucrose.

A 15-μl aliquot of this solution (i.e. 30 μg of protein) was then pipetted into a cuvette containing 900 μl of 318 mM sucrose, 1 mM Pipes/Tris, pH 6.5, and 20 μM Acridine orange. The 61-fold Na_2SO_4 concentration gradient (in to out) thus imposed, provided the driving force for proton uptake into the vesicles. In some assays K_2SO_4 or tetramethylammonium sulfate was substituted for sucrose in the dye solution, but the final pH gradient was larger when sucrose alone was used although a binding artefact had to be subtracted.

In some experiments, as detailed in the figure legends, the pH, cation concentration or temperature of the assay was varied. Inhibitors or ionophores, when present were either preincubated with the vesicles, or added in the dilution medium. The variables of interest were the initial slope of the optical change and the change in $A_{492-546}$ between the origin of the curve and the steady-state value reached, corresponding to the rate of acidification and the size of pH gradients achieved, respectively.

The initital rate of vesicle acidification was obtained by drawing a tangent to the initial portion of the optical signal which was linear for about the first 5 s. When the vesicles were diluted into sucrose, the initial change of absorbance occurred in less than 1 second and was a vertical excursion on the recorder. In this type of experiment the initial rate was taken as the tangent to the curve following the initial rapid binding of dye. The recorder response time was 300 ms and mixing was complete within 1 s. Since the presence of an ionophore such as gramicidin or nigericin produced a faster initial rate of vesicle acidification than Na^+ gradients alone, dye response was not rate limiting in the experiments. Moreover, whether ionophores were added to the vesicles prior to dilution or were present only in the dilution medium made little difference to the effects discussed. Hence ionophore binding at the concentrations used was fast enough so as not to be rate limiting in terms of the responses observed.

Experiments were run using a pH electrode as previously described [4] under identical conditions with or without 20 μM Acridine orange to ensure that the presence of dye did not contribute to the proton permeability of the vesicles.

Chloride/hydroxyl exchange assay

Vesicles were pre-equilibrated at 4°C as above in a solution containing 95 mM tetramethylammonium sulfate, 191 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5, and 20 μM Acridine orange. 50 μl (100 μg protein) of these vesicles were then diluted into 900 μl of a solution containing 190 mM tetramethylammonium chloride, 58 mM sucrose, 10 mM Pipes/Tris, pH 6.5, and 20 μM Acridine orange. The optical response was recorded. As necessary ionophores were added to give a final concentration of 1 μM TCS or 1 μg/ml valinomycin.

Materials

All chemicals were reagent grade. Gramicidin was obtained from Calbiochem, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and DCCD from Aldrich, amiloride from Merck, Sharpe and Dohme, valinomycin from Sigma and tetrachlorosalicylanilide from Eastman Kodak. The plastic cuvettes were purchased from BioRad.

Results

There are three general areas covered by the data: the interaction of Acridine orange with renal brush border membranes; the use of pH gradients and ionophores to determine the nature of ion pathways in these particles; and finally the properties of the Na^+/H^+ antiporter itself.

1. Linear range of dye response

Since the response of Acridine orange to pH gradients is concentration dependent and changes in concentration determine absorbance or fluorescence changes, it is essential to work in a linear range of dye response to pH gradients. Moreover since the dye also binds to the membranes, protein concentration is also relevant. As shown in Fig. 1,

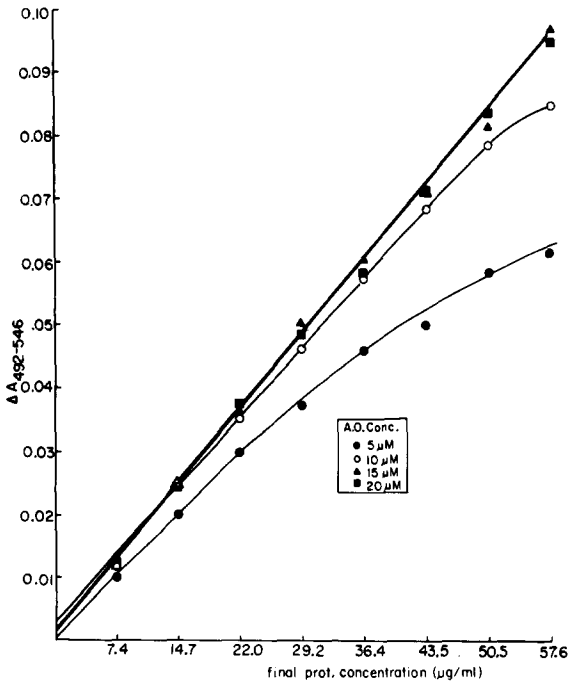

Fig. 1. Vesicles were preloaded with 95 mM K_2SO_4, 123 mM sucrose, and 5.4 mM Mes/Tris, pH 5.5 at a protein concentration of 2 mg/ml. The dilution medium consisted of 95 mM K_2SO_4, 110 mM sucrose, 10 mM Pipes/Tris, pH 7.5 and (●——●) 5 μM, (○——○) 10 μM, (▲——▲) 15 μM, or (■——■) 20 μM Acridine orange. Between 10 and 80 μl of vesicles were added to 2.7 ml dilution medium to vary the protein concentration between 7.4 and 57.6 μg/ml. The optical signal was recorded within 3 s of addition.

where a constant pH gradient of 2 units was applied across the membrane, both 5 and 10 μM Acridine orange gave a linear response up to about 22 μg protein/ml but the response fell off at higher protein concentrations. 15 or 20 μM dye gave a linear response up to about 60 μg protein/ml. We therefore chose 20 μM dye for all future experiments.

At this concentration of dye, with a pH gradient of 1.8 (the theoretical maximum for an Na^+ gradient of 61 and 1:1 Na^+/H^+ exchange) the internal dye concentration would reach 1.2 mM. If the protonated form were significantly permeable, the dye itself would shunt the pH gradient. In experiments not shown here, alkalinisation of the medium was measured using a pH electrode in the presence or absence of 20 μM Acridine orange. Neither the initial signal nor its return to baseline was affected by the presence of the dye. Hence the permeability of the protonated form of Acridine orange does not contribute to the conclusions derived below.

2. Calibration of the dye response

Using low ionic strength media for dilution of the vesicles results in a ΔpH-independent binding of Acridine orange that varies as a function of ionic strength. Hence to calibrate the dye response as a unique function of a pH gradient across the membrane vesicles, high ionic strength dilution media were used. As shown in Fig. 2A, dilution of K^+-loaded vesicles into solutions at identical K^+ concentrations at the same pH gave a change in absorbance of 0.0025 whereas a pH difference of 2.0 gave a change in absorbance of 0.048. Thus for small pH differences (i.e. less than 0.4 units) the method is not suitable as used here. However, as also shown in Fig. 2A, the signal due to a 61-fold dilution of Na^+-loaded vesicles into a K^+ medium is 0.035 units, comfortably within the range of the method. It may also be noted that the rapid reversal of the signal by gramicidin returns the baseline not to the original level, but to the level of the spurious response. The return of the signal due to the imposed pH gradient is faster in the presence of K_2SO_4 than with tetramethylammonium sulfate indicating interaction of K^+ with H^+ pathways which will be discussed below.

Calibration of the dye signal as a function of

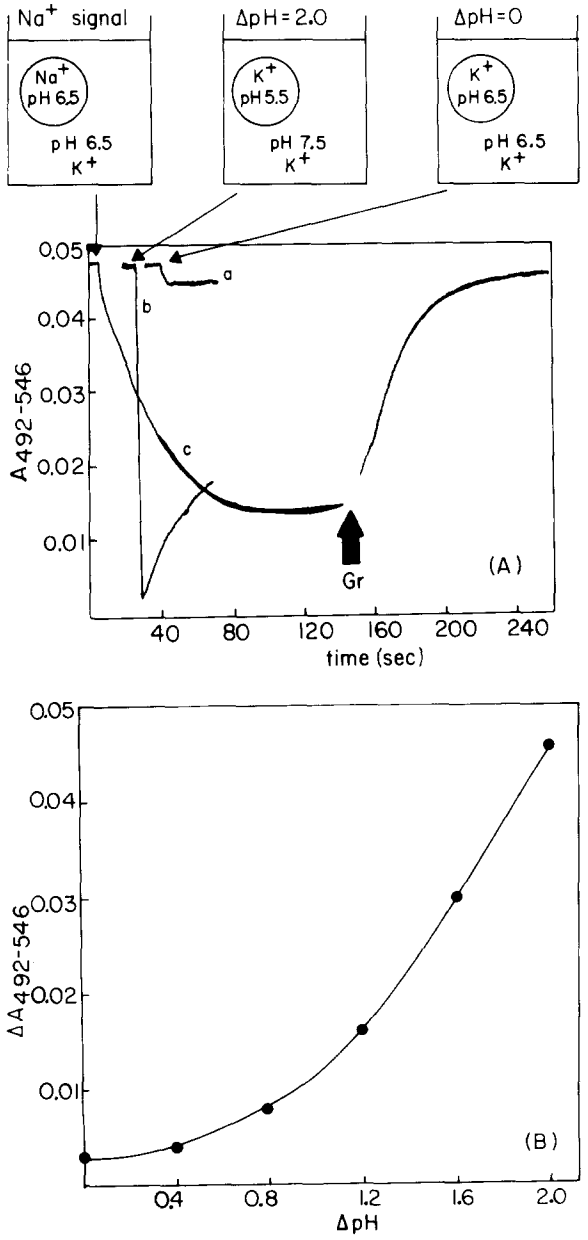

Fig. 2. (A) Vesicles were preloaded with 95 mM K_2SO_4 or 95 mM Na_2SO_4, 123 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5 or 5.4 mM Mes/Tris, pH 5.5, and 20 μM Acridine orange. The protein concentration was 2 mg/ml. 45 μl of these vesicles were diluted into 2.7 ml of stirred dye medium at pH values ranging from 5.5 to 7.5 as detailed in Materials and Methods. Gramicidin (Gr) was added to the solution where indicated by the arrow (↓). The final concentration of gramicidin was 1 μg/ml. (B) A plot of $\Delta A_{492-546}$ versus the ΔpH imposed by dilution into K^+ at varying pH of vesicles preloaded with K^+ at pH 5.5 was obtained under the conditions described in Fig. 2A and in Materials and Methods under Calibration. The maximum signal was used for the calibration.

the pH gradient showed reasonable linearity between a ΔpH 0.8 and 2.0. Hence it was possible to conclude that a 61-fold dilution of Na_2SO_4 loaded vesicles resulted in a pH gradient of 1.64 ± 0.015 ($n = 4$) units. An equivalent dilution of NaCl loaded vesicles gave a pH gradient of 1.2 units from the calibration curve. In the presence of gramicidin added to the vesicles before dilution, a pH gradient of close to 1.8 was reached when 95 mM tetramethylammonium sulfate was in the diluting solution.

In the absence of ionophore, dilution of Na_2SO_4 containing vesicles gave a response curve resolvable into two exponentials, one with a rate constant of 1.11 min^{-1} which accounted for 95% of the signal and a second slower rate constant of 0.139 min^{-1} which represented only 3% of the signal.

Accordingly uptake of dye can be equated with the development of pH gradients across renal brush border membrane vesicles, due to either ion gradients or imposed pH gradients. A signal unrelated to the pH gradient is present but can be dissociated from the gradient dependent component due to the very rapid time course of the latter and is much reduced in high ionic strength diluting medium. The vesicles also appear to consist largely of a single population in terms of Na^+ gradient dependent H^+ uptake.

3. Ion pathways in renal brush border vesicles

By changing the ion gradients imposed (i.e. Na^+, K^+, Cl^- or H^+) and measuring either the rate and magnitude of the pH gradient or its rate of dissipation either in the absence of cation specific ionophores such as TCS or valinomycin or in their presence, the relative permeabilities and conductances for Na^+, K^+, Cl^- or H^+ could be estimated. For example, if the protonophore, TCS, increased the rate of pH gradient formation due to an imposed Na^+, K^+ or Cl^- gradient, we conclude that a gradient of any of these ions produces a potential difference across the vesicle membrane and that the inherent proton conductance (at pH 6.5) is lower than that for these ions (at 190 mM).

The rate of pH gradient development due to dilution of Na_2SO_4 loaded vesicles was diminished by 20% in the presence of 2.5 mM tetraphenylphosphonium or 1 μg/ml valinomycin in the presence of 1 mM K_2SO_4. At least two interpretations

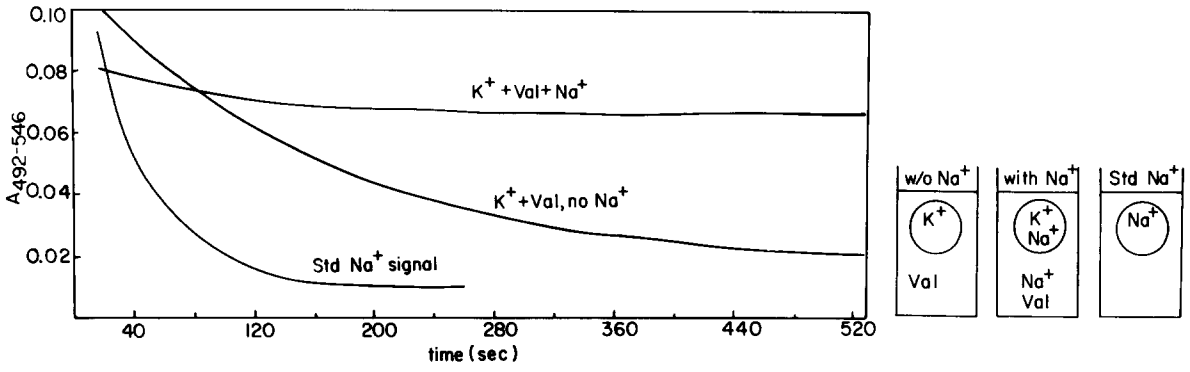

Fig. 3. H^+ conductance. The curve designated 'Std Na^+ signal' was as described in Materials and Methods under Na^+/H^+ exchange. In the curve designated '$K^+ + Val$, no Na^+', vesicles were preloaded with 86 mM K_2SO_4, 138 mM sucrose and 5.4 mM Pipes/Tris, pH 6.5. 15 μl (30 μg protein) of these were diluted into 900 μl of dilution medium containing 318 mM sucrose, 20 μM Acridine orange, 1 mM Pipes/Tris, pH 6.5, and 1 μg/ml valinomycin (Val). In the curve designated '$K^+ + Val + Na^+$' vesicles were preloaded with 32 mM Na_2SO_4, 86 mM K_2SO_4, and 5.4 mM Pipes/Tris, pH 6.5. 15 μl (30 μg protein) of these were diluted into a dilution medium containing 32 mM Na_2SO_4, 252 mM sucrose, 20 μM Acridine orange, 1 mM Pipes/Tris, pH 6.5, and 1 μg/ml valinomycin.

are possible for this inhibition of H^+ gradient development by the presence of lipid permeable cations. If the vesicles contain an H^+ conductance but no Na^+ or SO_4^{2-} conductance, the addition of a conductance pathway will allow an electrically coupled efflux of H^+ in exchange for the lipid permeable cation. Alternatively, if both an Na^+ conductance and an H^+ conductance are present, then a part of the proton gradient may be due to electrical coupling of the Na^+ diffusion potential to H^+ uptake. The effect of lipid permeable cations thus suggests the presence of a proton conductance or both a Na^+ and a H^+ conductance.

Fig. 3 demonstrates that a proton conductance is indeed present in these vesicles. Thus when the vesicles were loaded with K_2SO_4 and diluted, only a small H^+ uptake could be detected. However, if valinomycin was added, the same final pH gradient was achieved as for the Na^+ dilution experiment, but at about 30% of the rate. Moreover if Na^+ was present on both sides of the membrane, virtually no pH gradient was detected. This showed that the Na^+/H^+ exchange could shunt the electrically coupled H^+ gradient and hence both pathways were present in the same vesicle population.

If both Na^+ and H^+ conductances were present, then perhaps the H^+ conductance was limiting for Na^+ diffusion potential driven H^+ uptake. An increase of proton conductance induced by TCS increased the rate of H^+ gradient formation due to a Na^+ gradient as shown in Fig. 4. This was pronounced in the presence of amiloride. It may be noted that the effect of TCS is more pronounced at low temperatures (e.g. 12°C) showing a differential sensitivity of conductance and Na^+/H^+ exchange to temperature. Hence the

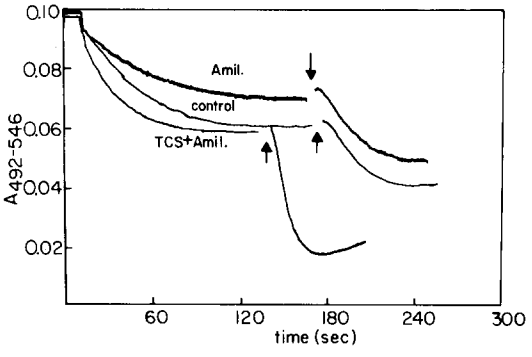

Fig. 4. Na^+ conductance. Vesicles were preloaded with 95 mM Na_2SO_4, 191 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5, and 20 μM Acridine orange. 45 μl of these were pipetted into 2.7 ml of stirring dilution medium containing 95 mM tetramethylammonium sulfate, 173 mM sucrose, 10 mM Pipes/Tris, pH 6.5 and 20 μM Acridine orange. As indicated, the dilution medium also contained 0.1 mM amiloride (Amil.), 0.1 mM amiloride and 1 μM tetrachlorosalicylanilide, or was without addition (control). At the arrows, 1 μg/ml gramicidin was added to the cuvette.

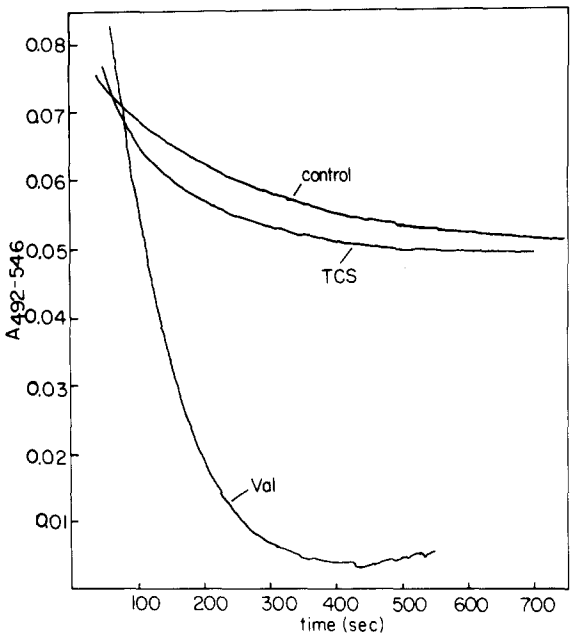

Fig. 5. Vesicles were preloaded with 95 mM K_2SO_4, 191 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5, and 20 μM Acridine orange. 50 μl of these (100 μg protein) were diluted into 900 μl of dilution medium containing 95 mM tetramethylammonium sulfate, 173 mM sucrose, 10 mM Pipes/Tris, pH 6.5, 20 μM Acridine orange, and as indicated 1 μg/ml valinomycin (Val), 1 μM tetrachlorosalicylanilide (TCS) or no ionophore (control).

Na^+ conductance which was present in the vesicle membrane was greater than the H^+ conductance.

For determination of K^+ or Cl^- related pathways higher protein concentrations were used as noted in the figure legends. Dilution of K_2SO_4-loaded vesicles into tetramethylammonium sulfate solutions resulted in the development of a ΔpH at 10% of the rate due to an Na^+ gradient (Fig. 5). This was inhibited by 21% by 0.1 mM amiloride. This can be taken as evidence that K^+ is able to substitute for Na^+ on the Na^+/H^+ antiporter, but with much lower efficacy. The rate of the K^+ gradient dependent signal was increased 2-fold by tetrachlorosalicylanilide but 4-fold by valinomycin. Since this provides evidence on one hand that H^+ conductance is rate limiting and on the other that K^+ conductance is rate limiting, it would appear that two vesicle populations are present, one (the larger, 75%) where H^+ conductance exceeds K^+ conductance, the other where H^+ conductance is less than K^+ conductance.

The presence of anionic pathways able to interact with H^+ (or OH^-) was shown in several ways. In Fig. 6, it can be seen that the pH gradient either due to Na^+ gradients, or to Na^+ gradients in the presence of gramicidin (or nigericin, data not shown) was significantly affected if Cl^- or SCN^- were substituted for SO_4^{2-}. Thus both the rate and magnitude of the ΔpH decreased in the order $SO_4^{2-} > Cl^- > SCN^-$. With ionophore present the magnitude of the ΔpH decreased in the same sequence. Hence either Cl^- or SCN^- was able to shunt the H^+ gradient due to Na^+/H^+ exchange via the antiporter, the rheogenic ionophore gramicidin or the electroneutral ionophore, nigericin. In data not shown, pH gradients due to

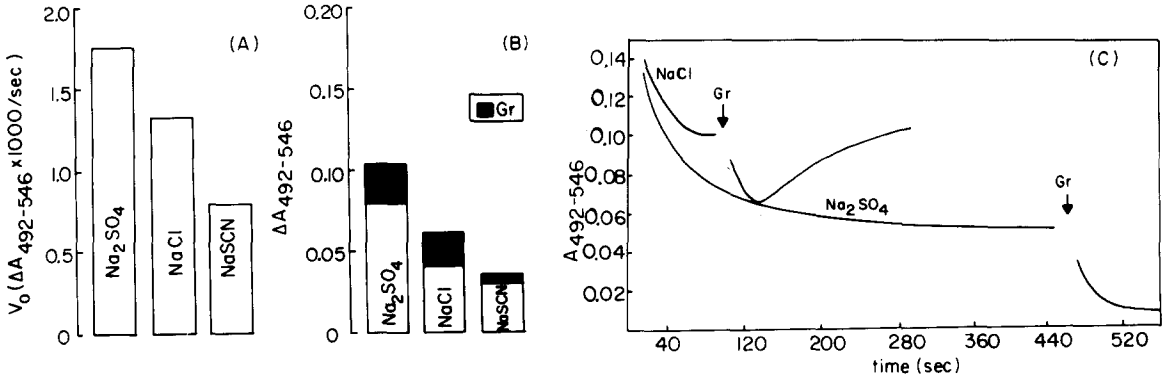

Fig. 6. Anion effects. Na^+/H^+ exchange signals were run as described in Materials and Methods except that, where indicated, 190 mM NaCl or 190 mM NaSCN were substituted for 95 mM Na_2SO_4. The solid portions of the bars represent the gramicidin (Gr) effect on the $\Delta A_{492-546}$.

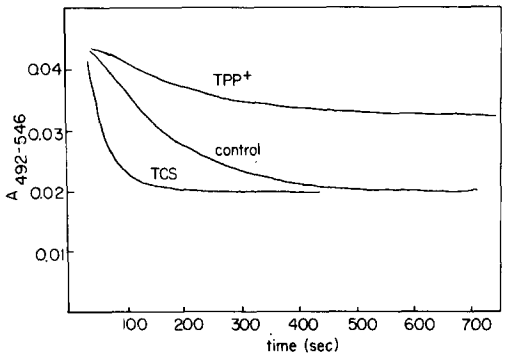

Fig. 7. Cl^-/OH^- exchange. Vesicles were preloaded with 95 mM tetramethylammonium sulfate, 191 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5 and 20 μM Acridine orange. 50 μl of these vesicles (100 μg protein) were diluted into 900 μl of 190 mM tetramethylammonium chloride, 10 mM Pipes/Tris, pH 6.5, 58 mM sucrose and 20 μM Acridine orange. The plastic cuvettes were mixed by inversion and the chart pen placed at the top of the page.

K^+ dilutions in the presence of ionophores were also reduced by Cl^-. Thus either a Cl^-/OH^- exchange or a Cl^- conductance was of sufficient magnitude to reduce even an ionophore mediated ΔpH. Loss of cation gradient via a directly coupled cation chloride cotransport system would demand non-selectivity between Na^+ and K^+, and hence a larger K^+ leak in the proximal tubule cell. This is unlikely to be the case physiologically.

When inward Cl^- gradients are imposed, acidification of the vesicle interior occurs as shown in Fig. 7. Partial inhibition of the acidification occurs in the presence of tetraphenylphosphonium and an increase occurs with the addition of TCS. Hence a Cl^- conductance is present, greater than the inherent H^+ conductance. The fraction of the signal insensitive to shunting by lipid permeable cations is then presumably due to Cl^-/OH^- exchange (or HCl cotransport). It should be noted however, that the sum of both of these is much less, by a factor of 0.05, than the Na^+ dependent pathways.

Hence the renal brush border contains Na^+/H^+ exchange, a Cl^-/OH^- exchange and Na^+, H^+, Cl^- and K^+ conductances. The conductance sequence in one group of vesicles is $Na^+ > Cl^- > H^+ > K^+$, and in another $Na^+ > Cl^- > K^+ > H^+$.

4. General properties of Na^+/H^+ antiporter

The cation selectivity of the exchange is shown in Fig. 8. It can be seen that in terms of magnitude of the pH gradient developed, the sequence is $Li > Na \gg K^+$, Rb^+, Cs^+. The lower gradient with the last three cations is not due to loss of cation gradient since with the addition of gramicidin, the same final pH gradient is reached even 6 min after dilution. The larger gradient in the presence of Li^+ may be due to partial loss of the Na^+ gradient as opposed to Li^+ due to the efflux of Na_2SO_4 by the Na^+-dependent SO_4^{2-}

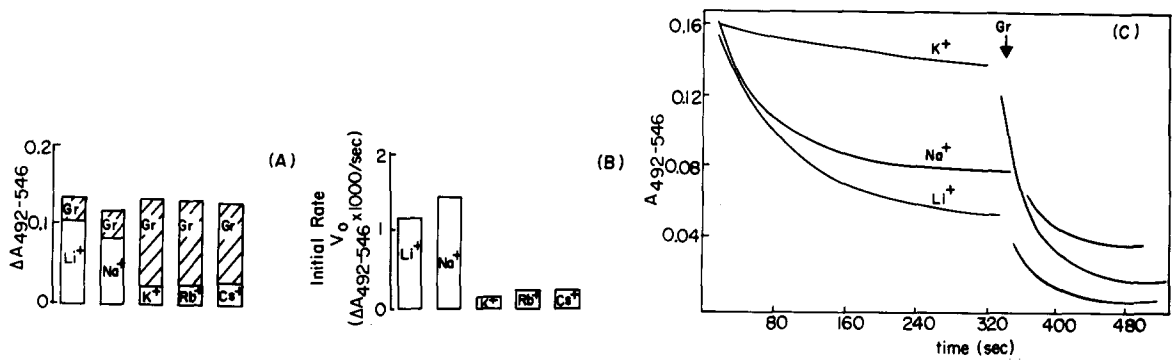

Fig. 8. Cation selectivity. Vesicles were preloaded as in the standard Na^+/H^+ exchange except that 95 mM Na_2SO_4 was replaced by 95 mM Li_2SO_4, K_2SO_4, Rb_2SO_4 or Cs_2SO_4 as indicated. In (A) the lower section of each bar indicates the $\Delta A_{492-546}$ obtained with the cation indicated. The upper section represents the addition $\Delta A_{492-546}$ which occurred when 1 μg/ml gramicidin (Gr) was added. In (B) the bars represent the initial rates obtained with the indicated cations. (C) Representative spectrophotometer tracings from vesicles preloaded with the indicated cations. Gramicidin was added at the time indicated by the arrow (↓) at a final concentration of 1 μg/ml.

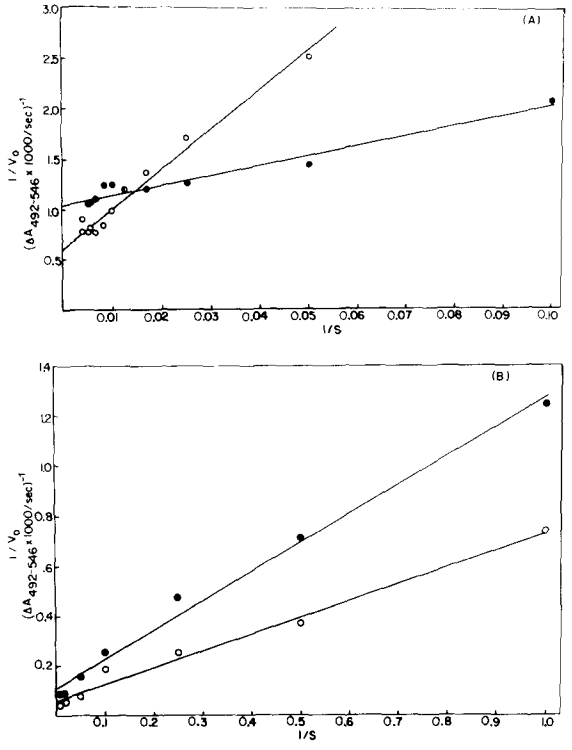

Fig. 9. Michaelis-Menten kinetics. (A) Vesicles were preloaded with Na_2SO_4 or Li_2SO_4 in concentrations ranging from 10 mM to 127 mM sulfate salt. Sucrose was added to maintain osmolarity. Otherwise, conditions were the same as described in Materials and Methods for Na^+/H^+ exchange. K_m values: Na^+, O——O, 80 mM; Li^+, ●——●, 7 mM. (B) The procedure was the same as for pH pulse calibration except that the dilution medium contained tetramethylammonium instead of K^+. The $\Delta A_{492-546}$ were reversed after the pH pulse by adding 200 μl of a solution containing 20 μM Acridine orange and Na^+ or Li^+ (as the sulfate salt) such that final concentrations in the cuvettes were 1, 2, 4, 10, 20, 60 or 130 mM cations. K_m values: Na^+, O——O, 11.5 mM; Li^+, ●——●, 10.5 mM.

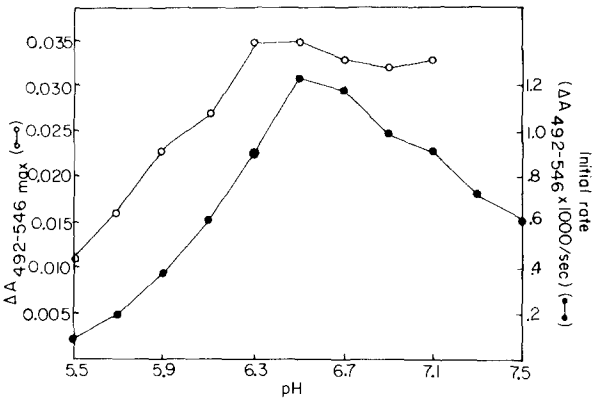

Fig. 10. pH optimum. Vesicles were preloaded with 40 mM Na_2SO_4, 230 mM sucrose and 5.4 mM buffer. At pH 6.3 and below, the buffer was Mes/Tris. Above pH 6.3 the buffer was Pipes/Tris. The dilution medium was 318 mM sucrose, 20 μM Acridine orange, and 1 mM buffer. The buffers were the same as in the preloading solution. 15 μl (30 μg protein) of vesicles were added to 900 μl of dilution medium to initiate proton uptake.

transport system [19]. This is also consistent with the lower pH gradient achieved by the addition of gramicidin in the Na^+ experiment as compared to the Li^+ experiment.

As shown in Fig. 9, the apparent affinity of the transport system as measured by the initial rate of gradient formation due to dilution or by the initial rate of dissipation of a preformed gradient, was different with Na^+ and Li^+. The latter cation had similar affinities, whether the cation was used to generate or to dissipate a gradient. In the case of Na^+, an apparent K_m of 11.5 mM was found for pH gradient reversal, but the surprising result was that a value of 80 mM was found for pH gradient formation. Since Li^+ did not exhibit this rectification, many trivial explanations are excluded. Presumably there are additional Na^+ selective sites on the vesicle interior which affect the apparent affinity of the antiporter for Na^+. Many of the brush border transport systems discriminate between Na^+ and Li^+, in contrast to the antiporter; hence the concept of sites which are Na^+ selective which interfere with the K_m estimates is not unreasonable. For either side however, the V for Na^+/H^+ exchange is greater than that of Li^+/H^+ exchange, as also seen in Fig. 8.

The Na^+/H^+ antiport could be a neutral site channel or carrier or a charge site system. As shown in Fig. 10, however, the exchange shows the presence of a pH optimum. From this, a simple conclusion is that a protonatable group with a pK_a of 6.5 is involved in the translocation of Na^+ and H^+ and hence a neutral site model is unlikely.

In Fig. 11, an Arrhenius plot of the rate of Na^+/H^+ exchange shows that the activation energy (E_A) is 14.3 kcal per mol. However, there is no evidence of any transition temperature, as might be expected of fixed site rather than mobile site behavior.

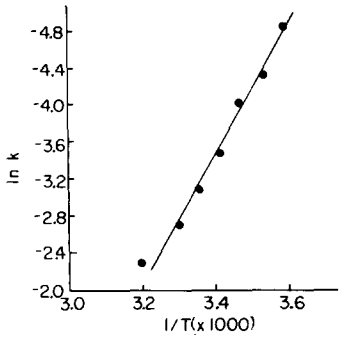

Fig. 11. Arrhenius plot of Na^+/H^+ exchange. The standard Na^+/H^+ exchange experiment was carried out at temperatures varying from 5°C to 40°C. Temperature was regulated using a circulating water bath.

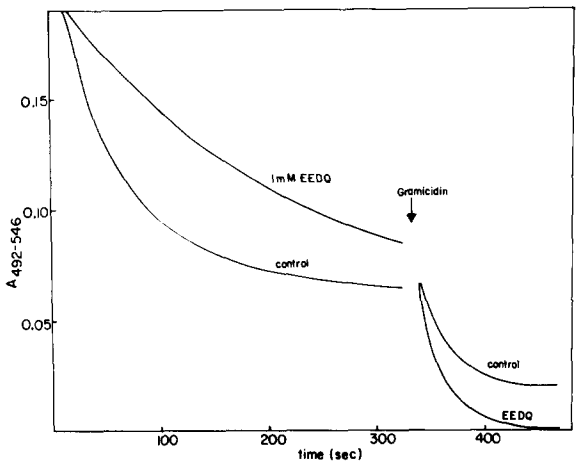

Fig. 12. Effect of EEDQ on Na^+/H^+ exchange. Vesicles preloaded as for the standard Na^+/H^+ exchange were incubated with 1 mM EEDQ for 1 h at room temperature. The methanol concentration in the incubation medium was 1%. Control vesicles were incubated 1 h at room temperature with 1% methanol alone. Under both control and experimental conditions, the dilution medium was the same as for the standard Na^+/H^+ exchange. 15 μl (30 μg protein) of vesicles were diluted into 900 μl dilution medium at pH 6.5. 1 μg/ml gramicidin was added where indicated by the arrow (↓).

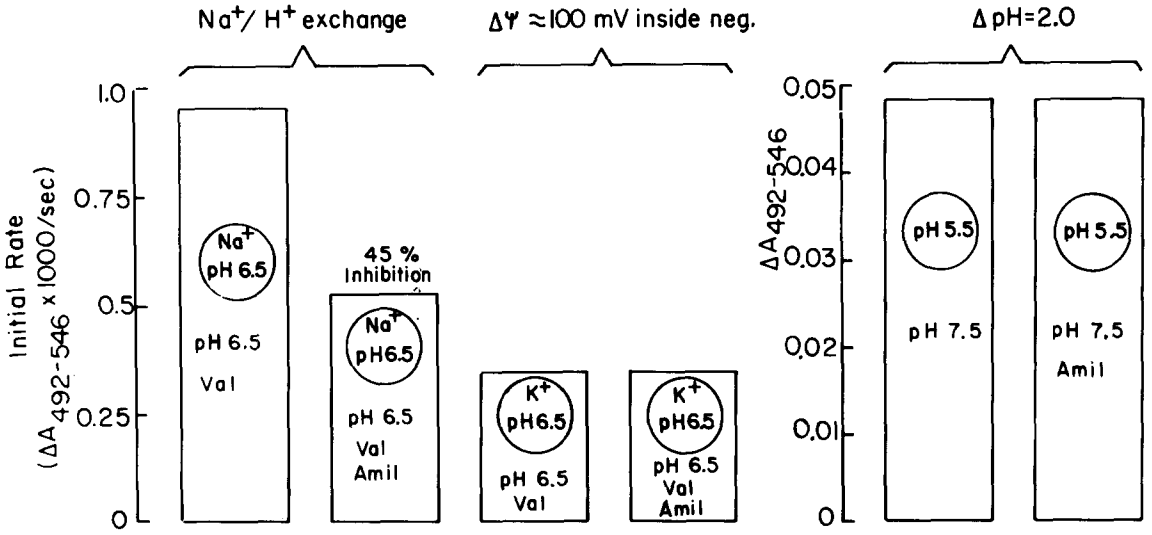

Fig. 13. Amiloride inhibition. The bars represent, from left to right: (1) the initial rate of Na^+/H^+ exchange at pH 6.5 in the presence of 1 μg/ml valinomycin (Val); (2) the initial rate of Na^+/H^+ exchange at pH 6.5 in the presence of 1 μg/ml valinomycin and 0.1 mM amiloride (Amil); (3) the initial rate of voltage-dependent H^+ uptake into K^+-preloaded vesicles in the presence of 1 μg/ml valinomycin; (4) the initial rate of voltage-dependent H^+ uptake into K^+ preloaded vesicles in the presence of 1 μg/ml valinomycin and 0.1 mM amiloride; (5) the $\Delta A_{492-546}$ observed in a 2-unit pH pulse, and (6) the $\Delta A_{492-546}$ observed in a 2-unit pH pulse in the presence of 0.1 mM amiloride. In each case the dilution medium contained 95 mM tetramethylammonium sulfate, 123 mM sucrose, 10 mM Pipes/Tris buffer (pH as indicated), and 20 μM Acridine orange. Vesicles for Na^+/H^+ exchange were preloaded with 95 mM Na_2SO_4, 123 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5, and 20 μM Acridine orange. Vesicles for voltage dependent H^+ uptake were preloaded with 95 mM K_2SO_4, 123 mM sucrose, 5.4 mM Pipes/Tris, pH 6.5 and 20 μM Acridine orange. Vesicles for pH pulses were preloaded with 95 mM K_2SO_4, 123 mM sucrose, 5.4 mM Mes/Tris, pH 5.5, and 20 μM Acridine orange. In all cases, 45 μl (90 μg protein) of vesicles were diluted into 2.7 ml of dilution medium.

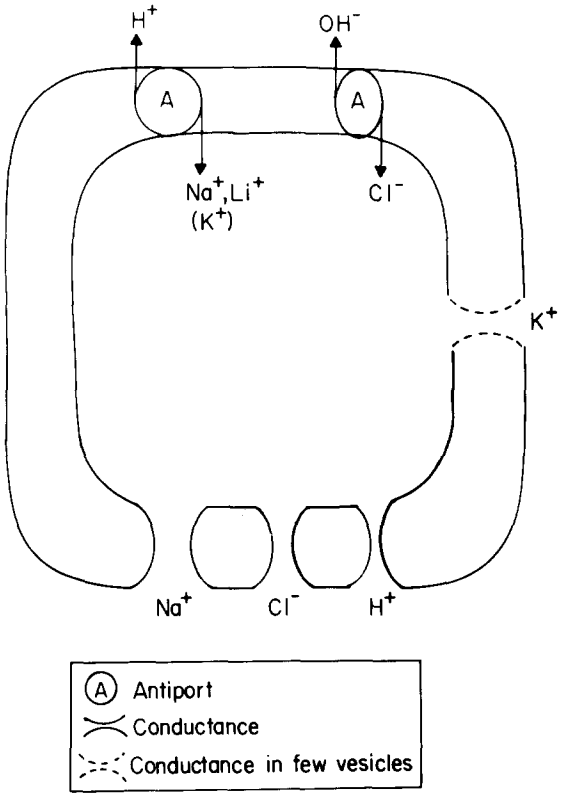

Fig. 14. Schematic diagram of ion pathways found in rabbit renal brush border membrane vesicles. The present study indicates the presence of two exchange proteins, a relatively more active Na^+/H^+ antiporter and a smaller Cl^-/OH^- antiporter. Conductive pathways for Na^+, Cl^- and H^+ are also observed. A small population of vesicles appears to leave a K^+ conductance.

The hydrophobic carboxyl reagent, DCCD did not alter the activity of the Na^+/H^+ antiporter. An alternative carboxyl reagent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) however did inhibit the initial rate of Na^+/H^+ exchange by 70% as shown in Fig. 12. It should be noted that this was not due to either H^+ or Na^+ leaks induced by the reagent since the gramicidin effect was retained. An EEDQ-activated carboxyl group can react with an amino, OH or SH group. Hydrophobic carboxyl groups may have a high pK_a, but usually react with DCCD. Hence the final site of attack of EEDQ may be on an amino group essential for Na^+/H^+ antiporter function.

It has been noted previously that amiloride inhibits Na^+/H^+ exchange [11]. Fig. 13 confirms and extends these findings. Thus amiloride, but not its analog, sulfaguanine, inhibits Na^+/H^+ exchange. However, 0.1 mM amiloride does not affect a preformed pH gradient nor a pH gradient due to the H^+ conductance elicited by a K^+-valinomycin induced diffusion potential; hence amiloride appears to be specific for the antiporter.

Discussion

Several studies have been carried out on Na^+/H^+ exchange in renal or intestinal brush borders as well as on other ion pathways using techniques other than dye probes, as discussed in the introduction. The data presented above are in agreement with many but not with all of the previous findings.

Using either pH electrodes or weak acid uptake, no evidence was found for the presence of a H^+ conductance [10,11]. Since the presence of Acridine orange itself did not result in any added conductance, it is not likely to be a dye induced artefact. What seems likely is that the dye technique which measures intravesicular pH is considerably more sensitive than the pH electrode method and perhaps either stirring or the pH electrode response was rate limiting in previous studies. In agreement with our data, a recent study on rat proximal tubule has suggested the presence of a H^+ conductance [20].

Another conclusion, that the major Cl^- pathways in these membrane vesicles are small, with both a Cl^- conductance and a Cl^-/OH^- exchange, is only in partial agreement with previous work using different techniques [21]. Although Cl^- gradients induced a ΔpH in our experiments, this was much smaller than that due to Na^+ gradients. Recent work in salamander using intracellular pH measurements is supportive of Na^+/H^+ exchange across both luminal and contraluminal membrane, but of Cl^-/HCO_3^- exchange only across the contraluminal membrane [22]. This is consistent with our data. The positive effect of protonophores on Cl^- induced gradients showed that $G_{Cl} > G_H$. Intestinal brush borders may have different pathways [12].

The measurable K^+ conductance is in agreement with results of others who have recently also used Acridine orange [17]. We have, however, noted

that K^+ permeability increased with aging of the vesicles and effects of ionophores suggest two groups of vesicles, those with high and those with low K^+ conductance.

With the techniques used, we have evidence for the presence of an Na^+ conductance, since the effects of lipid permeable cations and protonophores on the proton gradient induced by Na^+ efflux can be explained by proton and Na^+ conductances with $G_{Na^+} > G_{H^+}$. Evidence has also been obtained for Na^+ conductance using carbocyanine dyes [23].

H^+ and Cl^- conductances present in the brush border at -40 mV potential would tend to reduce the pH gradient due to Na^+/H^+ exchange. However, the loss of gradient forming ability with NaCl gradients is apparently not due entirely to the combination of H^+ and Cl^- conductances since the magnitudes of these are relatively small compared to the Na^+ pathways found.

The physiological role of the Na^+/H^+ antiporter has been considered to be alteration of pH gradients in the proximal tubule. Evidently with a transmembrane potential oriented so that cell interior is negative, and Cl^- at electrochemical equilibrium, the proton conductance would reduce the gradient developed by the antiporter. It would appear to be of insufficient magnitude to abolish the pH gradient. Hence Na^+ entry is in part coupled to H^+ flux and in part uncoupled by the H^+ and Cl^- conductances. Na^+ flux through the Na^+/H^+ antiporter and Cl^- flux through the Cl^-/OH^- antiporter has been suggested to be a major pathway for NaCl entry. The low activity of the latter exchange argues against this idea in renal membranes. Nevertheless a rapid NaCl pathway may exist, allowing then for two ion-coupled pathways across renal brush border membranes, Na^+/H exchange and Na^+/Cl^- cotransport. In addition there are the Na^+-coupled solute porters which are often rheogenic. When flux through this class of pathways is rheogenic, it can be directly coupled in the same membrane to H^+ or Cl^- flux through their respective conductances.

The lack of selectivity between Na^+ or Li^+ of this channel structure is of interest. It can be safely assumed that Na^+/H^+ exchange is one of the more important pathways for cell pH regulation and is widely distributed. The regulation of its activity is unexplored. Hyperactivity of Na^+/Li^+ exchange in red cells has been associated with hypertension [14]. If this exchange in fact reflects Na^+/H^+ exchange, then the increased Na^+ entry would, other transport systems being constant, increase cellular Na^+. In time this would reduce the effectiviness of Na^+/Ca^{2+} exchange with elevation of cytosolic Ca^{2+}. If this occurred in vascular smooth muscle, an increase in vascular tone might be anticipated. The antiporter may also be a significant route for Li^+ entry in the central nervous system and hence play a role in the effectiveness of Li^+ therapy in manic depressive illness.

Acknowledgements

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