Biochimica et Biophysica Acta, 1107 (1992) 150–158

BBAMEM 75649

Trans-potassium effects on the chloride/proton symporter activity of guinea-pig ileal brush-border membrane vesicles

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(Received 5 December 1991)

Key words: Brush-border membrane; Chloride/proton symport; Potassium ion/proton antiport; pH regulation; Proton flux; Pyranine; (Guinea-pig ileum)

To investigate the inhibitory effect of *trans* potassium on the Cl^-/H^+ symporter activity of brush-border membrane vesicles from guinea pig ileum, we measured both ^{36}Cl uptake and, by the pyranine fluorescence method, proton fluxes, in the presence of appropriate H^+ and K^+ gradients. In the absence of valinomycin, a time-dependent inhibitory effect of chloride uptake by *trans* K^+ was demonstrated. This inhibition was independent of the presence or absence of any K^+ gradient. Electrical effects cannot be invoked to explain these inhibitions because the intrinsic permeability of these vesicles to Cl^- and K^+ is negligibly small. Rather, our results show that, in the absence of valinomycin, the inhibitory effect of intravesicular K^+ involves an acceleration of the rate of dissipation of the proton gradient through an electroneutral exchange of *trans* K^+ for *cis* H^+, catalyzed by the K^+/H^+ antiporter also present in these membranes. Valinomycin can further accelerate the rate of pH gradient dissipation by facilitating an electrically-coupled exchange between K^+ and H^+. To evaluate the apparent rate of pH-dissipating, downhill proton influx, we measured chloride uptake by vesicles preincubated in the presence of alkaline-inside pH gradients ($pH_{out}/pH_{in} = 5.0/7.5$), charged or not with K^+. In the absence of intravesicular K^+, proton influx exhibited monoexponential kinetics with a time constant $k = 11\ s^{-1}$. Presence of 100 mM K^+ within the vesicles significantly increased the rate of pH gradient dissipation which, furthermore, became bi-exponential and revealed the appearance of an additional, faster proton influx component with $k = 71\ s^{-1}$. This new component we interpret as representing the sum of the electroneutral and the electrically-coupled exchange of *trans* K^+ for *cis* H^+, mentioned above. Finally, by using the pH-sensitive fluorophore, pyranine, we demonstrate that, independent of the absence or presence of a pH gradient, either vesicle acidification or alkalinisation can be generated by adding, respectively, Cl^- or K^+ to the extravesicular medium. Such results confirm the independent existence of both Cl^-/H^+ symporter and K^+/H^+ antiporter activities in our vesicle preparations, the relative activity of the former being larger under the conditions of the present experiments. The possible interplay of these two proton-transfer mechanisms in the regulation of the intracellular pH is discussed.

Introduction

We have shown recently that an alkaline-inside pH gradient can furnish the energy necessary for the uphill transport of chloride ions across brush-border membrane vesicles from guinea pig and rabbit ileum [1,2]. In addition to this pH gradient-dependent chloride transport which involves mainly an electroneutral Cl^-/H^+ symporter (or its physicochemical equivalent, a Cl^-/OH^- antiporter), these vesicles exhibit a small, rheogenic Cl^- uniport component, but neither Cl^-/Na^+, Cl^-/K^+ or $2Cl^-/Na^+/K^+$ symporter nor obligatory Cl^-/Cl^- or Cl^-/HCO_3^- antiporter activities.

Our observation that moderately-high intravesicular K^+ and/or Na^+ concentrations cause a time-dependent inhibition on pH gradient-dependent Cl^- uptake suggested that both *trans* K^+ and Na^+ increase the rate of pH-gradient dissipation [1]. Under short-circuiting conditions (imposed with $[K^+]_{out}/[K^+]_{in} = 100/100$ mM in the presence of valinomycin), the initial rate of pH gradient-dependent Cl^- uptake is inconsistently affected whereas the total uptakes measured at longer time intervals (10 to 60 s) are strongly diminished [1].

Because the inhibitory effects of intravesicular K^+ become consistent only at 10 s or more, the suggestion

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonate; TMA, tetramethylammonium hydroxyde pentahydrate; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid (trisodium salt); EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid. The suffixes 'out' and 'in' indicate, respectively, the extra- and the intravesicular media.

seems warranted that K^+ efflux accelerates H^+ influx, thereby indirectly causing a time-dependent decrease in the driving force for Cl^-/H^+ cotransport. Independently, Steffens et al. [3] and Kleinman et al. [4] have also concluded that voltage clamping accelerates pH-gradient dissipation in isolated brush-border vesicles. Taken as a whole, these observations serve to call attention to the difficulties inherent to using voltage clamping as a strategy to establish whether a given transport process is either electrogenic (rheogenic) or electroneutral.

By using brush-border membrane vesicles from guinea pig ileum, in the present work we address the question whether or not, in the presence of an alkaline-inside pH gradient, the inhibition of Cl^- uptake by *trans* K^+ results from an accelerated dissipation of the pH gradient necessary for chloride uphill transport. In order to do this, we investigated the effect of *trans* K^+ on pH gradient-dependent Cl^- uptake as a function of both the absolute potassium concentration and the potassium Nernst potential, E_K. In parallel, we measured proton fluxes across ileal brush border membrane vesicles by monitoring changes in the fluorescence intensity of the pH-sensitive dye, pyranine, previously trapped within the vesicles. Finally, we have examined the chloride-driven transport of protons under different conditions of transmembrane K^+ gradient.

A preliminary account of this work has been given [5].

Materials and Methods

Materials. $H^{36}Cl$ (0.4 mCi/mmol, Amersham) was neutralized with Tris base before use. Valinomycin and nigericin were from Sigma; tetramethylammonium hydroxide pentahydrate (TMA) from Aldrich; pyranine from Eastman-Kodak; Sephadex G-25, medium, from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were also of the highest purity available.

Buffers. In all the experiments, both the extra- and the intravesicular media contained the same metal-free buffer consisting of 20 mM Hepes and 40 mM of either citric acid or Mes, adjusted to the desired pH with Tris base. The extravesicular media were always pH-adjusted by taking into account the incvitable carry-over of a known fraction of intravesicular pH 7.5 medium at the beginning of the transport assay. When, within each experiment, the effect of certain ions was tested, the total ionic strength was maintained as nearly constant as possible by adding as needed the appropriate amount of 'inert' acid and/or base; respectively, gluconic acid and either TMA or Tris. Sorbitol was used to maintain the total osmolarity ratio, (out)/(in), at 650/550 mosmol/l.

Valinomycin and nigericin, all pre-dissolved in ethanol, were allowed to evaporate to dryness before mixing with the membrane preparation, at least 15 min prior to assaying for Cl^- transport [1]. Both ionophores were used at the same dose, 10 μg/mg of protein.

Membrane vesicle preparation. Brush-border membrane vesicles from guinea pig ileum were prepared and handled as previously described [1,6]. Loading of the vesicles with a given buffer was accomplished either by entrapment during vesicle formation or by membrane resealing, e.g., by applying two consecutive cycles of freezing and thawing [1]. Protein was measured by using the Bio-Rad assay kit.

Chloride transport measurement. Chloride uptake was assayed by using ^{36}Cl and a rapid filtration technique [7]. Initial uptake rate measurements (0.5 to 2 s) were carried out with a Short-Time Incubation apparatus (Innovativ Labor AG, Zürich, Switzerland), in a room thermostated at 23 ± 2°C, as previously described [1].

Results are expressed as either (i) absolute uptakes (units: nmol(mg protein)$^{-1}$, (ii) absolute velocities (nmol(mg protein)$^{-1}$s^{-1}) or (iii) relative velocities (nl(mg protein)$^{-1}$s^{-1}) [8]. Whenever possible, data are presented as the means (±S.D.) of several experiments performed with at least two different membrane preparations. Uptake data were statistically compared by applying a one-way analysis of variance [9]. Initial absolute velocities as a function of the chloride concentration were fitted by non-linear least-squares regression analysis to an equation containing one saturable Michaelian transport system plus a diffusional component, as described [1]. All calculations were done by using an Apple MacIntosh microcomputer.

Preparation of pyranine-loaded vesicles. Vesicles were loaded with pyranine as described by De Vrij et al. [10]. Briefly, 2 ml of membrane vesicles (about 40 mg total protein) were mixed with an aqueous 5 mM pyranine solution and distributed into two equal batches. One batch was subjected to a double cycle of freezing in liquid nitrogen, followed by slow thawing to room temperature. The second batch was not subjected to freezing but was maintained during the same time interval in the ice bath. In the first batch, assumed to have undergone vesicle opening and resealing (see Ref. 11), pyranine was expected to be present both inside and outside the vesicles ($MV_{Pyr(out+in)}$), whereas the second batch would contain only pyranine bound to extravesicular sites ($MV_{Pyr(out)}$). External free pyranine was removed from both vesicle batches by Sephadex G-25 filtration [11]. The filtered vesicles were then concentrated by centrifugation, suspended in the appropriate buffer and stored at 4°C until the time of experiment which took place the same day.

Proton flux evaluation. Vesicles (400–500 μg of protein) loaded with pyranine in the desired buffer were mixed with 2.5 ml medium in a continuously-stirred,

1-cm polystyrene cuvette maintained at 25 + 1°C. Fluorescence measurements (excitation at 469 nm; emission at 512 nm) were carried out in a Jobin-Yvon JY3C spectrofluorimeter connected to a Hewlett-Packard 9815 A recorder. The variation of fluorescence intensity with time was followed continuously from 3.6 to 300 s. At the end of each run, the vesicles were lysed with 25 μl of 3% Triton X-100, and the total fluorescence intensity determined.

The fluorescence intensity of vesicle-entrapped pyranine as a function of time, $F(t)$, was first normalized to account for variations in protein concentration, then standardized to the equilibrium value, F_e, determined after Triton X-100 lysis. The final pH of each sample was determined with a pH-meter and, because of the large excess of external over internal medium, assumed to correspond exactly to the initial pH of the external medium. Because F and pH are non-linearly related through sigmoid titration curves [12], F_t was converted into proton concentrations according to the relationship:

$$-\log[H^+]_t = (pK - \log[(F_{max} - F_t)/F_t]^a) \quad (1)$$

where $[H^+]$ is expressed in moles per mg protein. pK, F_{max} and a (the reciprocal of the Hill coefficient) were derived from fluorescence intensity-versus-pH plots by using either 1 μM pyranine or pyranine-loaded vesicles as reference. A pyranine pK value of 7.2 was calculated by linear regression.

Results and Discussion

^{36}Cl uptake experiments

As we shall see, the inhibitory effect of *trans* potassium on pH gradient-dependent chloride uptake is time-dependent. In the present paper, therefore, we shall distinguish between rapid effects taking place during the initial 1–2-s and long-term effects observable between 10 and 60 s.

Effect of intravesicular potassium on the initial chloride entry rates. For reasons that will become evident as we go along, and sharply contrasting with the effects at long time intervals, which are more consistent, the effects of *trans* potassium on the initial chloride entry rate are inconsistent, i.e., they can be seen with certain vesicle preparations, but not with all. To illustrate this point, we shall begin by presenting a series of positive results obtained with a given vesicle preparation (Table I). These results extend and are qualitatively similar, although quantitatively not identical with, those obtained before with a different vesicle preparation (Table 1 in Ref. 1). Negative results obtained with still other vesicle lots will not be illustrated.

As shown in Table I, the initial chloride entry rate under zero *trans* K^+ conditions and in the absence of any pH gradient ($pH_{out}/pH_{in} = 7.5/7.5$) increased by 3.6-times when a 2.5-unit, alkaline-inside pH gradient ($pH_{out}/pH_{in} = 5.0/7.5$) was imposed. Superposition of high, equilibrated potassium concentrations ($[K^+]_{out} = [K^+]_{in} = 200$ mM), with or without valinomycin and/or nigericin, had no effect when no pH gradient was present. However, when a pH gradient was present, a 27% inhibition of chloride uptake was found which increased to 44% when valinomycin was added and to 69% upon addition of nigericin (with and without valinomycin). With nigericin, the level of chloride uptake dropped to that observed in the absence of a pH gradient, consistent with the notion that, in the presence of *trans* K^+, irrespective of the presence or absence of valinomycin, nigericin can abolish any existing pH gradient, practically instantaneously [2,13].

TABLE I

Effects of a pH gradient and/or trans potassium on the absolute chloride uptakes at 2 and 60 s, in the absence and in the presence of ionophores

Cl^- uptake was measured with 14 mM ^{36}Cl as substrate. Both the extra- and the intra-vesicular spaces contained: (i) a 20 mM Hepes/40 mM citric acid buffer adjusted with Tris base to give the indicated pH gradients; and (ii) a 200 mM mixture of the gluconate salt of either Tris or potassium to obtain the indicated $[K^+]_{out}/[K^+]_{in}$ ratios. The absolute Cl^- uptakes at both 2 and 60 s are given in nmoles per mg protein ± S.D. (n). Identical low-case letters identify results that are indistinguishable according to a one-way analysis of variance, at the $P < 0.01$ level.

$[K^+]_{out}$ / $[K^+]_{in}$ ratios (mM)	Ionophore		Absolute Cl^- uptakes	
	valino-mycin	niger-icin	2 s	60 s
$pH_{out}/pH_{in} = 7.5/7.5$				
0/0	–	±	1.0 ± 0.1 (24) [a]	3.0 ± 0.2 (5) [c,h]
200/200	±	±		
$pH_{out}/pH_{in} = 5.0/7.5$				
0/0	–	–	3.6 ± 0.3 (5) [b]	16.7 ± 1.0 (5) [c]
200/200	–	–	2.6 ± 0.4 (14) [c]	9.4 ± 1.5 (14) [f]
200/200	+	–	2.0 ± 0.2 (6) [d]	4.5 ± 0.2 (5) [g]
200/200	–	+	1.1 ± 0.1 (3) [a]	3.8 ± 0.6 (3) [b,g,h]
200/200	+	+	1.1 ± 0.1 (3) [a]	4.0 ± 0.4 (3) [b,g]

In order to clarify the mechanism of *trans* potassium inhibition of chloride uptake, a kinetic analysis of Cl transport in the presence and absence of intravesicular K^+ was performed. We showed previously that the kinetics of chloride uptake across the brush-border membrane can be adequately described by an equation involving a single Michaelian transport term plus a diffusional component [1]. The results summarized in Table II reveal that preloading the vesicles with 100 mM K^+ leads to a significant, 17% decrease of V, with no effect on K_T. Addition of valinomycin caused an additional, 28% significant increase in this inhibitory V

TABLE II

Chloride transport in the presence of an alkaline-inside pH gradient: noncompetitive effect of trans potassium in the absence and in the presence of valinomycin

Chloride saturation curves were performed by using chloride concentrations ranging from 4 to 84 mM. Buffer composition as in Table I, with a pH gradient $pH_{out}/pH_{in} = 5.0/7.5$. The kinetic parameters, V (nmol(mg protein)$^{-1}$ s^{-1}) and K_T (mM) were estimated by nonlinear regression analysis. The kinetic diffusion constant, K_d, was fixed to the common value, 6.7 nl(mg protein)$^{-1}$ s^{-1}, before running each iteration. For the F test, the degrees of freedom [df] for pure error and for lack of fit, in that order, are indicated between brackets. P = n.s. means that the data points do not differ statistically from the theoretical fit of the equation under study. The three sets of results were statistically compared by using an F' test as described by Van Melle and Robinson [14,15]. Further details in the text.

Experiment:	A	B	C
$[K^+]_{out}/[K^+]_{in}$ ratios (mM):	0/0	100/100	100/100
Valinomycin:	−	−	+
V	4.8 ± 0.4	4.0 ± 0.3	2.9 ± 0.2
K_T	11.8 ± 1.9	13.1 ± 1.7	9.5 ± 1.6
F-tests			
F, [df], P	1.37 [29,4], n.s.	1.99 [29,5], n.s.	1.4 [33,4], n.s.
		F', [df]	
(A, B, C)		25.5 [104,4] $P < 0.005$	
(A, B)		15.6 [67,2] $P < 0.005$	
(B, C)		12.4 [71,2] $P < 0.005$	
(A, C)		44.1 [70,2] $P < 0.005$	

effect *. Such results indicate that *trans* K^+ behaves in practice as a non-competitive inhibitor of pH gradient-dependent Cl transport.

Taken together, the results just described suggest the existence of interactions between the fluxes of chloride, potassium and hydrogen ions across the brush-border membrane, thereby raising questions of physiological interest. In principle, these interactions could be explained in terms of either: (i) strictly indirect, electrical coupling between ion gradients (henceforth, we shall refer to this as the 'electrical hypothesis' or 'interpretation'); or (ii) functional interactions between carrier-mediated, electroneutral fluxes (the 'electroneutral hypothesis'). Two such mediated transport systems come to the fore in this regard, namely, the electroneutral Cl^-/H^+ symporter recently demonstrated in this laboratory [1,2] and the K^+/H^+ antiporter recently evidenced in apical intestinal vesicles by Binder and Murer [16]. It seems quite easy to conceive that, through the interplay of these two electroneutral mechanisms, *trans* K^+ may cause Cl^- uptake inhibition by accelerating the rate of dissipation of the proton gradient energizing chloride uphill transport. An analogous suggestion has recently been made by Knickelbein et al. [17] who showed that K^+ influx can accelerate H^+ efflux, thereby causing a decrease in the driving force for Na^+/H^+ antiport in rabbit ileum brush-border membrane vesicles.

The electrical hypothesis may seem the simplest and therefore the most attractive at first sight. However, the very low intrinsic permeability of the intestinal brush-border membrane to both Cl^- and K^+ (Refs. 1, 16 and 18–20 plus Alvarado and colleagues, unpublished results **) casts much doubt on its validity, which would render the electroneutral interpretation more credible. In the remainder of this paper, the two hypotheses will be experimentally compared.

Effect of intravesicular potassium on the 60-s chloride uptakes. With time, the inhibitory effects of *trans* K^+, observed with some but not all vesicle preparations at 2 s, become stronger and more consistent. Thus, with a given vesicle preparation, 200 mM *trans* K^+ inhibited 27% at 2 s but 43% at 60 s (Table I). With *trans* K^+ plus valinomycin, the inhibition was 44% at 2 s versus 73% at 60 s. Particularly worth noticing here is that the inhibition caused by valinomycin was statistically identical with that caused by nigericin at 60 s, whereas, at 2 s, valinomycin was significantly less efficient than nigericin. Such results warrant the interpretation that both ionophores accelerate the rate of dissipation of the pH gradient, but the effect of nigericin is much faster. The reason for this difference probably depends on the mechanism of action of each ionophore, which seems to be different. According to current knowledge, nigericin may be expected to act directly by catalyzing the electroneutral exchange of K^+ for H^+ [13]. To the contrary, as we will show further below in this paper, valinomycin appears to act indirectly by facilitating an electrically-coupled exchange of these two ions.

* The statistical analysis of the data in Table II has involved both the F and the F' tests of Van Melle and Robinson [14,15]. First, the F test shows for each curve that the data points do not deviate significantly from the equation used to fit them. Second, the F' test demonstrates that the three sets of curves are statistically different from one another, both if treated as a group or by pairs. Because the estimated K_T values overlap whereas the V results do not, the conclusion is warranted that V, not K_T, is the parameter affected by the various treatments employed.

** The low intrinsic permeability to K^+ and Cl^- of brush-border membrane vesicles from both pig and guinea pig jejunum and ileum has been demonstrated in experiments on rheogenic D-glucose/Na^+ cotransport. These largely unpublished results show that: (i) a potassium potential ranging from −60 to +60 mV has no effect whatsoever on the uptake of D-glucose, unless valinomycin is added; and (ii) the electrical effectiveness of various sodium salts to activate D-glucose uptake yields the anion permeability sequence: $SCN^- > NO_3^- > I^- > Br^-$ > gluconate = SO_4^{2-} = F^- = Cl^-, indicating that, similar to gluconate and sulfate, Cl^- is a poorly permeable anion, in these vesicles.

Effect of the absolute intravesicular potassium concentration under conditions of equilibrated K^+ on pH gradient-dependent chloride uptake. We have shown that *cis* K^+ has no effect on pH gradient-dependent chloride uptake [1]. The purpose of studying the effect of the absolute *trans* K^+ concentration under conditions of $[K^+]_{out} = [K^+]_{in}$ was to eliminate the potassium gradient as a factor in Cl uptake inhibition by *trans* K^+. The results (Fig. 1) show that increasing $[K^+]_{in}$ leads to a progressive decrease in the 60-s absolute uptakes, an effect that is potentiated by valinomycin. These effects appear to be linearly proportional up to $[K^+]_{in} = 75$ mM.

At first sight, a likely interpretation of these results is that the proton gradient gives an inside-positive membrane potential capable of energizing Cl uptake: *trans* K^+ would inhibit by short-circuiting this potential, an effect that would increase upon addition of valinomycin. Although this electrical interpretation is partially correct when valinomycin is present (see below), it should be rejected when in its absence, for two main reasons. First, we have shown that more than 85% of the total Cl uptake driven by a pH gradient is electroneutral, meaning that a proton potential cannot satisfactorily explain Cl uptake [1]. Second, this interpretation presupposes that the permeability of K^+ is high, which is not true. As mentioned above, all the evidence available to us demonstrates that, in the absence of valinomycin, P_K is negligibly small in intestinal brush-border membrane vesicles.

Consequently, as an alternative to the electrical explanation, we would propose here that, in the absence of valinomycin, the inhibitory effect of *trans* K^+ involves mainly a time-dependent dissipation of the pH

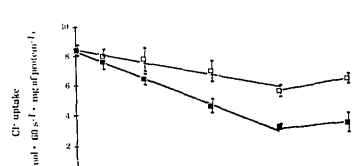

Fig. 1. Long-term effects of intravesicular potassium on the pH gradient-dependent uptake of chloride in the absence of a membrane potential. Chloride uptake was measured for 60 s with 4 mM ^{36}Cl as substrate, in the presence of a pH gradient $pH_{out}/pH_{in} = 5.0/7.5$. The buffer was as in Table I. A 200 mM mixture of the gluconate salt of either Tris or potassium was used to obtain the indicated K^+ concentrations under equilibrated ($[K^+]_{out} = [K^+]_{in}$) conditions, both in the absence (□) and in the presence (■) of valinomycin ($n = 3$–9 per point, except for the 100 mM $[K^+]$ condition where $n = 21$ and 20, respectively). The chloride absolute uptakes are expressed in nmol/mg protein (±S.D.) per 60 s.

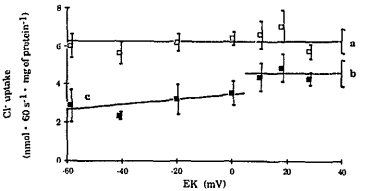

Fig. 2. Long-term effects of intravesicular potassium on the pH gradient-dependent uptake of chloride, as a function of the potassium diffusion potential. Chloride uptake was measured under the same conditions as in Fig. 1, except that $[K^+]_{in}$ was kept constant at 100 mM and $[K^+]_{out}$ was varied as needed to obtain the indicated E_K values, both in the absence (□) and in the presence (■) of valinomycin ($n \geq 9$ per point). The slope of lines a and b is indistinguishable from zero, but the average uptake of each line (respectively, 6.3 ± 0.6 ($n = 70$) and 4.6 ± 0.7 ($n = 17$)) is significantly different ($P \ll 0.005$). The slope of line c ($y = 3.5 + 0.014\ E_K$) differs significantly from zero ($F = 13$ [54,1]; $P \ll 0.005$). Further details in the text.

gradient through the intervention of an electroneutral exchange of K^+ for H^+. The electroneutral K^+/H^+ antiporter recently found to exist in intestinal apical membrane vesicles [16] would provide the physical basis for this exchange.

Role of the membrane potential at variable $[K^+]_{out}$ but constant $[K^+]_{in}$ on pH gradient-dependent chloride uptake. pH gradient-dependent Cl^- uptake was assayed in the presence of constant (100 mM) $[K^+]_{in}$ and variable $[K^+]_{out}$ to secure E_K values ranging from +30 to −60 mV. The results indicate that total Cl uptake is independent from the transmembrane K^+ gradient, further weakening the electrical hypothesis. In effect, if both P_K and P_{Cl} were high, net Cl uptake should be activated when $[K^+]_{in} < [K^+]_{out}$. Cl uptake, however, was perfectly horizontal (Fig. 2, line a).

Valinomycin significantly increased the inhibitory effect of *trans* K^+, a result also incompatible with the electrical hypothesis. In effect, when $[K^+]_{in} < [K^+]_{out}$, valinomycin can be expected to generate an inside-positive membrane potential that should have increased the Cl uptake rate if such an uptake involved a rheogenic Cl uniport. Instead, valinomycin increased the inhibition caused by *trans* K^+ (Fig. 2, line b). This result, furthermore, strongly supports the tenet that P_{Cl} is negligibly small in these vesicles [1]. We conclude that valinomycin increases the inhibitory effect of *trans* K^+ because, to the electroneutral K^+/H^+ exchange observed in the absence of the ionophore, it adds an electrically-coupled K^+/H^+ exchange, further accelerating H^+ influx.

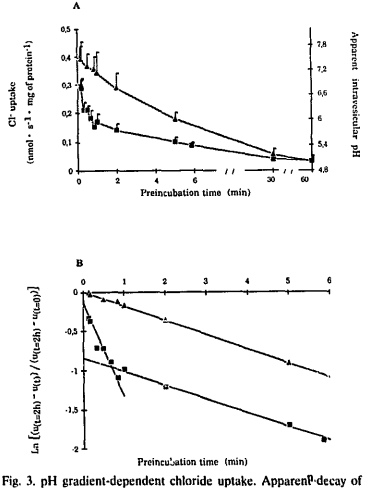

Fig. 3. pH gradient-dependent chloride uptake. Apparent decay of the pH gradient as a function of time. Vesicles in Hepes/citric acid/Tris buffer (pH 7.5) were charged with valinomycin and 100 mM of either Tris- or K gluconate. 10-μl aliquots were mixed with 35 μl of the Hepes/citrate buffer containing 100 mM K gluconate to obtain a pH gradient (pH_{out}/pH_{in} = 5.0/7.5) with ($[K^+]_{out}/[K^+]_{in}$ = 100/100 mM, ■); or without ($[K^+]_{out}/[K^+]_{in}$ = 100/0 mM, ▲) *trans* K^+. After each indicated preincubation time, 5 μl of the same buffer containing 100 mM K gluconate and ^{36}Cl were added to measure uptake for 2 s at 4 mM, zero *trans* chloride. In panel A, the results are illustrated as initial chloride entry rates in nmol/mg protein per s ± S.D. ($n \geq 9$ per point). The right-hand ordinate illustrates the conversion of the data into apparent intravesicular pH values, according to Eqn. 2. Panel B illustrates the fit of the data up to 6 min, according to Eqn. 3. The lower curve was decomposed as shown into an initial, fast component (0.1 to 0.8 min; $k_1 = 71.3\ s^{-1}$) and a second, slower component with a time constant $k_2 = 10.6\ s^{-1}$). Further details in the text.

Effect of intravesicular potassium on the time-dependent decay of the pH gradient. The electroneutral explanation of the mechanism of Cl uptake inhibition by *trans* K^+ implies that intravesicular K^+ accelerates the rate of pH gradient dissipation. The experiment in Fig. 3 was performed to test this point directly.

Valinomycin-loaded membrane vesicles were charged or not with potassium gluconate. Appropriate vesicle aliquots were then mixed with the same buffer containing K gluconate, in such a manner that preincubations could be performed at 23°C in presence of a pH gradient (pH_{out}/pH_{in} = 5.0/7.5), both in the absence and in the presence of *trans* K^+. After the preincubation time intervals indicated in Fig. 3A, ^{36}Cl was added to assay for chloride transport. The results show that the initial rate of chloride uptake rapidly decreases as a function of the preincubation time, indicating that the pH gradient energizing Cl uptake decays in an apparently exponential manner. The rate of this decay was strongly accelerated by the presence of *trans* K^+, as we had expected.

Knowledge of the intravesicular pH value at preincubation time zero ($pH_{in(t=0)}$ = 7.5) and at the end of the experiment ($pH_{in(t=120\ min)} = pH_{out}$ = 5.0) was used to compute the apparent intravesicular pH at each preincubation interval, $pH_{in(t)}$, by applying the equation:

$$pH_{in(t)} = pH_{out} - [(pH_{out} - pH_{in(t=0)}) \cdot (t u_{(t=2h)} - u_t)/(u_{(t=2h)} - u_{(t=0)}))] \quad (2)$$

where $u_{(t=0)}$ and $u_{(t=2h)}$ are, respectively, the absolute Cl^- uptake values at zero and 2 h of preincubation, and u_t represents the experimentally-determined, absolute Cl^- uptake data at time t. The estimated pH_{in} values as a function of the preincubation time are indicated in the right-hand ordinate of Fig. 3A.

The two curves in Fig. 3A looking quite different to the naked eye, it seemed of interest to evaluate them quantitatively. To do this, we applied to the first 6-min results the following relationship [12] derived from Eqn. 2:

$$\ln[(u_{(t=2h)} - u_t)/(u_{(t=2h)} - u_{(t=0)})] = a - kt \quad (3)$$

where k is the decay time constant.

This treatment revealed that, in the absence of *trans* K^+, the results follow a single exponential with the time constant $k = 11.1\ s^{-1}$. In contrast, the results obtained in the presence of *trans* K^+ could be decomposed into two exponentials with $k_1 = 71.3\ s^{-1}$ and $k_2 = 10.6\ s^{-1}$, respectively (see Fig. 3B). It would appear that k and k_2 represent the same phenomenon, e.g., the non-mediated, downhill influx of protons. To the contrary, k_1 appears to constitute a new phenomenon depending on the presence of *trans* K^+. For the reasons stated earlier in this paper, we would propose here that k_1 represents the influx of H^+ catalyzed by the sum of two factors: the electroneutral K^+/H^+ antiporter and, in the presence of valinomycin, the electrically-coupled exchange of K^+ for H^+.

Proton flux experiments

To complement the preceding ^{36}Cl uptake studies, vesicles charged with the pH-sensitive fluorophore, pyranine, were used to acquire direct knowledge on the

flux of protons, the second cosubstrate in Cl^-/H^+ cotransport.

Properties of vesicle-entrapped pyranine. The amount of fluorophore incorporated within the vesicles was calculated from the difference in the fluorescence intensity between the two preparations, $MV_{Pyr(out+in)}$ and $MV_{Pyr(out)}$, described in Materials and Methods. According to these calculations, $76 \pm 12\%$ ($n = 17$) of the pyranine present in the $MV_{Pyr(out+in)}$ preparation was intravesicular and gave the emission spectrum characteristic of free pyranine. The changes of fluorescence intensity in these vesicles were linearly correlated to the concentration of entrapped pyranine in the range from 100 to 600 μg protein/ml of pyranised vesicles. Treating the vesicles with Triton X-100 at any given time during the run resulted in a fast, monophasic change in fluorescence intensity corresponding to the pH jump from internal to external pH, thus indicating the complete lysis of the vesicles. The pyranine fluorescence intensity did not significantly change when the ionic strength of the buffers was modified within the limits set in our experiments.

Spectrofluorometric evidence for Cl^-/H^+ symport and K^+/H^+ antiport activities in isolated brush-border membrane vesicles from guinea pig intestine. In a first series of experiments, the effects of either Cl^-, K^+, or both together on the dissipation of an alkaline-inside pH gradient were studied.

Fig. 4 illustrates some representative results. The 'standard', spontaneous decay of an imposed pH gradient ($pH_{out}/pH_{in} = 6.0/7.5$) was first established by using equilibrated Tris gluconate as the 'inert' salt replacement.

All the observed decay curves involved an initial rapid fall in intravesicular pH from 7.5 to about 6.8, presumably representing the practically instantaneous neutralization of the pyranine bound to the outer face of the vesicles. This rapid initial phase was then followed by a slower decrease towards the pH_{in} at equilibrium, whose exact limiting value was determined at the end of each experiment after Triton lysis of the vesicles.

In the absence of *trans* K^+, extravesicular K^+ gluconate significantly slowed down the dissipation of the pH gradient, indicating that K^+ influx causes H^+ efflux. This inverse relationship between the K^+ and H^+ fluxes we interpret as direct evidence for K^+/H^+ exchange. In sharp contrast, TMA Cl (results not illustrated) significantly increased the rate of pH gradient dissipation, thus confirming the presence of Cl^-/H^+ symport activity in these vesicles. Interestingly, *cis* KCl also significantly increased the rate of pH gradient dissipation, suggesting that the Cl^-/H^+ symporter is stronger than the K^+/H^+ antiporter and therefore can prevail under the conditions of these experiments (further evidence below).

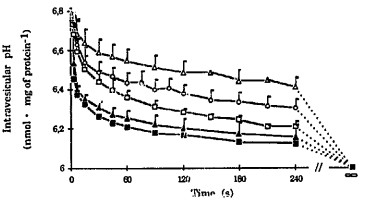

Fig. 4. The rate of dissipation of an alkaline-inside pH gradient, in the presence of *cis* chloride and/or potassium: Acceleration by the presence of intravesicular potassium. Vesicles were loaded with pyranine in the presence of a 20 mM Hepes/40 mM Mes buffer adjusted to $pH_{in} = 7.5$ with Tris base. In addition, half of the vesicles were loaded with 200 mM Tris gluconate (empty symbols); the other half with 100 mM Tris gluconate plus 100 mM K gluconate (black symbols). The reaction was initiated by mixing each vesicles aliquot with the same buffer adjusted to $pH_{out} = 6.0$ and containing the following salts: 200 mM Tris gluconate (○, $n = 18$ per point); or 100 mM Tris gluconate plus 100 mM of either K^+ gluconate (△, ▲, $n = 4$ and 3, respectively), or KCl (□, ■, $n = 3$). At 240 min, Triton was added to lyse the vesicles. As explained in the text, the fluorescence intensities have been transformed into the negative logarithm of intravesicular $[H^+]$, which are the units used to build the left-hand ordinate scale. Compared to the control condition (◇), the *trans* K^+ conditions were significantly different at any time, whereas for the conditions *cis* KGlu and *cis* KCl (zero *trans* K^+) the difference was significant only after 15 and 60 s, respectively.

Finally, our results further show that *trans* K^+ strongly accelerates the rate of pH gradient dissipation, fully confirming the proposal that intravesicular K^+ accelerates proton influx. This effect is independent of the presence of *cis* K^+ and is not significatively increased by valinomycin (Fig. 4, lower two curves).

Taken together, therefore, the results here summarized constitute a direct confirmation of all the premises and predictions contained in the electroneutral hypothesis discussed in previous sections of this paper.

Uphill proton transport induced by either cis chloride or by trans potassium. If our conclusion is correct that the above-described proton fluxes are catalyzed by specific cotransport mechanisms, it should be expected that appropriate Cl^- and K^+ gradients should each be able to create a proton gradient, although such gradients should have, by definition, opposite signs. To address this key question, pyranized vesicles were used where, at time zero, $pH_{out} = pH_{in} = 6.5$. Under such conditions, any ion-induced change in pH will take place, by definition, uphill.

As shown in Fig. 5, and fully in accord with prediction, an (out)/(in) = 100/0 mM Cl^- (TMA salt) gradient caused vesicle acidification whereas a similar gradient of K^+ gluconate caused the opposite effect, intravesicular alkalinisation. Because it acts as a K^+/H^+ exchanger, nigericin in the presence of *cis* K^+ glu-

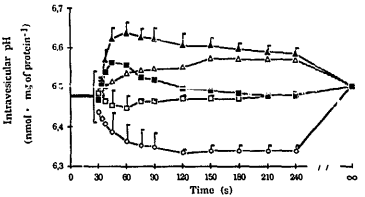

Fig. 5. pH gradient-generation by the extravesicular addition of either chloride or potassium ions to pH-equilibrated vesicles, as determined with the pyranine fluorescence method. Vesicles containing 100 mM TMA gluconate and a 20 mM Hepes/40 mM Mes buffer adjusted to pH 6.5 with Tris were mixed with media of identical composition and pH, supplemented (black symbols) or not (empty symbols) with valinomycin. After 30 s, 100 mM of either K^+ gluconate (△, $n = 7$ per point; ▲, $n = 6$), KCl (□, $n = 6$; ■, $n = 7$) or TMA Cl (○, $n = 11$) were added. At 240 s, Triton was added to lyse the vesicles. The results are expressed as in Fig. 4.

conate also caused a rapid, short-lived alkalinisation (results not illustrated).

When valinomycin was added in the presence of the same inside-directed K^+ gradient, the result was a significant increase in the rate of intravesicular alkalinization which, furthermore, lasted to last considerably longer than the one observed in the absence of valinomycin (Fig. 5). We conclude that a K^+ gradient, can cause a purely electroneutral, uphill proton transport catalyzed by the K^+/H^+ antiporter. In contrast, in the presence of valinomycin, superimposed to the electroneutral fluxes just described, there can also be an electrically-coupled exchange of K^+ for H^+.

Fig. 5 also illustrates that an inside-directed KCl gradient had a small, short-lived intravesicular acidifying effect. Such a result agrees with the notion that the Cl^-/H^+ symport and the K^+/H^+ antiport systems act in opposite directions, but the former is the strongest of the two and therefore can temporarily prevail. However, this advantage of Cl^- over K^+ disappears when valinomycin is added.

Concluding remarks

In conclusion, the inhibition of proton-driven chloride uptake caused by intravesicular potassium can be fully explained in terms of an acceleration of the rate of dissipation of an imposed pH gradient, resulting from a specific, electroneutral, K^+-driven proton influx through the K^+/H^+ antiporter. Valinomycin would act synergistically with *trans* K^+ by facilitating an additional, electrically-coupled exchange of these two ions.

Kleinman et al. [4] proposed a quite similar mechanism whereby voltage clamping can accelerate the dissipation of an acid-inside pH gradient, leading to a decrease in the driving force for Na^+/H^+ exchange in human jejunal brush-border membrane vesicles. Rentro et al. [21] showed that the uphill SO_4^{2-} uptake driven by a pH gradient in chick renal tubule brush-border vesicles was inhibited by equilibrated K^+ in the presence of valinomycin. Furthermore, from these experiments it appears that, under voltage-clamp conditions, all pH gradient effects on the uptake of either the sodium or the sulfate ions were greatly reduced at long time intervals (10 s to 5 min), but were practically unaffected at 2 s [4,21].

Together with our own, therefore, all of these observations indicate that the effects of short-circuiting with K^+ and valinomycin can be rather complex and, depending on the system under study, may involve net decreases in the gradient of a driver ion, e.g., H^+. Therefore, valinomycin should be used with great caution when experimentally trying to evaluate whether or not a given transport system is electrically silent.

We would conclude by mentioning that Cl^-/H^+ symporter as well as Na^+/H^+ and K^+/H^+ antiporter activities coexist in the intestinal brush-border membrane. As concerns at least the enterocyte, therefore, the functional interaction between all of these transporters will have to be taken into account to achieve a full understanding of the mechanism of intracellular pH regulation.

Acknowledgments

This work was supported in part by contracts 87.7001 and 489NS1 from the Institut National de la Santé et de la Recherche Médicale (INSERM), the Association Française de Lutte contre la Mucoviscidose (AFLM), and the Fondation pour la Recherche Médicale (Paris).

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