Mechanisms of Net Chloride Secretion During Rotavirus Diarrhea in Young Rabbits : Do Intestinal Villi Secrete Chloride?

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Key Words

Rotavirus • Diarrhea • Crypts • Villi • Brush border membrane • Cl⁻/H⁺ symporter • Cl⁻/anion exchange activity • Voltage-activated Cl⁻ conductance activity • SGLT1

Abstract

Rotaviral diarrheal illness is one of the most common infectious diseases in children worldwide, but our understanding of its pathophysiology is limited. This study examines whether the enhanced net chloride secretion during rotavirus infection in young rabbits may occur as a result of hypersecretion in crypt cells that would exceed the substantial Cl⁻ reabsorption observed in villi. By using a rapid filtration technique, we evaluated transport of ³⁶Cl and D-¹⁴C glucose across brush border membrane (BBM) vesicles purified from villus tip and crypt cells isolated in parallel from the entire small intestine. Rotavirus infection impaired SGLT1-mediated Na⁺-D-glucose symport activity in both villus and crypt cell BBM, hence contributing to the massive water loss along the crypt-villus axis. In the same BBM preparations, rotavirus failed to stimulate the Cl⁻ transport activities (Cl⁻/H⁺ symport, Cl⁻/anion exchange and voltage-activated

Cl⁻ conductance) at the crypt level, but not at the villus level, questioning, therefore, the origin of net chloride secretion. We propose that the chloride carrier might function in both normal (absorption) and reversed (secretion) modes in villi, depending on the direction of the chloride electrochemical gradient resulting from rotavirus infection, agreeing with our results that rotavirus accelerated both Cl⁻ influx and Cl⁻ efflux rates across villi BBM.

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Introduction

Rotavirus is the major cause of dehydrating gastroenteritis in children worldwide [1]. However, even though a considerable amount of research has been devoted to this subject over several decades, the mechanisms underlying the diarrheal illness remain unclear [2, 3]. Rotavirus has been reported to inhibit intestinal Na⁺-solute symport systems, hence contributing to the massive loss of water into the intestinal lumen [4]. Rotavirus also reduced intestinal disaccharidase activities both *in vivo* in young mice [5] and *in vitro* in human Caco-2 cells [6]. It has also been reported that rotavirus increased net Cl⁻ secretion at a time coinciding with the

appearance of mild diarrhea [7, 8]. However, the increase in luminal Cl^- concentrations was found to be moderate, which would seem to be in line with observations that the ionic concentrations in the stools of rotavirus-positive children are much lower than those found in the pure secretory diarrheas caused by secretagogues such as the enterotoxins of *Vibrio cholerae* and *Escherichia coli* [9]. Also, unlike enterotoxigenic diarrhea in which chloride absorption is impaired [10, 11], rotavirus selectively increased the capacity (V_{max}) parameter characterizing the Cl^-/H^+ symporter across intestinal BBM from young rabbits [7]. In principle, such activation would result in decreased rather than increased net chloride secretion. It was proposed, therefore, that the substantial Cl^- reabsorption in villi could partly overwhelm chloride secretion in crypt cells, which possibly (but not proven) increases during rotavirus diarrhea, the resulting imbalance leading to a moderate net chloride secretion [7].

In the present study, we evaluated the extent to which rotavirus can modify Cl^- transport in small intestine crypt cells. Because rotavirus still results in diarrhea in mice lacking the apical CFTR (Cystic Fibrosis Transmembrane conductance Regulator) chloride channel [12], alternative chloride transport pathways must be at work. We report the novel observation that rotavirus infection has no effect on all Cl^- uptake activities (Cl^-/H^+ symport, Cl^- /anion exchange and Cl^- conductance) observed across BBM vesicles purified from crypt cells isolated from rabbit small intestine. This absence of stimulation of Cl^- transport in crypt and the substantial Cl^- reabsorption in villi raises the question as to how rotavirus may cause a slight increase in net Cl^- secretion at the time of diarrhea. We propose that the carrier can mediate Cl^- transport in and out of the cell depending on the direction of the driving force, agreeing with our results that rotavirus accelerated both Cl^- influx and Cl^- efflux rates across villi BBM. Evidence that in certain circumstances intestinal villi can secrete Cl^- has been reported by other researchers [13–17].

Materials and Methods

Rabbit inoculations

Specific pathogen-free (SPF) four-week-old New Zealand albino hybrid rabbits were obtained from Charles River (France) and maintained at the animal house of the University of Paris-Sud, Châtenay-Malabry [4, 7]. At 4 weeks of age, rabbits were orally inoculated with 2 ml of the lapine rotavirus stock suspension, strain La/RR510 ($10^{6.2}$ infectious particles/rabbit) [4, 7].

Isolation and purification of villus tip and crypt cells from the small intestine

At appropriate times after infection, three to eight animals were killed by cervical dislocation after stunning. For each animal, the entire small intestine was removed, rinsed with saline at room temperature and villus and crypt cells were isolated by a modified calcium-chelation method [18] using everted intestine and a low temperature (22°C) isolation procedure. Briefly, fresh intestinal segments of 25–30 cm length were everted, rinsed with saline, filled with the cell buffer (115 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4 , 26 mM NaHCO_3 , 6 mM glucose, adjusted to pH 7.4), tied at both ends and incubated in the chelating buffer formed by the cell buffer supplemented with 0.5 mM DTT and 1.5 mM EDTA. Villus-to-crypt sequential fractions were isolated by constant shaking (250 rpm, Promax 2020, Heidolph) for timed intervals over 15 min at room temperature. Five successive fractions of cells were sedimented at 500 g for 8 min and washed with the cell buffer to remove the chelating buffer. Each cell fraction was further fractionated on a discontinuous 12/35/50% Percoll gradient (Amersham Pharmacia) at 100 g for 4 min. Cells at the 12/35% and 35/50% Percoll interfaces were collected as villus and crypt cells, respectively [19–21]. Villus tip cells in the first 30 min incubation and crypt cells obtained between 45 and 60–75 min incubation were pooled separately, washed with the cell buffer, frozen immediately in liquid nitrogen and stored at -80°C .

BBM vesicle preparation

BBM vesicles from villus tip and crypt cells isolated from the same animal were prepared by using the magnesium precipitation method as described previously [4, 7]. They were suspended at about 4 mg of membrane protein per ml in the appropriate membrane buffer, and stored in liquid nitrogen until the day of transport assay [4, 7, 22, 23]. For glucose uptake assays, the membrane buffer consisted of 20 mM Hepes/ 10 mM Tris HCl/ 0.02% LiN₃, supplemented with D-arabinose to a total osmolarity of 600 mOsm and adjusted to pH 7.4 with Tris base [4]. For chloride uptake assays, the membrane buffer consisted of 20 mM Hepes/ 40 mM citric acid/ 100 mM of either Tris gluconate, K^+ gluconate or KCl/ 0.02% LiN₃, supplemented to a total osmolarity of 560 mOsm with sorbitol and adjusted to pH 7.5 with Tris base [7, 22, 23]. Membrane protein concentrations were measured with a Bio-Rad protein assay kit, using bovine serum albumin as standard.

Transport assay and expression of results

Transport was assayed by using a rapid filtration technique and either D- ^{14}C glucose or ^{36}Cl as the substrate. Before use, H^{36}Cl (0.4 mCi mmol $^{-1}$, Amersham) was neutralized with Tris base. For glucose uptake assays, 10 μl of a BBM vesicle preparation were used for transport measurement by mixing with 40 μl of transport buffer formed by the membrane buffer supplemented with 0.1 mM of unlabeled substrate, ^{14}C -labeled glucose as a tracer and 100 mM NaSCN [4]. Cl^- influx experiments were carried out by mixing 5 μl of a BBM vesicle preparation with 45 μl of transport buffer formed by the membrane buffer supplemented with 10 mM unlabeled substrate, 5 mM ^{36}Cl , the amount of sorbitol necessary to secure

Fig. 1. Time course of D-glucose uptake by BBM purified of villus tip and crypt cells isolated from non-infected, control rabbit intestines. The uptake of 0.1 mM D-glucose was measured at 22°C in the presence of a zero trans, 100 mM cis NaSCN gradient, using BBM vesicles of villus (■) or crypt (●) cells isolated from non-infected control intestines. Results are expressed as absolute uptake values in nanomoles . milligrams of protein-1 $\pm$ SD, with n = 9 determinations per point derived from 3 different membrane preparations.

a total osmolality of 660 mOsm and adjusted to either pH 7.5 or pH 5.0 with Tris base (final concentrations and pH in the incubation mixtures) [7, 22-24]. When present, valinomycin (Sigma), a potassium specific ionophore, was dissolved in ethanol and allowed to evaporate to dryness before being mixed with the membrane preparation [22]. Cl⁻ efflux was determined after loading the vesicles with membrane buffer containing 5 mM ³⁶Cl, followed by mixing 5 μ l of the loaded vesicles with 95 μ l of transport buffer, as previously described [25].

Influx and efflux rates were measured at 22°C in an automatic, temperature-controllable short-time incubation apparatus. Initial influx rate measurements were carried out for either 2 sec (D-glucose) or 4 sec (Cl⁻) [4, 7, 22-24]. Influx results are shown as absolute uptake rates, v , in pmoles . sec⁻¹ . mg protein⁻¹ $\pm$ standard deviation (SD), of the pool of several experiments performed with 3-7 different membrane preparations. Cl⁻ efflux results are given in percent of intravesicular ³⁶Cl content (in nmoles . mg protein⁻¹) at zero time. These data were quantified by applying equation 3 of Vasseur (1992) [22]:

$$a - (kt) = \ln \{ [v(t=30 \text{ min}) - vt] / [v(t=30 \text{ min}) - v(t=0)] \} \quad (1)$$

where a is the y-axis intercept at $t = 0$, v is the intravesicular ³⁶Cl content at time t and k is the first-order decay time constant. Uptake data were statistically compared by using a global F test [26]. All calculations were performed on Apple Macintosh microcomputers.

SGLT1 protein identification in the BBM vesicles from villus and crypt cells isolated from the same rabbit intestine

Brush border membrane proteins (30 μ g per well) were separated by SDS-PAGE on 9% polyacrylamide gels and immunoblotted as previously described [4, 27]. The rabbit anti-SGLT1 antibody (Biogenesis, Poole, UK) was used at 1:2000 dilution.

Results

Measurement of SGLT1 as marker for enterocyte differentiation

SGLT1 protein has been described as restricted to rabbit mature enterocytes BBM when using immunocytochemical techniques [28]. On the other hand, Na⁺-D-glucose symport activity has been demonstrated to occur in both villus and crypt cell BBM of rabbit and rat [29-33]. SGLT1 activity strongly diminished from the villus to the crypt region, as did sucrase activity, reflecting the degree of enterocyte development [29, 32, 33]. Under the conditions of our experiments, we know that sucrase was inhibited by Tris [34], and for that reason we did not use it in the present work. Accordingly, sugar transport studies were undertaken to secure good cell separation.

We compared the time course of 0.1 mM D-glucose uptake in the presence of a zero trans, 100 mM cis NaSCN gradient in BBM vesicles from villus tip and crypt cells isolated from the same rabbit intestine. As expected in these conditions [29-33], D-glucose uptake by both villus and crypt cell BBM exhibited the characteristic overshoot phenomenon as demonstrated by the 60 s / equilibrium D-glucose concentration ratios, r_{60} , that are greater than unity (Fig. 1). These overshoots were, however, different in size, as both the initial absolute entry rates and the height of the overshoots were significantly decreased in crypt cell BBM. The r_{60} value dropped from 16 in villus cell BBM to 3 in crypt cell BBM. The same was true for the initial velocities where the uptake values were about 6 fold higher for villi than for crypt cell BBM (Table 1).

Table 1. Rotavirus effects on SGLT1 and Cl⁻/H⁺ symport activities in both villus and crypt cell BBM from rabbit intestine. Conditions are as defined in Figs. 1 and 2 for D-glucose and proton-coupled Cl⁻ uptakes, respectively. The initial entry rates are expressed in picomoles . mg protein⁻¹. s⁻¹ ± SD with n determinations per point derived from 3 to 8 different membrane preparations from non-infected controls or infected animals at 5 dpi. Identical letters indicate results found to be statistically indistinguishable according to a global F test (p < 0.01).

	Uptake at dpi:		
	0	5	
D-glucose uptake			% Inhibition
Villus cell BBM	210 ± 56 (9) a	119 ± 54 (9) b	57
Crypt cell BBM	33 ± 14 (9) c	15 ± 8 (15) d	47
Proton -coupled Cl ⁻ uptake			% Activation
Villus cell BBM	2232 ± 1409 (22) a'	4992 ± 2118 (8) b'	124
Crypt cell BBM	2013 ± 554 (15) a'	1995 ± 780 (12) a'	-

To detect possible changes in the total mass of Na⁺-D-glucose symporter per unit weight of membrane protein, the amount of SGLT1 antigen present in the purified BBM vesicles was investigated. As determined by densitometry, the amount of SGLT1 antigen present in the BBM vesicles was found to be higher for villi than for crypt cells (relative densities of 100 and 47, respectively, not shown).

Taken as a whole, the results demonstrate good separation of crypt and villus tip cells as evidenced by both the activity and expression of SGLT1 which increase as enterocytes migrate from the crypt to villus region.

Rotavirus infection impairs SGLT1 activity all along the crypt-villus axis.

We next investigated whether or not glucose uptake in BBM vesicles purified from isolated villus and crypt cells was affected by rotavirus at 5 days post infection (dpi), a time coinciding with the appearance of mild diarrhea [7].

In infected rabbits, D-glucose transport continued to occur uphill in both villus and crypt cell BBM, but again the size of the overshoots differed depending on the cell population. The r60 value dropped from 8.7 in villus cell BBM to 1.3 in crypt cell BBM (data not shown) and the initial velocities were over 7 fold higher for villi than for crypt cell BBM in infected rabbits (Table 1).

Rotavirus infection significantly inhibited SGLT1 activity in villus cell BBM (Table 1, first line). A similar conclusion was reached when studying the effect of rotavirus infection on Na⁺-solute symport activities across BBM isolated from the entire small intestine [4], further confirming that the intestinal BBM vesicles were derived mostly from villus enterocytes [7, 35]. In crypt cell BBM, rotavirus infection also inhibited SGLT1 activity (Table 1, second line). The inhibitions appeared to be significantly different in villus and crypt cell BBM, 57% and 47%

respectively (Table 1). The SGLT1 protein concentration present in either villus or crypt cell BBM, although higher for villi than for crypt cells, was unaffected by rotavirus infection (not shown).

Chloride transport systems in crypt cell BBM from control rabbits

We previously showed that total initial Cl⁻ uptake across intestinal BBM from guinea pig [22-24] and young rabbits [7] occurred with three distinct activities: Cl⁻/H⁺ symport, Cl⁻/anion exchange and rheogenic Cl⁻ conductance. By using a similar approach in the present study, we investigated the presence and participation of each activity in crypt cell BBM from control rabbits. The conditions for this investigation were selected from a diversity of experimental conditions aimed at modifying the alkali-metal ion, electrical, Cl⁻ and/or H⁺ gradients [7].

Evidence for pH gradient-dependent uphill transport of Cl⁻

Considering the reports concerning the presence of Cl⁻/H⁺ symporters in the enterocyte membranes of several animal species [7, 22-24, 36], we compared pH gradient-driven Cl⁻ overshoots in BBM vesicles from villus and crypt cells isolated in parallel from the small intestine of young rabbits.

In the presence of a pH_o/pH_i = 5.0/7.5 gradient (o (out) and i (in) indicate the extra- and intravesicular spaces, respectively), Cl⁻ uptake took place uphill in both villus and crypt cell BBM, confirming the existence of Cl⁻/H⁺ symport activity along the small intestinal crypt-villus axis (Fig. 2). Here, the overshoot phenomena appeared to be quantitatively similar in both BBM populations. The initial absolute entry rates, the height of the overshoots (5 min) and the Cl⁻ uptake values at equilibrium (3 hrs) did not differ significantly (Table 1

Fig. 2. Time course of proton-coupled Cl^- uptake by BBM of villus and crypt cells isolated from non-infected rabbit intestines. Cl^- uptake was determined with 15 mM cis ^{36}Cl as substrate. The extra- and intra-vesicular spaces contained a 20 mM Hepes/ 40 mM citric acid/ 100 mM Tris gluconate buffer adjusted with Tris base to give an initial $\text{pH}_o/\text{pH}_i = 5.0/7.5$ gradient. Symbols: (■) for villus cell BBM and (●) for crypt cell BBM. Results are expressed in nmoles . mg of protein $^{-1} \pm \text{SD}$, with $n = 9\text{--}21$ determinations per point obtained from 3 to 8 different membrane preparations.

and Fig. 2). These results contrast with the SGLT1 overshoots (Fig. 1), even though the same villus and crypt cell BBM preparation was used for glucose and chloride uptake measurements, hence confirming that SGLT1 was the best functional test for validating good separation of villus and crypt cells.

Evidence for electroneutral Cl^-/H^+ symport and Cl^-/Cl^- exchange activities and rheogenic Cl^- conductance in crypt cell BBM

To establish whether the uphill Cl^- uptake driven by a pH gradient in crypt cell BBM involved an electroneutral Cl^-/H^+ symporter, experiments were performed under voltage clamping conditions established by short-circuiting to zero the membrane potential with 100 mM equilibrated K^+ concentrations and valinomycin. Under voltage clamping conditions, the initial Cl^- entry rate obtained at equilibrated pH ($\text{pH}_o = \text{pH}_i = 7.5$) can be taken as the reference of basal, initial Cl^- entry rate (Table 2, condition C). With an alkaline-inside, 2.5-unit pH gradient, the initial Cl^- entry rate increased significantly (Table 2, condition E), indicating the functioning of an electroneutral Cl^-/H^+ symporter. In the absence of voltage clamping, cis proton further increased the initial Cl^- entry rate (2013 and 1505 picomoles . mg of protein $^{-1}$. sec $^{-1}$ in the absence (Table 1) and presence (Table 2) of voltage clamping, respectively), here indicating the proton effect to be purely electrical.

We next investigated the effects on Cl^- uptake of imposing gradients of either chloride, pH, or both together, in the presence of short-circuiting conditions to assess an electroneutral mechanism. Under equilibrated pH

conditions, cold trans Cl^- caused a significant increase in the initial Cl^- entry rate, which was further increased when an inside-directed $[\text{H}^+]$ gradient ($\text{pH}_o/\text{pH}_i = 5.0/7.5$) was superimposed (Table 2, conditions D and F versus C and E). These results confirm the existence of an electroneutral Cl^-/anion exchange activity in crypt cell BBM from rabbit intestine. Furthermore, and similar to previous reports with enterocyte membranes purified from the entire small intestine of guinea pigs and rabbits, the observation that external protons activated Cl^-/Cl^- exchange activity (Table 2, conditions D and F) provides evidence for the functioning of a symporter, rather than an antiporter, in crypt cell BBM [7, 22–24, 35].

We next specifically looked for the presence of electrical (rheogenic) Cl^- uptake in crypt cell BBM. Under equilibrated pH conditions, a positive-inside membrane potential created by the inward-directed K^+ gradient plus valinomycin had a significant but small effect on the initial Cl^- entry rate. The activation was fully abolished under voltage clamping conditions (Table 2, conditions B versus C), indicating the involvement of a Cl^- conductance pathway caused by a positive-inside membrane potential. In the absence or presence of zero trans, 100 mM cis of either K^+ , Na^+ or an equimolar mixture of Na^+ plus K^+ , the Cl^- data were statistically identical to those obtained with 100 mM equilibrated K^+ concentrations plus valinomycin (Table 2, conditions A and C). This lack of effect of the inward-directed alkali-metal ion gradient on Cl^- uptake provides clear evidence for the absence of any electroneutral Cl^-/Na^+ , Cl^-/K^+ or $2\text{Cl}^-/\text{Na}^+/\text{K}^+$ symport activity in the rabbit crypt cell BBM preparations, as was the case in intestinal (villus cell) BBM from rabbit [7].

Table 2. Chloride uptake by crypt cell BBM purified from non-infected controls and infected rabbits at 5 dpi: effect of electrical, chloride and/or proton gradients on the initial Cl⁻ entry rates. The extra- and intra-vesicular spaces contained a 20 mM Hepes/40 mM citric acid buffer and the indicated K⁺, electrical and Cl⁻ gradients with pH gradients (pHo/pHi) of 7.5/7.5 or 5.0/7.5. Valinomycin (Val) was at 10 µg .

pH gradient					
and condition	[K ⁺]o/[K ⁺]i mM	Val	[Cl ⁻]o/[Cl ⁻]i mM	Chloride uptake at dpi:	
				0	5
pHo/pHi = 7.5 / 7.5					
A	100 / 0	-	15 / 0	419 ± 171 (29) ab*	495 ± 238 (35)ab*
B	100 / 0	+	15 / 0	592 ± 244 (8)bc	775 ± 207 (12) c
C	100 / 100	+	15 / 0	366 ± 151 (19) a	492 ± 234 (25) ab
D	100 / 100	+	15 / 100	961 ± 535 (19) cd	938 ± 500 (18) cd
pHo/pHi = 5.0 / 7.5					
E	100 / 100	+	15 / 0	1505 ± 294 (16) e	1256 ± 377 (12) de
F	100 / 100	+	15 / 100	2378 ± 750 (20) f	2323 ± 881 (12) f

mg protein⁻¹. Positive-inside (Conditions B) or short-circuited to zero (Conditions C-F) membrane potentials were obtained with valinomycin and the indicated K⁺ gradients. The initial chloride entry rates, with 15 mM cis ³⁶Cl as substrate, are given in pmoles . mg protein⁻¹. s⁻¹ ± SD with n determinations per point derived from 4 to 8 different membrane preparations from either non-infected controls or infected animals at 5 dpi. Identical letters indicate results found to be statistically indistinguishable according to a global F test (p < 0.01). The asterisk means that the results obtained in the absence or presence of 100 mM cis of either K⁺, Na⁺ or an equimolar mixture of Na⁺ plus K⁺ are statistically identical and have been pooled.

Table 3. Quantification of electroneutral Cl⁻/H⁺ symport and Cl⁻/Cl⁻ exchange activities and rheogenic Cl⁻ conductance by crypt cell BBM vesicles purified from non-infected controls and experimentally infected rabbits. Conditions are as defined in Table 2. The data are given in terms of uptake increments (in pmoles . mg protein⁻¹. s⁻¹) obtained by the difference between the reference, basal, initial Cl⁻ entry rate. The quantitative contribution of any of the activities to the total initial Cl⁻ uptake is given in parentheses.

Effect	Conditions	Uptake increments at dpi:	
		0	5
Proton	E - C	1139 (58%)	765 (51%)
Trans chloride	D - C	595 (30%)	447 (30%)
Electrical	B - C	226 (12%)	283 (19%)

From the whole set of results, we conclude that total initial Cl⁻ uptake in rabbit crypt cell BBM occurred with three distinct activities: proton-coupled Cl⁻ transport, Cl⁻/anion exchange and voltage-activated Cl⁻ conductance.

Effect of rotavirus infection on Cl⁻ transport into villus and crypt cell BBM

By using the same (villus or crypt cell) BBM preparation that showed that rotavirus infection impaired SGLT1 activity at both villus and crypt levels (Table 1), we undertook to determine the proton-coupled Cl⁻ uptake at 5 dpi. In crypt cell BBM rotavirus had no significant effect on Cl⁻/H⁺ symport activity, whereas in villus cell BBM, rotavirus stimulated Cl⁻/H⁺ symport activity (Table 1).

To further confirm the absence of an effect of rotavirus on Cl⁻ transport into crypt cell BBM, chloride uptake experiments were repeated in the absence or presence of K⁺, electrical, Cl⁻ and/or H⁺ gradients. In all the study conditions, rotavirus infection had no significant effect on Cl⁻ uptake activities present in crypt cell BBM

(Table 2). Neither the rheogenic Cl⁻ conductance nor the electroneutral Cl⁻/anion exchange and Cl⁻/H⁺ symport activities were modified. Consequently, in both infected and control crypt cell BBM, the quantitative participation of any of the activities to total initial Cl⁻ uptake could be expected to be identical. To quantify the proportion of either activity, the effects elicited by either gradient when alone were calculated in terms of the uptake increments (Table 3). The results indicate that the electroneutral Cl⁻/H⁺ symport and Cl⁻/anion exchange activities contributed to a great extent (on average 85%) to total initial Cl⁻ uptake into crypt cell BBM, whereas the rheogenic Cl⁻ conductance activity was weakly operative (on average 15%). These percentages are very similar to those obtained in intestinal (villus cell) BBM of infected and control rabbits, where about 90% and 10% of total initial Cl⁻ uptake involved electroneutral and electrical mechanisms, respectively [7].

To further confirm that rotavirus infection did not affect Cl⁻ transport at the crypt cell level, the proton-gradient-driven Cl⁻ uptake was studied as a function of

Fig. 3. Rotavirus effects on crypt cell BBM Cl^-/H^+ symport as a function of time after infection. The initial Cl^- entry rates were determined with 15 mM cis ^{36}Cl . The extra- and intra-vesicular spaces contained a 20 mM Hepes/40 mM citric acid/100 mM Tris gluconate buffer adjusted with Tris base to give an initial $\text{pH}_o/\text{pH}_i = 5.0/7.5$ gradient. Results are expressed in nmoles. mg of protein $^{-1}$. s $^{-1} \pm$ SD, with $n = 6$ -15 determinations per point obtained from 2 to 5 different membrane preparations. Results were found to be statistically indistinguishable according to a global F test ($p < 0.01$).

time after infection. As illustrated in Fig. 3, all proton-coupled Cl^- uptakes into crypt cell BBM from infected animals were statistically indistinguishable from the control group. From the whole set of observations, we conclude that rotavirus infection did not modify chloride transport across crypt cell BBM up to 7 dpi, the longest time used.

Rotavirus activating effect on Cl^- efflux across villi BBM

Because rotavirus infection exclusively stimulated Cl^- uptake activities across villi BBM up to 7 dpi [7], we studied its effect on Cl^- efflux across these vesicles. The experiments were carried out under equilibrated pH conditions ($\text{pH}_o = \text{pH}_i = 7.5$) with vesicles loaded with 5 mM ^{36}Cl , in the absence of cold extravesicular (trans) Cl^- . Because of the carry-over of a fixed quantity of ^{36}Cl from the preincubation to incubation media (proportion of 1/20), the imposed initial $[\text{Cl}^-]_o/[\text{Cl}^-]_i$ ratios were 0.25/5 mM. In the presence of an outside-directed Cl^- gradient, the intravesicular Cl^- content decreased with time, indicating Cl^- efflux (Fig. 4, upper curve). Rotavirus infection strongly accelerated the time-dependent drop (Fig. 4, two lower curves). The intravesicular Cl^- contents were significantly different from those of the control group at times as short as 5 and 10 sec. To better characterize the differences, these data were quantified by applying equation 1. As illustrated in the inset of Fig. 4, the first 30-sec results of the control group followed a simple exponential with the time constant $k = 0.019 \text{ s}^{-1}$. In contrast, the results for infected animals at 1 and 3 dpi could be divided into two exponentials yielding, respectively, $k = 0.0078$ and 0.0067 s^{-1} for the first 5-sec results and similar k values of 0.012 sec^{-1} for the 5-30

sec results. Such results clearly indicate that rotavirus infection activated Cl^- efflux in villi BBM.

Discussion

This study examines whether the enhanced net Cl^- secretion during rotavirus infection may occur as a result of hypersecretion in crypt cells that would exceed the substantial Cl^- reabsorption in villi [7]. To address this question, we used an experimental approach aimed at isolating in parallel the villus tip and crypt cells from the rabbit small intestine. The present results indicate that both the activity and expression of SGLT1 were much higher in villi than in crypt cell BBM, confirming this symporter to reflect the degree of enterocyte development along the small intestinal crypt-villus axis [29-33]. On the other hand, Cl^-/H^+ symport activity appeared to be unchanged along the crypt-villus axis (Fig. 2), even though the same villus and crypt cell BBM preparation was used for the glucose and Cl^- uptake experiments. Using the same approach, Knickelbein *et al.* reported that Cl^-/H^+ symport and $\text{Cl}^-/\text{HCO}_3^-$ exchange activities were relatively constant along the crypt-villus axis from rabbit intestine [32]. Coon and Sundaram also demonstrated that $\text{Cl}^-/\text{HCO}_3^-$ exchange activity was unaffected in crypt but inhibited in villus cell BBM during chronic ileitis in rabbit [36].

We show that infection of young rabbits by lapine rotavirus, La/RR510 strain, did not alter Cl^- transport in crypt cell BBM. Neither the electroneutral systems nor the electrogenic system was modified. Both in infected and control crypt cell BBM, the electroneutral Cl^-/H^+

Fig. 4. Effect of rotavirus infection on Cl⁻ efflux from Cl⁻ loaded villi BBM vesicles under equilibrated pH conditions. Villi BBM vesicles from non-infected controls (■) or infected animals at either 1 (▲) or 3 (●) dpi were loaded with 5 mM ³⁶Cl as described earlier [25]. The extra- and intra-vesicular spaces contained a 20 mM Hepes/ 40 mM citric acid/ 100 mM Tris gluconate buffer adjusted with Tris base to give an initial pH_o/pH_i = 7.5/7.5 gradient and [Cl⁻]_o/[Cl⁻]_i ratios of 0.25/5 mM. The Cl⁻ efflux rates are given in percent ± SD of intravesicular ³⁶Cl content (nmoles · mg protein⁻¹) at zero time (n = 3-4 determinations per point). Because the efflux values at 30 min were identical for control and infected rabbits, data have been pooled (n = 9 at the arrow). Inset: quantification of the first 30-sec data by applying equation 1. Further details in the text.

symport and Cl⁻/anion exchange activities predominated, representing on average 55% and 30% of the total initial Cl⁻ uptake, respectively, whereas the rheogenic Cl⁻ conductance activity was weakly operative. The absence of rotavirus effect on Cl⁻ transport contrasts with the strong inhibitory effect on SGLT1 symport activity in the same crypt cell BBM preparation. Taken as a whole, the present results provide compelling evidence that SGLT1 and chloride carriers are present in crypt cell BBM and are differently affected by rotavirus. The same is true at the villus cell level, although alterations by rotavirus were different. The present results confirm previous observations that rotavirus both impaired SGLT1 [4] and stimulated Cl⁻ transport [7] in (villus cell) BBM purified from the entire small intestine.

While it is widely accepted that loss of Cl⁻ in the stools can be due to decreased absorption in the villus cells and/or increased secretion in the crypt cells [10, 37-39], the secretory mechanisms of rotavirus-mediated diarrhea appear to be rather different. As shown here, rotavirus failed to stimulate Cl⁻ transport at the crypt level, whereas it stimulated Cl⁻ reabsorption in villi. If this is so,

the origin of Cl⁻ secreted into the intestinal lumen at the onset of rotavirus diarrhea is called into question. We suggest, therefore, that additional mechanisms might be involved. A likely explanation relies on several reports providing evidence that in certain circumstances villus cells can secrete chloride [13-17, 40]. The possibility that the chloride secretory mechanism is operative in villi implies that the membrane carrier operates in reverse. We earlier proposed that the same transport entity, namely a single, mobile carrier acting as a random (non-ordered), non-obligatory Cl⁻/H⁺ symporter, can account for Cl⁻/Cl⁻ exchange and Cl⁻ conductance activities, in addition to symport of both H⁺ and Cl⁻ [7, 22-24, 35, 41]. Also, by definition, the symporter is reversible, meaning that the protein may transport Cl⁻ in and out of the cell, depending on the direction of the driving force [25, 41]. Our study provides evidence that, similar to the Cl⁻ influx results across villi BBM (Table 1), Cl⁻ efflux rates are accelerated by rotavirus infection (Fig. 4), strongly indicating that Cl⁻ influx and efflux both involve the same carrier system across these membranes. The capability for bidirectional transport, in both normal (outside to inside) and reversed

(inside to outside) modes, has been recently demonstrated for SGLT1 [42, 43], the H⁺/peptide carrier PEPT1 [44] and the H⁺/amino acid symporter PAT1 [45], each expressed in *Xenopus laevis* oocytes. While all these symporters are known to function as absorptive carriers in small intestinal epithelial cells, the physiological significance of their reverse transport mode is currently not known. It has been proposed that, under certain (non-physiological) conditions, such as an accumulation of substrate concentrations into the enterocyte, they may serve as efflux transporters, favoring backward transport of substrates out of the cell [42-44]. In the crypt epithelium, it is well known that accumulation of intracellular Cl⁻ above its electrochemical equilibrium is a required condition for promoting Cl⁻ secretion across the apical CFTR chloride channel [38, 46] and see Fig. 2 in [10]. Because rotavirus still results in diarrhea in CFTR knockout mice [12], the symporter which is present in the apical membranes of both villus and crypt cells may explain why either cell type can secrete chloride [36, 47, 48]. Hence, the symporter might function in both normal (absorption) and reversed (secretion) modes in villi, depending on the direction of the chloride electrochemical gradient resulting from rotavirus infection, agreeing with our results that rotavirus accelerated both Cl⁻ influx and Cl⁻ efflux rates across villi BBM. The question concerning the molecular basis of the protein(s) that mediate Cl⁻ transport in the small intestine has been largely debated elsewhere and will not be discussed in the present paper [7, 35]. To date, very little is known about the membrane localization and the villus-crypt distribution of the AE2 isoforms throughout the intestine [49].

In conclusion, the mechanisms used by rotavirus to induce diarrheal disease might involve generalized inhibition of Na⁺-solute symport systems, hence contributing to the massive water loss all along the small intestinal crypt-villus axis [4]. With regard to the overall chloride secretory response, the substantial Cl⁻ reabsorption in villi and the absence of stimulation of Cl⁻ transport in crypt cells appear to be mechanisms unable to explain the moderate increase in net Cl⁻ secretion at the onset of rotavirus diarrhea. The villus enterocytes might be the secreting cells as a result of rotavirus infection. Progress in the understanding of the pathogenic processes of rotaviral diarrheas may serve as a basis for a rational approach in the design of novel therapeutic strategies and the development of new drugs.

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