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Modulation of salt permeabilities of intestinal brush-border membrane vesicles by micromolar levels of internal calcium

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A possible modulation of ion permeabilities of rat intestinal brush-border membrane vesicles by Ca^{2+}, a putative second messenger of salt secretion, was explored by three independent methods: (1) measurements of [^{3}H]glucose accumulation driven by a Na^+ gradient; (2) stopped-flow spectrophotometry of salt-induced osmotic swelling; (3) $^{86}Rb^+$, $^{22}Na^+$ and $^{36}Cl^-$ flux measurements. Cytoskeleton-deprived membrane vesicles were prepared from isolated brushborders by thiocyanate treatment. Intravescicular Ca^{2+} levels were varied by preincubating vesicles in Ca-EGTA buffers in the presence of the Ca^{2+}-ionophore A23187. At $Ca^{2+}_{free} > 10^{-5}$ M, initial Na^+-dependent glucose uptake in the presence of a 0.1 M NaSCN gradient (but not in its absence) was inhibited by about 50 per cent as compared to EGTA alone ($ED_{50} \cong 10^{-6}$ M Ca^{2+}). By contrast, initial rates of $^{22}Na^+$ uptake and reswelling rates of vesicles exposed to a NaSCN gradient were increased at least 2-fold by 10^{-5} M Ca^{2+}_{free}. Both observations are compatible with a Ca^{2+}-induced increase of the Na^+-permeability of the vesicle membrane. The modulation of ion transport was fully reversible and critically dependent on internal Ca^{2+}, suggesting a localization of Ca^{2+}-sensor sites at the inner surface of the microvillous membrane. As shown by radiotracer and osmotic swelling measurements, micromolar Ca^{2+} additionally increased the flux rate of K^+, Rb^+, Cl^- and NO_3^- but did not change the membrane permeability for small uncharged molecules, including glucose and mannitol. The effect of Ca^{2+} on ion permeabilities could be blocked by Ba^{2+} (10^{-3} M) or Mg^{2+} (10^{-2} M), but not by amiloride (10^{-3} M), apamin ($2 \cdot 10^{-7}$ M), trifluoperazine (10^{-4} M) or quinine ($5 \cdot 10^{-4}$ M). At present it is unclear whether Ca^{2+} activates a nonselective cation and anion channel or multiple highly selective channels in the vesicle membrane.

Introduction

Studies of stimulus-secretion coupling in intestinal epithelium have revealed a crucial role for cyclic AMP, cyclic GMP and Ca^{2+} as modulators of transepithelial Na^+ and Cl^- transport [1–4]. The major cyclic nucleotide- and Ca^{2+}-sensitive ion transport systems have been localized in the apical membrane of the enterocyte and consist of (i) an electroneutral Na^+-Cl^- cotransport system in the brush border of the mature villous cell, presumably composed of separate Na^+/H^+ and Cl^-/HCO_3^- exchangers coupled by circular proton movements [5–8] and (ii) an electrogenic Cl^- channel, apparently enriched in intestinal crypt cells [1,9]. The molecular nature of the transporters and of the signal transduction mechanism is largely unknown. An intermediate step in the

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coupling mechanism shared by both cyclic nucleotides is the cophosphorylation of a 25 kDa proteolipid in the microvillous membrane by the type II-isoenzyme of cyclic AMP-dependent protein kinase and by a unique isoenzyme of cyclic GMP-dependent protein kinase previously discovered in the intestinal brush border [3,4,10–13]. High-affinity receptors for Ca^{2+} in the brush-border region may include calmodulin, associated with the cross-filaments [14] and with a Ca^{2+}-dependent protein kinase [3,4,15]; a Ca^{2+}/phospholipid-dependent protein kinase [3,4]; polyphosphoinositides of the lipid bilayer [16]; intrinsic Ca^{2+}-activatable phospholipases [17]; and Ca^{2+} regulatory sites on the transport protein itself.

Studies of ion transport modulation at the level of brush-border membrane vesicles, avoiding the complexity of the intact cell, in principle allow a more detailed analysis of the kinetic and regulatory properties of the ion channels and carriers in the apical membrane. A preliminary characterization of ion transport pathways in intestinal brush-border membrane vesicles have sofar provided direct evidence for the occurrence of (1) Ca^{2+}-calmodulin inhibition of Na^+-Cl^- cotransport in rabbit brush borders [18]; (2) allosteric regulation of the Na^+/H^+ exchanger by intravesicular pH [19]; (3) Cl^--OH^- exchange in rat ileal but not in jejunal vesicles [6,7]; (4) selective conductance pathways for Na^+, K^+ and Cl^- [20]; (5) various cotransport systems for Na^+ and non-electrolytes, e.g. glucose [21]; and (6) activation of an anion conductance in rat brush-border membrane vesicles loaded with cyclic AMP and an ATP-regenerating system [13]. The latter study carried out at millimolar levels of Ca^{2+} prompted us to investigate a possible modulation of ion transport pathways by intravesicular Ca^{2+} alone and varied within a physiological concentration range. In order to avoid complications arising from interaction of Ca^{2+} with the microvillar cytoskeleton [22], most experiments were carried out with brush-border membrane vesicles deprived of their cytoskeleton by KSCN exposure as described by Hopfer et al. [23]. The results provide evidence for a fully reversible modulation of both cation- and anion-permeabilities of the vesicle membrane by intravesicular high-affinity Ca^{2+}-receptors apparently different from calmodulin, Ca^{2+}/phospholipid-dependent protein kinase or phospholipase.

Materials and Methods

Materials. D-[1(n)-^{3}H]Glucose (15.7 Ci/mmol), ^{22}NaCl (11 Ci/mmol), ^{86}RbCl (0.48 Ci/mmol) and H^{36}Cl (0.35 mCi/mmol) were purchased from Amersham International. Valinomycin and Ca^{2+}-ionophore A23187 were obtained from Boehringer. Phlorizin came from Roth, trifluoperazine from Röhm Pharma, apamin and quinine from Sigma. Amiloride was a gift from Merck, Sharp and Dohme. All other chemicals were analytical grade.

Animals. Adult male Wistar rats weighing 300–350 g and fed normal laboratory chow were used throughout this study.

Preparation of vesicles. Each batch of brush-border membrane vesicles originated from jejunal and ileal segments of small intestine freshly obtained from two or three rats. Under light ether anaesthesia, the intestine was removed and rinsed three times with 20 ml icecold 0.9% NaCl. All further steps were performed at 0–4°C. Intact brush borders were obtained by mechanical vibration (50 Hz, amplitude 1.5 mm) of everted intestine for 25 min in Tris-buffered EDTA (2.5 mM), removal of nuclear aggregates with glass-wool and low speed centrifugation as described by Harrison and Webster [24]. Removal of terminal web and cytoskeletal elements, and vesiculation of the microvillous membrane was effected by exposing the brush border to 0.52 M KSCN as described by Hopfer et al. [23]. Vesicles isolated according to this procedure (hereafter referred to as 'KSCN-brush-border membrane vesicles') are virtually depleted of cytoskeletal proteins (e.g. actin, myosin, the 110 kDa cross filament [22] and calmodulin [14]) as judged by protein staining patterns of SDS-acrylamide gels and a ^{125}I-calmodulin radioimmunoassay (results not shown).

Alternatively, intestinal villous cells were released by mechanical vibration and microvillous vesicles were generated by a freeze-thaw technique as described previously [13,25]. Vesicles obtained by this method (hereafter refered to as 'Mg-brush-border membrane vesicles') were purified by differential Mg^{2+} precipitation and a washing step as desribed in Ref. 25. For most experiments,

vesicles were finally resuspended in buffer A (10 mM Hepes-Tris/0.3 M mannitol (pH 7.0)) by means of a Potter-Elvehjem homogenizer.

Radiotracer flux measurements. Initial rates of [^{3}H]glucose uptake into the vesicles in the presence of an inwardly directed gradient of Na^+ (0.1 M) and the half-filling time for [^{3}H]glucose uptake under isotope equilibrium exchange conditions were determined as described previously [25] using a semi-automatic rapid mixing and stopping apparatus (constructed according to Kessler et al. [26]) and nitrocellulose filters (Millipore, pore size 0.45 μm) to separate vesicles from the medium.

The uptake of $^{22}Na^+$, $^{86}Rb^+$ and $^{36}Cl^-$ radiotracers was measured by mixing 50–200 μl of a brush-border membrane vesicle suspension in buffer A (1–2 mg protein per ml) with an equivolume of Na^+, K^+ or Cl^- salts (0.2 M) in buffer A containing 1–4 μCi of the various isotopes. With various time intervals, samples of the mixture (50 μl) were loaded on minicolumns (0.6 ml of packed resin) of Dowex AG 50W-X8 (Tris form, 50–100 mesh) or Dowex AG 1-X8 (gluconate form, 50–100 mesh) to promote rapid and quantitative binding of labeled cations or anions present in the vesicle medium. Vesicles were quickly eluted with 1 ml icecold buffer and processed for liquid scintillation counting (^{36}Cl, $^{86}Rb^+$) or γ-emission spectroscopy ($^{22}Na^+$). The ion exchange separation technique was found superior to Millipore filtration in respect to reproducibility, radioactivity of blanks obtained in the absence of vesicles (5% of vesicle uptake), and separation time (less than 15 s) limiting the efflux of radiotracers during the separation phase.

Light-scattering measurements. Osmotically induced volume changes in brush-border membrane vesicles were detected by measuring the changes in light-scattering intensity at 470 nm (slit width 15 nm) at right angle to the incident beam using an Aminco DW2 spectrophotometer operated in the dual beam mode. Brush-border membrane vesicles suspended in buffer B (10 mM Hepes-Tris/0.1 M mannitol (pH 7.0)) to a concentration of 0.4–0.6 mg protein per ml were mixed at 25°C in a stopped-flow apparatus (Aminco) with an equivolume (100 μl) of buffer B containing 0.1 M salt or 0.2 M additional mannitol. The light-scattering signal was plotted graphically and recorded simultaneously by a data storage system (Dasar) for subsequent mathematical analysis [27].

Manipulation of intravesicular Ca^{2+} levels. Intra- and extravesicular Ca^{2+} levels were equilibrated by incubating brush-border membrane vesicles at 0°C for at least 15 min in the presence of 1 mM EGTA/Ca buffers and 20 μM Ca^{2+}-ionophore A23187 (cf. Ref. 13). Free Ca^{2+} concentrations of the buffers were calculated as described by Bers [28] and verified with a Ca^{2+}-electrode (Radiometer).

Biochemical assays. Protein was determined by the procedure of Lowry et al. [29] using bovine serum albumin as a standard.

Data evaluation. All values shown in the figures and tables represent means of triplicate experiments with a single batch of vesicles and the outcome was representative of at least two other batches of freshly isolated vesicles. The significance of the differences between two conditions was calculated by the (unpaired) Student's *t*-test.

Results

Effects of Ca^{2+} on Na^+-driven glucose uptake

In brush-border membrane vesicles, the initial rates of Na^+-dependent glucose transport are linearly related to the electrochemical driving force for Na^+, which in turn is a function of the membrane potential and the Na^+ concentration gradient across the membrane [30]. The transient accumulation of [^{3}H]glucose by the Na^+-symport carrier could therefore be used as a diagnostic tool to analyze Ca^{2+}-induced permeability changes of the vesicle membrane towards Na^+ and anions.

As shown in Fig. 1, the initial rates of glucose-uptake expressed per mg of protein, but not the glucose 'overshoot' defined as: $[glucose]_{in}^{max}/[glucose]_{in}^{equilibrium}$, was considerably higher in KSCN- as compared to Mg-brush-border membrane vesicles, apparently due to the removal of cytoskeletal proteins by the KSCN treatment (cf. Refs. 23 and 31). Raising the intra- and extravesicular free Ca^{2+} levels in KSCN-vesicles from 10^{-7} to 10^{-5} M led to a 50% reduction in the initial rate of glucose uptake and in glucose overshoot driven by a NaSCN gradient, but did not affect the equilibrium concentration of intravesicular glucose. Under isotope equilibrium conditions how-

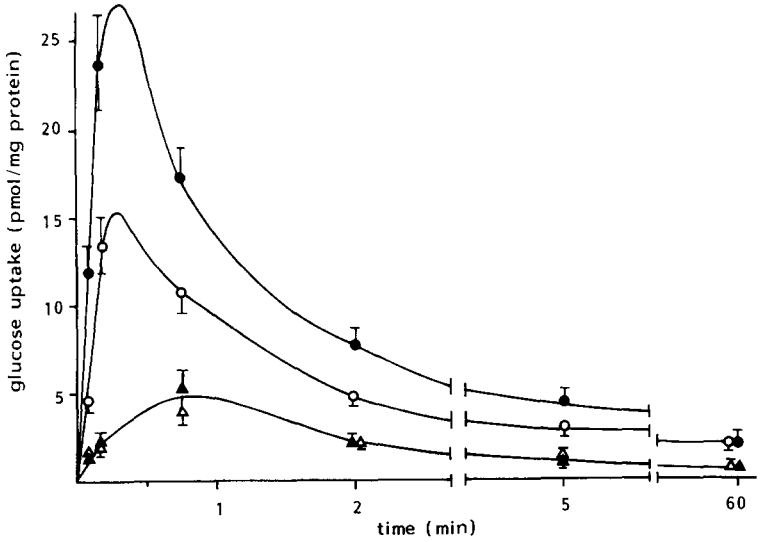

Fig. 1. Na^+-dependent glucose transport in intestinal brush-border membrane vesicles plotted as a function of time. KSCN-vesicles (●, ○) or Mg-vesicles (▲, △) in buffer A were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and 1 mM EGTA/Ca mixtures buffered at p*Ca* 7.0 (●, ▲) or p*Ca* 5.0 (○, △). Na^+-driven glucose uptake was measured in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M NaSCN (see Materials and Methods). Vertical bars indicate S.E.

ever, the half-time ($t_{1/2}$) for maximal uptake of labeled glucose (determined according to Ref. 25) appeared insensitive to variations in Ca^{2+} levels (9.6 ± 1.8 s at p*Ca* 7.0; 9.4 ± 2.0 s at p*Ca* 5.0; $n = 4$) arguing against a direct effect of Ca^{2+} on the glucose carrier itself. In contrast to these findings in KSCN-vesicles, Na^+-dependent glucose uptake in Mg-brush-border membrane vesicles appeared unresponsive over a wide range of free Ca^{2+} concentrations (10^{-7}–10^{-3} M; Fig. 1).

Half-maximal inhibition of glucose uptake in KSCN-vesicles by Ca^{2+} was reached at 10^{-6} M (Fig. 2). If Ca^{2+}-depleted vesicles, preloaded with 0.2 mM EGTA during the vesiculation step and incubated in a medium containing 0.2 mM EGTA and Ca^{2+}-ionophore, were exposed to Ca^{2+} (10^{-3}

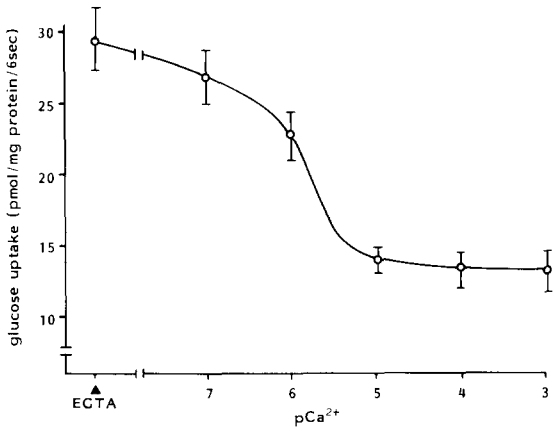

Fig. 2. Na^+-dependent glucose transport in KSCN-brush-border membrane vesicles plotted as a function of p*Ca*. KSCN-vesicles in buffer A were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and 1 mM EGTA/Ca buffers covering a broad range of p*Ca*. Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M NaSCN (see Materials and Methods). Vertical bars indicate S.E.

TABLE I

INHIBITION OF Na^+-DEPENDENT GLUCOSE TRANSPORT IN KSCN-BRUSH-BORDER MEMBRANE VESICLES BY Ca^{2+}: RAPID ONSET IN THE PRESENCE OF Ca^{2+}-IONOPHORE

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M NaSCN (see Materials and Methods) following preincubation of KSCN-vesicles (prepared in buffer A containing 0.2 mM EGTA) for various times at 25°C in the presence or absence of Ca^{2+}-ionophore A23187 (20 μM) and 1 mM Ca^{2+}. In case of preincubation 0 min, Ca^{2+} was added to the radiotracer mixture instead of to the vesicle suspension. Data are means of triplicate experiments ± S.E.

Preincubation time (min)	Addition of Ca^{2+} (1 mM)	[^{3}H]Glucose uptake (pmol/0.1 min per mg protein)	
		−A23187	+A23187
0	−	21 ± 2	21 ± 2
0	+	23 ± 2	11 ± 1 [a]
0.5	+	21 ± 2	10 ± 1 [a]
2.5	+	20 ± 2	11 ± 1 [a]
30	+	13 ± 1	11 ± 1

[a] $P < 0.05$ compared to data in absence of ionophore.

TABLE II

INHIBITION OF Na^+-DEPENDENT GLUCOSE TRANSPORT IN KSCN-BRUSH-BORDER MEMBRANE VESICLES BY Ca^{2+}: DEMONSTRATION OF ITS REVERSIBILITY

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M NaSCN (see Materials and Methods) following preincubation of KSCN-vesicles in buffer A plus Ca^{2+}-ionophore A23187 (20 μM) for 15 min at 0°C in the presence or absence of 1 mM EGTA/Ca buffers. Data are means of triplicate experiments ± S.E.

Preincubation conditions	[^{3}H]Glucose uptake (pmol/0.1 min per mg protein)
No additions	9 ± 1
EGTA/Ca (p*Ca* 5.0)	9 ± 1
EGTA/Ca (p*Ca* 7.0)	17 ± 2 [a]
EGTA/Ca (p*Ca* 7.0) followed by 1 mM Ca^{2+} (1 min)	9 ± 1 [b]

[a] $P < 0.05$ compared to p*Ca* 5.0.
[b] $P < 0.05$ compared to p*Ca* 7.0.

M), full inhibition of glucose transport was reached without a detectable lag phase (Table I). In the absence of Ca^{2+}-ionophore, however, a similar inhibition was obtained only after 30 min, confirming the low basal Ca^{2+} permeability observed earlier in Mg-brush-border membrane vesicles loaded with a Ca^{2+}-probe [13]. The acceleration of Ca^{2+} inhibition in the presence of ionophore clearly demonstrates that the Ca^{2+}-inhibitory site is localized at the interior of the vesicles.

As shown in Table II, KSCN-brush-border membrane vesicles preincubated in the absence or presence of EGTA/Ca buffer (p*Ca* 5.0) plus Ca^{2+}-ionophore displayed similar rates of glucose uptake, suggesting that free Ca^{2+} levels at the interior of freshly isolated KSCN-vesicles were at least in the micromolar range. Such a level was anticipated in view of the rather high concentrations of Ca^{2+} ($5 \cdot 10^{-6}$–10^{-5} M) measured in buffer A. The reversibility of Ca^{2+}-triggered transport inhibition is demonstrated in the same table by showing that initial exposure of KSCN-brush-border membrane vesicles to an EGTA/Ca buffer (p*Ca* 7.0) plus ionophore led to a 2-fold increase of the glucose uptake rate whereas subsequent titration with excess Ca^{2+} (10^{-3} M) again lowered

TABLE III

Na^+-DEPENDENT GLUCOSE TRANSPORT IN INTESTINAL BRUSH-BORDER MEMBRANE VESICLES; EFFECT OF ANION REPLACEMENT, VOLTAGE CLAMPING AND Ca^{2+}

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M Na^+-anion (see Materials and Methods). KSCN-vesicles in buffer A were preincubated for 30 min at 0°C in the presence of the Ca^{2+}-ionophore A23187 (20 μM) plus 1 mM EGTA/Ca buffer (p*Ca* 5.0 or 7.0). Voltage clamping was achieved by loading the vesicles during preincubation (60 min, 0°C) with 50 mM potassium gluconate plus 10 μM valinomycin (final concentration of potassium gluconate in transport medium: 25 mM). Mg-vesicles in buffer A were preincubated and voltage clamped in the absence of Ca buffers. Data are means of triplicate experiments ± S.E. n.d., not determined.

Anion	Voltage clamping	[^{3}H]Glucose uptake (pmol/0.1 min per mg protein)		
		KSCN-vesicles		Mg-vesicles
		p*Ca* = 7.0	p*Ca* = 5.0	
SCN^-	–	23 ± 2	12 ± 1 [a]	4.5 ± 0.5
	+	21 ± 1	15 ± 2 [a]	2.5 ± 0.3
NO_3^-	–	14 ± 1	8.1 ± 0.7 [a]	1.7 ± 0.2
	+	15 ± 2	16 ± 2	1.8 ± 0.2
Cl^-	–	4.0 ± 0.5	2.7 ± 0.3 [a]	0.5 ± 0.1
	+	11 ± 1	11 ± 1	1.5 ± 0.2
Gluconate$^-$	–	2.4 ± 0.3	2.4 ± 0.3	n.d.
	+	9.0 ± 1.1	9.2 ± 1.0	n.d.

[a] $P < 0.05$ compared to p*Ca* 7.0.

the transport rate to a value seen in the absence of Ca^{2+} buffers.

Table III shows a comparison of the effects of anion replacement and voltage clamping on initial rates of Na^+-driven glucose uptake in KSCN-brush-border membrane vesicles, exposed to 10^{-7} or 10^{-5} M free Ca^{2+}, and in Mg-vesicles (no Ca^{2+} buffers added). Clamping of the vesicle membrane at a slightly negative potential (inside) was accomplished by preincubation in the presence of potassium gluconate plus valinomycin and diluting the vesicle suspension 2-fold in the transport assay. The replacement of a monovalent anion by a more permeable species is expected to result in a change in membrane potential (inside negative) leading to acceleration of Na^+-driven glucose transport (cf. Ref. 30). The anion permeability sequence of conductance pathways suggested by the anion replace-

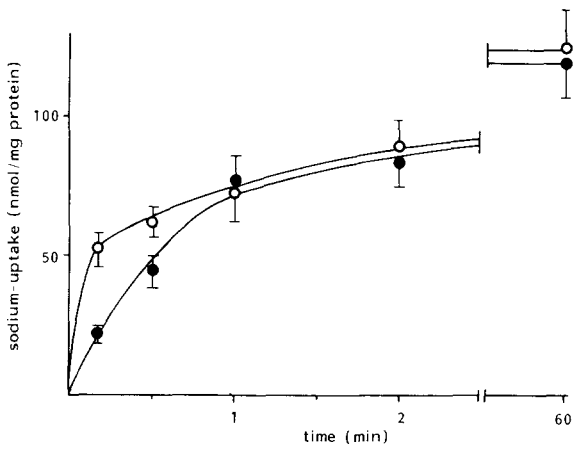

Fig. 3. Uptake of $^{22}Na^+$ in KSCN-brush-border membrane vesicles plotted as a function of time. KSCN-vesicles in buffer A were preincubated for 15 min at 0°C in the presence of 1 mM amiloride, Ca^{2+}-ionophore A23187 (20 μM) and either 1 mM EGTA alone (●) or 1 mM EGTA/Ca buffered at p*Ca* 5.0 (○). Vesicular $^{22}Na^+$ uptake in the presence of 0.1 M NaSCN was measured at 25°C as a function of time by a method involving rapid removal of $^{22}Na^+$ from the medium by cation exchange on minicolumns of Dowex AG 50W-X8 as described in Materials and Methods. Vertical bars indicate S.E.

ment data in Table III (first and third column; no voltage clamping) was similar for Ca^{2+}-poor KSCN-vesicles and Mg^{2+}-vesicles ($P_{SCN^-} > P_{NO_3^-} > P_{Cl^-} > P_{gluconate}$). In the clamped condition, Na^+-driven glucose uptake was slightly decreased (NaSCN), strongly increased (NaCl, sodium gluconate) or maintained at the same level ($NaNO_3$). This behaviour is consistent with a permselectivity sequence $P_{SCN^-} > P_{NO_3^-} > P_{Na^+} > P_{Cl^-} > P_{gluconate}$ creating an inside negative (NaSCN, $NaNO_3$) or inside positive (NaCl, sodium gluconate) diffusion potential across the vesicle membrane. Interestingly, a shift in p*Ca* from 7.0 to 5.0 substantially lowered the rate of glucose uptake in response to a NaSCN, $NaNO_3$ and NaCl gradient but failed to affect glucose transport rates in the voltage clamped condition (except in case of NaSCN, possibly due to incomplete clamping in the presence of the highly permeable SCN^- ion). Both observations are diagnostic for a Ca^{2+}-induced increase of the P_{Na^+}/P_{anion} ratio of the vesicle membrane leading to membrane depolarization. Such a change may originate from an increase of P_{Na^+} or a decrease of P_{anion}. Less indirect measurements of cation and anion permeabilities were needed to discriminate between both possibilities.

Effect of Ca^{2+} on $^{22}Na^+$, $^{86}Rb^+$ and $^{36}Cl^-$ uptake

In the presence of amiloride, an inhibitor of the Na^+/H^+ antiporter [19], and in the absence of Na^+-cotransport, sodium mainly enters the brush-border membrane through conductance pathways [7,20]. Using SCN^- as the counterion, the initial uptake of $^{22}Na^+$ into KSCN-brush-border membrane vesicles under these conditions showed a 2-fold increase in response to 10^{-5} M Ca^{2+} (Fig. 3). Considering the Ca^{2+} inhibition of NaSCN-

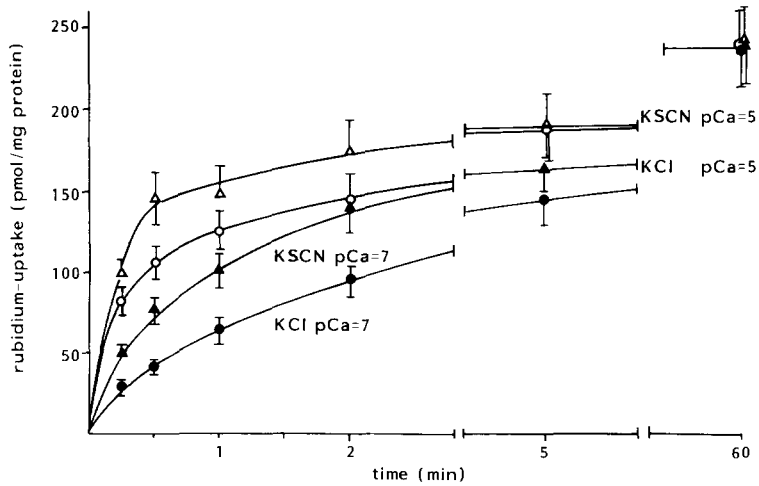

Fig. 4. Uptake of $^{86}Rb^+$ in KSCN-brush-border membrane vesicles plotted as a function of time. KSCN-vesicles in buffer A were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and either 1 mM EGTA alone (▲, ●) or 1 mM EGTA/Ca buffered at p*Ca* 5.0 (△, ○). Vesicular $^{86}Rb^+$ uptake in the presence of 0.1 M KSCN (△, ▲) or 0.1 M KCl (○, ●) was measured at 25°C as a function of time by a method involving rapid removal of $^{86}Rb^+$ from the medium by cation exchange on minicolumns of Dowex AG 50W-X8 as described in Materials and Methods. Vertical bars indicate S.E.

TABLE IV

UPTAKE OF $K^{36}Cl^-$ INTO KSCN-BRUSH-BORDER MEMBRANE VESICLES: EFFECTS OF Ca^{2+} AND K^+-IONOPHORE

KSCN-vesicles were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and either 1 mM EGTA or 1 mM EGTA/Ca^{2+} buffer (p*Ca* 5.0) plus or minus the K^+-ionophore valinomycin (10 μM). Vesicular $^{36}Cl^-$ uptake in the presence of 0.1 M KCl was measured at 25°C as a function of time of a method involving rapid removal of $^{36}Cl^-$ from the medium by anion exchange on minicolumns of Dowex AG1-X8 as described in Materials and Methods. Data are means of triplicate experiments ± S.E.

Preincubation condition	$^{36}Cl^-$ uptake (nmol/mg protein)	
	0.25 min	0.5 min
EGTA	4.4 ± 0.5	8.3 ± 0.8
EGTA/Ca (p*Ca* 5.0)	11.3 ± 1.2 [a]	17 ± 2.0 [a]
EGTA + valinomycin	8.1 ± 0.9	15 ± 1.4
EGTA/Ca (p*Ca* 5.0) + valinomycin	15.3 ± 1.4 [b]	22 ± 2.0 [b]

[a] $P < 0.5$ compared to EGTA alone.
[b] $P < 0.05$ compared to EGTA + valinomycin.

driven glucose uptake shown above (Figs. 1, 2; Table III), its effect on $^{22}Na^+$ uptake seems more consistent with an increase of P_{Na^+} than with an increase of P_{SCN^-}. As shown in Fig. 4, Ca^{2+} caused an additional increase of the influx of $^{86}Rb^+$ measured in the presence of a KSCN or KCl gradient, without changing equilibrium uptake. Since K^+ and Rb^+ usually share the same transport system (cf. Ref. 7), these experiments are indicative for a Ca^{2+}-triggered increase of the K^+ permeability of the brush-border membrane. In the presence of 0.1 M KCl, Ca^{2+} was also able to increase the initial rate of $^{36}Cl^-$ uptake in KSCN-brush-border membrane vesicles (Table IV). This increase could not be explained entirely by an increase of P_{K^+}, since the K^+-ionophore valinomycin was unable to abolish the effect of Ca^{2+} on $^{36}Cl^-$ uptake. The results of radiotracer experiments taken together point to a general enhancement of both cation and anion permeabilities of the vesicle membrane by micromolar Ca^{2+}.

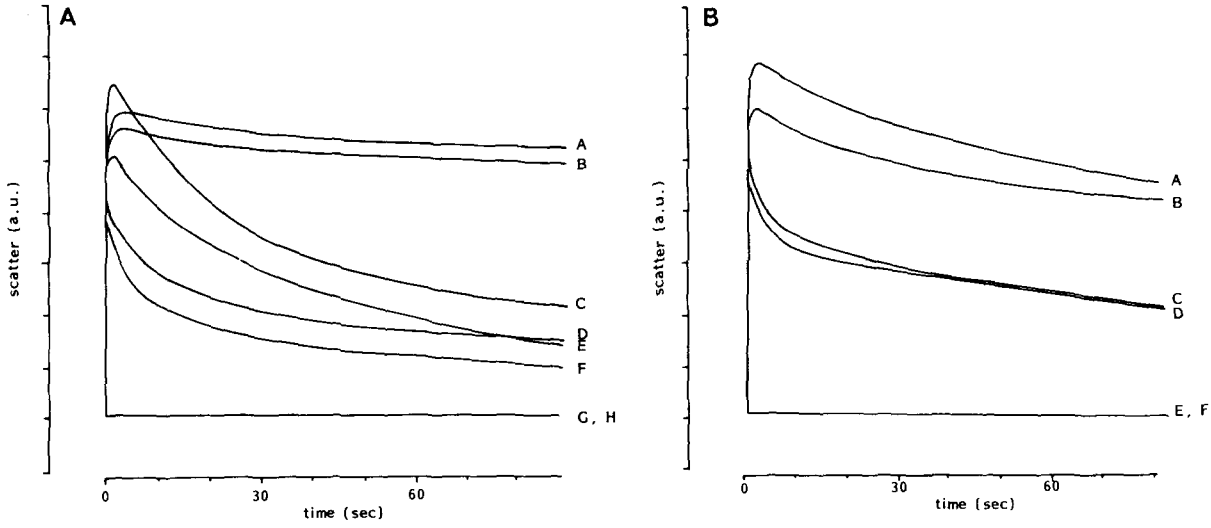

Fig. 5. Effects of Ca^{2+} on osmotic volume changes of KSCN-brush-border membrane vesicles in response to salt or mannitol gradients. KSCN-vesicles in buffer B were preincubated for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and either 1 mM EGTA alone (−) or 1 mM EGTA/Ca buffered at p*Ca* 5.0 (+). Shrinkage-reswelling of vesicles in response to 0.05 M salt or 0.1 M mannitol in buffer B was monitored by registration of changes in light-scatter (plotted as arbitrary units) in a stopped-flow apparatus attached to a spectrophotometer as described in Materials and Methods. The plots shown were representative for at least two other batches of vesicles. Conditions in Fig. 5A: A (−, mannitol); B (+, mannitol); C (−, KSCN); D (+, KSCN); E (−, NaSCN); F (+, NaSCN); G (−, buffer B alone); H (+, buffer B alone). Conditions in Fig. 5B: A (−, KCl); B (−, NaCl); C (+, KCl); D (+, NaCl); E (−, buffer B alone); F (+, buffer B alone).

Effect of Ca^{2+} on salt-induced osmotic swelling

To further assess the influence of Ca^{2+} on the permeability of KSCN-brush-border membrane vesicles for monovalent ions and uncharged solutes, osmotic volume changes of a vesicle suspension in response to hypertonic salt or mannitol solutions were measured by monitoring rapid changes in light-scattering. For a restricted range of vesicle osmolarities (below 1 osM), a linear relationship between scattered light intensity and vesicle volume has been established with apical membranes from various sources [27,32]. A fast increasing phase in scattering intensity (less than 0.5 s), corresponding to shrinkage of the vesicles as a result of water outflow, is followed by a slower reswell phase, dependent on solute entry into the intravesicular space. Therefore the velocity of the reswell phase in response to a salt gradient is a function of the total permeability (P') of the vesicle membrane to the least permeable cation or anion.

The results of the osmotic experiments, shown in Figs. 5 and 6, confirm and replenish the glucose transport and radiotracer studies.

(1) In the presence of a highly permeable anion (SCN^-), initial rates of reswelling in response to Na^+ or K^+ gradient were dramatically increased at 10^{-5} M Ca^{2+} as compared to EGTA alone (Fig. 5A) confirming a Ca^{2+}-triggered increase of P'^{+}_{Na} and P'^{+}_{K}. Apparently, P'^{+}_{Na} is slightly higher than P'^{+}_{K} under both conditions.

(2) Ca^{2+} did not simply increase the leakiness of the membrane to low-molecular weight solutes, as demonstrated by the lack of Ca^{2+} effects on the permeability for mannitol (Figs. 5A and 6A; curve A versus B) and its minor effect on the permeability for gluconate (measured in the presence of K^+ plus valinomycin; Fig. 6B, curve A versus B).

(3) As shown by comparison of the reswell curves A and B in Fig. 5B with curves C and E in Fig. 5A, the P'_{Cl^-} of the brush-border membrane vesicles must be much lower than P'_{SCN^-}, and P'_{Na^+} or P'_{K^+} are apparently also larger than P'_{Cl^-}, in agreement with the permselectivity sequence suggested by the glucose uptake study. The reswell rates in response to NaCl and KCl gradients were

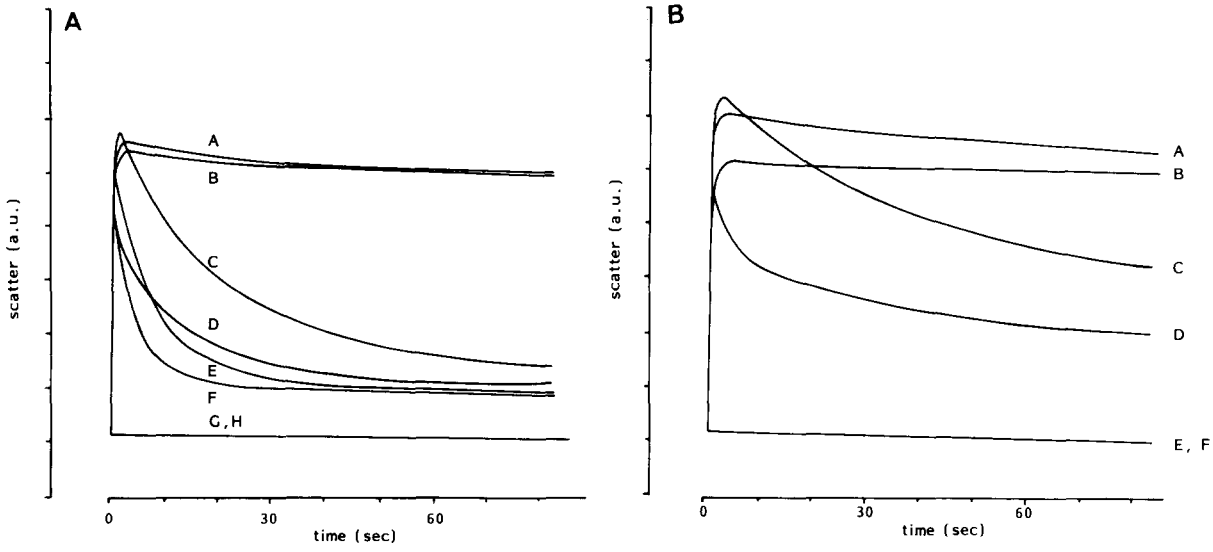

Fig. 6. Effect of Ca^{2+} on osmotic volume changes of KSCN-brush-border membrane vesicles in response to K^+-salts in the presence of K^+-ionophore. KSCN-vesicles in buffer B were preincubated for 15 min at 0°C in the presence of the K^+-ionophore valinomycin (10 μM) and the Ca^{2+}-ionophore A 23187 (20 μM) and either 1 mM EGTA alone (−) or 1 mM EGTA/Ca buffered at p*Ca* 5.0 (+). Shrinkage-reswelling of vesicles in response to 0.05 M K^+ salt or 0.1 M mannitol in buffer B was monitored by registration of changes in light-scatter (plotted as arbitrary units) in a stopped-flow apparatus attached to a spectrophotometer as described in Materials and Methods. The plots shown are representative for at least two other batches of vesicles. Conditions in Fig. 6A: A (−, mannitol); B (+, mannitol); C (−, NO_3^-); D (+, NO_3^-); E (−, SCN^-); F (+, SCN^-); G (−, buffer B alone); H (+, buffer B alone). Conditions in Fig. 6B: A (−, gluconate$^-$); B (+, gluconate$^-$); C (−, Cl^-); D (+, Cl^-); E (−, buffer B alone); F (+, buffer B alone).

strongly increased in the presence of Ca^{2+} (Fig. 5B, curves C and D), suggesting an activating effect of Ca^{2+} on P'_{Cl^-}.

(4) If it is assumed that reswelling rates in the presence of K^+ plus valinomycin mainly reflect the anion permeability of the vesicle membrane, the anion permselectivity sequence suggested by comparison of curves C and E in Fig. 6A and curves C and A in Fig. 6B was again similar to the electrogenic conductance sequence concluded from the glucose transport measurements, i.e., $P'_{SCN^-} > P'_{NO^-_3} > P'_{Cl^-} > P'_{gluconate^-}$. Moreover, Ca^{2+} caused a profound acceleration of the reswell process in the presence of Cl^- (Fig. 6B, curve D versus C) and NO_3^- (Fig. 6A, curve D versus C), directly confirming a Ca^{2+} provoked increase of the anion permeability of the vesicle membrane. A partial selectivity of the Ca^{2+}-sensitive anion permeability was suggested by the much smaller Ca^{2+}-induced changes observed in the presence of SCN^- (Fig. 6A, curve F versus E) and gluconate$^-$ (Fig. 6B, curve B versus A).

TABLE V

EFFECT OF VARIOUS COMPOUNDS ON THE INHIBITION OF Na^+-DEPENDENT GLUCOSE TRANSPORT IN KSCN-BRUSH-BORDER MEMBRANE VESICLES BY Ca^{2+}

Na^+-driven glucose uptake was measured at 0.1 min in the presence of 1.0 μM [^{3}H]glucose plus 0.1 M NaSCN (see Materials and Methods) following preincubation of KSCN-vesicles in buffer A for 15 min at 0°C in the presence of Ca^{2+}-ionophore A23187 (20 μM) and either 1 mM EGTA or 1 mM EGTA/Ca buffer (p*Ca* 5.0). Compounds to be tested were present during preincubation. Data are means of triplicate experiments ± S.E.

Compound	Concn. (M)	[^{3}H]Glucose uptake (pmol/0.1 min per mg protein)		
		EGTA	EGTA/Ca p*Ca* 5.0	% inhibition of Ca^{2+} effect
–	–	23 ± 2	13 ± 1	0
Amiloride	10^{-3}	24 ± 3	13 ± 1	0
Apamin	$2 \cdot 10^{-7}$	23 ± 1	14 ± 1	0
Ba^{2+}	10^{-3}	22 ± 2	23 ± 2 [a]	100
Ba^{2+}	$3 \cdot 10^{-4}$	23 ± 2	18 ± 2 [a]	50
Mg^{2+}	10^{-3}	23 ± 2	17 ± 2 [a]	40
Mg^{2+}	10^{-2}	21 ± 2	22 ± 2 [a]	100
Quinine	$5 \cdot 10^{-4}$	23 ± 2	13 ± 1	0
Trifluoperazine	10^{-4}	24 ± 3	12 ± 1	0

[a] $P < 0.05$ compared to control (–).

Blockade of the Ca^{2+} effect by Mg^{2+} and Ba^{2+}

Using Na^+-dependent glucose transport as a probe to monitor Ca^{2+} modulation of vesicular ion permeability, various compounds were tested for their ability to antagonize this Ca^{2+} effect (Table V). Amiloride, a blocker of Na^+ channels in colon epithelium and of Na^+-H^+ exchange in ileal brush-border membrane vesicles appeared ineffective. Specific blockers of Ca^{2+}-activated K^+ channels, i.e. quinine and apamin, and trifluoperazine, a calmodulin antagonist, were likewise incapable of counteracting Ca^{2+} inhibition of glucose uptake (Table V) or Ca^{2+}-triggered $^{36}Cl^-$ uptake (results not shown). In contrast, Ba^{2+} ions at a rather low concentration (10^{-3} M) completely antagonized the effect of Ca^{2+} on glucose uptake (Table V) as well as on $^{36}Cl^-$ and $^{86}Rb^+$ influx and salt-induced osmotic swelling (not shown). Another bivalent cation, Mg^{2+}, only partially inhibited Ca^{2+} modulation at 10^{-3} M (30–50% inhibition) but was completely inhibitory at 10^{-2} M. Most likely, this Mg^{2+} 'brake' (cf. Ref. 33) is also responsible for the lack of Ca^{2+} effects on ion permeabilities in Mg-brush-border membrane vesicles since (i) KSCN-vesicles transiently exposed twice to 10^{-2} M Mg^{2+} in order to mimic the isolation conditions of Mg-vesicles, had lost their Ca^{2+} sensitivity; and (ii) extraction of residual Mg^{2+} from Mg-vesicles by incubation for 30 min at room temperature in the presence of 5 mM EDTA and 20 μM A23187, unmasked a similar Ca^{2+}-sensitivity of the Na^+-dependent glucose transport process as found routinely in KSCN-vesicles (results not shown). The dependence on Ca^{2+}-/Mg^{2+}-ionophore for sensitization is diagnostic for a localization of the Mg^{2+}-inhibitory site at the vesicle interior.

Discussion

By using three independent techniques to characterize salt permeabilities of rat intestinal brush-border membrane vesicles prepared by the thiocyanate method, a Ca^{2+}-triggered increase of both cation and anion permeabilities of the vesicle membrane by micromolar levels of Ca^{2+} has been

clearly demonstrated. On the basis of Na^+-dependent glucose uptake measurements, the following conclusions could be drawn: (i) Ca^+ apparently acts through a high-affinity receptor ($K_d \cong 10^{-6}$ M; fig. 2) localized at the interior of the vesicle membrane (Table I); (ii) in view of the depletion of cytoskeletal elements in KSCN-vesicles, interaction of Ca^{2+} with Ca^{2+}-binding proteins of the microvillar core e.g. villin [22] seems unlikely; (iii) the action of Ca^{2+} was fully reversible (Table II), arguing against the involvement of a Ca^{2+}-activatable phospholipase (rapid resynthesis of phospholipids is hampered by a lack of energy source, e.g. ATP or CTP, at the vesicle interior); (iv) the Ca^{2+} effect on transport is blocked partially by 10^{-3} M Mg^{2+} and completely by 10^{-3} M Ba^{2+} or 10^{-2} M Mg^{2+}, suggesting that these bivalent cations compete with Ca^{2+} for a common binding site (cf. the effect of Ba^{2+} on Ca^{2+}-activated K^+ channels in renal apical membranes; Ref. 33). The Mg^{2+}-brake may also explain why Ca^{2+} modulation of glucose or ion transport has not been observed earlier by us and others in Mg-brush-border membrane vesicles; depletion of Mg^{2+} with EDTA and the Ca^{2+}/Mg^{2+}-ionophore A23187, however, resensitized the ion channels to micromolar levels of Ca^{2+}. Because the physiological level of free Mg^{2+} at the interior of the microvilli is unknown (probably around 1–2 mM), the efficacy of the Mg^{2+}-brake in the intact enterocyte is difficult to assess; (v) no evidence was found of a direct effect of Ca^{2+} on the Na^+/glucose symporter; moreover, Ca^{2+} did not induce a general leakiness of the vesicle membrane to small molecules, e.g., glucose (Fig. 1, equilibrium uptake) or mannitol (Figs. 5 and 6); (vi) the reduction of Ca^{2+} effects on Na^+-driven glucose transport under voltage clamped conditions (Table III) indicated a major effect of Ca^{2+} on Na^+ and/or anion conductance pathways in the vesicle membrane (increase of P^+_{Na} or decrease of P_{anion}). Because the radiotracer and osmotic swelling experiments provided evidence for a Ca^{2+}-provoked increase of both anion and cation permeabilities, the inhibitory effect on glucose uptake can only result from an increase of P^+_{Na}, apparently not fully compensated by a concomitant rise in P_{anion} (Ca^{2+}-triggered permselectivity change).

Osmotic shrinkage-swelling experiments (Figs. 5 and 6) in principle enabled us to study the effects of Ca^{2+} on both carrier-mediated and conductance pathways for ions, avoiding complications arising from ion binding to the vesicle membrane. As shown earlier for gastric apical membrane vesicles [27], salt-induced reswelling rates measured in Ca^{2+}-depleted KSCN-brush-border membrane vesicles could be fitted to a double exponential (Vaandrager, A.B., unpublished data), suggesting a functional heterogeneity of the vesicle population. Upon exposure to Ca^{2+}, the first phase, in contrast to the second (slow) phase, was accelerated dramatically under conditions that either Na^+ and K^+ (Fig. 5A) or NO_3^- and Cl^- (Figs. 6A and B) were the rate-limiting ions. The fall in peak height of the shrinkage-swelling curve additionally indicated that the salt permeability in the presence of Ca^{2+} was no longer orders of magnitude different from the water permeability of the vesicle. Although a quantitative description of the Ca^{2+} effect in terms of rate constants and ion permeability coefficients is hampered by the functional heterogeneity of the vesicle population, the data do suggest the existence of nonselective cation and anion channels unmasked by micromolar Ca^{2+} and presumably confined to a subpopulation. By comparison with Ca^{2+} inhibition of Na^+-dependent glucose uptake, a specific fucntion of the burshborder membrane, at least the change in Na^+ conductance is apparently localized in a subpopulation of vesicles originating with certainty from the intestinal microvilli.

It should be pointed out that, independent of the intravesicular Ca^{2+} level, the permeability sequence of Na^+ and Cl^- ions observed in both KSCN- and Mg-brush-border membrane vesicles ($P^+_{Na} > P^-_{Cl}$) was clearly opposite to the sequence reported for rabbit intestinal brush-border membrane vesicles prepared by the Ca^{2+}-precipitation method [20]. The reason for this discrepancy is not clear but may be related to the species difference or to the different vesicles isolation procedure.

At present we can only speculate about the molecular nature of the high-affinity Ca^{2+} receptor. Considering its insensitivity to quinine, apamin and trifluoperazine, the receptor does not seem to belong to the major class of Ca^{2+}-activatable K^+ channels (quinine- and apamin-inhibitable) neither can it be identical to calmodulin. This does not

disqualify calmodulin as a potential regulator of vesicular ion transport, e.g. neutral Na^+-Cl^- cotransport [18], because (i) the residual calmodulin content in KSCN-treated vesicles was extremely low ($2 \cdot 10^{-5}$ M) compared to Mg-vesicles ($5 \cdot 10^{-4}$ M; cf. Ref. 14); (ii) the conditions in our ion transport experiments (0.5–0.1 M salt) were unfavorable for the detection of saturable non-conductance pathways [7,20]. More complicated mechanisms of Ca^{2+} regulation, e.g. involving a Ca^{2+}-dependent phosphorylation process [2–4], can be excluded in view of a lack of ATP in KSCN-vesicles. Instead, the possible presence of Ca^{2+}-regulatory sites on the channels itself might well explain the survival of Ca^{2+} regulation in 'empty' brush-border membrane vesicles, its reversibility and its inhibition by Mg^{2+} and Ba^{2+}. Alternatively, the Ca^{2+} sensor site could be identical to a high-affinity Ca^{2+} binding component of the lipid bilayer, e.g. polyphosphoinositides [16,34], recently identified in intestinal brush-borders and KSCN-vesicles (Vaandrager, A.B. et al., unpublished data).

To our knowledge, no physiological equivalent of a Ca^{2+}-triggered nonselective cation channel has been assigned to the apical membrane of the enterocyte. Nonselective cation channels displaying a similar Ca^{2+}-sensitivity have been characterized in cardiac [35], neuroblastoma [36] and pancreatic acinar cells by a patch-clamp technique [37] but their relationship to the cation conductance in intestinal brush-border membrane vesicles is unclear. A Ca^{2+}-opened cation channel found in the apical membrane of rat colonocytes seemed rather specific for K^+ [38]. In some tissues, silent conductive channels for K^+ and Cl^- may be activated by intracellular Ca^{2+} to promote KCl efflux in response to osmotic swelling [39]. However, the existence of a similar mechanism of volume regulation in the enterocyte has not been demonstrated sofar. In contrast, a Ca^{2+}-triggered anion channel probably enriched in the apical membrane of the intestinal crypt cell, has been postulated as a major target for intestinal secretagogues acting through intracellular Ca^{2+} signals [1–4]. Although the vesicles used in the present study mainly originate from mature villous cells, it could be speculated that a silent anion channel in the brush-border membrane may become reactivated by the removal of inhibitory factors during the vesicle isolation procedure.

Regardless of its possible physiological significance, the finding of Ca^{2+}-activatable and Mg^{2+}-inhibitable conductance pathways for cations and anions in intestinal brush-border membrane vesicles may have important implications for other vesicles studies: first, dependent on the intravesicular Mg^{2+} level, the channels may become spontaneously activated by trace amounts of Ca^{2+} in the medium, leading to changes in permeability of the vesicle membrane and to diminished and variable rates of Na^+-driven cotransport processes; secondly, in the absence of a Mg^{2+}-brake, a possible inhibitory effect of Ca^{2+} on a Na^+-Cl^- cotransport system in the brush-border membrane [1,8] could become masked by a concomittant opening of Na^+ and Cl^- conductance pathways; finally, a better knowledge of the effect of physiological Ca^{2+} levels on ion transport characteristics of 'empty' brush-border membrane vesicles is a prerequisite for the subsequent study of Na^+ and Cl^- transport regulation by other physiological regulatory factors (e.g. ATP, cyclic nucleotides, calmodulin, protein kinases) entrapped at the vesicle interior during vesiculation [13] or by osmotic shock [40]. Studies along this line are in progress in our laboratory.

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