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Characterization of Mg^{2+} transport in brush border membrane vesicles of rabbit ileum studied with mag-fura-2

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Abstract

Mg^{2+} transport in rabbit ileal brush border membrane vesicles (BBMV) was characterized by means of a modified mag-fura-2 technique. In the presence of an $i > o$ Na^+ gradient, BBMV showed a saturable Mg^{2+} uptake with a K_m of 1.64 mmol l^{-1}. There was no evidence of an overshoot. K^+, Li^+, and choline$^+$ were as effective as Na^+ in stimulating Mg^{2+} transport. In contrast, only a small amount of Mg^{2+} transport was observed in the presence either of an $o > i$ Na^+ gradient, or in an Na^+ equilibrium or in the absence of Na^+. Moreover, the findings that Na^+ efflux was not stimulated but inhibited by outside Mg^{2+} and that the nonfluorescent amiloride-analogues DMA and EIPA did not affect Mg^{2+} transport do not favour the idea of an Mg^{2+}/Na^+ antiport system. At Cl^- equilibrium, independent of the Na^+ gradient, the rate of Mg^{2+} transport was markedly suppressed compared with the transport rate noted in the presence of an $i > o$ Cl^- gradient. The stimulating effect of inside anions could be enhanced by SCN^- and decreased by SO_4^{2-}. Furthermore, nonfluorescent anion transport antagonist H_2-DIDS stimulated Mg^{2+} transport. These findings indicate that Mg^{2+} transport can be modulated by inside anions. Mg^{2+} transport appeared to be electroneutral because it was not dependent on membrane potential. Mg^{2+} transport was neither stimulated by Bay K8644, a Ca^{2+} channel agonist, nor inhibited by verapamil, diltiazem, nifedipine and imipramine, the Ca^{2+} channel antagonists. It, therefore, seems unlikely that Mg^{2+} uses the Ca^{2+} transport system. © 1998 Elsevier Science B.V.

Keywords: Ileum; Brush border membrane vesicle; Magnesium transport; Mag-fura-2; (Rabbit)

1. Introduction

Although intestinal Mg^{2+} transport has long been studied under a wide variety of conditions, the actual mechanism of intestinal Mg^{2+} absorption has not yet been adequately characterized [1–3]. Evidence of a concentration gradient or solvent drag driven paracellular intestinal Mg^{2+} uptake representing the main route has been presented [4,5]. However, additional cellular-mediated intestinal Mg^{2+} transport is sup-

Abbreviations: BBMV, brush border membrane vesicles; H_2-DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid, disodium salt; DMA, 5-(*N*,*N*-dimethyl)amiloride, hydrochloride; DMSO, dimethyl sulfoxide; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride, hydrochloride; $i > o$ or $o > i$, from intra- to extravesicular directed ion gradient, or vice versa; LDH, lactate dehydrogenase; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; MET, mannitol in concentrations as indicated in 5 mM EGTA, 12 mM Tris, pH 7.4; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; X_i or X_o, concentration of substance X intravesicular or extravesicular BBMV

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ported by the following findings:

(a) the saturation of Mg^{2+} absorption with increasing Mg^{2+} as shown in perfusion studies in rats [6], human intestine [7], short circuited rat ileum [8] and colon [9]; and

(b) the existence of an inherited disorder of intestinal Mg^{2+} absorption [10,11] with a decrease of Mg^{2+} absorption in the lower saturable range of the curvilinear relationship between rate of Mg^{2+} absorption and Mg^{2+} concentration [11].

In order to define the cellular route, Mg^{2+} transport has been studied in rat intestinal BBMV [12–14] using the short-lived isotope ^{28}Mg. Unfortunately, with this method it is difficult to segregate transport, on the one hand, and the Mg^{2+} binding to BBMV on the other, which in case of the intestinal BBMV [13] and renal BBMV [15] can make up for as much as half of the total Mg^{2+} uptake. Isolated BBMV using a suitable indicator for the free Mg^{2+} offer advantages for the study of cellular Mg^{2+} uptake mechanisms in either intestine or kidney; but, to-date, no such studies have been carried out. In the present study, we found that BBMV were surprisingly able to hydrolyze and accumulate the membrane permeant esters of fluorescence indicators such as mag-fura-2/AM. We succeeded in applying the mag-fura-2 technique so that it was possible to characterize Mg^{2+} transport in isolated ileal BBMV. This fluorescence method which is sensitive for free intravesicular Mg^{2+} allows continuous monitoring of Mg^{2+} transport.

2. Materials and methods

The indicator mag-fura-2/tetrapotassium salt and its membrane permeant acetoxymethylester (mag-fura-2/AM) were obtained from Molecular Probes (Eugene, OR). Stock solutions of mag-fura-2/AM in DMSO were prepared with a final concentration in the transport assay of 0.02% or less. All other chemicals were purchased at the highest grade of purity available from Merck (Darmstadt, Germany), Serva Feinbiochemica GmbH (Heidelberg, Germany) or from Sigma (St. Louis, MO). Filtered, de-ionized water with a resistance of 15–18 MΩ cm, which was virtually Ca^{2+}- and Mg^{2+}-free, was used for solutions. ^{22}Na was obtained from DuPont NEN (Bad Homburg, Germany).

2.1. Isolation of BBMV

BBMV were prepared from the ileum of decapitated adult rabbits (2.0–2.5 kg). The distal third of the small intestine (60–90 cm) was removed and rinsed thoroughly with 0.9% saline, followed by gently scrapping the ileal mucosa. BBMV were prepared by means of an Mg^{2+}-EGTA precipitation method as originally developed by Hopfer et al. [16] and modified by Kikuchi et al. [17]. Briefly, the scrapped mucosa was homogenized, using a Waring commercial blender, in a hypoosmolal solution of 12 mmol $^{-1}$ Tris, pH 7.4, containing 60 mmol l^{-1} mannitol and 5 mmol l^{-1} EGTA, followed by precipitation with 10 mmol l^{-1} $MgCl_2$. After stirring for 30 min, the homogenate was centrifuged at $3500 \times g$ for 20 min. The supernatant was centrifuged at $49\,000 \times g$ for 25 min and the pellet resuspended in 60 mmol l^{-1} MET. After a second precipitation with 10 mmol l^{-1} $MgCl_2$, the two steps of centrifugation were repeated. The final pellet of BBMV pellet was resuspended in 300 mmol l^{-1} MET at a concentration of 4–8 mg protein ml^{-1} and stored in liquid nitrogen until used. The protein content was assayed using bicinchoninic acid [18] (BCA Protein Assay Reagent, Pierce) and bovine serum albumin as standard.

2.2. Purity and functional integrity of the BBMV

The purity of the BBMV fraction was routinely examined by measuring marker enzyme activities one day after isolation. Sucrase (EC 3.2.1.26), leucine aminopeptidase (EC 3.4.11.2) and alkaline phosphatase (EC 3.1.3.1) for BBMV and adenosine triphosphate phosphohydrolase (Na^+/K^+-ATPase, EC 3.6.1.3) for the basolateral membranes were measured as previously described [19]. A Glucoquant kit (Boehringer Mannheim GmbH, Germany) and saccharose as substrate were used for sucrase activity. Lysosomal β-glucuronidase (EC 3.2.1.31) and cytosolic lactate dehydrogenase (EC 1.1.1.27) were determined with Sigma kits. The functional integrity of the BBMV was tested by measuring the phlorizin sensitive Na^+/D-glucose transport [20].

The specific enrichment of the brush border membrane marker enzyme activity as compared with the

homogenate was 20.8 ± 1.1 ($n = 9$) for sucrase, 15.6 ± 1.1 ($n = 10$) for aminopetidase and 8.4 ± 0.5 ($n = 11$) for alkaline phosphatase. The activity enrichment for the basolateral marker enzyme Na^+/K^+-ATPase was 0.45 ± 0.07 ($n = 10$). Enrichment of the activity of β-glucuronidase, the marker enzyme of lysosomal contamination was 0.41 ± 0.05 ($n = 10$). The BBMV were deemed free of cytosolic contamination because of the lack of activity of lactate dehydrogenase (0.043 ± 0.014, $n = 8$). Preparations with an enrichment of < 13 for aminopeptidase or seven for alkaline phosphatase were discarded. For Na^+/D-glucose transport, the transport rate obtained was 572 pmol mg^{-1} protein 10 s^{-1} ± 19 ($n = 8$), which is consistent with earlier reported transport rates in small intestinal rat BBMV [21]. Glucose equilibrium experiments showed an intravesicular space of 3.61 ± 0.27 μl mg^{-1} protein ($n = 8$).

2.3. Incorporation of mag-fura-2 into the BBMV

Samples of brush border membrane vesicles BBMV suspensions (1 mg protein 200 μl^{-1}) were incubated with the membrane permeant ester of the indicator mag-fura-2, having a final concentration of 50 μmol l^{-1}. Incubation was performed at 25°C for 60 min. In order to remove extravesicular mag-fura-2 (generated by esterases on the external surface of the vesicles or by diffusion out of unsealed vesicles), BBMV were washed twice in the particular equilibrium solution, and resuspended at 1 mg protein ml^{-1}.

2.4. Measurement of mag-fura-2 fluorescence

Fluorescence measurements were performed in a luminescence spectrometer LS 50B (Perkin–Elmer, England) with an excitation and emission monochromator. The cuvette holder was stirred and thermostatically regulated.

2.5. Mg^{2+} transport measurements

Frozen BBMV were rapidly thawed at 37°C and equilibrated for 60 min at 25°C in the desired solution, which contained different salt concentrations in 5 mmol l^{-1} EGTA, 12 mmol l^{-1} Tris pH 7.4, balanced with mannitol to 300 mosmol kg^{-1}. BBMV were then sedimented and loaded with the indicator, followed by washing in 1 ml of the particular equilibrium solution in order to remove extravesicular generated indicator. Immediately after the resuspension of the BBMV (1 mg protein ml^{-1}), Mg^{2+} was added by rapid injection through a light proof cover of the cuvette holding. After mixing on line fluorescence recording was started. Since ~3 s were needed for adequate mixing and for stabilizing the scattering signal, recordings were not used before 5 s.

2.6. Determination of free intravesicular Mg^{2+} with mag-fura-2

The fluorescence of mag-fura-2 can be calibrated in terms of the free intravesicular Mg^{2+} concentration by using the following expression [22,23]:

$$\left[Mg^{2+}\right]_i = K_D\left((R - R_{min})/(R_{max} - R)\right)\left(S_{f2}/S_{b2}\right)$$

where K_D is the dissociation constant for mag-fura-2 with Mg^{2+}, R is the ratio of the emission fluorescence at the excitation wavelengths of 335 nm and 349 nm, R_{min} and R_{max} are the fluorescence ratios for uncomplexed mag-fura-2 at zero Mg^{2+} and for mag-fura-2 saturated with Mg^{2+}. S_{f2} and S_{b2} are the fluorescence intensities at 349 nm for mag-fura-2 with zero and excess Mg^{2+}, respectively. Parameters R_{max} and R_{min} were measured during each experiment. Fluorescence was corrected by subtracting autofluorescence for each corresponding point. R_{max} values were typically close to 1.25 and R_{min} 0.7. It should be pointed out that, for higher resolution, the second excitation wavelength used was the isobestic point rather than the other excitation maximum at 380 nm. When using excitation wavelengths of 335/349 nm, resolution was as high as 10 Hz compared with only 0.1 Hz for wavelenghts of 335/380 nm. Thus, it was possible to measure low noise Mg^{2+} transport rates in the time range of seconds that is needed for vesicle transport studies. Furthermore, at the isobestic point, the relative fluorescence should be independent of the Mg^{2+} and the value for the S_{f2}/S_{b2} term was near unity.

K_D values of mag-fura-2 hydrolyzed by BBMV were calculated in accordance with Raju et al. [23], using a Hill plot. On the assumption of a 1 : 1 binding between mag-fura-2 and Mg^{2+}, this plot yielded a

straight line with a slope of one. For calibration experiments, 50 μmol l^{-1} digitonin and a defined Mg^{2+} were used, the free Mg^{2+} being set with EGTA as described [24,25]. Since the K_D values critically depend on (1) the salt used, (2) the ionic strength and (3) the BBMV properties, it was necessary to determine the K_D value for different experimental conditions. To do this, BBMV were equilibrated with the specific intravesicular solution. For mag-fura-2 generated in the BBMV, the following K_D values (mmol l^{-1}) were obtained (salt concentrations in mmol l^{-1}): for 300 MET 0.65 ± 0.13, for 100 NaCl plus 100 MET 2.55 ± 0.21, for 100 KCl plus 100 MET 2.65 ± 0.17, for 100 LiCl plus 100 MET 3.15 ± 0.28, for 100 cholineCl plus 100 MET 2.12 ± 0.32, and for 100 NaSCN plus 100 MET 2.51 ± 0.31, $n = 10–12$. Depending on the experimental conditions, a 1–5 mmol l^{-1} variation in the K_D of mag-fura-2 from has been reported in the literature [26].

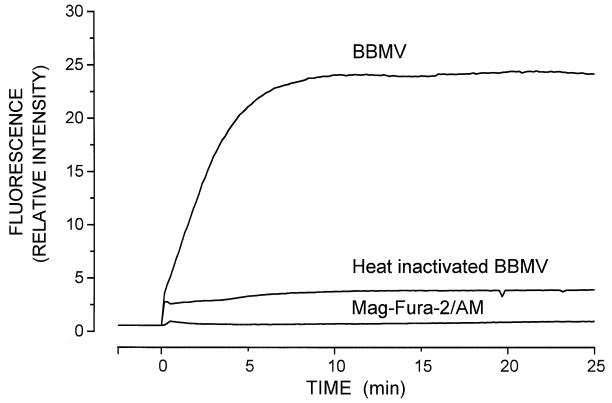

Fig. 1. Generation of mag-fura-2 from the AM form (50 μmol l^{-1}) by native and by heat-inactivated BBMV (1 mg protein l^{-1}) at 25°C. For heat inactivation, BBMV were preincubated at 70°C for 30 min. BBMV were equilibrated in 100 mmol l^{-1} NaCl, 100 mmol l^{-1} MET, pH 7.4. The excitation wavelength was 335 nm (slit 5 nm) and emission was measured at 500 nm (slit 10 nm). Records from a typical single experiment.

2.7. ^{22}Na efflux

^{22}Na efflux from BBMV (1 mg protein ml^{-1}) was measured with and without extravesicular $MgCl_2$. The intravesicular medium contained 50 mmol l^{-1} NaCl, 92.5 kBq ^{22}Na mg^{-1} protein and 200 mmol l^{-1} MET, pH 7.4. The extravesicular medium contained 5 mmol l^{-1} $MgCl_2$ and 300 mmol l^{-1} MET, pH 7.4. After equilibration with the intravesicular medium, BBMV were diluted 1:100 with the extravesicular medium and the efflux measured. Efflux at various times was terminated by rapid vacuum filtration on 0.45 μm nitrocellulose filters that were rinsed twice with 300 mmol l^{-1} MET. Efflux was calculated from the difference between the measured BBMV activity at the set times and the ^{22}Na activity at time zero.

3. Results

3.1. Incorporation of mag-fura-2 into BBMV

Incorporation of the Mg^{2+}-sensitive indicator mag-fura-2 into BBMV was performed by using the membrane-permeant ester. As indicated by a significant increase in the relative fluorescence (excitation at 335 nm, emission at 500 nm) the BBMV were able to hydrolyze the ester (Fig. 1) and to accumulate the mag-fura-2 indicator. In contrast, heat inactivated BBMV (preincubated for 30 min at 70°C) showed no esterase activity. The AM form exhibited only a minimal fluorescence signal and showed no autohydrolysis.

After washing, the intensity of BBMV fluorescence decreased to $14.2 \pm 0.5\%$ ($n = 8$) of the fluorescence intensity immediately after dye generation. This large decrease in fluorescence could be caused by hydrolysis of the AM form through enzymes on the external surface of the BBMV or through leakage from some unsealed vesicles. Localization of the hydrolyzed mag-fura-2 within the BBMV was confirmed by the observation that, after two additional washings, there was no further decrease in the fluorescence intensity ($13.9 \pm 0.6\%$, $n = 8$). Depending on the preparation, the intravesicular concentration of mag-fura-2 varied from 0.4–1.2 nmol mg^{-1} protein, which equals $\approx$ 120–330 μmol l^{-1}. At an Mg^{2+} transport in the range of up to 5 nmol mg^{-1} protein which is equivalent to 1.4 μmol l^{-1} intravesicular fluid, this dye concentration is high enough to detect Mg^{2+} in the range of nmol mg^{-1} protein. Autofluorescence of unloaded BBMV was 0.25%.

An efflux of mag-fura-2 would overestimate the calculated transport rates for Mg^{2+}, but a significant efflux of the dye could be excluded for two reasons. First of all, dye-loaded BBMV were centrifuged at different times following generation of mag-fura-2. Fluorescence was measured in the supernatant and in the sedimented BBMV. As a function of time, the indicator concentration slowly increased in the supernatant and decreased in the BBMV. Leak flux per min was estimated to be only 0.08% of the intravesicular indicator concentration ($n = 8$). Secondly, leak flux was determined by the difference in the increase of the fluorescence ratio of BBMV incubated with Mg^{2+} for 1 min, and for 90 min following the generation of indicator. Overestimation of the Mg^{2+} transport caused by a reaction of the leaked mag-fura-2 with extravesicular Mg^{2+} was equivalent to 1.55 pmol Mg^{2+} mg^{-1} protein 10 s^{-1}. After 10 s, the Mg^{2+} transport rate was ≈ 1.5 nmol Mg^{2+} mg^{-1} protein. Thus, at 10 s, the error produced by the leak flux is $< 0.1\%$, and is easily tolerable for transport measurements lasting up to 1 min. However, this method is not valid for the BBMV determination of Mg^{2+} equilibrium distribution where periods of 2 h are involved.

3.2. Transport of Mg^{2+} into BBMV

The time course of Mg^{2+} transport was initially determined and since Na^+/Mg^{2+} antiport has been described in various cells [for review see Ref. [27]], differently directed NaCl gradients were used. BBMV with a NaCl $i > o$ gradient in absence of Mg_0^{2+} were used as a control. As can be seen in Fig. 2, the control BBMV showed no measurable content of Mg_i^{2+} at any time of the investigation. Following the addition of Mg_o^{2+}, some Mg^{2+} transport could be registered in isotonic MET and with an $o > i$ directed 100 mmol l^{-1} NaCl gradient, but the rate was slow. However, in the presence of an $i > o$ NaCl gradient there was a marked increase in Mg^{2+} transport. The transport rate appeared to be linear within the first 5 s. In order to avoid overestimation of the Mg^{2+} transport rate by dye efflux, measurement of the transport rate was limited to 1 min. At 10 s, the transport rate was 2.04 ± 0.35 nmol Mg^{2+} mg^{-1} protein ($n = 8$). No overshoot was found. The K_m value for NaCl activation was 16 mmol l^{-1} ($n = 4$).

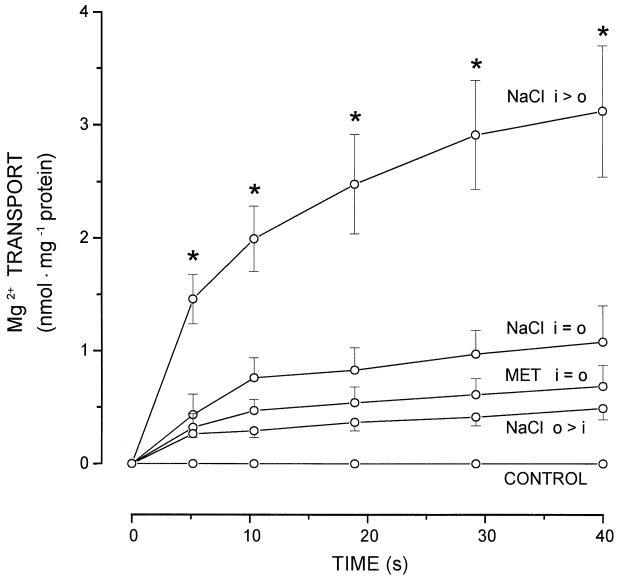

Fig. 2. Time-dependence of Mg^{2+} transport with different NaCl gradients. BBMV (1 mg protein ml^{-1}) were equilibrated with 100 mmol l^{-1} NaCl and 100 mmol l^{-1} MET or in 300 mmol l^{-1} MET, pH 7.4. The extravesicular medium contained 100 mmol l^{-1} NaCl and 100 mmol l^{-1} MET or 300 mmol l^{-1} MET, pH 7.4. The initial NaCl gradient was 100 mmol l^{-1}. Mg^{2+} transport was initiated with 5 mmol l^{-1} $MgCl_2$. Control was BBMV equilibrated with 100 mmol l^{-1} NaCl and resuspended in 300 mmol l^{-1} MET, pH 7.4 without addition of Mg_o^{2+}. Mean ± SEM, $n = 10$; $^* p < 0.05$ compared with MET $i = o$.

3.3. Transport kinetics

After the initial experiments, the Mg^{2+} transport was determined at different extravesicular Mg^{2+} in the presence of an $i > o$ 100 mmol l^{-1} NaCl gradient. A transport time of 5 s was used for calculating the K_m value. Fig. 3 shows that transport saturated with increasing Mg_o^{2+}. The inset of Fig. 3 depicts the Hanes–Woolf plot of Mg^{2+} transport at different Mg_o^{2+}. It can be seen that Mg^{2+} transport followed the Michaelis–Menten kinetics with $K_m = 1.64$ mmol l^{-1} Mg^{2+} ($= 2.59$ mmol l^{-1} total Mg^{2+}) and $V_{max} = 1.79$ nmol Mg^{2+} mg^{-1} protein 5 s^{-1}.

3.4. Unspecific stimulation by intravesicular NaCl

In order to investigate the specificity of Mg^{2+} transport stimulation by an $i > o$ NaCl gradient in-

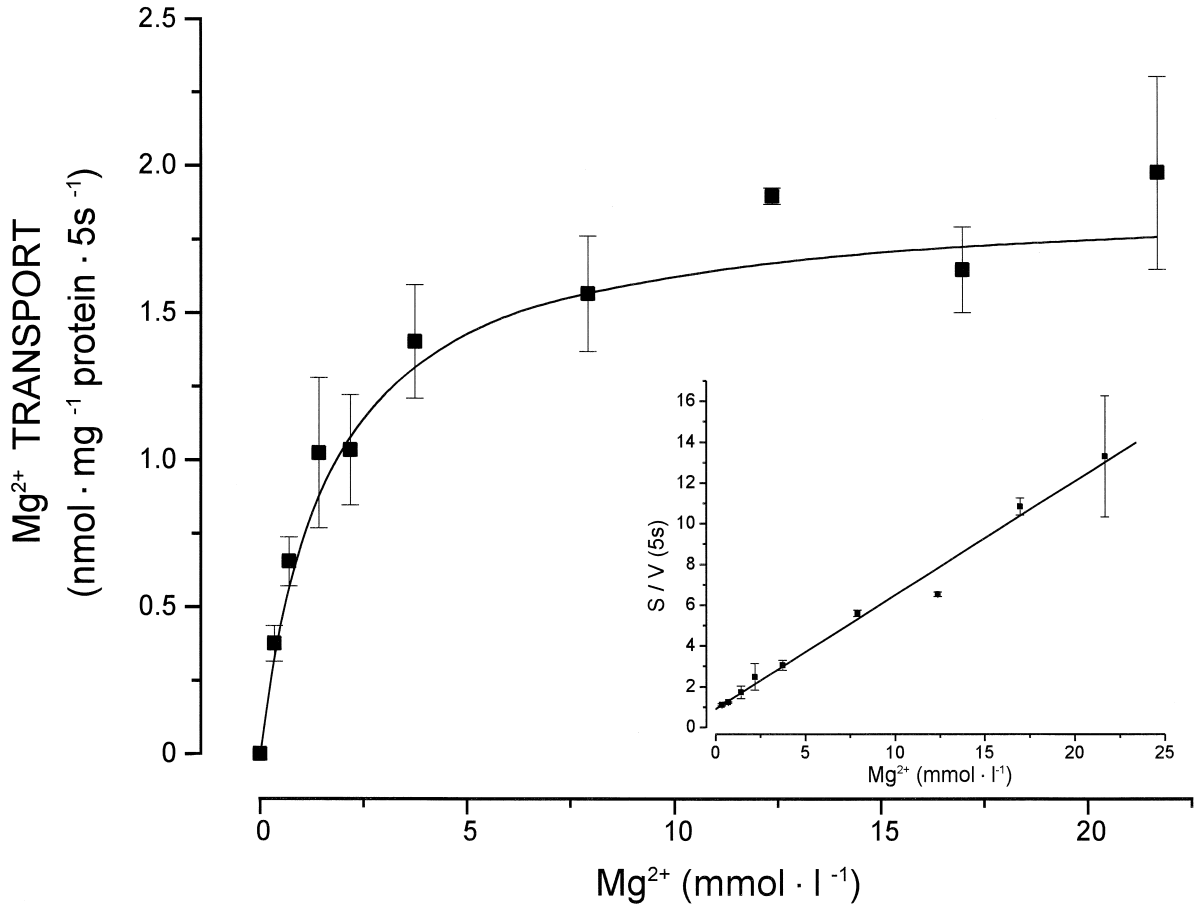

Fig. 3. Mg^{2+} transport into BBMV (1 mg protein ml^{-1}) at different Mg^{2+} concentrations. The intravesicular medium contained initially 100 mmol l^{-1} NaCl and 100 mmol l^{-1} MET at pH 7.4, the extravesicular medium contained 300 mmol l^{-1} MET at pH 7.4 and the desired $MgCl_2$ concentrations. Inset: Hanes–Woolf plot with $r = 0.993$, $K_m = 1.64$ mmol l^{-1}, $V_{max} = 1.79$ nmol mg^{-1} protein $5\,s^{-1}$. Mean ± SEM, $n = 5–7$.

side, NaCl was replaced with KCl, LiCl or cholineCl. As Table 1 shows, Mg^{2+} transport in the presence of these different $i > o$ cation gradients was the same, both at 10 s and at 60 s. This finding indicates that, in rabbit ileal BBMV, there could be a cation/Mg^{2+} antiporter, but no specific Na^+/Mg^{2+} antiporter.

3.5. Absence of an Na^+/Mg^{2+} antiporter

As a coupled Na^+/Mg^{2+} antiporter will also show Na^+ movements, Na^+ efflux from BBMV was determined in the absence, and presence of Mg^{2+}. As seen in Fig. 4, ^{22}Na efflux decreased in the presence of Mg^{2+}, probably indicating that extravesicular Mg^{2+} reduces Na^+ permeability. This finding contradicts the presence of an Na^+/Mg^{2+} antiporter which should show an increase in Na^+ efflux in presence of the Mg^{2+} transport.

The typical erythrocyte Na^+/Mg^{2+} antiporter is amiloride-sensitive [28]. In this study, the nonfluorescent amiloride-analogues DMA (10 μmol l^{-1}) and EIPA (10 μmol l^{-1}) did not influence the Mg^{2+} transport in the presence of an $i > o$ or $i = o$ NaCl gradient (data not shown). However, under an inside negative membrane potential DMA but not EIPA slightly stimulated (9%, $n = 8$, $p = 0.0278$) Mg^{2+} transport. The transmembrane voltage gradient in the presence of NaCl $i > o$ 50 mmol l^{-1} was produced by KCl $i > o$ 50 mmol l^{-1} followed by the addition of valinomycin (20 μmol l^{-1}). Also at 60 s, the stimulation was present (+11%, $n = 8$, $p < 0.002$).

Table 1
Mg^{2+} transport into BBMV (1 mg protein ml^{-1}) with different $i > o$ cation gradients

Salt	Mg^{2+} transport (nmol mg^{-1} protein)	
	in 10 s	in 60 s
MET $i = o$	0.66 ± 0.03	1.42 ± 0.05
NaCl $i > o$	1.23 ± 0.13	2.56 ± 0.29
CholCl $i > o$	1.27 ± 0.13	2.42 ± 0.17
KCl $i > o$	1.07 ± 0.06	2.48 ± 0.14
LiCl $i > o$	1.33 ± 0.14	2.49 ± 0.18

The intravesicular medium contained 100 mmol l^{-1} cation as chloride and 100 mmol l^{-1} MET, pH 7.4. The extravesicular medium contained 300 mmol l^{-1} MET, pH 7.4 and 5 mmol l^{-1} $MgCl_2$. CholCl – choline chloride. Mean ± SEM, $n = 11$.

These results do not support the presence of a Na^+/Mg^{2+} antiporter in rabbit ileal BBMV. Moreover, since the main effect of amiloride and its analogues is inhibition of Na^+/H^+ exchange, a coupling of Mg^{2+} transport with Na^+/H^+ exchange, apparently, can also be excluded.

3.6. Activation by intravesicular anions

Since Mg^{2+} transport could be stimulated by an $i > o$ gradient of cations with Cl^- as the anion, the role of anions in possibly activating Mg^{2+} transport was investigated. Three series of experiments were performed. Firstly, the role of Cl^- itself in activating Mg^{2+} transport was investigated by measuring Mg^{2+} transport with, and without an $i < o$ Na^+ gradient while maintaining equilibrium for Cl^- across the vesicles. This was achieved by applying an $i > o$ Na^+ and an $o > i$ K^+ gradient in the presence of Cl^- $i = o$. The results illustrated in Table 2 show that maintaining Cl^- across the vesicles at equilibrium reduced the activating effect of an $i > o$ NaCl gradient on Mg^{2+} transport, a finding that could indicate that Mg^{2+} transport is activated by an $i > o$ Cl^- gradient.

Secondly, the effect of anions with different membrane permeabilities was investigated. The anions were tested namely, SO_4^{2-} with a relatively low permeability, Cl^- with a moderate permeability and SCN^- with a high permeability. As can be seen in Fig. 5, Mg^{2+} transport was influenced by anion gradients in both directions. For an $o > i$ anion gradient, SCN^- and Cl^- were equal to MET $i = o$, but SO_4^{2-} was significantly lower (Fig. 5(A)), giving a ranking order of $SCN^- = Cl^- > SO_4^{2-}$. With an $i > o$ SCN^- or Cl^- gradient, Mg^{2+} transport was several times more intense than with MET $i = o$. In the presence of an $i > o$ SO_4^{2-} gradient Mg^{2+} transport was inhibited (Fig. 5(B)). Thus, the ranking order for an $i > o$ anion gradient was $SCN^- > Cl^- > SO_4^{2-}$. In anion equilibrium, the ranking order was $SCN^- > Cl^- = SO_4^{2-}$ (data not shown, $n = 8$). Thus, anion effects vs. transmembrane voltage effects are ruled out. Taken together, these findings indicate that Mg^{2+} transport is activated by an $i > o$ gradient of permeable anions.

Thirdly, the effect of anion transport inhibitors was investigated. As can be seen in Fig. 6, nonfluorescent H_2-DIDS (100 μmol l^{-1}) stimulated Mg^{2+} transport in NaCl equilibrium. The same was found at KCl equilibrium (data not shown). These findings point towards a certain relationship between anion and Mg^{2+} transport.

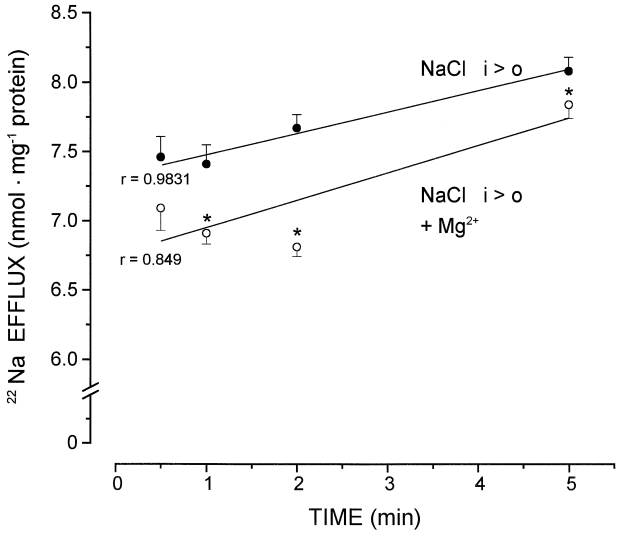

Fig. 4. ^{22}Na efflux from BBMV (1 mg protein ml^{-1}) with, and without extravesicular $MgCl_2$. The intravesicular medium contained 50 mmol l^{-1} NaCl (180 nmol mg^{-1} protein NaCl, 92.5 kBq ^{22}Na mg^{-1} protein) and 200 mmol l^{-1} MET, pH 7.4. The extravesicular medium contained 5 mmol l^{-1} $MgCl_2$ and 300 mmol l^{-1} MET, pH 7.4. Efflux was calculated from the difference between the measured ^{22}Na activity within the BBMV at the set times and the ^{22}Na activity at time zero. Mean ± SEM, $n = 8$. $^*p < 0.05$.

Table 2
Effect of Cl^- equilibrium with or without a maintained $i > o$ NaCl gradient on Mg^{2+} transport into BBMV (1 mg protein ml^{-1})

Salt	Mg^{2+} transport (nmol mg^{-1} protein)		
	5 s	10 s	60 s
MET $i = o$	0.37 ± 0.04	0.56 ± 0.05	1.27 ± 0.06
NaCl $i > o$	1.92 ± 0.19	2.45 ± 0.22	4.78 ± 0.32
NaCl $i > o$, KCl $o > i$	0.94 ± 0.04 *	1.21 ± 0.04 *	2.28 ± 0.11 *
NaCl $i = o$	0.79 ± 0.06 *	1.05 ± 0.12 *	2.31 ± 0.09 *

The intravesicular medium contained 300 mmol l^{-1} MET or 100 mmol l^{-1} NaCl and 100 mmol l^{-1} MET. The extravesicular medium contained 300 mmol l^{-1} MET, pH 7.4 and 5 mmol l^{-1} $MgCl_2$ or 100 mmol l^{-1} NaCl and 100 mmol l^{-1} MET, pH 7.4 or 100 mmol l^{-1} KCl and 100 mmol l^{-1} MET, pH 7.4. Mean ± SEM, $n = 6$, * $p < 0.05$ compared with NaCl $i > o$.

Other reagents tested had no effect on Mg^{2+} transport. These were SITS (100 μmol l^{-1}), NPPB (250 μmol l^{-1}), sulfinepyrazone (250 μmol l^{-1}) and probenicid (500 mol l^{-1}). Here, to exclude interference with the mag-fura-2 fluorescence, BBMV were preincubated with the inhibitors and, thereafter, the unbound extravesicular inhibitor was washed out.

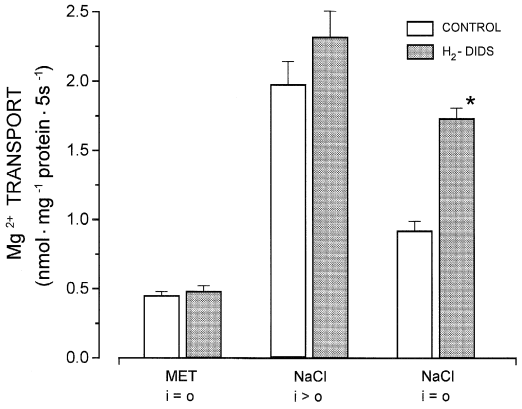

Fig. 6. Effect of nonfluorescent H_2-DIDS (100 μmol l^{-1}) on Mg^{2+} transport into BBMV (1 mg protein $^{-1}$) at 100 mmol l^{-1} NaCl $i > o$ and 100 mmol l^{-1} NaCl $i = o$. Control is 300 mmol l^{-1} MET. * $p < 0.05$, mean ± SEM, $n = 6$.

3.7. Lack of effect of voltage

It has been previously shown that, in intestinal BBMV, transport of ^{28}Mg was electrogenic [13]. To evaluate the effect of membrane potential on Mg^{2+}

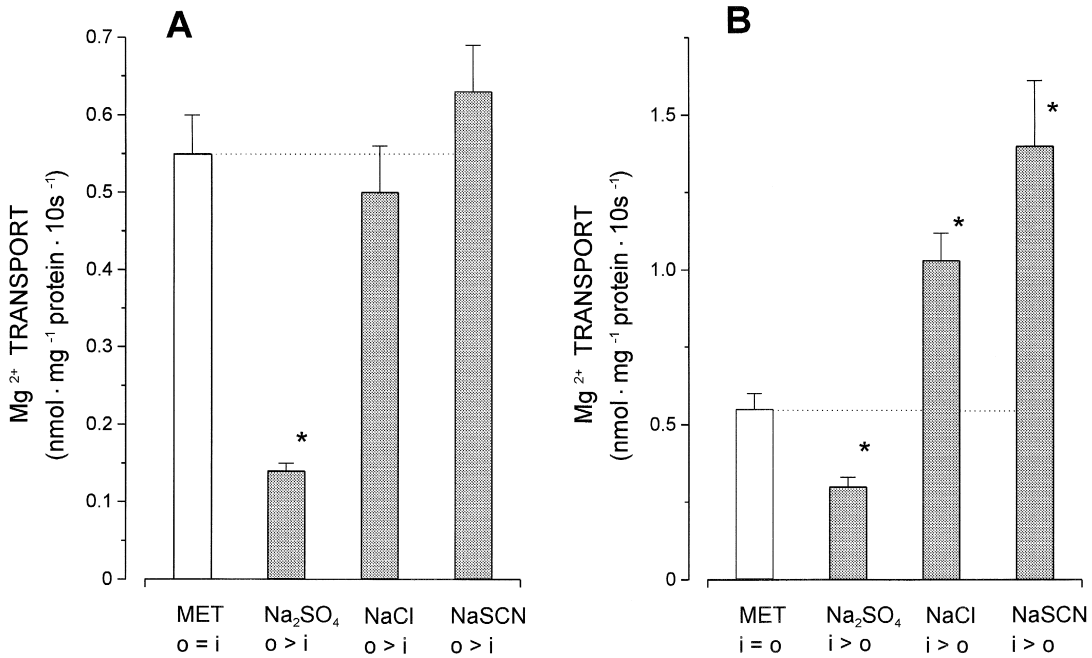

Fig. 5. Effect of different anions, with different permeabilities, with $o > i$ and $i > o$ gradients on Mg^{2+} transport into BBMV (1 mg protein ml^{-1}). (A) $o > i$ gradient; the intravesicular medium contained 300 mmol l^{-1} MET, pH 7.4 and the extravesicular medium contained 100 mmol l^{-1} of Na^+ salt and 100 mmol l^{-1} MET, pH 7.4; and (B) $i > o$ anion gradient; the intravesicular medium contained 100 mmol l^{-1} of sodium salt and 100 mmol l^{-1} MET, pH 7.4; the extravesicular medium contained 300 mmol l^{-1} MET and 5 mmol l^{-1} $MgCl_2$. Mean ± SEM, $n = 8$. * $p < 0.05$ compared with MET $i = o$.

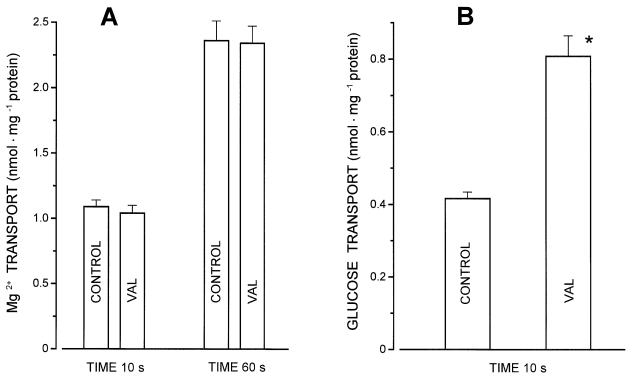

Fig. 7. Effect of a negative intravesicular potential on transport of (A) Mg^{2+} and (B) Na^+/D-glucose into BBMV (1 mg protein ml^{-1}). The negative diffusion potential was generated by valinomycin (= VAL, 20 $\mu mol\,l^{-1}$) with an $i > o$ K^+ gradient. The intravesicular medium contained 100 $mmol\,l^{-1}$ KCl and 100 $mmol\,l^{-1}$ MET, pH 7.4. (A) For Mg^{2+} transport, the extravesicular medium contained 300 $mmol\,l^{-1}$ MET, pH 7.4. Transport was initiated with 5 $mmol\,l^{-1}$ $MgCl_2$. (B) For D-glucose transport the extravesicular medium contained 100 $mmol\,l^{-1}$ NaCl, 100 $mmol\,l^{-1}$ mannitol in 10 $mmol\,l^{-1}$ HEPES-Tris, pH 7.4 with, and without valinomycin. Transport was initiated by 100 $\mu mol\,l^{-1}$ D-glucose. For further details see Section 2. Mean ± SEM, $n = 8$. * $p < 0.05$.

transport into ileal BBMV, three sets of experiments were performed.

Initially the effect of an inside negative diffusion potential generated by a K^+ gradient ($i > o$, 100 $mmol^{-1}$) in the presence of valinomycin (25 $\mu mol\,l^{-1}$) was tested. As can be seen in Fig. 7(A), $i > o$ NaCl-dependent Mg^{2+} transport was not influenced, in contrast to Na^+/D-glucose transport which was stimulated (Fig. 7(B)). In a second series of experiments, the membrane potential was short-circuited by valinomycin in KCl equilibrated BBMV in the presence of an $i > o$ NaCl or NaSCN gradient (Table 3). No effect on Mg^{2+} transport could be detected. Analogous experiments with different NaCl gradients in the presence of natriophorous gramicidin D also had no effect on Mg^{2+} transport (data not shown).

The third experiment, illustrated in Fig. 5(a), showed that $o > i$ SCN^- and $o > i$ Cl^- were equal in their effect on Mg^{2+} transport. Thus, the more negative diffusion potential with $o > i$ SCN^- had no additional effect on Mg^{2+} transport. Taken together, all these findings indicate a lack of effect of membrane potential on the Mg^{2+} transport system.

3.8. Effect of Ca^{2+} channel effectors

In kidney cells and hepatocytes, Mg^{2+} transport can be inhibited by verapamil and other Ca^{2+} transport/channel effectors [29–31]. In our rabbit ileal BBMV, verapamil (25 $\mu mol\,l^{-1}$), diltiazem (50 $mol\,l^{-1}$), nifedepine (50 $\mu mol\,l^{-1}$), imipramine (100 $\mu mol\,l^{-1}$) and Bay K8644 (10 $\mu mol\,l^{-1}$) had no effect on Mg^{2+} transport under an $i > o$ NaCl gradient. On the contrary, only imipramine, and not the other inhibitors, slightly stimulated Mg^{2+} transport (16%) under an inside negative diffusion potential. In the control, Mg^{2+} transport averaged 1.51 ± 0.09 nmol mg^{-1} protein 10 s^{-1} while with imipramine 1.79 ± 0.06 nmol mg^{-1} protein 10 s^{-1} were registered ($n = 8$, $p = 0.0406$). The inside negative membrane potential at NaCl $i > o$ 50 $mmol\,l^{-1}$ was created with an $i > o$ KCl gradient in the presence of valinomycin as described above for DMA.

Table 3
Mg^{2+} transport into zero potential clamped BBMV (1 mg protein ml^{-1}) with an $i > o$ NaCl or NaSCN gradient

Salt	Mg^{2+} transport (nmol mg^{-1})		
	5 s	10 s	60 s
MET $i = o$	0.47 ± 0.04	0.71 ± 0.04	1.49 ± 0.06
NaCl $i > o$, KCl $i = o$	0.94 ± 0.12	1.38 ± 0.11	3.06 ± 0.18
NaCl $i > o$, KCl $i = o$ + valinomycin	0.76 ± 0.05	1.23 ± 0.07	2.90 ± 0.13
NaSCN $i > o$, KCl $i = o$	1.17 ± 0.11	1.60 ± 0.08	3.21 ± 0.14
NaSCN $i > o$, KCl $i = o$ + valinomycin	1.06 ± 0.10	1.56 ± 0.10	3.12 ± 0.13

In the control, BBMV was equilibrated in 300 $mmol\,l^{-1}$ MET, pH 7.4. To zero clamp, BBMV was equilibrated in 50 $mmol\,l^{-1}$ KCl and 200 $mmol\,l^{-1}$ MET, pH 7.4 and valinomycin 25 $\mu mol\,l^{-1}$. The $i > o$ NaCl or NaSCN gradient was 50 $mmol\,l^{-1}$. The extravesicular medium contained 300 $mmol\,l^{-1}$ MET and 5 $mmol\,l^{-1}$ $MgCl_2$. Mean ± SEM, $n = 6$.

4. Discussion

4.1. Loading of BBMV with mag-fura-2

The present study is the first to examine Mg^{2+} transport in rabbit ileal BBMV with the Mg^{2+}-sensitive indicator mag-fura-2; the indicator being loaded into the vesicles in the ester form. In BBMV, a significant hydrolysis of the esterified indicator could not have been expected since cytosolic contamination was small. Native, but not heat inactivated, rabbit ileal BBMV were able to hydrolyze mag-fura-2/AM so that significant amounts of mag-fura-2 were produced; the intravesicular dye concentration being high enough to detect free Mg^{2+} in the range of $nmol\,mg^{-1}$ protein. Leak flux of the indicator was minimal, so that overestimation of Mg^{2+} transport by a reaction of the leaked dye with extravesicvular Mg^{2+} could be neglected up to 1 min. The method allowed continuous monitoring of the intravesicular free Mg^{2+} when free Mg^{2+} is transported into BBMV. This finding, that purified BBMV contain esterases could mean that other fluorescent indicators could also be loaded into BBMV with the ester technique.

4.2. Evidence for Mg^{2+} transport

In the presence of an initial $i > o$ NaCl gradient, the BBMV accumulated Mg^{2+}. The Mg^{2+} uptake was nonlinear with time and showed no overshoot. At 10 s the Mg^{2+} transport rate of rabbit ileal BBMV was $\approx 2\,nmol\,mg^{-1}$ protein. Using $1\,mmol\,l^{-1}$ ^{28}Mg, an approximately five-times greater uptake rate was measured in rat duodenal BBMV [12,13]. This value was not, however, corrected for Mg^{2+} binding to the vesicles, and as calculated from Fig. 4(b) of Baillien et al. [13] in rat duodenal BBMV, binding of Mg^{2+} accounted for as much as half of the total ^{28}Mg uptake at 60 s. Taking this binding into account, the true transport rate of free Mg^{2+} would be within the range found in these experiments.

Another argument supporting the transport of Mg^{2+} into BBMV is the saturation with increasing Mg_o^{2+} concentrations. It could be assumed that the observed saturation may be explained by saturation of mag-fura-2 with Mg^{2+}. Since the concentration of accumulated Mg_i^{2+} was in the order $\mu mol\,l^{-1}$ but the K_D value of mag-fura-2 was in the order of $mmol\,l^{-1}$, saturation of Mg^{2+} transport can not be explained by saturation of mag-fura-2.

For an $i > o$ NaCl gradient a K_m value of $1.64\,mmol\,l^{-1}$ was measured, which is within the range of the sparce data reported in literature. ^{28}Mg uptake in rat duodenal BBMV gave a K_m value close to $1\,mmol\,l^{-1}$ [13], and in vivo intestinal perfusion and total Mg^{2+} uptake in rat colon a K_m value of $1.2\,mmol\,l^{-1}$ [8]. A curvilinear concentration-dependent component of unidirectional mucosa-to-serosa ^{28}Mg flux was found in stripped short-circuited mucosa of rat ileum and colon. While the K_m value could not be calculated for the ileum, the K_m in the colon was $\sim 0.5\,mmol\,l^{-1}$ [10,11]. These findings on saturable Mg^{2+} transport have all been hampered by the fact that total Mg^{2+} uptake was not corrected for binding. Moreover, it cannot be excluded that the saturable Mg^{2+} transport obtained in the in vivo intestinal perfusion experiments may have been caused by a reduction of the tight junctional permeability at the higher Mg^{2+} concentration [4]. These findings in rabbit ileal BBMV show conclusively that the uptake of free Mg^{2+} by the luminal membrane is saturable.

It could be argued that the observed increase in Mg_i^{2+} in the presence of $i > o$ NaCl does not represent Mg^{2+} transport but displacement of internally bound residual Mg_i^{2+} by Na_i^+. This hypothesis could be excluded from the following reasons:

a) Before starting Mg^{2+} transport, the BBMV were equilibrated with Na^+ and allowed to accumulate mag-fura-2 and, subsequently, were sedimented. Thus, the Mg_i^{2+} bound to the internal surface would be desorbed by Na_i^+ and washed away before the experiment.

b) When NaCl equilibrated, BBMV were resuspended in MET without addition of Mg_o^{2+}, no Mg_i^{2+} could be registered.

c) Furthermore, when some Na_i^+ was really bound before Mg_o^{2+} had been added, it is more reasonable to assume that some Na_i^+ might be displaced by accumulated Mg_i^{2+} but not the other way around.

d) The observed time-dependent increase in intravesicular free Mg_i^{2+} cannot be explained by Mg_i^{2+} desorption, because binding and displacement of ions are usually faster processes.

4.3. Exclusion of a Na^+ and Mg^{2+}/anion-symporter

In rat hepatocytes an amiloride, verapamil and pCMBS inhibitable Na^+ and Mg^{2+}/anion-symport system has been postulated [31,32]. This transport system can be excluded in rabbit ileal BBMV because Mg^{2+} transport was neither activated by extravesicular NaCl nor inhibited by amiloride analogues and verapamil. Furthermore, pCMBS did not inhibit Mg^{2+} transport in rabbit ileal BBMV (not shown, $n = 12$).

4.4. Exclusion of a Mg^{2+}/2 Na^+ antiporter

Since Mg^{2+} transport into rabbit ileal BBMV was stimulated by an $i > o$ NaCl gradient and, apparently, was electroneutral, an $Mg^{2+}/2Na^+$ antiporter could be a possibility. In several tissues, e.g. squid axon, heart muscle and red cells of different species, an Na^+-dependent Mg^{2+}-efflux system has been described [for review, see Ref. [33]]. While in erythrocytes, evidence has been presented favouring an electroneutral $2Na^+/Mg^{2+}$ antiport as the mechanism [34,35], in heart muscle the evidence for an Na^+/Mg^{2+} antiport is not conclusive [for review, see Ref. [36]].

In the intestine and the kidney, where there is a net Mg^{2+} reabsorption, the $2Na^+/Mg^{2+}$ antiporter system would operate in the reverse mode. Our finding of a stimulation of the Mg^{2+} transport into rabbit ileal BBMV by an $i > o$ NaCl gradient confirms previous data on a borderline stimulation of ^{28}Mg uptake in BBMV of flounder kidney at an $i > o$ 100 mmol l^{-1} Na^+ gradient [15]. However, several findings do not support the hypothesis of an $Mg^{2+}/2Na^+$ antiport in rabbit ileal BBMV:

(a) In rabbit ileal BBMV, Mg^{2+} transport in the presence of KCl, LiCl and cholineCl was similar to that with NaCl. By contrast, in erythrocytes [35,37], Mg^{2+} efflux was Na^+-dependent and neither KCl, LiCl nor cholineCl could be substituted for NaCl. Also, in rat sublingual acini, Mg^{2+} efflux was largely dependent on NaCl [38].

(b) An electroneutral $Mg^{2+}/2Na^+$ antiporter would require the efflux of $2Na^+$ coupled to the influx of $1Mg^{2+}$ ion. In these experiments, this was not the case, since Mg_o^{2+} reduced the efflux of Na_i^+. This result is in agreement with previous findings that Mg_i^{2+} inhibited Na_o^+ uptake in apical membrane vesicles of the rat colon [39].

(c) Amiloride has been reported to inhibit the red cell $2Na^+/Mg^{2+}$ antiporter at the relatively high concentration of 1 mmol l^{-1} [34,35], while in rabbit ileal BBMV Na^+/H^+ antiport was half maximally inhibited at ≈ 10 μmol l^{-1} [40]. In our experiments, 10 μmol l^{-1} of the more potent nonfluorescent amiloride analogues DMA and EIPA did not inhibit Mg^{2+} transport in rabbit ileal BBMV. Such results are in agreement with the findings that, in Mg^{2+} depleted kidney cell cultures [30] and in renal BBMV of the rainbow trout, Mg^{2+} uptake was not affected by amiloride [41].
Interestingly, under an inside negative diffusion potential, DMA slightly stimulated Mg^{2+} transport in rabbit ileal BBMV, which confirms the previous finding on amiloride stimulated Mg uptake of MDCT cells [42] and shows that this effect may be localized at the cell membrane outsite.

(d) Na^+ activated BBMV antiporter exhibit an ‘‘overshoot phenomena’’ in the presence of an excess of the counterion that is to have a transport rate several times greater than the equilibrium value. This phenomena could not be observed for the NaCl activated Mg^{2+} transport of rabbit ileal BBMV. It was also absent in rat duodenal and jejunal BBMV [14], when Mg^{2+} uptake was measured with ^{28}Mg. It could be argued that the overshoot was masked by Mg^{2+} binding to the intravesicluar surface following transmembrane transport. However, to explain the absence of the overshoot, the major part – if not all – of the transported Mg^{2+} must be bound. While this cannot be excluded with certainty it seems unlikely since free intravesicular Mg^{2+} was always detectable under all experimental conditions.

4.5. Exclusion of electrogenic Mg^{2+} transport

Recently, Freire et al. [41] postulated a Mg^{2+} channel for electrogenic Mg^{2+} uptake into renal BBMV of the rainbow trout, a channel distinct from the Ca^{2+}-dependent Mg^{2+} channel of *Paramecium* [43]. An Mg^{2+} uniporter or a channel would be

expected to exhibit electrogenic Mg^{2+} transport, as was the case in rat jejunal BBMV [14]. However, in that study, the binding component of Mg^{2+} uptake was not allowed for. In contrast, in rabbit ileal BBMV, we found that Mg^{2+} transport was electroneutral. No effect on transport was found to change the intravesicular potential by altering the K^+ gradients in the presence of valinomycin. The Mg^{2+} transport was also unaffected by an intravesicular negative potential created by an $o > i$ SCN^- gradient. This finding of electroneutrality supports previous findings on short-circuited rat ileum [10]. These results on Mg^{2+} transport into rabbit ileal BBMV would seem to exclude electrogenic Mg^{2+} transport via a transport protein or through a channel.

4.6. Mg^{2+} transport in rabbit ileal BBMV is distinct from Ca^{2+} transport

The following arguments may be relevant to the fact that, in rabbit ileal BBMV, the Mg^{2+} transport mechanism is distinct from that of Ca^{2+}.

(a) ^{45}Ca transport into BBMV of rabbit distal tubule cells was inhibited by trans NaCl as well as by trans KCl [44]. In our rabbit ileal BBMV, Mg^{2+} was stimulated by trans NaCl and by trans KCl.

(b) While Ca^{2+} transport into rat ileal cells could be inhibited by verapamil and diltiazem [45], in rabbit ileal BBMV different Ca^{2+} channel inhibitors did not inhibit Mg^{2+} transport. Only in the presence of an inside negative diffusion potential was Mg^{2+} transport in rabbit ileal BBMV slightly stimulated by imipramine. Our finding is supported in literature where a different sensitivity to these inhibitors has been reported. In Mg^{2+}-depleted isolated renal TAL cells [30] and in MCDK cells [29], verapamil and diltiazem inhibited Mg^{2+} transport. No effect of Ca^{2+} transport antagonistic to Mg^{2+} transport could be found in BBMV of flounder kidney cortex [41], rat duodenal BBMV [13] and in the rat sublingual acini [38].

4.7. Role of intravesicular anions

We have shown that Mg^{2+} transport into rabbit ileal BBMV is modulated by an $i > o$ anion gradient. The finding that H_2-DIDS, which inhibits the outside binding of Cl^-, abolished the inhibiting effect of anion equilibrium on Mg^{2+} transport is in line with this assumption. Mg^{2+} may permeate as a temporary Mg^{2+}/anion complex, as suggested for cation transport through Cl^- channels [46] and for Zn^{2+} transport into rat BBMV [47]. This is in accord with the finding that the Mg^{2+} transport rate was dependent on anion permeability, which was in the ranking order of $SCN^- > Cl^- > SO_4^{2-}$.

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