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The effects of 1,25-dihydroxyvitamin D-3 deficiency on Ca^{2+}-transport and Ca^{2+}-uptake into brush-border membrane vesicles from pig small intestine

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BBMV were prepared from duodenal segments of untreated, 1,25-$(OH)_2D_3$- or vitamin D-3-treated rachitic piglets and from non-rachitic controls by the Mg^{2+} precipitation method. The rachitic piglets were offspring from the 'Hannover Pig Strain' which suffer from pseudo vitamin D-deficiency rickets, type I (no renal 1-hydroxylase activity). Initial uptake of Ca^{2+} (up to 35 s) at low [Ca^{2+}] (between 0.02–0.25 mmol/l) into isolated BBMV consisted of a saturable and non-saturable component. The apparent V_{max} of the saturable component was significantly lower in rachitic piglets than in control piglets. In the presence of an inside negative potassium diffusion potential, the difference in uptake extended over at least 15 min. The apparent K_m of Ca^{2+}-uptake was not influenced by the rachitic condition. Treatment of rachitic piglets with sequential doses of 1,25-$(OH)_2D_3$ for three days (1 μg/day) or with a single dose (2.5 mg) of vitamin D-3 elevated the saturable Ca^{2+}-uptake component to values similar to those of control piglets. Addition of 1 mmol/l verapamil to the vesicular suspension inhibited Ca^{2+}-uptake in BBMV of control piglets by 40–60% but was without effect on preparations from rachitic piglets. It was concluded from the study that 1,25-$(OH)_2D_3$ dependent Ca^{2+}-uptake into BBMV constituted a saturable process which can be inhibited by a known Ca^{2+}-channel blocking agent. It appears that 1,25-$(OH)_2D_3$ increases the number of verapamil sensitive Ca^{2+}-transport components in brush-border membranes. The vitamin D-dependent changes in vesicular Ca^{2+}-uptake were paralleled by the expression of an active transmural Ca^{2+}-transport across the mucosa.

Introduction

The principal effect of 1,25-$(OH)_2D_3$ on intestinal mucosa is stimulation of active Ca^{2+}-transport. This transport is thought to be a three-step process, involving entry of Ca^{2+} into the enterocyte across the luminal membrane, its translocation across the cytosol and finally, its active extrusion via the basolateral membranes [1–3]. Hence, at least one of these steps must to be under control of 1,25-$(OH)_2D_3$. Studies of van Corven et al. [4] and Kaune et al. [5] with rat and pig duodenal enterocytes have shown that the ATP-dependent transport of Ca^{2+} across basolateral membranes was not influenced by 1,25-$(OH)_2D_3$. The uptake of calcium into isolated brush-border membrane vesicles of chicks and rats, however, has been shown to depend on 1,25-$(OH)_2D_3$. This was concluded from its 30% depression in vitamin-D depleted animals [6–10].

Identification of vitamin D-dependent factors which led to reduced uptake of calcium provided no clear answers. While a decrease in the K_m of the uptake process and an increase in equilibrium value after 1,25-$(OH)_2D_3$ was reported in some studies [6,7], an increase in V_{max} was observed in others [8–10]. In addition, the Ca^{2+} concentrations used in these studies were rather high and apparent K_m values of about 1 mmol/l were calculated. No distinction was made between saturable and non-saturable processes. In more recent studies, it has been demonstrated, however, that Ca^{2+}-uptake into BBMV constituted of a saturable and

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Abbreviations: BBMV, brush-border membrane vesicle(s); 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D-3; 25-$(OH)D_3$, 25-hydroxyvitamin D-3; [Ca^{2+}], total Ca^{2+} concentration in medium; AP, activity of alkaline phosphatase; EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

a non-saturable component [11–14] and that the K_m of the saturable component is much below 1 mmol/l [13,14]. Thus, we reexamined the influence of 1,25-$(OH)_2D_3$ upon uptake of Ca^{2+} into BBMV at low [Ca^{2+}] (0.02–0.25 mmol/l) and over short incubation times (2–35 s). We used a pig model with inherited rickets. Due to defective renal production of 1,25-$(OH)_2D_3$ [15], these piglets possess unphysiological low circulating 1,25-$(OH)_2D_3$ concentrations [16] and cannot absorb calcium in amounts sufficient to maintain normocalcemia [17]. The animals regularly develop symptoms of florid rickets when they are 5–12 weeks old.

Materials and Methods

Animals and treatment

A number of 25 homozygote piglets with type-I pseudo vitamin D-deficiency and symptoms of florid rickets and an equal number of age-matched heterozygote controls of both sexes were used for the experiments [16]. The animals were cross breeds of 'German Landrace' and 'Göttinger Miniature Pigs' with 3.5–10.5 kg body weight and were 5–12 weeks old. They were weaned at an age of 4–6 weeks and, after weaning, offered a commercial pig starter diet with 0.9% calcium, 0.65% phosphorus and 50 μg vitamin D-3 per kg feed.

For vesicle preparation, the piglets were killed by stunning and bleeding from the carotids. The proximal part of the small intestine (duodenum and part of proximal jejunum) was removed, immediately rinsed with ice-cold saline and prepared for incubation of stripped mucosa in Ussing-type chambers. For vesicle preparations the intestine was cut into 15-g pieces and stored at −70°C.

Three rachitic piglets were treated 1 week before tissue preparation with an intramuscular injection of 2.5 mg vitamin D_3 in water soluble form (Sanofi/Ceva, Düsseldorf, Germany). Three other rachitic piglets were treated three days before tissue preparation with six intravenous injections of 0.5 μg 1,25-$(OH)_2D_3$ (1 μg/day) via a catheter placed into the internal jugular vein. The 0.5-μg doses of 1,25-$(OH)_2D_3$ were dissolved in 50 μl ethanol and diluted with 1 ml plasma obtained from the receiver animal.

Measurement of Ca^{2+} fluxes

Mucosal to serosal (ms) and serosal to mucosal (sm) fluxes of Ca^{2+} were measured in Ussing-type chambers in the absence of electrochemical gradients by the aid of a computer controlled automatic voltage clamp device (AC Microclamp, Aachen, Germany). About 10-cm segments from the duodenum were opened along the mesenteric border and fixed mucosal surface upside on a pyrex plate. Epithelial sheets of tunica mucosa (6–8 cm^2) were scraped off the underlying muscle layers with a spatula and mounted in Ussing chambers with an exposed area of 1 cm^2. To minimize edge damage and to prevent the mucosa from bulging, silicon rubber rings and polyester nettings with open areas > 70% (Reichelt, Heidelberg, Germany) were placed at both sides of the mucosa. The isolated mucosae were bathed on both sides with 13 ml of buffer solution containing (mmol/l): 137 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 12 $NaHCO_3$, 1.0 $MgCl_2$, 0.4 NaH_2PO_4, 0.01 indomethacin and 5.0 Hepes-Tris (pH 7.4). In addition, the serosal solution contained 10 mmol/l D-glucose and the mucosal solution 10 mmol/l mannitol. The bathing solutions were continuously circulated and gassed by a gas lift device with 95% O_2 and 5% CO_2. The incubation temperature was 39°C. The transepithelial potential difference was continuously measured with 3 mol/l KCl agar bridges connected to calomel electrodes. Under short circuited conditions the potential difference was clamped to zero by a computer controlled current via another pair of agar bridges connected to Ag/AgCl electrodes. The tissue conductance was calculated from super-imposed 1-s bipolar electrical pulses of 50 μA amplitude and was recorded along with the potential difference and the short circuit current at 2.5-min intervals. Approx. 30 min after mounting the tissues, 300 kBq $^{45}CaCl_2$ was added to one of either side of the mucosa. Samples (0.1 ml) were taken from the labelled side 30 min later and subsequently in 10 min intervals from the unlabelled side (0.5 ml) of the Ussing chamber. The samples drawn from the unlabelled side of the mucosa were replaced by equal volumes of isosmotic bathing fluid. Two to three samples were collected under control conditions before 130 μl of verapamil dissolved in incubation medium was added to the luminal surface to give a final concentration of 1 mmol/l. 10–30 min after addition of verapamil, Ca^{2+} fluxes were measured and compared to those obtained during the control period. The differences of ms to sm fluxes were tested between tissues of paired chambers whose conductances did not differ by more than 25%. Net fluxes were expressed as differences between ms and sm fluxes obtained from paired chambers.

Preparation of BBMV

The BBMV were prepared by a modification of the Mg^{2+}-EGTA precipitation method originally described by Biber et al. [18] and Binder and Murer [19]. Frozen intestines of 20 to 30 g wet weight were thawed and the following procedure was carried out at 4°C. Epithelial cells were harvested after 10 min vibration with a vibro mixer type E1 (Chemap, Männedorf, Switzerland) in 30 ml of 12 mmol/l Tris-HCl buffer (pH 7.1) with 300 mmol/l mannitol and 5 mmol/l EGTA. The suspended cells were diluted 5-fold with 120 ml of ice-cold distilled water and homogenized for 3 min with a

blender (Mulinex, type 530.02, Germany). After removal of foam, $MgCl_2$ was added to a final concentration of 10 mmol/l. The solution was kept on ice for 15 min and centrifuged at 9000 × *g* for 15 min. The supernatant was centrifuged at 27000 × *g* for 30 min and the pellet was resuspended in 35 ml of 5 mmol/l EGTA/Tris buffer (pH 7.1) with 60 mmol/l mannitol in a glass-teflon-potter (Braun Melsungen, Germany). Precipitation of membranes by Mg^{2+} addition and the two-step centrifugation procedure were repeated. The resulting pellet was suspended with a glass-teflon-potter in 35 ml of either a 10 mmol/l Hepes-Tris buffer (pH 7.4) with 100 mmol/l KCl and 200 mmol/l mannitol or in a 20 mmol/l Hepes-Tris buffer (pH 7.4) with 150 mmol/l KCl and 1 mmol/l $MgCl_2$. This suspension was centrifuged at 27000 × *g* for 40 min and the pellet was resuspended in 0.5–1.0 ml of the same buffer by passing the mixture several times through a hypodermic needle (0.45 × 25 mm).

Enzyme assays and protein determination

The activity of alkaline phosphatase (AP) (EC 3.1.3.1) was calculated from photometric measurements of the rate of hydrolysis of *p*-nitrophenyl phosphate according to an assay kit from Boehringer-Mannheim. The activity of the Na^+/K^+-ATPase (EC 3.6.1.37) was determinated by the method of Mircheff and Wright [20]. Activities of KCN-resistant NADH–oxidoreductase (EC 1.6.99.2) and succinate–cytochrome-*c* oxidoreductase (EC 1.3.99.1) were measured following modified methods of Sottocasa et al. [21] and Fleischer and Fleischer [22], respectively. Protein was determined with a Coomassie blue kit from Bio-Rad (Munich, Germany) using bovine γ-globulin as standard protein.

Ca^{2+}- and glucose-uptake into BBMV

Uptakes of $^{45}Ca^{2+}$ and [^{14}C]glucose into BBMV were quantified by using the rapid filtration technique with cellulose nitrate filters of 0.45 μm pore size (Sartorius, Göttingen, Germany). 50–300 μl portions of vesicle suspension containing 50–750 μg protein were mixed with 300 μl transport buffer containing 85 kBq $^{45}Ca^{2+}$ or 10 kBq [^{14}C]glucose and unlabelled Ca^{2+} or glucose. The mixtures were incubated at 25 or 37°C. At intervals, 100 μl of the BBMV solution was mixed with 5 ml ice-cold stop buffer, immediately filtered by suction and the filters were washed three times with 3 ml of the stop buffer to remove extravesicular activity. The stop buffer was isosmotic to the incubation medium and contained (mmol/l) either: 150 NaCl, 50 mannitol, 1 EGTA and 10 Hepes-Tris (pH 7.4) or 150 KCl, 1 $MgCl_2$, 1 EGTA, and 20 Hepes-Tris (pH 7.4). The washed vesicles of the filter surface were then transferred into scintillation vials with 4 ml scintillator (Quickscint 2000, Zinsser Analytic, Frankfurt, Germany) for radioactivity measurement with an accuracy of 1% standard deviation. Appropriate blanks were obtained from incubations of radioactive calcium and glucose without vesicular membranes. At the end of the experiment total radioactivity was measured in 100 μl of the BBMV solution.

The Ca^{2+}-uptake in presence of a K^+ diffusion potential was estimated using a modified method of Garty and Asher [23]. BBMV were suspended in 150 mmol/l KCl, 1 mmol/l $MgCl_2$, 20 mmol/l Hepes-Tris (pH 7.4). The extravesicular K^+ was removed by passing the suspended vesicles through small cation exchange columns (Dowex, 50 × 8, 200–400 mesh, H-form, 0.5 ml void volume) which were equilibrated with 350 mmol/l Tris (pH 7.4). The vesicles were eluted from the column with isosmotic buffer containing 325 mmol/l mannitol, 30 mmol/l Tris (pH 7.4). Immediately after potassium removal incubation was started at 25°C by mixing 400 μl vesicle suspension with preincubated elution buffer. The incubation mixture contained 0.5 mmol/l EGTA, 5 μmol/l valinomycin, 120 kBq/ml $^{45}Ca^{2+}$ and 30 μmol/l free Ca^{2+} in a final volume of 1000 μl. Serial samples of 100 μl were taken at 1, 2, 3, 4, 5, 6, 10 and 15 min after starting the incubation. The samples were stopped and filtered as described above.

For short term incubations (< 30 s), 50 μl transport buffer with radioisotopes and 50 μl vesicular suspension were pipetted to separate places at the bottom of a 20-ml vial and incubated for 1 min without mixing. The two solutions were then mixed by vortexing at zero time and stopped at times by adding 5 ml of ice-cold stop buffer. Filtration of BBMV and measurement of radioactivity were the same as described above, except that 50 μl of labelled transport buffer was used for counting total radioactivity. All uptake measurements were carried out in duplicates.

Free Ca^{2+} concentrations

In the experiments which were carried out at 37°C, the [Ca^{2+}] in the incubation medium was 0.25 mmol/l. In the other experiments, the desired free Ca^{2+} concentration was obtained by adding appropriate amounts of calcium to 0.5 mmol/l EGTA. The amounts of Ca^{2+} were calculated according to formulas and binding constants given by Van Heeswijk et al. [24].

Chemicals

$^{45}Ca^{2+}$ (spec. act. 28 GBq/mmol) and D-[1-^{14}C]glucose (spec. act. 2 GBq/mmol) were obtained from Amersham Buchler (Braunschweig, Germany). (±)-Verapamil, valinomycin and Ca^{2+} ionophore A23187 were purchased from Sigma (Deisenhofen, Germany) and saponin was from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Calculations

Differences between means from different experimental groups were tested for significance with Student's *t*-test. The kinetic values (V_{max} and K_m) for Ca^{2+}-uptake were calculated by fitting Ca^{2+} uptake vs. free Ca^{2+} concentration to a rectangular hyperbola relationship using the computer program 'Graph Pad' (ISI, Philadelphia, USA). The algorithm for the relationship was:

$$Ca^{2+}\text{-uptake} = \frac{V_{max} \cdot [Ca^{2+}]_f}{K_m + [Ca^{2+}]_f} + U \cdot [Ca^{2+}]_f$$

where $[Ca^{2+}]_f$ = concentration of free Ca^{2+} (mmol/l), U = nonspecific binding [(nmol/mg protein)/(mmol/l)], V_{max} = maximum of saturable Ca^{2+}-uptake (nmol/mg protein), K_m = concentration at half saturation (mmol/l).

Statistics

The uptake of Ca^{2+} in the presence of a diffusion potential at different times of incubation was described by fitting the data of each experiment to a saturation curve of the form $Y = A \cdot (1 - e^{-kt})$. The Sigma Plot, Version 4.0, was used as curve fitter (Jandel Scientific, USA) to obtain the parameters A and k. The appropriate A and k values from rachitic and control piglets were tested for significance using the *t*-test for independent (unpaired) data sets.

Results

Ca^{2+}- and glucose-uptake into BBMV

On protein basis enrichment of the brush-border membrane marker enzyme AP was 16- to 18-fold, and 2- to 3-fold for the basolateral membrane marker, Na^+/K^+-ATPase. This indicated satisfactory concentration of the brush-border membranes. The specific activities of NADH:oxidoreductase and succinate–cytochrome-*c* oxidoreductase decreased, showing enrichment factors below 0.1, which indicated the virtual absence of endoplasmic reticular and mitochondrial membranes from the BBMV preparations. Enrichment factors of marker enzymes were the same for rachitic piglets and control piglets and piglets treated with vitamin D-3 or 1,25-$(OH)_2D_3$.

Functional integrity of the vesicles were tested by measuring Na^+-coupled glucose transport. A characteristic Na^+-gradient dependent overshoot of glucose uptake was found to occur in preparations of both control and rachitic piglets. The overshoot, however, was quantitatively less in rachitic piglets, whereas equilibrium values were not different from those of control piglets (data not shown).

Typical plots of Ca^{2+}-uptake into BBMV vs. time of incubation from rachitic and control piglets are shown in Fig. 1. In control piglets, the initial rate of uptake (< 1 min) was significantly higher than in rachitic piglets (Fig. 1). The difference disappeared when incubation was extended to 10 min (Fig. 1) or up to 40 min (data not shown). The amount of Ca^{2+} taken up by the vesicles at the same time near equilibrium (40 min) was about 10-fold higher than one would predict from the intravesicular glucose space (Table II). This indicated considerable unspecific binding.

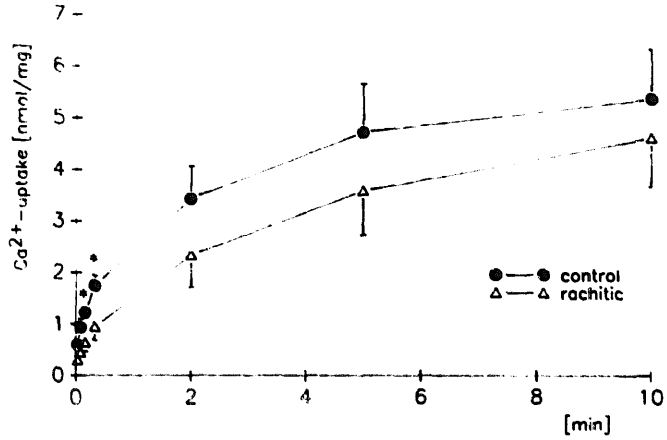

Fig. 1. Uptake of Ca^{2+} into duodenal BBMV of rachitic ($n = 4$) and control ($n = 6$) piglets from 2 s up to 10 min of incubation. Vesicles were incubated with (mmol/l): 100 KCl, 200 mannitol, and 10 Hepes-Tris. Measurements of Ca^{2+}-uptake were started by addition of a $^{45}Ca^{2+}$ containing buffer. The $[Ca^{2+}]$ was 0.25 mmol/l and the incubation temperature 37°C. Values are presented as means ± S.E.; * $P < 0.05$.

Unspecific binding of calcium

It was attempted to obtain a further estimate on the extent of nonspecific binding by varying the concentration of free Ca^{2+} and temperature [11]. In addition,

TABLE I

Uptake of Ca^{2+} into duodenal BBMV of control piglets at 0.03 and 2.0 mM free calcium

Vesicles were preincubated with 150 mmol/l KCl, 1 mmol/l $MgCl_2$, 20 mmol/l Hepes-Tris and (where specified) with 10 μg/ml A23187. The incubation time was 35 s at 25°C. Values are expressed as means ± S.E. The number of preparations is given in parentheses. ** $P < 0.01$; * $P < 0.05$, compared to control conditions at 25°C.

Conditions	Ca^{2+}-uptake (nmol/mg protein)	
	0.03 mmol/l Ca^{2+}	2.0 mmol/l Ca^{2+}
Control, 25°C	1.48 ± 0.25 (8)	3.92 ± 0.38 (8)
Control, 7°C	0.22 ± 0.06 (3) *	1.86 ± 0.27 (3) **
0.5% saponin, 25°C	0.19 ± 0.03 (3) *	0.94 ± 0.07 (4) **
1 mmol/l verapamil, 25°C	0.64 ± 0.09 (7) *	3.57 ± 0.54 (5) n.s
10 μg/ml A23187, 25°C	7.18 ± 1.14 (5) **	38.4 ± 13.1 (5) **

n.s., not significant.

TABLE II

Uptake of Ca^{2+} and glucose space of duodenal BBMV from control, untreated and 1,25(OH)$_2$D$_3$ or vitamin D-treated rachitic piglets

BBMV were incubated for 20 s with 0.25 mmol/l radioactive calcium at 37°C. Glucose space was calculated from radioactivity measurements after incubation with 0.1 mmol/l of [^{14}C]glucose at 37°C for 60 min. Vesicles were preincubated with (mmol/l): 100 KCl, 200 mannitol, 10 Hepes-Tris (pH 7.4). Values are expressed as means ± S.E. The number of piglets is given in parentheses.
* $P < 0.05$ compared to controls.

	Ca^{2+}-uptake (nmol mg^{-1} 20 s^{-1})	Glucose-space (μl mg^{-1})
Control	1.74 ± 0.20 (6)	3.97 ± 0.67 (5)
Rachitic	0.94 ± 0.26 (4) *	3.66 ± 0.47 (4)
Rachitic treated with 1,25-(OH)$_2$D$_3$	1.43 ± 0.38 (3)	2.76 ± 1.05 (3)
Rachitic treated with vitamin D-3	1.94 ± 0.47 (3)	4.70 ± 1.12 (3)

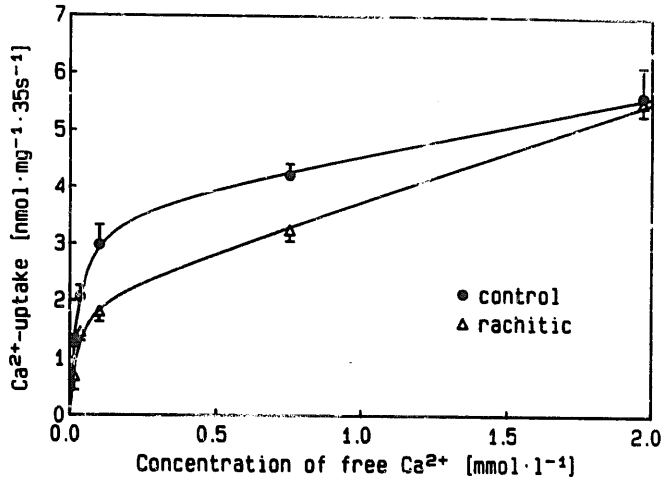

Fig. 3. Uptake of Ca^{2+} into duodenal BBMV of rachitic piglets and control piglets in the presence of increasing free [Ca^{2+}]. For experimental conditions, see legend of Fig. 2. Each value represents the mean value of two different piglets. The V_{max} and K_m values are given in the text. The error bars represent the range of the two preparations.

the effects of A23187, verapamil and saponin on initial phase Ca^{2+}-uptake, when transmembranal concentrations of Ca^{2+} were far from equilibrium, were studied [11]. Initial Ca^{2+}-uptake (35 s) was inhibited at 0.03 and 2.0 mmol/l free Ca^{2+} by both lowering the temperature from 25 to 0°C and by addition of 0.5% saponin (Table I). Relative inhibition of uptake was greater at 0.03 mmol/l than at 2.0 mmol/l free Ca^{2+}. Verapamil depressed Ca^{2+}-uptake significantly only at 0.03 mmol/l. Addition of A23187 stimulated Ca^{2+}-uptake markedly at both calcium concentrations (Table I).

Effects of verapamil

Addition of 1 mmol/l verapamil significantly inhibited Ca^{2+}-uptake under control conditions, whereas it was without effect in rachitic piglets (Fig. 2). In the presence of verapamil the difference in Ca^{2+}-uptake between rachitic and control piglets at low Ca^{2+} concentrations was practically abolished. In the presence of 2.0 mmol/l free Ca^{2+}, the difference in Ca^{2+}-uptake between control and rachitic piglets was also abolished.

Fig. 3 demonstrates the presence of a saturable component in the uptake of calcium at low free [Ca^{2+}] (< 0.5 mmol/l). At higher [Ca^{2+}], nonspecific binding of Ca^{2+} appeared to be the predominating process. The apparent V_{max} of the 35-s Ca^{2+}-uptake for control piglets was 3.63 ± 0.10 nmol/mg protein per 35 s and was reduced to 2.04 ± 0.24 nmol/mg protein per 35 s in rachitic piglets, while the K_m was unaltered be-

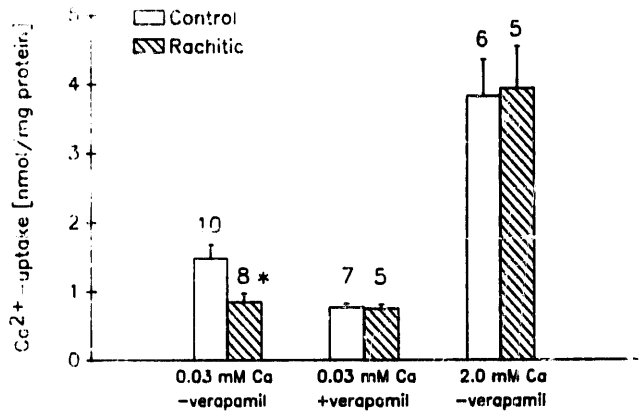

Fig. 2. Uptake of Ca^{2+} into duodenal BBMV of rachitic and control piglets at 0.03 mmol/l free [Ca^{2+}] in presence and absence of 1 mmol/l verapamil and in the presence of 2.0 mmol/l free [Ca^{2+}]. Vesicles were preincubated with 150 mmol/l KCl, 1 mmol/l $MgCl_2$ and 20 mmol/l Hepes-Tris. The incubation time was 35 s at 25°C. Values are presented as means ± S.E.; n = number of piglets; * $P <$ 0.02 compared to control.

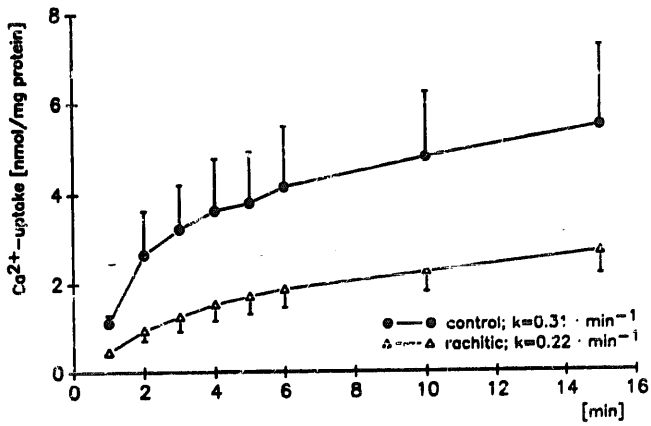

Fig. 4. Ca^{2+}-uptake into isolated brush-border membrane vesicles of rachitic ($n = 4$) and control ($n = 6$) piglets over 15 min in the presence of a K^+-diffusion potential (means ± S.E.). The rate constants of Ca^{2+}-uptake between the two sets of experiments differed with $P < 0.05$ (Table III). The concentration of free Ca^{2+} was 0.03 mmol/l.

TABLE III

Rate constants, k, of Ca^{2+}-uptake (mean ± S.E.; (min^{-1})) in the presence of a K^+-diffusion potential from 10 vesicle preparations (four from rachitic piglets, six from control piglets)

The k values were calculated by fitting eight data points from individual incubations to the equation $Y = A \cdot (1 - e^{-kt})$. $P < 0.05$.

	k	
	control	rachitic
	0.25 ± 0.030	0.28 ± 0.012
	0.37 ± 0.026	0.25 ± 0.008
	0.29 ± 0.015	0.15 ± 0.015
	0.33 ± 0.041	0.22 ± 0.011
	0.28 ± 0.036	
	0.35 ± 0.075	
Mean:	0.31 ± 0.018	0.22 ± 0.028

tween the two groups (0.029 ± 0.002 mmol/l in control and 0.025 ± 0.009 mmol/l in rachitic piglets).

Effect of a potassium diffusion potential

In another series of experiments, vesicular Ca^{2+}-uptake was measured in rachitic and control piglets in the presence of an intravesicular negative potassium-diffusion potential. Under these conditions, the difference in uptake between the groups was maintained for at least 15 min (Fig. 4). Ca^{2+}-uptake by control piglets after 2 min of incubation was 278% of that of rachitic piglets. In the absence of a potential, this difference had already begun to decline and was only 175% (data not shown). The rate constants of Ca^{2+}-uptake between rachitic and control piglets were significantly different ($P < 0.05$) while the A values, which represent maximum uptake, were not different (Table III). The time course of Ca^{2+}-uptake in the presence of a diffusion potential is illustrated in Fig. 4.

Treating of rachitic piglets with either 1,25-$(OH)_2D_3$ or vitamin D-3 elevated vesicular Ca^{2+}-uptake to values found in control piglets (Fig. 5, Table II). The equilibrium glucose spaces which were measured in the same preparations did not differ significantly between rachitic and control piglets (Table II), indicating that the difference in Ca^{2+}-uptake between rachitic and control piglets and the effects of treatment with 1,25-$(OH)_2D_3$ or vitamin D-3 were not caused by changes of vesicle volumes.

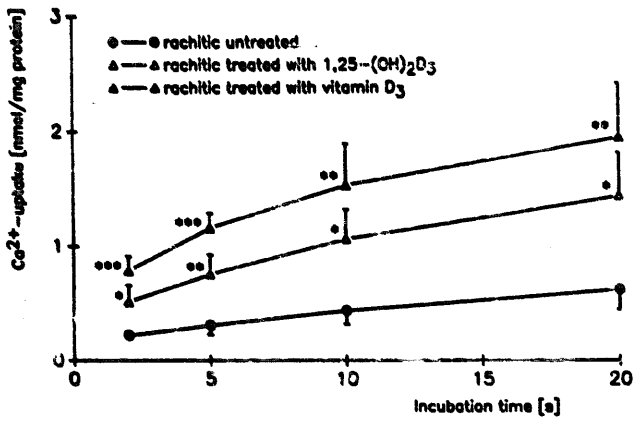

Fig. 5. Uptake of Ca^{2+} into duodenal BBMV of untreated rachitic piglets ($n = 8$) and rachitic piglets treated with either 1,25-$(OH)_2D_3$ ($n = 3$) or a pharmacological dose of vitamin D-3 ($n = 3$). The experimental conditions were as described in Fig. 1; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with untreated piglets.

TABLE IV

Effects of verapamil (1 mmol/l, mucosal) on unidirectional and net fluxes of calcium across stripped duodenal mucosa of rachitic and control piglets

For experimental details, see Materials and Methods. Values are expressed as means ± S.E.; $n = 4$.
* $P < 0.01$ compared to control.

	Ca^{2+} flux (nmol cm^{-2} h^{-1})	
	control period	verapamil
Control piglets		
ms	116.9 ± 8.8	83.5 ± 6.1 *
sm	41.8 ± 2.6	48.2 ± 2.4
net	75.1 ± 9.3	48.9 ± 6.0 *
Rachitic piglets		
ms	54.8 ± 10.4	48.9 ± 5.6
sm	48.5 ± 5.5	51.5 ± 5.7
net	6.2 ± 11.9	−2.6 ± 7.5

Kinetic experiments with vesicles from three 1,25-$(OH)_2D_3$-treated rachitic piglets by varying free Ca^{2+} concentrations resulted in apparent V_{max} values of 3.07 ± 0.44 nmol/mg protein per 35 s and K_m values of 0.053 ± 0.027 mmol/l. A comparison of these values with those of control and untreated rachitic piglets (see above and Fig. 3) showed that V_{max} increased due to the treatment with 1,25-$(OH)_2D_3$ whereas K_m did not change significantly.

Ca^{2+} fluxes across stripped intestinal mucosa

The stripped short-circuited duodenal mucosa from control piglets actively transported Ca^{2+} from lumen to blood. The mucosa from rachitic piglets showed no active transport of calcium (Table IV). In control piglets, verapamil partly inhibited ms flux but did not influence sm flux. The drug, however, exerted no effect on unidirectional fluxes of rachitic piglets (Table IV). Verapamil had also no effect on tissue conductance (24 ± 3 mS cm^{-2}), potential difference (0.29 ± 0.22 mV) and short-circuit currents (0.25 ± 0.20 μmol cm^{-2} h^{-1}) in both control and rachitic piglets.

Discussion

Rates of Ca^{2+}-uptake described in this study were similar to those reported for pig intestine by Maenz and Forsyth [25]. Though sodium-dependent overshoot in glucose uptake revealed functional integrity of the

vesicular membranes from both +D and −D animals, the overshoot was consistently smaller with vesicles from rachitic piglets. This was not caused by a difference in vesicular volumes, since equilibrium uptake of glucose was not affected by the vitamin D status. Similar observations were also made, however, by Bikle et al. [6] and Peterlik et al. [26] in chick vesicular preparations.

Uptake of Ca^{2+} into BBMV may be superimposed by nonspecific binding. Other investigators have shown, however, that calcium bound to the extravesicular surface can be removed to a great extent by washing with EGTA, whereas calcium bound to the intravesicular surface cannot [6,7,27]. We have addressed these issues by measuring the effects of lowering temperature, of adding saponin, verapamil and A23187 on vesicular uptake of calcium. The results of these experiments indicated that a considerable portion of Ca^{2+} associated with the vesicles was transported across the membrane, rather than being attached to it by nonspecific mechanisms. And it is worth noting that the transported portion was always greater at low [Ca^{2+}] (Table I).

Disrupting vesicular membranes by saponin (no glucose space) markedly inhibited uptake. Addition of saponin, however, appeared to affect both Ca^{2+}-transport and binding. Apparently, under these conditions, the vesicles were not only opened, but also removal of certain membrane proteins might have been achieved by the detergent. The stimulatory effect of the Ca^{2+} ionophore, A23187, at low and high [Ca^{2+}] demonstrated that the vesicles possessed the ability to accumulate Ca^{2+}.

The extent of nonspecific binding in preparations from rachitic piglets tended to be even higher than in those from control piglets (Fig. 3). This means that the differences in real uptake between both groups, reported in this study, may even be underestimated.

It appears of interest that an inhibitory effect of verapamil on both vesicular uptake and transmural transport of calcium was only present in control piglets Verapamil did not inhibit passive bidirectional fluxes across stripped mucosa. Thus, we suggest that a verapamil sensitive portion of uptake was related to the active intestinal Ca^{2+}-transport component and that this uptake represented the vitamin D-dependent portion. The concentration of verapamil which was used in this study, as well as in studies of other authors, appears high. But the necessity of such high concentrations added to intestinal mucosa is well documented [8,28,29]. In this respect, the mechanism of Ca^{2+}-uptake probably differs from that through Ca^{2+}-channels of excitable cells.

The demonstration of a saturable component of Ca^{2+}-uptake in our studies at low [Ca^{2+}] agrees with findings made with rat intestine [11,13] where uptake also declined between 1 and 60 s and was highest at incubation times below 1 s [11]. We measured uptake from 2 to 60 s and found that uptake was more consistent between 20 and 40 s than at 2 s. Since we know from other experiments that the K_m of Ca^{2+}-uptake remains unchanged when extending the incubation time up to 60 s, we expected to obtain suitable values of Ca^{2+}-uptake between 20 to 40 s, being not too far away from the initial rate at zero time.

While vesicular uptake in the absence of a diffusion potential was only related to vitamin D at incubation times < 35 s, the vitamin D-dependent difference persisted for 15 min in the presence of an inside negative K^+ diffusion potential. The electrical driving force generated by the potential resulted in a longer lasting Ca^{2+}-influx but not in a markedly higher influx rate of calcium. It is possible that the additional preparative steps which were required to generate the potassium diffusion potential have led to a reduction in the number of calcium channel molecules in the membranes.

Despite some contradictory findings, stimulation of Ca^{2+}-uptake into chick [6,7,9,30,31] and rat [8,10] BBMV by treating the animals with 1,25-$(OH)_2D_3$ has been reported earlier. However, the kinetics of uptake reported in those studies differ in several ways from the findings reported here. First, the reported apparent K_m values are always higher than 0.2 mmol/l compared to 0.025–0.029 mmol/l which we found. Second, the vitamin D-related difference in vesicular Ca^{2+}-uptake was also present at considerably higher extravesicular Ca^{2+} concentrations and it lasted for a much longer time. Finally, no discrimination was made in these studies between saturable and non-saturable mechanisms of uptake. At present, we have no explanation for the differences in Ca^{2+}-uptake between those reported in the literature and our findings. The physiological significance of the low K_m of Ca^{2+}-uptake which we found remains to be determined. Free calcium concentrations in duodenal chyme of 7-week-old piglets are in the range of 1 mmol/l 3 h after feeding (unpublished observation). The apparent K_m value of the saturable component which is described in our study is, however, in good agreement with reports from Ghishan et al. for humans [12] and from others for rats and hamsters [13,14]. These investigators also made a distinction between saturable and non-saturable uptake components. Despite some differences between our findings and other observations, there is agreement with results in chicks [7,9,31] and rats [10] that only V_{max} responded to 1,25-$(OH)_2D_3$ and that the K_m value was not influenced.

We also demonstrated that the reduced vesicular uptake of calcium coincided with the absence of the active transmural Ca^{2+}-transport. Furthermore, verapamil inhibited both active transmural Ca^{2+}-transport and vesicular uptake of Ca^{2+} in control piglets. Con-

versely, treatment of rachitic piglets with 1,25-$(OH)_2D_3$ or vitamin D-3 stimulated influx of Ca^{2+} into BBMV. Previous studies have shown that treatment of rachitic piglets with pharmacological doses of vitamin D-3 resulted in a significant rise of 1,25-$(OH)_2D_3$ in plasma, despite the absence of renal 1-hydroxylation [16]. We, therefore, conclude that 1,25-$(OH)_2D_3$ stimulates active intestinal Ca^{2+}-transport by increasing the number of verapamil-sensitive transport components in the brush-border membrane.

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