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ATP-DEPENDENT CALCIUM TRANSPORT AND ITS CORRELATION WITH Ca^{2+}-ATPase ACTIVITY IN BASOLATERAL PLASMA MEMBRANES OF RAT DUODENUM

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Isolated basolateral plasmamembrane vesicles from rat duodenum epithelial cells exhibit ATP-dependent calcium-accumulation and Ca^{2+}-dependent ATPase activity. Calcium accumulation stimulated by ATP is prevented by the calcium ionophore A23187, inhibited 80% by 0.1 mM orthovanadate but is not effected by oligomycin. Calcium accumulation is not observed with the substrate β-γ-(CH_2)-ATP, ADP and *p*-nitrophenyl phosphate. Kinetic studies reveal an apparent K_m of 0.2 μM Ca^{2+} and a V_{max} of 5.3 nmol Ca^{2+}/min per mg protein for the ATP-dependent calcium-uptake system. Calmodulin and phenothiazines have no effect on calcium accumulation in freshly prepared membranes, but small effects are inducable after a wash with a 5 mM EGTA. The kinetic parameters of Ca^{2+}-ATPase are: $K_m = 0.25$ μM Ca^{2+} and $V_{max} = 19.2$ nmol P_i/min per mg protein. Three techniques, osmotic shock, treatment with Triton X-100 or the channel-forming peptide alamethacin, reveal that about 40% of the vesicles are resealed. Assuming that half of the resealed vesicles have an inside-out orientation, the V_{max} of ATP-dependent calcium uptake amounts to 25 nmol Ca^{2+}/min per mg protein and of the Ca^{2+}-ATPase to 23 nmol P_i/min per mg protein. The close correlation between kinetic parameters of Ca^{2+}-ATPase and ATP-dependent calcium-transport strongly suggests that both systems are expressions of a Ca^{2+}-pump located in duodenal basolateral plasma membranes.

Introduction

Present knowledge of calcium transport mechanisms in epithelial cells is rather limited when compared with the vast literature on calcium transport in nerve, muscle and red blood cells. However, intestinal Ca^{2+} absorption is important in whole body Ca^{2+} homeostasis and it is therefore of interest to understand in detail the mechanism of Ca^{2+} absorption and its regulation by the seco-steroid hormone 1,25-dihydroxy vitamin D-3 [1–5]. Rat duodenum and chicken small intestine have been useful models to study effects of $1,25(OH)_2D_3$ on Ca^{2+} absorption [1–5], but studies with isolated plasma membrane preparations are needed to gain more insight into the mechanisms behind Ca^{2+} absorption.

In a previous study we demonstrated Ca^{2+}-ATPase activities with high affinity for Ca^{2+} in plasmamembranes of rat duodenum [6]. Further studies led to the conclusion that Ca^{2+}-induced ATP hydrolysis was the result of two enzymatic activities, namely alkaline phosphatase present in brush borders as well as basolateral membranes, and a more specific Ca^{2+}-ATPase exclusively located in basolateral plasma membranes [7]. This

Abbreviations: EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; K_m, apparent half maximal saturation constant; V_{max}, maximal velocity.

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latter conclusion was confirmed by phosphorylation studies with duodenal plasma membranes [8]. In the study described here, we report the presence of an ATP-dependent Ca^{2+}-pumping system in basolateral membranes of rat duodenum, which properties closely parallel those of Ca^{2+}-ATPase activity in the same membrane preparation.

Methods and Materials

Membrane preparation

Four male Wistar rats (180–210 g) were decapitated and the proximal 10–15 cm of the small intestine were removed and rinsed with ice-cold isotonic saline solution. Epithelial cells were isolated according to Stern [9] and collected after centrifugation at $400 \times g$ for 5 min. Further isolation was performed at 0–4°C. The cells were homogenized during 1 min using a Polytron (Braun) in 30 ml medium containing 25 mM NaCl, 1 mM Hepes-Tris (pH 8.0) and the protease inhibitor phenylmethanesulphonyl fluoride (0.2 mM). The homogenate was diluted to 75 ml with the same solution and centrifuged at $550 \times g$ for 15 min to remove brush borders and cell debris. The supernatant was centrifuged at $90000 \times g$ for 20 min. This pellet was suspended gently in a loose-fitting Dounce apparatus (±100 strokes) in 15 ml of an isotonic sucrose buffer containing 250 mM sucrose, 5 mM Hepes-Tris (pH 7.4) and 5 mM $MgCl_2$. The basolateral membranes in this suspension were separated from mitochondria and residual brush borders by centrifugation in 40% sorbitol buffered with 5 mM Hepes-Tris (pH 7.4), as described before [7]. Finally, the purified basolateral plasma membrane fraction was pelleted at $100000 \times g$ for 15 min and resuspended in a buffer containing 20 mM Hepes-Tris (pH 7.4), 100 mM KCl, and 5 mM $MgCl_2$ (standard uptake medium) and used for uptake studies within 6 h after the start of isolation. For experiments in which the effect of calmodulin was studied the $90000 \times g$ pellet was homogenized in 250 mM sucrose buffer containing 5 mM EGTA. For enzyme studies, membranes were used which had been frozen in liquid nitrogen immediately after isolation and stored at −80°C as long as 5 days. In this period no decrease in Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities could be detected when compared to freshly prepared membranes.

Ca^{2+}-uptake measurements

Ca^{2+}-uptake experiments were done at 25°C in the standard uptake medium which contained 0.05 to 5 μM free Ca^{2+} and 1.5 μCi/ml ^{45}Ca. Free Ca^{2+} was buffered with 0.5 mM EGTA. Ca^{2+} uptake was measured in the presence or absence of 3 mM Tris-ATP in a final volume of 300 μl. The free Ca^{2+}-concentration was calculated from the different equilibria of H^+, Ca^{2+} and Mg^{2+} with the ligands EGTA and ATP at an ionic strength of 0.1 M at 25°C. The appropriate dissocation constants were taken from Sillén and Martell [10] and from Scharff [11]. The calculations were carried out on a programmable pocket calculator (TI-58).

The Ca^{2+}-uptake reaction was started by adding membranes (1 mg protein/ml) to the uptake medium. At appropriate times, 50-μl aliquots were quenched into 1 ml ice-cold stop solution (standard uptake medium plus 1 mM $LaCl_3$). Membranes were collected on 0.45 μm Sartorius filters (type SM 11306) which were precooled with 2 ml ice-cold stop solution. The filter with sample was washed twice with 2 ml stop solution and filtration was completed within 15 s. Filters were placed in 5 ml scintillation cocktail and radioactivity was counted in a scintillation counter (Philips). The kinetics of Ca^{2+}-uptake were studied by measuring initial rates of uptake. In these experiments, membranes were prewarmed at 25°C for 5 min before starting Ca^{2+} uptake.

To study effects of calmodulin on ATP-dependent Ca^{2+} uptake, membranes were preincubated with 10 μg/ml calmodulin. Calmodulin was purified from bovine brain as described before [8]. Solutions of trifluoperazine and penfluridol were freshly prepared and preincubation of membranes with phenothiazines was carried out in the dark to prevent free radical formation [12].

The solutions used for Ca^{2+} uptake studies and enzyme assays were made with water which had been distilled three times in the presence of EDTA. Calcium ionophore A23187, oligomycin, Triton X-100, alamethicin and penfluridol were added to the uptake medium or enzyme assay mixtures as 100% ethanol solutions. The control experiments contained the same amount of ethanol (< 0.1%).

Enzyme studies

Ca^{2+}-induced ATP hydrolysis was assayed in the standard uptake medium and was assayed as the difference between the rates of ATP hydrolysis with and without 0.1–10 μM Ca^{2+} as described before [7]. For Ca^{2+}-stimulated *p*-nitrophenyl phosphate hydrolysis the same medium was used with *p*-nitrophenyl phosphate instead of ATP and NaCl instead of KCl to avoid interference of K^+-stimulated *p*-nitrophenylphosphatase activity. Replacement of KCl by NaCl did not influence Ca^{2+}-ATPase activity. $(Na^+ + K^+)$-ATPase, sucrase, succinate dehydrogenase, NADPH-cytochrome *c* reductase and *p*-nitrophenylphosphatase were assayed as described before [7,13]. Protein was determined with a commercial reagent kit (BioRad). For membrane sidedness studies, basolateral membranes were suspended in 250 mM sucrose, and 20 mM Hepes-Tris (pH 7.4) instead of 100 mM KCl to get optimal osmotic shock effects. For these experiments vesicles (0.25 mg protein) were diluted 20–50-fold in distilled water (osmotic shock) or sucrose buffer (control) just prior to the assay. Membranes were also preincubated with Triton X-100 or alamethicin for 10–15 min at 25°C. These pretreated membranes were diluted 20–25-fold in $(Na^+ + K^+)$-ATPase assay medium and incubated for 15 min at 37°C.

Materials

Tris-ATP, β-γ-(CH_2)-ATP, oligomycin, theophylline, ADP, AMP and EGTA were obtained from Sigma (St. Louis, U.S.A.). All other chemicals used were of the purest grade. $^{45}CaCl_2$ (15 m Ci/mg) was obtained from New England Nuclear (Dreieich, F.R.G.), Orthovanadate was from ICN Pharmaceuticals Inc. (Plainview, U.S.A.), Alamethicin was a gift from Dr. J.E. Grady, Upjohn Comp. (Kalamazoo, U.S.A.), trifluoperazine dihydrochloride was a gift from Dr. E. Lefkof, Jr., Smith Kline and French Laboratories (Philadelphia, U.S.A.) and penfluridol was kindly provided by Janssen Pharmaceutica (Beerse, Belgium).

Results

Previously, we have used basolateral membranes for enzyme studies, which had been isolated from enterocytes homogenized in 5 mM EDTA [7,8]. These membranes were not resealed for Ca^{2+} and ATP [7]. In the study described here membrane vesicles are used which are resealed for Ca^{2+} (Fig. 1). The factors leading to resealing are homogenization in the presence of 25 mM NaCl with no EDTA. The purification factors of different marker enzymes and their recoveries in this basolateral membrane preparation are shown in Table I. $(Na^+ + K^+)$-ATPase is purified 10-fold with respect to the initial homogenate but 17-, 15- and 9-fold with respect to mitochondria, brush borders and smooth endoplasmic reticulum, respectively. This means a better purity than previously reported, especially with respect to contamination with smooth endoplasmic reticulum [7,13].

ATP-dependent Ca^{2+} uptake

The temperature dependency of Ca^{2+} uptake in the presence of ATP in basolateral membrane vesicles is given in Fig. 1. At 25°C as well as 37°C, a rapid uptake of Ca^{2+} is observed but with a faster initial rate at 37°C. After 5 min at 37°C the Ca^{2+} content of the vesicles appears to decrease, while at 25°C a plateau is reached between 10 to 20 min and therefore we studied Ca^{2+} uptake at 25°C.

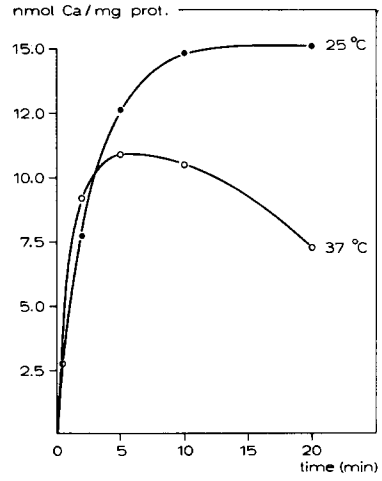

Fig. 1. Effect of temperature on Ca^{2+} uptake in the presence of ATP by basolateral plasma membrane vesicles of rat duodenum. Free Ca^{2+} concentration in the uptake medium is 1 μM. ATP concentration is 3 mM.

TABLE I

RECOVERY AND PURITY OF BASOLATERAL MEMBRANES OF RAT DUODENAL EPITHELIUM

The recovery is expressed as percentage of the total enzyme activity present in the initial homogenate. The purification factor gives the ratio between the specific activities of the final membrane preparation and the initial homogenate. The data are given as mean values with the standard error of the mean and the number of observations in parentheses.

	Protein	$(Na^+ + K^+)$-ATPase	Succinate dehydrogenase	Sucrase	NADPH-cyt. *c* reductase
% recovery	1.5±0.2 (8)	15.4±2.0 (7)	0.9±0.3 (4)	1.1±0.3 (3)	1.9±0.5 (3)
Purification factor	–	10.3±1.6 (7)	0.6±0.3 (4)	0.7±0.1 (3)	1.2±0.3 (3)

In Fig. 2 the effect of ATP and the Ca^{2+}-ionophore A23187 on calcium uptake is shown. At 1 μM Ca^{2+}, a 10-fold stimulation by ATP is observed above the plateau reached in the absence of ATP. In the presence of A23187, ATP-dependent Ca^{2+} accumulation is abolished, while addition of A23187 after Ca^{2+} had been accumulated induces a rapid release of Ca^{2+}. These results indicate that Ca^{2+} is transported into the intravesicular space and is not superficially bound. Moreover, the ionophore effect proves uptake of Ca^{2+} against a concentration gradient.

Fig. 3 shows the kinetic properties of ATP-dependent Ca^{2+} uptake in basolateral membranes. Between 0.05 and 5 μM Ca^{2+}, ATP-dependent Ca^{2+}-uptake is linear with time the first two minutes. In Fig. 3 initial rate values are given which have been determined from Ca^{2+} uptake values at 1 and 2 min. ATP-dependent Ca^{2+} uptake saturates at Ca^{2+} concentrations between 0.5 and 1 μM and kinetic analysis yields an apparent K_m of 0.20 μM Ca^{2+} and a V_{max} value of 5.3 nmol Ca^{2+}/min per mg protein.

The substrate dependency of Ca^{2+}-accumulation is shown in Fig. 4. *p*-Nitrophenyl phosphate has no effect while ADP slightly stimulates Ca^{2+}-uptake. This may be explained by presence of adenylate kinase activity which produces ATP and AMP from ADP. The non-hydrolysable

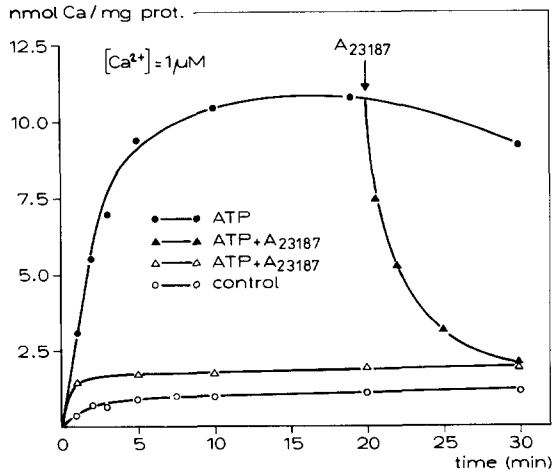

Fig. 2. Effect of calcium-ionophore A23187 on ATP-dependent Ca^{2+} uptake by basolateral membranes of rat duodenum. Free Ca^{2+} concentration in the uptake medium is 1 μM. A23187 concentration is 10 μg/ml.

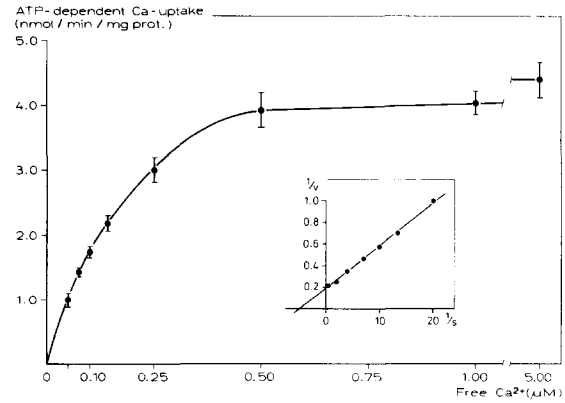

Fig. 3. Kinetics of ATP-dependent Ca^{2+} uptake in basolateral plasmamembranes of rat duodenum. Mean values with standard errors of four experiments are given. Inset shows a Lineweaver-Burk plot of the data. Initial rate values for ATP-dependent Ca^{2+} uptake have been corrected for ATP-independent Ca^{2+} uptake.

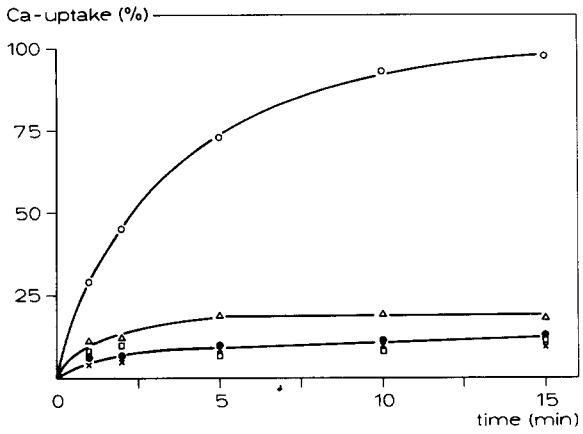

Fig. 4. Substrate dependency of Ca^{2+} uptake in basolateral membranes. × ——— ×, 3 mM β-γ-(CH_2)-ATP; ○ ——— ○, 3 mM ATP; △ ——— △, 3 mM ADP; □ ——— □, 3 mM *p*-nitrophenyl phosphate; ● ——— ●, control.

ATP-analog β-γ-(CH_2)-ATP has no effect on Ca^{2+} uptake which proves that ATP hydrolysis is needed for Ca^{2+}-accumulation. This specificity of the transport system for ATP is similar to the ATP preference of Ca^{2+}-ATPase described previously [7].

In Table II the effects are demonstrated of different inhibitors on ATP-dependent Ca^{2+} uptake. Oligomycin at a concentration which completely blocks Ca^{2+} uptake in mitochondria from chick intestine [14] has no effect which excludes a possible mitochondrial contribution to Ca^{2+} uptake. Furthermore, ATP-dependent Ca^{2+} uptake is insensitive to theophylline, a good inhibitor of alkaline phosphatase, also present in basolateral membranes [7,15]. Orthovanadate, a potent inhibitor of Ca^{2+}-ATPase and ATP-dependent Ca^{2+} transport in sarcoplasmic reticulum [16], red cell [17] and heart sarcolemna [18], inhibits more than 80% of ATP-driven Ca^{2+} uptake in basolateral membranes.

TABLE II

EFFECTS OF INHIBITORS ON ATP-DEPENDENT Ca^{2+} UPTAKE IN BASOLATERAL MEMBRANES OF RAT DUODENUM

The ATP-dependent Ca^{2+} uptake was determined after 10 min of incubation in the standard uptake medium at 1 μM Ca^{2+} (see Methods and Materials) with the uptake value in the absence of ATP subtracted from the uptake in the presence of ATP. Mean values are given with the standard error of the mean and the number or experiments in parentheses.

	ATP-dependent Ca^{2+} uptake (% of control)
Control	100
Oligomycin (10 μg/ml)	96.9±5.2 (4)
Theophylline (1 mM)	100.4±9.7 (4)
Vanadate (0.1 mM)	18.6±2.1 (4)

Ca^{2+}-induced ATP hydrolysis

Fig. 5 shows the rate of ATP-hydrolysis by basolateral membranes induced with Ca^{2+} at concentrations between 0.1 and 10 μM. Since 75% of this Ca^{2+}-dependent ATP-hydrolysis is readily inhibited by theophylline it is obvious that most of this activity is due to activation of alkaline phosphatase by Ca^{2+} [7]. In order to find out to what extent alkaline phosphatase is inhibited by theophylline its effect on Ca^{2+}-induced *p*-nitrophenyl phosphate hydrolysis was studied.

Table III gives a comparison between Ca^{2+}-induced ATP and *p*-nitrophenyl phosphate hydrolysis and the effect of theophylline. Since theophylline inhibits completely *p*-nitrophenyl phosphate hydrolysis, it seems justified to conclude that theophylline-insensitive ATP hydrolysis gives us the specific Ca^{2+}-ATPase activity of basolateral mem-

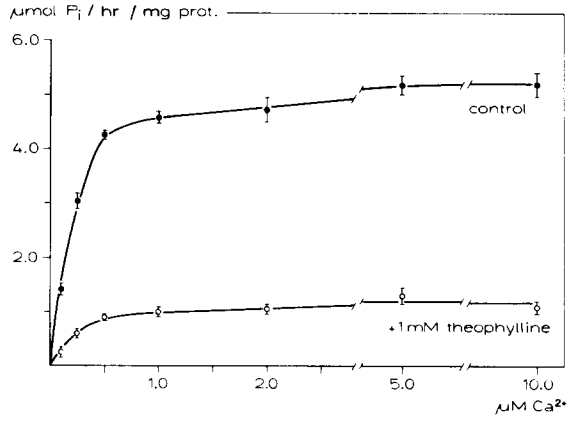

Fig. 5. ATP hydrolysis by basolateral membranes induced by Ca^{2+} in the presence and absence of theophylline.

TABLE III

EFFECT OF INHIBITORS ON Ca^{2+}-STIMULATED ATP AND *p*-NITROPHENYL PHOSPHATE HYDROLYSIS IN BASOLATERAL MEMBRANE VESICLES OF RAT DUODENUM

The results are expressed as μmol P_i/h per mg protein. ATP and *p*-nitrophenyl phosphate hydrolysis are assayed in the presence and absence of 1 μM free Ca^{2+} at pH 7.4 (see Methods and Materials). Mean values are given with the standard error of the mean and the number of experiments in parentheses.

	Ca^{2+}-stimulated ATP hydrolysis	Ca^{2+}-stimulated *p*-nitrophenyl phosphate hydrolysis
Control	4.78±0.43 (7)	3.32±0.30 (5)
Theophylline (1 mM)	1.15±0.12 (4)	0.08±0.06 (5)
Vanadate (0.1 mM)	2.70±0.32 (3)	2.37±0.15 (4)
Vanadate+theophylline	0.41±0.06 (6)	n.s. [a] (3)

[a] No significant stimulation (n.s.) above the residual *p*-nitrophenyl phosphate hydrolysis in the absence of Ca^{2+}.

branes. From Fig. 5 and Table III it follows that Ca^{2+}-ATPase has an apparent K_m of 0.25 μM Ca^{2+} and a V_{max} of 1.15 μmol P_i/h per mg protein. This K_m value is not significantly different from the one for ATP-dependent Ca^{2+} uptake. Since orthovanadate effects both Ca^{2+}-ATPase and alkaline phosphatase (Table III) this inhibitor is of no use in discriminating between the two enzymes.

Orientation of basolateral plasma membrane vesicles

To compare V_{max} values of ATP-dependent Ca^{2+} uptake and Ca^{2+}-ATPase activity in basolateral membranes we need information on the orientation of the vesicle population. Only vesicles which have an inside-out orientation and which are sealed for Ca^{2+} will exhibit ATP-dependent Ca^{2+} uptake but all leaky plus inside-out vesicles will contribute to Ca^{2+}-ATPase activity. Right-side-out vesicles which are sealed for ATP will not contribute to either process. In Table IV results are summarized of experiments on latency of $(Na^+ + K^+)$-ATPase activity in basolateral membrane vesicles. Assuming that resealed vesicles are impermeable for ATP and ouabain, then $(Na^+ + K^+)$-ATPase activity of untreated vesicles reflects the leaky vesicle fraction. Osmotic shock and treatment with detergents should unmask latent $(Na^+ + K^+)$-ATPase activity. Stimulation of $(Na^+ + K^+)$-ATPase activity by osmotic shock, Triton X-100, osmotic shock plus Triton X-100 is between 50 and 60%, without a significant difference ($P > 0.6$) among the three treatments. Treatment of vesicles with the channel-forming antibiotic

TABLE IV

EFFECTS OF OSMOTIC SHOCK AND DETERGENTS ON $(Na^+ + K^+)$-ATPase ACTIVITY IN BASOLATERAL MEMBRANE VESICLES OF RAT DUODENUM

$(Na^+ + K^+)$-ATPase activities are expressed as μmol P_i/h per mg protein±S.E. (the number of experiments in parentheses). The percentage sealed vesicles is calculated from the stimulation in $(Na^+ + K^+)$-ATPase activity. The vesicles are osmotically shocked or preincubated with Triton X-100 or alamethicin as described in Methods. The dilution factors and concentration range giving maximal stimulation are given in parentheses.

Treatment	$(Na^+ + K^+)$-ATPase activity	Percentage resealed vesicles
Control	29.70±3.37 (14)	
Osmotic shock (25–50x)	45.14±6.24 (12)	34
Triton X-100 (0.3–0.75 mg/mg protein)	48.71±4.75 (21)	39
Triton X-100+shock	53.40±2.86 (3)	44
Alamethicin (0.25–0.50 mg/mg protein)	43.36±4.46 (14)	32

TABLE V

EFFECTS OF CALMODULIN AND PHENOTHIAZINES ON THE INITIAL RATE OF ATP-DEPENDENT Ca^{2+} UPTAKE IN BASOLATERAL MEMBRANES OF RAT DUODENUM

Basolateral membranes have been pretreated with 5 mM EGTA (see Methods). Mean values are given with S.E. and number of observations in parentheses.

Phenothiazines (10^{-4} M)	ATP-dependent Ca^{2+} uptake (nmol Ca^{2+}/min per mg protein)			
	0.5 μM Ca^{2+}		1.0 μM Ca^{2+}	
	–	+calmodulin (10 μg/ml)	–	+calmodulin (10 μg/ml)
Control	2.53±0.22 (6)	3.98±0.17 (6)	4.22±0.30 (4)	5.04±0.45 (4)
Trifluoperazine	2.10±0.06 (3)	2.71±0.13 (3)	2.94±0.70 (3)	3.95±0.70 (3)
Penfluridol	2.33±0.16 (4)	2.59±0.24 (4)	4.41±0.10 (3)	4.56±0.26 (3)

alamethicin gives a somewhat smaller stimulation of ($Na^+ + K^+$)-ATPase activity. From Table IV we conclude that roughly 40% of the vesicles is resealed for either ATP or ouabain. Assuming that the resealed vesicles have for 50% an inside-out orientation then only 20% of the total population is involved in ATP-driven Ca^{2+} uptake. Hence, the V_{max} of ATP-dependent Ca^{2+} uptake should increase to 25 nmol Ca^{2+}/min per mg protein. The same reasoning makes it likely that the measured Ca^{2+}-ATPase activity is 80% of the total activity. Therefore, the V_{max} of theophylline-insensitive Ca^{2+}-ATPase amounts to 24 nmol P_i/min per mg protein (Table III), which equals the V_{max} of the Ca^{2+}-uptake system.

Effects of calmodulin and phenothiazines

The effects of calmodulin and phenothiazines (trifluoperazine, penfluridol) were studied on ATP-dependent Ca^{2+} uptake and on Ca^{2+}-ATPase activity in basolateral membranes. With freshly prepared vesicles, no significant effects could be found on both systems, even after 10 min preincubation with calmodulin or phenothiazines. However, when after the initial homogenization, 5 mM EGTA is added to the isolation buffers, then effects of calmodulin could be demonstrated. These effects are summarized in Table V. Calmodulin increases the initial rate of Ca^{2+} uptake by more than 50% at 0.5 μM Ca^{2+} but only by 20% at 1 μM free Ca^{2+}. This suggests an effect on affinity rather than on the V_{max} of the Ca^{2+}-uptake system. The stimulatory effects of calmodulin are completely inhibitable by phenothiazines (Table V).

Discussion

This study provides strong evidence for the identity of Ca^{2+}-ATPase activity and the ATP-dependent Ca^{2+}-uptake system in basolateral membranes of duodenal epithelium. The affinity of both systems for Ca^{2+} is identical (0.2 μM Ca^{2+}) and this affinity is well suited for a Ca^{2+}-pump functioning at Ca^{2+} concentrations well below 1 μM.

A significant stimulation of ATP-dependent Ca^{2+}-uptake by calmodulin could not be demonstrated with freshly prepared, untreated membranes. This observation suggests that endogenous calmodulin is still present after isolation of the membranes, which is not surprising in view of the fact that osmotically shocked and EDTA-treated basolateral plasma membranes still have abundant calmodulin (10 to 20 μg/mg membrane protein) [8]. Similar findings were reported for Ca^{2+}-ATPase in plasmamembranes of smooth muscle [19] and of rat corpus luteum [20]. An effect of calmodulin became visible after washing basolateral membranes with 5 mM EGTA, but it was relatively small compared to stimulatory effects of calmodulin reported in red blood cells [12,21], heart sarcolemna [18] and brain synaptic

plasmamembranes [22]. This small effect may be explained by uncomplete release of calmodulin by EGTA. Hypotonic as well as hypertonic shocks in the presence of EGTA were used with heart sarcolemna to release calmodulin [18]. These treatments made basolateral vesicles completely leaky for Ca^{2+}, so attempts to induce greater calmodulin effects failed. With freshly prepared basolateral membranes, trifluoperazine had only a small effect on Ca^{2+}-ATPase and ATP-dependent Ca^{2+} uptake, comparable to rat corpus luteum plasma membranes [20]. This result suggests tightly bound endogenous calmodulin, not susceptible to phenothiazines. Previously, we have shown that Ca^{2+}-ATPase and phosphorylated intermediate formation could be inhibited with chlorpromazine [7,8]. However, these membranes were isolated from cells homogenized in 5 mM EDTA. Obviously, the osmotic shock in the presence of EDTA during cell disruption, renders the calmodulin-Ca^{2+}-ATPase association more susceptible to phenotiazines. On the other hand, these membranes failed to accumulate Ca^{2+} due to leakiness [7]. Summarizing the results with calmodulin and phenotiazines, it is most likely that the small effects on Ca^{2+} transport and Ca^{2+}-ATPase can be explained by tightly associated endogenous calmodulin. It remains puzzling that 10^{-4} M trifluoperazin has only a very small effect on Ca^{2+} transport, since at this concentration basal Ca^{2+}-ATPase activity, in the absence of calmodulin, can be inhibited in red cells and heart sarcolemna [12,18]. It may be possible that in duodenal cells an inherently different mechanism regulates the Ca^{2+} pump, since Ca^{2+} absorption is under control of 1,25-dihydroxy vit. D_3 [1–5]. This possibility evolves from a study in which we demonstrated that Ca^{2+}-ATPase activity as well as ATP-dependent Ca^{2+} uptake in basolateral membranes significantly increased after repletion of vitamin D-3-deficient rats with 1,25-dihydroxy vitamin D-3 [23]. Moreover, Thomasset et al. [24] demonstrated that $1,25(OH)_2D_3$ has no effect on calmodulin content of duodenal cells [24].

Very recently, Nellans and Popovitch [25] also reported ATP-dependent Ca^{2+} uptake in small intestinal basolateral membranes. The affinity for Ca^{2+} of the Ca^{2+}-transport system was one order of magnitude higher than the affinity we found. The difference between the two affinities is possibly due to a difference in EGTA: Ca^{2+} association constants used to calculate free Ca^{2+} concentrations. The V_{max} reported by these authors was smaller by a factor of 5, when compared with our results [25]. This difference may be explained either by the Percoll-isolation technique or by the use of a different small intestinal segment than we have used. Moreover, Nellans and Popovitch did not report on a correlation between Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake in intestinal basolateral membranes. In a recent review on calcium and phosphate transport across intestinal epithelium, Murer and Hildmann [26] reported preliminary results on Ca^{2+} transport in intestinal basolateral plasma membranes. These authors claimed two Ca^{2+}-transporting systems in parallel; an ATP-dependent Ca^{2+} transport and an Na^+-Ca^{2+} exchange mechanism, a similar situation as in heart sarcolemna [18]. Ca^{2+}-uptake studies reported in the present study were done in Na^+-free solutions. When 70 mM Na^+ was present in the uptake medium, ATP-dependent Ca^{2+} uptake was suppressed by 30%, while no effect on Ca^{2+}-ATPase activity was observed (results not shown). This observation supports the presence of Na^+-Ca^{2+} exchange as claimed by Murer and Hildmann [26], but further studies are needed to characterize this transport system and to evaluate its contribution to Ca^{2+} extrusion across the basolateral membrane.

A direct comparison of V_{max} values of Ca^{2+}-ATPase and ATP-dependent Ca^{2+} transport is complicated by two problems. Firstly, 75% of Ca^{2+}-induced ATP hydrolysis is inhibitable by theophylline, pointing at nonspecific contribution to ATP-hydrolysis due to the presence of alkaline phosphatase [7]. Secondly, the degree of resealing and the orientation of the membrane vesicles have to be known. The first problem could be solved by demonstrating that 1 mM theophylline inhibits completely alkaline phosphate, hence theophylline-insensitive ATP hydrolysis reflects the Ca^{2+}-pump activity. The second complication gave more difficulties. Using three techniques, osmotic shock, treatment with Triton X-100 or alamethicin, we demonstrated that the latency in $(Na^+ + K^+)$-ATPase was 50 to 60%. The channel-forming peptide alamethicin [27] has been used as a tool to

unmask latent ($Na^+ + K^+$)-ATPase activity in heart sarcolemna [28,29]. Since the three techniques gave comparable results, the conclusion is justified that about 40% of the basolateral vesicles is resealed for ATP or for ouabain. If all of the resealed vesicles would have an inside-out orientation, then 100% of Ca^{2+}-ATPase activity should be observed and the Ca^{2+}-transport capacity would be underestimated by a factor of 2.5. The stoichiometry of the Ca^{2+} pump would than be Ca:ATP = 2:3, which israther unlikely [18,21]. If only half of the resealed vesicles have an inside-out orientation, then the stoichiometry would be 1:1 which is in agreement with Ca^{2+}-pumps present in other tissues [18,21]. Therefore, it is most likely that only 20% of the vesicles is involved in ATP-dependent Ca^{2+} accumulation and that the V_{max} of this system amounts to 25 nmol Ca^{2+}/min per mg protein, a value comparable to the Ca^{2+}-pump capacity of heart sarcolemna [18]. For some unknown reasons, direct attempts to estimate the percentage of inside-out vesicles failed. Both, binding studies with [^{3}H]ouabain as well as latency studies on K^+-stimulated *p*-nitrophenylphosphatase were unreliable (results not shown). Latency studies of the latter enzyme have been proven to be useful with heart sarcolemna vesicles [29]. Nevertheless, the close correlation between the affinities and maximal velocities of Ca^{2+}-ATPase and ATP-dependent Ca^{2+} uptake presents strong evidence for the presence of a Ca^{2+} pump in rat duodenal basolateral plasma membranes. This adds duodenal epithelium to a wide variety of tissues where a Ca^{2+}-pump has been demonstrated in plasma membranes as for example in proximal tubular epithelium [30,31], adipocytes [32], pancreatic island cells [33], heart sarcolemna [18], red blood cell [21], smooth muscle [19], squid axon [34], brain synaptosomes [22], and corpus luteum [20].

When the intravesicular space is known and the amount of accumulated Ca^{2+}, the concentration of Ca^{2+} in the vesicular space can be calculated. D-Glucose uptake studies with basolateral membranes give an apparent intravesicular space of 2.5 μl/mg protein (data not shown). Similar values have been reported before for glucose and alanine spaces [35,36]. Making the assumption that the same volume is resealed for Ca^{2+} and that half of these vesicles are oriented inside-out, then a steady-state Ca^{2+} accumulation of 12.5 nmol Ca^{2+}/mg protein (Figs. 1, 2) means that the intravesicular Ca^{2+} concentration amounts to 10 mM, when the extravesicular concentration is 1 μM. This gradient across the vesicular membrane is close to the one across cell membranes in general. The possibility that most of the accumulated Ca^{2+} is bound to the membrane is unlikely since the calcium-ionophore readily releases accumulated Ca^{2+}. Therefore, the Ca^{2+} pump realizes Ca^{2+}-concentration gradients of physiological magnitude, which indicates that the resealed and inside-out vesicles in the basolateral membrane preparation must have a comparable Ca^{2+} permeability as duodenum epithelial cell membranes in situ.

An interesting observation is the high affinity for Ca^{2+} of alkaline phosphatase in basolateral plasma membranes. The Ca^{2+}-induced ATP-hydrolysis sensitive to theophylline has a K_m value identical to the K_m of the Ca^{2+} pump. Previously, we have reported that intestinal alkaline phosphatase can be activated at Ca^{2+} concentrations in the micromolar range, but these effects were ascribed to depletion of Zn^{2+} by the EDTA isolation procedure [7,8]. Basolateral membranes used in the present study are not Zn^{2+} depleted, but the stimulatory effects of low Ca^{2+} concentrations are even more pronounced. Despite the high-affinity Ca^{2+} sites and its ability to hydrolyze ATP, it is highly unlikely that alkaline phosphatase has a role in Ca^{2+} transport since theophylline inhibits alkaline phosphatase activity completely without an effect on ATP-dependent Ca^{2+} uptake in basolateral membranes.

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