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DISSOCIATION BETWEEN Ca^{2+}-ATPase AND ALKALINE PHOSPHATASE ACTIVITIES IN PLASMA MEMBRANES OF RAT DUODENUM

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Summary

The presence of Ca^{2+}-ATPase activities with high-affinity sites for Ca^{2+} in brush border as well as basolateral plasma membranes of rat duodenal epithelium has been reported previously (Ghijsen, W.E.J.M. and van Os, C.H. (1979) Nature 279, 802—803). Since both plasma membranes contain alkaline phosphatase (EC 3.1.3.1), which also can be stimulated by Ca^{2+}, the substrate specificity of Ca^{2+}-induced ATP-hydrolysis has been studied to determine whether or not alkaline phosphatase and Ca^{2+}-ATPase are two distinct enzymes. In basolateral fragments, the rate of Ca^{2+}-dependent ATP-hydrolysis was greater than that of ADP, AMP and *p*-nitrophenylphosphate at Ca^{2+} concentrations below 25 μM. At 0.2 mM Ca^{2+} the rates of ATP, ADP, AMP and *p*-nitrophenylphosphate hydrolysis were not significantly different. In brush border fragments the rates of ATP, ADP and AMP hydrolysis were identical at low Ca^{2+}, but at 0.2 mM Ca^{2+}, Ca^{2+}-induced hydrolysis of ADP and AMP was greater than either ATP or *p*-nitrophenylphosphate. Alkaline phosphatase in brush border and basolateral membranes was inhibited by 75% after addition of 2.5 mM theophylline. Ca^{2+}-stimulated ATP hydrolysis at 1 μM Ca^{2+} was not sensitive to theophylline in basolateral fragments while the same activity in brush border fragments was totally inhibited. At 0.2 mM Ca^{2+}, Ca^{2+}-induced ATP hydrolysis in both basolateral and brush border membranes was sensitive to theophylline. Oligomycin and azide had no effect on Ca^{2+}-stimulated ATP hydrolysis, either at low or at high Ca^{2+} concentrations. Chlorpromazine fully inhibited Ca^{2+}-stimulated ATP hydrolysis in basolateral fragments at 5 μM Ca^{2+}, while it had no effect in brush border fragments. From these results we conclude that, (i) Ca^{2+}-ATPase and alkaline phosphatase are two distinct

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

enzymes, (ii) high-affinity Ca^{2+}-ATPase is exclusively located in basolateral plasma membranes, (iii) alkaline phosphatase activity, present on both sides of duodenal epithelium, is stimulated slightly by low Ca^{2+} concentrations, but this Ca^{2+}-induced activity is inhibited by theophylline and shows no specificity with respect to ATP, ADP or AMP.

Introduction

Active absorption of Ca^{2+} in the upper part of the small intestine is an important factor in the Ca^{2+}-homeostasis of the body. Ca^{2+}-absorption is regulated by 1α,25-dihydroxycholecalciferol but the mechanism by which the epithelial cells transport Ca^{2+} is poorly understood [2]. A Ca^{2+}-stimulated ATPase has been reported in brush border membranes, and this activity was increased by vitamin D-3 [3,4]. Mircheff and Wright [5] also found Ca^{2+}-ATPase activity present in basolateral membranes of small intestinal cells. Moreover, a good correlation has been reported between net Ca^{2+}-absorption and Ca^{2+}-ATPase activity along the small intestinal tract [6,7]. Recently, we have reported the presence of high-affinity sites for Ca^{2+} on Ca^{2+}-ATPases in brush border and basolateral membranes [1]. This observation makes the Ca^{2+}-ATPase of intestinal plasma membranes a likely candidate for a Ca^{2+}-extrusion mechanism in analogy with Ca^{2+} pumps in sarcoplasmic reticulum and red blood cell membranes [8,9]. However, alkaline phosphatase is also present in brush border and basolateral membranes [3—5], and this enzyme lyses organic ortho- and pyrophosphates [10]. It is known that alkaline phosphatase can be stimulated by divalent ions, including Ca^{2+} [11,12]. Moreover, closely correlated responses between alkaline phosphatase and Ca^{2+}-ATPase to vitamin D [4,7] and to treatment with several inhibitors [4] have been reported. Also, co-purification of both enzymatic activities in chick brush broder membranes [13] and rat basolateral membranes [14] suggests that alkaline phosphatase and Ca^{2+}-ATPase may be different manifestations of the same enzyme.

In this study we examined the substrate specificity of Ca^{2+}-induced ATP hydrolysis in brush border and basolateral membranes of rat duodenum to find out whether Ca^{2+}-dependent hydrolysis of ATP is due to activation of alkaline phosphatase or to a specific Ca^{2+}-ATPase. We conclude that only the basolateral membranes contain a high-affinity Ca^{2+}-ATPase.

Methods and Materials

Male Wistar rats (140—160 g) were killed by a blow on the head and the proximal 15 cm of the small intestine were removed and rinsed with ice-cold isotonic saline solution. Mucosal scrapings were homogenized and brush border as well as basolateral membranes were isolated from the same homogenate according to the method of Mircheff and Wright [5]. Briefly, brush border membranes were separated from basolateral membranes and mitochondria by differential centrifugation while further purification of basolateral and brush border membranes was obtained by centrifugation on density gradients. Similar purification factors for $(Na^+ + K^+)$-ATPase and sucrase were found as reported

by Mircheff and Wright [5]. Mitochondrial contamination, expressed as percentage succinic dehydrogenase of the initial activity, was lower than 0.2% in both plasma membrane preparations. When isolated basolateral membranes are stored at 0°C, the Ca^{2+}-stimulated ATPase activity decreases and 2—3 days after isolation no further activity could be detected. Therefore, in most experiments a more rapid procedure was used for isolating basolateral membranes. Instead of overnight centrifugation on a sorbitol gradient, basolateral membranes were separated from mitochondria by centrifugation in 40% sorbitol for 1 h at 200 000 × *g* as described by Mircheff et al. [15]. Finally, basolateral membranes were collected in a band at the interface between 15 and 40% sorbitol. This basolateral fraction contained 13.4 ± 2.5% (n = 5) of the initial ($Na^+ + K^+$)-ATPase, 1.2 ± 0.3% (n = 5) of the sucrase, 1.5 ± 0.4% (n = 8) of the succinic dehydrogenase and 1.4 ± 0.1% (n = 7) of the protein. Immediately after isolation, brush border and basolateral fragments were washed twice with EDTA-free isolation buffer and suspensions in the same buffer were rapidly frozen in liquid N_2 and stored at −80°C. No loss of activity was observed for up to 2 weeks.

Enzyme assays

Ca^{2+}-stimulated ATPase activities were assayed in a standard medium containing 100 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP and 50 mM Tris-maleate buffer (pH 7.4). Ca^{2+}-ATPase was measured as the difference between the rates of ATP hydrolysis in the presence and absence of Ca^{2+}. Below 50 μM Ca^{2+} EGTA-buffering was necessary (see below); at higher Ca^{2+} concentrations 0.5 mM EGTA was present and to obtain the desired free Ca^{2+} concentration we assumed a Ca^{2+}-EGTA binding ratio of 1 : 1. Ca^{2+}-free controls contained 0.5 mM EGTA. In experiments where Zn^{2+} stimulation of the ATP hydrolysis was studied, Zn^{2+}-EGTA buffers were used to obtain 1 μM Zn^{2+}. All solutions used for the Ca^{2+}-ATPase assay were prepared with water which had been distilled three times in the presence of EDTA to remove traces of divalent cations. Enzyme reactions were started by adding aliquots of 25 μl basolateral or brush border membranes (5—10 μg protein) to 0.5 ml standard assay medium. After incubation for 30 min at 25°C, the reaction was stopped with 0.5 ml 10% trichloroacetic acid and phosphate was analyzed as described before [1]. All determinations were performed in triplicate. Stimulation of hydrolysis of ADP, AMP and *p*-nitrophenylphosphate by Ca^{2+} or Zn^{2+} was determined in a similar standard medium as described above but with 3 mM of the different substrates instead of ATP. In experiments with *p*-nitrophenylphosphate as substrate, the reactions were terminated by addition of 1.5 ml 1 N NaOH to the assay medium and *p*-nitrophenylphosphate hydrolysis was measured as the absorbance at 410 nm.

Protein, sucrase, ($Na^+ + K^+$)-ATPase and succinic dehydrogenase were assayed as described previously [1]. Statistical analysis of the difference between two mean values was carried out using the Student's *t*-test.

Ca^{2+}-EGTA buffers

The free Ca^{2+} concentration in Ca^{2+}-EGTA buffers was calculated as described by Katz et al. [16]. For Ca^{2+}-EGTA, a stability constant of $10^{6.83}$

was used, calculated from the value determined by Ogawa [17] at pH 6.8. The stability constant used is strictly correct in media with an ionic strength of 0.1 to 0.15 at 20—25°C and at pH 7.4. The assumptions made by Katz et al. in calculating free Ca^{2+} concentrations in media containing ATP, Mg^{2+}, Ca^{2+} and EGTA were checked by calculations in which all possible equilibria were included. This was done by making use of a computer program published by Perrin and Sayce [18]. Differences between the two calculated free Ca^{2+} concentrations were maximally 20%, which justified the simplified method given by Katz et al. [16]. Free Zn^{2+} concentrations of 1 μM were also buffered with EGTA assuming a binding ratio of total Zn^{2+} to total EGTA of unity. This is justified by the high stability constant of Zn^{2+}-EGTA, which is equal to $10^{14.3}$ (Ref. 19).

Materials

ATP, ADP, AMP, *p*-nitrophenylphosphate, oligomycin and theophylline were obtained from Sigma (St. Louis, MO). All chemicals used were of the purest grade (Merck). Chlorpromazine was obtained from Specia (Amstelveen, The Netherlands).

Results

Previously, we have shown that Ca^{2+} concentrations between 10^{-7} and 10^{-4} M were able to stimulate ATPase activities in basolateral and brush border membranes in the presence of 5 mM Mg^{2+} [1]. Two affinity sites for Ca^{2+} were found in basolateral and brush border membranes, one with a K_m value of about 1 μM and the other with a K_m value of about 50—70 μM. In basolateral membranes 40% of the total Ca^{2+}-sensitive ATP hydrolysis was reached at 1 μM Ca^{2+}, while in brush border membranes only 5% stimulation occurred at the same Ca^{2+} concentration. This difference between basolateral and brush border membranes will be discussed later (see Fig. 2). In order to find out whether alkaline phosphatase plays a role in Ca^{2+}-stimulated ATP hydrolysis, we have studied the effect of Ca^{2+} on hydrolysis of ATP, ADP, AMP and *p*-nitrophenylphosphate. The results are shown in Fig. 1. With basolateral fragments there was a significantly greater stimulation of ATP-hydrolysis than hydrolysis of ADP, AMP and *p*-nitrophenylphosphate at Ca^{2+} concentrations below 25 μM ($P < 0.001$). At 0.2 mM Ca^{2+}, ADP seemed to be the preferred substrate, but no significant difference between ATP, ADP, AMP and *p*-nitrophenylphosphate was observed ($P > 0.2$). With brush border fragments no clear preference for ATP was detectable at low Ca^{2+} concentrations. At 0.2 mM Ca^{2+}, ADP and AMP were better substrates than ATP and *p*-nitrophenylphosphate ($P < 0.05$). In Table I, Ca^{2+}-stimulation of ATP, ADP, AMP and *p*-nitrophenylphosphate hydrolysis at physiologically important Ca^{2+} concentrations is shown. In basolateral membranes, only ATP hydrolysis occurred at 0.5 μM Ca^{2+}. At 1 and 5 μM Ca^{2+} some stimulation of ADP and AMP hydrolysis was seen but ATP hydrolysis was significantly greater ($P < 0.001$). In this concentration range no stimulation of *p*-nitrophenylphosphate hydrolysis was observed. Although with brush border membranes some stimulation of ATP hydrolysis was observed at 0.5 μM Ca^{2+}, no significant preference for ATP com-

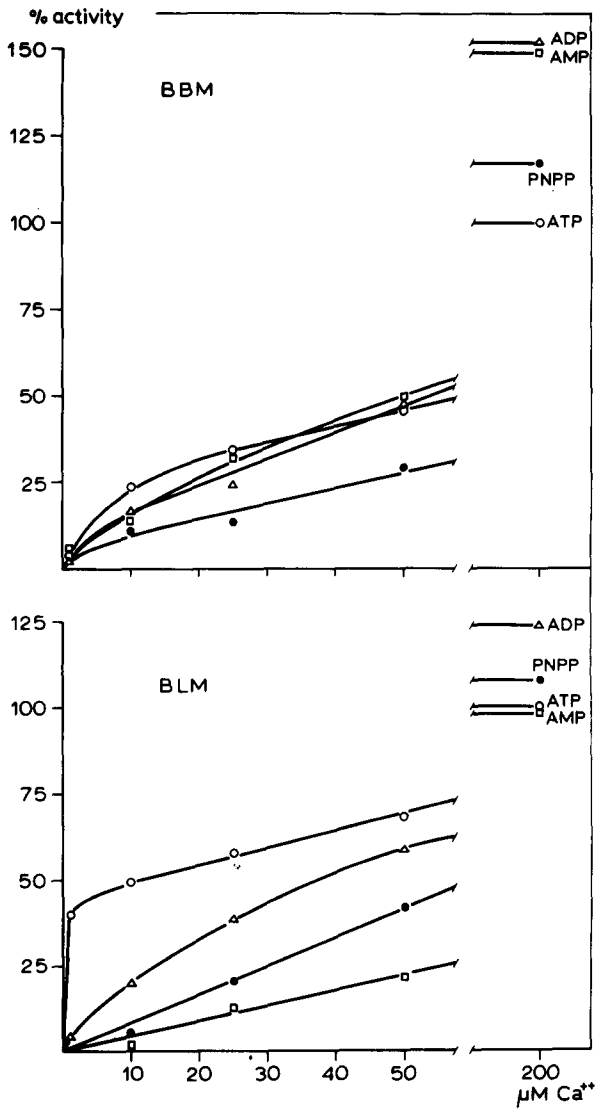

Fig. 1. Effect of Ca^{2+} on hydrolysis of ATP, ADP, AMP and *p*-nitrophenylphosphate (PNPP) in brush border (BBM) and basolateral membranes (BLM). The activities are expressed as percentages of maximal ATP hydrolysis obtained at 0.2 mM Ca^{2+}, i.e., 18.1 ± 1.4 (n = 9) and 3.3 ± 0.2 (n = 14) μmol P_i/h per mg protein in brush border and basolateral membranes, respectively. Results are given as mean values of at least four determinations.

pared to the other substrates could be found. Only at 5 μM Ca^{2+} was some specificity seen for ATP ($P < 0.01$).

These results suggest a specific Ca^{2+}-ATPase with high affinity for Ca^{2+} in basolateral fragments. The Ca^{2+}-stimulated hydrolysis of ATP, ADP, AMP and *p*-nitrophenylphosphate at 0.2 mM Ca^{2+} is most likely due to stimulation of an aspecific phosphatase, e.g., alkaline phosphatase. In brush border membranes, stimulation of ATP hydrolysis at low as well as high Ca^{2+} concentrations, may be due to stimulation of alkaline phosphatase. To provide more evidence for

TABLE I

EFFECT OF LOW Ca^{2+} CONCENTRATIONS ON HYDROLYSIS OF ATP, ADP, AMP AND *p*-NITROPHENYLPHOSPHATE IN BRUSH BORDER AND BASOLATERAL MEMBRANES

The values are expressed as percentages of maximal ATP hydrolysis obtained at 0.2 mM Ca^{2+}. Mean values are given with the standard error of the mean and the number of experiments in parentheses.

	$[Ca^{2+}]$ (μM)	Substrate hydrolysis (%)			
		ATP	ADP	AMP	*p*-Nitrophenylphosphate
Brush border membrane	0.5	2.0 ± 0.8 (6)	n.s. * (5)	n.s. * (5)	n.s. * (5)
	1.0	4.6 ± 1.5 (10)	2.2 ± 1.6 (8)	5.1 ± 1.8 (8)	n.s. * (5)
	5.0	16.1 ± 0.6 (12)	9.0 ± 2.2 (5)	7.4 ± 1.0 (5)	8.1 ± 2.7 (6)
Basolateral membrane	0.5	28.6 ± 4.9 (9)	n.s. * (5)	n.s. * (5)	n.s. * (5)
	1.0	40.0 ± 4.2 (13)	4.5 ± 3.9 (5)	n.s. * (5)	n.s. * (6)
	5.0	42.9 ± 3.0 (11)	15.9 ± 1.7 (3)	3.1 ± 2.1 (5)	n.s. * (5)

* No significant stimulation (n.s.) above the residual ATP hydrolysis at zero $[Ca^{2+}]$.

this conclusion we have studied the effect of alkaline phosphatase inhibitors. In Table II the effects are shown of two well-known inhibitors on alkaline phosphatase activities in brush border and basolateral membranes, i.e., L-phenylalanine [20] and theophylline [21], both added at a concentration causing maximal inhibition, which is 75% with 2.5 mM theophylline and 30% with 10 mM phenylalanine at pH 7.4. The relative inhibitions were the same for both membrane fractions. Theophylline is clearly the best inhibitor of alkaline phosphatase and was therefore used in further studies. The effects of theophylline on the Ca^{2+}-stimulated hydrolysis of ATP and *p*-nitrophenylphosphate in both plasma membrane fractions are presented in Table III. At 1 μM Ca^{2+}, theophylline had no effect on ATP hydrolysis in basolateral membranes, while complete inhibition occurred of the small but significant stimulation of ATP hydrolysis in brush border membranes. In basolateral membranes the increase in ATP as well as in *p*-nitrophenylphosphate hydrolysis at 100 μM Ca^{2+} was inhibitable by theophylline. In brush border membranes the inhibitory effects of theophylline on the hydrolysis of both substrates at 200 μM Ca^{2+} were similar. The intestinal alkaline phosphatase is known to be a Zn^{2+}-containing enzyme [22]. Inactivation of alkaline phosphatase occurs upon treatment with

TABLE II

EFFECT OF L-PHENYLALANINE AND THEOPHYLLINE ON ALKALINE PHOSPHATASE ACTIVITY IN BRUSH BORDER AND BASOLATERAL PLASMA MEMBRANES OF RAT DUODENUM

Enzyme activities are expressed as μmol P_i/h per mg protein and were assayed in the presence of 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 3 mM *p*-nitrophenylphosphate, 100 mM NaCl and 50 mM Tris-maleate (pH 7.4). Mean values are given with the standard error of the mean and the number of experiments in parentheses.

	Basolateral membrane	Brush border membrane
Control	4.28 ± 0.39 (6)	47.76 ± 5.74 (5)
+2.5 mM theophylline	1.01 ± 0.15 (10)	12.71 ± 1.00 (6)
+10 mM L-phenylalanine	2.92 ± 0.13 (4)	33.62 ± 1.01 (4)

TABLE III

EFFECT OF THEOPHYLLINE ON Ca^{2+} STIMULATION OF ATP AND *p*-NITROPHENYLPHOSPHATE HYDROLYSIS IN BRUSH BORDER AND BASOLATERAL MEMBRANES

The values are expressed as percentages of ATP hydrolysis obtained at 0.2 mM Ca^{2+}. Mean values are given with the standard error of the mean and the number of experiments in parentheses.

			ATP hydrolysis (%)		*p*-Nitrophenylphosphate hydrolysis (%)	
		$[Ca^{2+}]$:	1 μM	200 μM	1 μM	200 μM
Brush border membrane	control		4.0 ± 1.3 (8)	100	n.s. * (5)	117.3 ± 15.5 (9)
	+2.5 mM theophylline		n.s. * (5)	33.2 ± 5.7 (5)	n.s. * (5)	27.7 ± 7.2 (8)
Basolateral membrane	control		37.8 ± 6.5 (8)	100	n.s. * (5)	117.0 ± 7.0 (13)
	+2.5 mM theophylline		37.8 ± 8.6 (5)	55.7 ± 8.1 (10)	n.s. * (5)	21.1 ± 1.0 (10)

* No significant stimulation (n.s.) above the residual ATP hydrolysis at zero $[Ca^{2+}]$.

TABLE IV

EFFECT OF THEOPHYLLINE ON Zn^{2+} STIMULATION OF ATP AND *p*-NITROPHENYLPHOSPHATE HYDROLYSIS IN BRUSH BORDER AND BASOLATERAL MEMBRANES

The values are expressed as percentages of ATP hydrolysis obtained at 0.2 mM Ca^{2+}. Mean values are given with the standard error of the mean and the number of experiments in parentheses.

			ATP hydrolysis (%)		*p*-Nitrophenylphosphate hydrolysis (%)	
		$[Zn^{2+}]$:	1 μM	200 μM	1 μM	200 μM
Brush border membrane	control		71.2 ± 9.6 (4)	185.6 ± 17.6 (6)	88.5 ± 5.0 (4)	331.8 ± 28.2 (5)
	+2.5 mM theophylline		14.3 ± 3.9 (4)	37.8 ± 6.3 (4)	27.1 ± 10.3 (4)	86.4 ± 33.9 (4)
Basolateral membrane	control		82.7 ± 6.5 (5)	134.1 ± 21.1 (6)	70.8 ± 7.6 (5)	331.8 ± 27.0 (7)
	+2.5 mM theophylline		42.7 ± 9.7 (5)	48.6 ± 17.3 (4)	8.1 ± 2.2 (8)	25.4 ± 10.8 (4)

EDTA due to extraction of Zn^{2+} [12,22,23]. Since the basolateral and brush border membranes are isolated in EDTA-containing solutions, alkaline phosphatase activities are most likely inactivated. For these reasons we studied also the effect of Zn^{2+} on the hydrolysis of ATP and *p*-nitrophenylphosphate in both membrane fractions in combination with the inhibitory effects of theophylline. The results are shown in Table IV. At 1 μM Zn^{2+}, a marked stimulation of hydrolysis of ATP and *p*-nitrophenylphosphate was observed in brush border as well as in basolateral membranes. In basolateral membranes Zn^{2+}-induced ATP hydrolysis was inhibited 50% by theophylline ($P < 0.01$), while the stimulation of *p*-nitrophenylphosphate hydrolysis was almost completely inhibited. The relative stimulation of ATP hydrolysis obtained at 1 μM Zn^{2+} or 1 μM Ca^{2+} in the presence of theophylline was not significantly different in basolateral plasma membranes ($P > 0.7$). This observation suggests that theophylline-insensitive Ca^{2+}-ATPase in basolateral membranes can also be stimulated by Zn^{2+}. In both membrane fractions a maximal stimulation of *p*-nitrophenylphosphate hydrolysis at 200 μM Zn^{2+} was found, which could be inhibited by theophylline. In brush border membranes the relative inhibitions of *p*-nitrophenylphosphate and ATP hydrolysis were equal at both Zn^{2+} concentrations.

From the results presented in Tables III and IV three important conclusions can be drawn: (i) alkaline phosphatase in brush border and basolateral membranes is reactivated by Zn^{2+} as well as by Ca^{2+}; (ii) a high-affinity Ca^{2+}-ATPase is exclusively located in basolaterals; (iii) activation of alkaline phosphatase and stimulation of Ca^{2+}-ATPase are clearly distinguishable by using theophylline as inhibitor of alkaline phosphatase.

Chlorpromazine has been described as an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum [24] and in erythrocytes [9]. For this reason the effect of chlorpromazine on ATP hydrolysis in brush border and basolateral membranes was studied. To rule out any contribution of mitochondrial contamination to ATP hydrolysis the effects of oligomycin and azide were studied. Table V gives the results. Neither oligomycin nor azide, added at concentrations which strongly inhibit mitochondrial ATPase activity, had significant effects on Ca^{2+}-stimulated ATP hydrolysis in basolateral and brush border membranes ($P > 0.3$). In basolateral membranes, chlorpromazine inhibits the high-affinity Ca^{2+}-ATPase completely, while at 200 μM Ca^{2+}, an inhibition of 35% of the control activity was observed. No effect of chlorpromazine was found on Ca^{2+}-stimulation of ATP hydrolysis in brush border membranes. These results support the conclusions described above, namely, a high-affinity Ca^{2+}-ATPase exclusively located in basolateral membranes. The absence of an effect of L-phenylalanine on ATP-hydrolysis in basolateral membranes at low Ca^{2+} concentrations also supports this conclusion (Table V). L-Phenylalanine did have an effect on ATP hydrolysis in basolateral and in brush border membranes at high Ca^{2+} concentrations, which can be interpreted as inhibition of alkaline phosphatase. No significant effect of ouabain on Ca^{2+}-ATPase activity in basolateral membranes could be detected, which indicates that ($Na^+ + K^+$)-ATPase has no contribution to the ATP-hydrolysis reported above.

Additional support for the presence of a high-affinity Ca^{2+}-ATPase in addition to alkaline phosphatase in basolateral membranes of rat duodenum is

TABLE V

EFFECTS OF SEVERAL INHIBITORS ON Ca^{2+}-STIMULATED ATP HYDROLYSIS IN BRUSH BORDER AND BASOLATERAL MEMBRANES

The results are expressed as percentages of maximal ATP hydrolysis obtained at 0.2 mM Ca^{2+}. Mean values are given with the standard error of the mean and the number of experiments in parentheses.

	ATP hydrolysis (%)			
	Brush border membrane		Basolateral membrane	
$[Ca^{2+}]$:	5 μM	200 μM	5 μM	200 μM
Control	24.4 ± 2.2 (5)	100	50.3 ± 5.3 (9)	100
Oligomycin (10 μg/ml)	24.5 ± 2.3 (4)	105.3 ± 2.2 (4)	54.3 ± 5.4 (4)	98.8 ± 2.6 (7)
Azide (1 mM)	24.8 ± 7.6 (4)	101.2 ± 7.1 (4)	44.4 ± 2.0 (5)	98.5 ± 4.4 (5)
Chlorpromazine (0.1 mM)	21.5 ± 1.4 (4)	99.8 ± 1.5 (4)	n.s. * (6)	66.6 ± 13.1 (11)
L-Phenylalanine (10 mM)	15.2 ± 1.7 (4)	65.8 ± 3.1 (4)	44.9 ± 5.9 (4)	64.2 ± 2.5 (4)

* No significant stimulation (n.s.) above the residual ATP hydrolysis at zero $[Ca^{2+}]$.

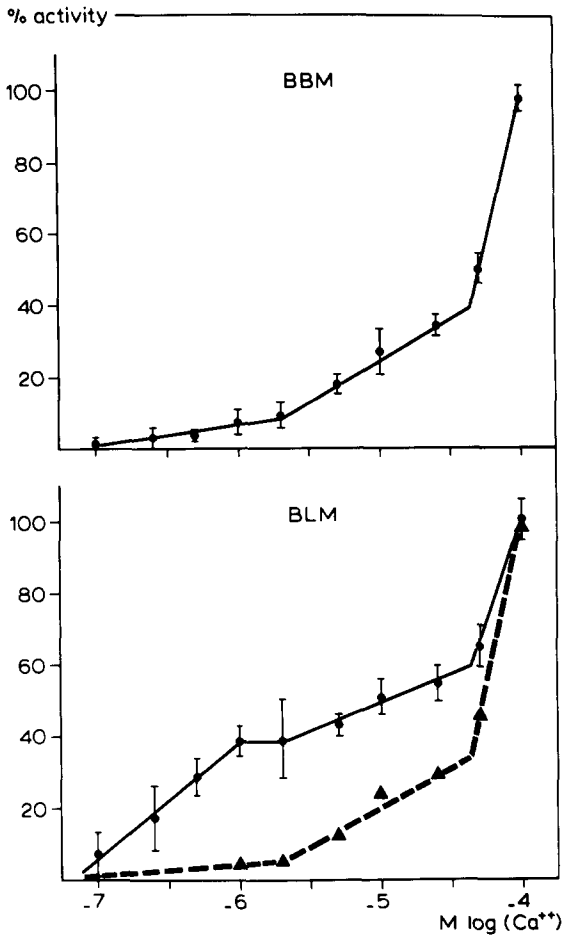

Fig. 2. Activities of Ca^{2+}-induced ATP hydrolysis vs. log[Ca^{2+}] (M) in brush border (BBM) and basolateral membranes (BLM) (solid lines). The dashed line with basolateral fragments depicts the activity obtained after subtraction of the specific Ca^{2+}-APTase activity (for further details see text).

provided in Fig. 2. In this figure, total Ca^{2+}-induced ATP hydrolysis is plotted against log[Ca^{2+}] in the concentration range 10^{-7} to 10^{-4} M. In brush border membranes the curve has three distinct slopes which may reflect different affinity sites for Ca^{2+}. The same plot for basolateral membranes differs from that of brush border fragments. However, in basolateral membranes the ATPase activity at 1 μM represents a specific Ca^{2+}-ATPase. When this activity, approx. 35% of the maximal stimulation, is subtracted from the total activity at 200 μM Ca^{2+} and when the residual activity is replotted (dashed line), then we obtain a strikingly similar curve as obtained with brush border fragments. This suggests that the alkaline phosphatases in brush border and basolateral membranes are very similar to one another, which is in support of a recent study by Hanna et al. [25].

Discussion

This study provides evidence for three important conclusions. Firstly, a high-affinity Ca^{2+}-ATPase, which may be the enzymatic expression of a Ca^{2+} pump, is exclusively located in the basolateral aspect of the duodenal cell. Secondly, alkaline phosphatase, present in brush border as well as basolateral membranes of intestinal cells, can be reactivated by Ca^{2+} in the concentration range from 10^{-7} to 10^{-4} M, even in the presence of 5 mM Mg^{2+} and at pH 7.4. Thirdly, a clear differentiation between Ca^{2+}-ATPase and alkaline phosphatase activities becomes possible when different inhibitors and substrates are used.

The first conclusion is physiologically important, but the second is rather surprising. The interaction of alkaline phosphatase with divalent cations is complex in nature [22]. On treatment with EDTA, alkaline phosphatase loses its activity due to extraction of Zn^{2+} [11,12,22]. In solutions with Mg^{2+} and Zn^{2+} 100% reactivation occurs [12]. Some activation has been reported with Co^{2+}, Ca^{2+}, Mn^{2+} and Ni^{2+} [11,12]. The plasma membranes of rat duodenum were isolated in the presence of 0.5 mM EDTA and some inactivation of alkaline phosphatase must have taken place. Therefore, reactivation with divalent cations can be anticipated. Moreover, alkaline phosphatase is known to be able to hydrolyse organic mono-, di- and triphosphate esters, including ATP [10]. A novel finding is that Ca^{2+} concentrations as low as 1 μM partly reactivate alkaline phosphatase in the presence of 5 mM Mg^{2+}. This we conclude from the following observations; ATP hydrolysis by brush border fragments induced by 1 μM Ca^{2+} is inhibited by L-phenylalanine and theophylline, shows no specificity with respect to ATP, ADP or AMP and is not sensitive to chlorpromazine. The fact that 1 μM Zn^{2+} stimulates ATP hydrolysis better than does 1 μM Ca^{2+} supports the conclusion that we are dealing with alkaline phosphatase reactivation. Zn^{2+} at 0.2 mM fully reactivates the enzyme which is in agreement with other studies on reactivation of alkaline phosphatase [11,12]. In a recent study by Hanna et al. [25] on rat small intestine, the ratio for the distribution of alkaline phosphatase in brush border compared to basolateral membranes was 10 : 1. In our study, Ca^{2+}-induced ATP hydrolysis in brush border membranes amounts to 18.1 ± 1.4 (n = 9) μmol P_i/h per mg protein. In basolateral membranes maximal Ca^{2+}-induced ATP hydrolysis is 3.3 ± 0.2 (n = 14) μmol P_i/h per mg protein. After subtracting the activity of the high-affinity Ca^{2+}-ATPase, the residual activity in basolateral membranes becomes 2.0 μmol P_i/h per mg protein. This gives us a ratio of about 10 : 1 for the Ca^{2+}-induced ATP hydrolysis in brush border compared to basolateral membranes, which is in support of the conclusion that ATP hydrolysis stimulated by 0.2 mM Ca^{2+} is due to activation of alkaline phosphatase.

In our study the substrate specificity of alkaline phosphatase varied with the concentration of the divalent cations. At low Ca^{2+} concentrations, adenosine phosphates were hydrolyzed but not p-nitrophenylphosphate, while at high Ca^{2+} concentrations all substrates were hydrolyzed. At high Zn^{2+} concentrations, p-nitrophenylphosphate was a far better substrate than ATP. An explanation for this change in substrate specificity is not clear but it may be sought in the fact that there are two different binding sites for Zn^{2+}, one involved in substrate hydrolysis and the other in an ordered association of the

monomers [12]. It was not the aim of this study to examine indetail the reactivation and substrate specificity of alkaline phosphatase in rat intestine. The point we make is, intestinal alkaline phosphatase can be activated under conditions which are typical for stimulation of Ca^{2+}-ATPases in muscle and red cell membranes, namely at low Ca^{2+} concentrations in the presence of 5 mM Mg^{2+} at pH 7.4. A clear distinction between the two events is possible by making use of the inhibitors theophylline and chlorpromazine. Several attempts are known in the literature to dissociate Ca^{2+}-ATPase from alkaline phosphatase activities in isolated brush border membranes as well as in whole homogenates of intestine [4,13,26]. The major flaw in these studies is that Ca^{2+}-ATPase activities were determined at Ca^{2+} concentrations between 1.0 and 40 mM at pH values of 8–10, hence, under conditions which are optimal for alkaline phosphatase. Our study is the first to report that high-affinity Ca^{2+}-ATPase and alkaline phosphatase are two distinct enzymes in plasma membranes of rat duodenum. The high-affinity Ca^{2+}-ATPase in basolateral plasma membranes has a K_m value for Ca^{2+} activation of approx. 0.5 μM [11], which is comparable to the value reported for erythrocyte Ca^{2+}-ATPase [9]. The specific activity is also similar to red cell Ca^{2+}-ATPase activity [9,27]. Chlorpromazine has been reported as a fairly active inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum [24] and in red blood cells [9]. We find total inhibition of Ca^{2+}-ATPase in basolateral membranes at 10^{-4} M chlorpromazine. In red blood cells, Ca^{2+}-ATPase can be partially stimulated by Mn^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} [9]. In basolateral membranes of rat duodenum Zn^{2+} fully replaces Ca^{2+}, since activities at 1 μM Zn^{2+} or Ca^{2+} are identical in the presence of theophylline (Tables III and IV). However, it is likely that ATPase activity stimulated by 1 μM Zn^{2+} is an overestimation since Zn^{2+} reactivates alkaline phosphatase better than Ca^{2+} does and theophylline inhibits not more than 75% of alkaline phosphatase activity.

A Ca^{2+} pump function for Ca^{2+}-ATPase in basolateral membranes of rat duodenum has yet to be established. In support of a pump function is a recent study of Gmaj et al. [28] on Ca^{2+} transport in basolateral plasma membranes of rat kidney cortex. These authors report an ATP-dependent Ca^{2+} accumulation with an apparent K_m value for Ca^{2+} of 0.5 μM. This affinity is strikingly similar to the Ca^{2+}-ATPase affinity in basolateral membranes of rat duodenum. So far, only a low-affinity Ca^{2+}-ATPase has been reported in basolateral membranes of rat kidney cortex [29], but this Ca^{2+}-ATPase was not identical to red cell Ca^{2+}-ATPase. In the presence of Mg^{2+} it could not be stimulated by Ca^{2+} and only when Mg^{2+} was absent did Ca^{2+} in the millimolar range give stimulation of ATP-hydrolysis. In view of the affinity of ATP-dependent Ca^{2+} accumulation in basolateral membranes of kidney cortex it is likely that a high-affinity Ca^{2+}-ATPase is present, but the extremely high background of Mg^{2+}-ATPase activity in these membranes makes it hard to demonstrate a relatively low Ca^{2+}-ATPase activity. Also in basolateral membranes of duodenum, stimulation by 1 μM Ca^{2+} is less than 10% of the Mg^{2+}-ATPase activity at zero Ca^{2+} concentration. After establishing Ca^{2+}-ATPase activity in basolateral plasma membranes we postulate a model for Ca^{2+} transport across duodenal epithelium. In Fig. 3 the model is outlined. It consists of passive Ca^{2+} influx across the brush border membrane and active extrusion across the basolateral side. It is known that 1α, 25-dihydroxycholecalciferol, increases the brush border permeability to Ca^{2+}

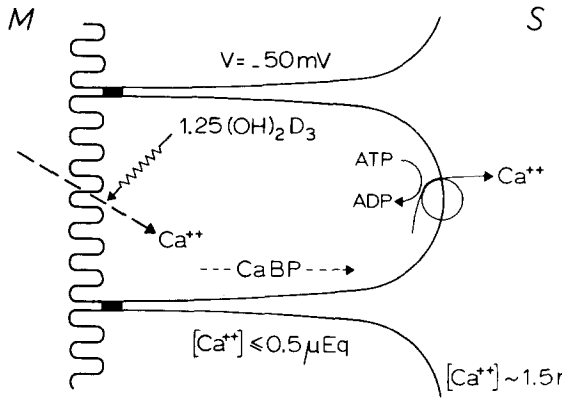

Fig. 3. Model for transcellular Ca^{2+} transport across rat duodenal epithelium (for details see text).

[30—32]. Hence, the rate of Ca^{2+} absorption can be controlled at the brush border level. In this model the activity of Ca^{2+}-ATPase must be high enough to maintain low intracellular Ca^{2+} concentrations. Enhancement of intestinal Ca^{2+} transport by 1α,25-dihydroxycholecalciferol is accompanied by the appearance of a Ca^{2+}-binding protein [33] and this Ca^{2+}-binding protein has been postulated to play a role in intracellular Ca^{2+}-buffering after the enhanced Ca^{2+} influx [34,35]. The apparent association constant of Ca^{2+}-binding protein for Ca^{2+} is about 2 μM [30]. This affinity implies that Ca^{2+}-binding protein can supply Ca^{2+} to the Ca^{2+}-ATPase in the basolateral membranes, which has a higher affinity for Ca^{2+} than Ca^{2+}-binding protein. We would like to emphasize that this postulated model for Ca^{2+} transport is very similar to current models for transepithelial Na^+ transport [37], except for the role of Ca^{2+}-binding protein. The energetics of the extrusion mechanism are as follows: intracellular Ca^{2+} concentration can be expected to be less or equal to 0.5 μM, since Ca^{2+}-ATPase has a K_m value of 0.5 μM; extracellular Ca^{2+} concentration is 1.5 mM and the cell potential of rat duodenum is about —50 mV with respect to the serosal side [38]. The difference in electrochemical potential between intracellular and extracellular Ca^{2+} can be calculated from the equation:

$$\Delta\tilde{\mu}_{Ca^{2+}} = RT \cdot \ln \frac{a^{o}_{Ca^{2+}}}{a^{i}_{Ca^{2+}}} + ZF\,\Delta\psi$$

where R, T, Z and F have their usual meanings, $\Delta\psi$ is the electrical potential difference and $a^{o}_{Ca^{2+}}$ and $a^{i}_{Ca^{2+}}$ are the extracellular and intracellular Ca^{2+} activities, respectively. Inserting the above values we arrive at $\Delta\tilde{\mu}_{Ca^{2+}} = 7.24$ kcal · mol^{-1}. This value implies that hydrolysis of 1 M ATP, which renders 7.2 to 12.0 kcal · mol^{-1} dependent on intracellular ATP, ADP and P_i concentrations [39], provides enough energy for the extrusion of 1 M Ca^{2+}. Further work is needed along the lines of the model depicted in Fig. 3 in order to obtain a more detailed understanding of Ca^{2+} transport in the samll intestine.

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