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Ca^{2+}-dependent ATP hydrolysis of the porcine intestinal brush-border membranes

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The brush-border membrane from the porcine small intestine possesses Ca^{2+}-dependent ATPase activity. The Ca^{2+} stimulation of ATP hydrolysis by the membranes is biphasic with a high affinity (K_m = 0.38 μM) and a low affinity (K_m = 98.3 μM). Treatment of the membrane vesicles with *n*-heptylthioglucoside did not cause further increase of the Ca^{2+}-ATPase activity. Mg^{2+} also stimulates the ATP hydrolysis in the absence of Ca^{2+} but decreases the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+}. The Ca^{2+}-ATPase activities are not inhibited by addition of vanadate, ouabain, sodium azide and alkaline phosphatase inhibitors (theophylline and L-phenylalanine), irrespective of the Ca^{2+} concentrations in medium. A specific calmodulin-inhibitor W-7 (up to 30 μM) also did not influence on the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+}. The Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+} show no specificity for ATP. ADP, GTP and CTP could also be used as substrates. From these results, it is suggested that the porcine intestinal brush-border membrane possesses Mg^{2+}-independent Ca^{2+}-ATPase activity and that the Ca^{2+}-ATPase activities with biphasic responses for Ca^{2+} stimulation observed in the present study reside on the same protein. The physiological functions of the Ca^{2+}-ATPase in the membranes, however, remain unknown at present.

Introduction

The small intestine plays an important role in calcium homeostasis of the body. Especially the entry step of Ca^{2+} into the epithelial cells across the brush-border membrane may be rate-limiting in transcellular Ca^{2+} transport that is regulated by $1\alpha,25\text{-}(OH)_2D_3$ [1–4].

Several investigators have reported the presence of Ca^{2+}-stimulated ATPase in the brush-border membranes as well as in the basolateral membranes [5–10]. On the other hand, alkaline phosphatase in the brush-border membranes has been demonstrated to be in a close correlation with Ca^{2+}-ATPase in the response to vitamin D [6–8]. Recently it has been demonstrated that $1\alpha,25\text{-}(OH)_2D_3$ increases the activities of alkaline phosphatase [11] and Ca^{2+}-ATPase [12] in the intestinal mucosa as well as synthesis of the calcium-binding protein [11,13]. However, the enzymatic properties of Ca^{2+}-ATPase in the brush-border membranes are poorly understood. Ghijsen et al. [10] have recently demonstrated that Ca^{2+}-ATPase and alkaline phosphatase in the brush-border

Abbreviations: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate.

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membranes of rat duodenal epithelium are distinct enzymes and Ca^{2+}-ATPase with a specifically high affinity for Ca^{2+} locates in the basolateral membranes [10,14].

In the present study, we examined the properties of Ca^{2+}-dependent ATP hydrolysis by the porcine intestinal brush-border membranes and demonstrated that the membrane possesses Mg^{2+}-independent Ca^{2+}-ATPase activity which has a wide substrate specificity and no sensitivities for calmodulin-inhibitors, vanadata, sodium azide and ouabain.

Materials and Methods

Materials

p-Nitrophenyl phosphate (disodium salt), sodium orthovanadate and *n*-heptylthioglucoside were purchased from Wako Pure Chemical Co. (Tokyo, Japan). ATP (2K and 2Na), ADP, CTP (2Na), GTP (3Na), AMP (1Na), Ap_5A (5Na) and trifluoperazine were obtained from Sigma Chemical Co. Chlorpromazine hydrochloride was obtained from Yoshitomi Seiyaku (Osaka, Japan). W-7 was a generous gift from Professor H. Hidaka, Mie University, School of Medicine. All other materials were the purest grade obtainable from commercial sources.

Preparation of membrane vesicles

Brush-border membrane vesicles were prepared from the porcine small intestine according to the calcium-precipitation method described in our previous paper [15] and suspended in 10 mM Tris-HCl buffer (pH 7.4). Protein concentration was assayed by method of Lowry et al. [16] using bovine serum albumin as standard.

Enzyme assays

The ATPase activities were assayed in 1 ml of the reaction medium containing 30 mM Tris-HCl buffer (pH 7.4), 3 mM ATP (dipotassium salt), 0.1 mM ouabain, 0.2 mM EGTA and 0.15 or 0.4 mM $CaCl_2$ (for Ca^{2+}-ATPase), or 3 mM $MgCl_2$ (for Mg^{2+}-ATPase) at 37°C for 30 min unless otherwise specified. For studies of substrate specificity, sodium salts of ATP, ADP, AMP, GTP and CTP (3 mM of each) were used. The membrane protein concentration was 10 μg per sample. Inorganic phosphate liberated was measured by the method of Fiske and SubbaRow [17]. The Ca^{2+}- or Mg^{2+}-ATPase activity was determined by subtracting the activity obtained with EGTA alone from that obtained in the presence of divalent cations. In calculation of the free Ca^{2+} concentrations, the dissociation constant of EGTA and Ca^{2+} was assumed to be $2 \cdot 10^{-7}$ M [18].

p-Nitrophenyl phosphate hydrolysis was measured in a medium containing 30 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$ and 2 mM *p*-nitrophenyl phosphate (disodium salt) unless otherwise specified. The reaction was started by the addition of the membrane vesicles (5 μg protein) and incubated at 37°C for 30 min. The reaction was terminated by the addition of 5 ml of 0.05 M NaOH to the reaction mixture (1 ml) and the absorbance of liberated *p*-nitrophenol was measured at 420 nm.

Results

Ca^{2+} concentration dependence of ATPase activity

As shown in Fig. 1A, ATP was hydrolyzed by the membranes in a Ca^{2+} concentration-dependent fashion over the free Ca^{2+} concentration range from 0.0286 to 300 μM. Analysis of the data according to Eadie [19] yielded distinct two slopes, suggesting the presence of two kinetic forms with a high affinity ($K_m = 0.38$ μM) and a low affinity ($K_m = 93.3$ μM) for Ca^{2+}-stimulation of the ATPase activity (Fig. 1B).

Effect of Mg^{2+}

The addition of Mg^{2+} caused a further stimulation of the ATPase activities at low and high concentrations of Ca^{2+} (data not shown). Since Mg^{2+} is also an effective activator of ATP hydrolysis by the membranes in the absence of Ca^{2+} (Table II), the Ca^{2+}-ATPase activities in the presence of Mg^{2+} were corrected for the basal Mg^{2+}-ATPase activity. As can be seen in Fig. 2, the Ca^{2+}-ATPase activities in the presence of 0.59 and 200 μM free Ca^{2+} were decreased with increasing Mg^{2+} concentration, indicating that Mg^{2+} is not required on further stimulation of the Ca^{2+}-ATPase activities.

Effect of n-heptylthioglucoside

Effect of *n*-heptylthioglucoside, a good solubi-

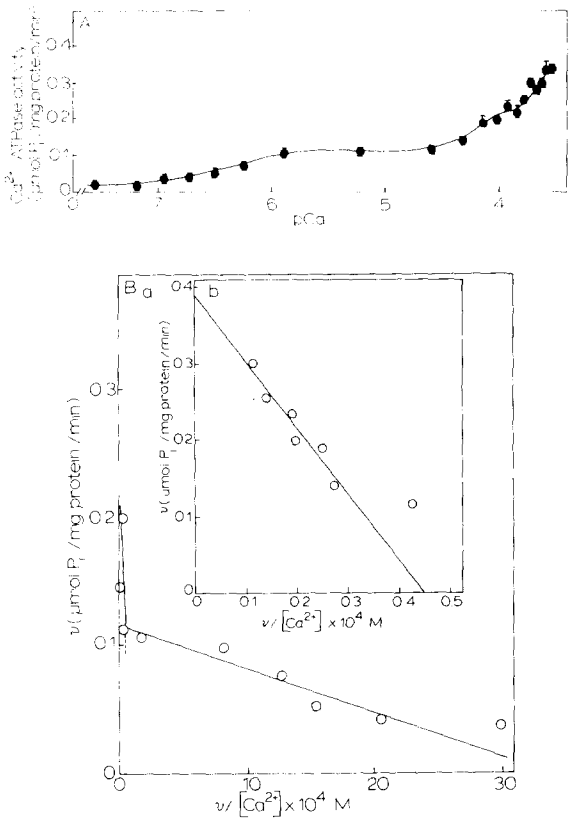


Fig. 1. (A) Ca^{2+} concentration dependence of ATP hydrolysis of the membranes. Free Ca^{2+} concentration was varied from 0.0286 to 300 μM. Other experimental conditions are given in Materials and Methods. The values are expressed as means ± S.E. for triplicate determinations. (B) Plots of data from Fig. 1A according to Eadie [19]. The (a) and (b) in Fig. 1B represent the plots in the free Ca^{2+} concentration range of 0.12–75.5 and 26.5–250 μM, respectively.

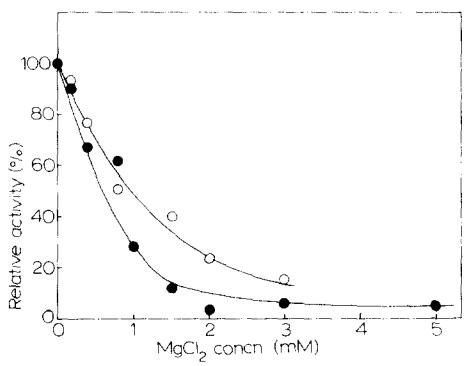


Fig. 2. $MgCl_2$ concentration dependence of Ca^{2+}-dependent ATP hydrolysis of the membranes. The relative ATPase activities were expressed as $[(Ca^{2+}+Mg^{2+})\text{-ATPase}-(Mg^{2+}\text{-ATPase})]/(Ca^{2+}\text{-ATPase})\times 100$ at each $MgCl_2$ concentration indicated. ○, 0.59 μM free Ca^{2+}; ●, 200 μM free Ca^{2+}. The values are expressed as means of triplicate determinations.

lizing agent of membrane proteins [20,21], on the Ca^{2+}-ATPase activities in the presence of 0.59 and 200 μM free Ca^{2+} was studied. In this experiment, the membrane vesicles (1 mg protein/ml) were preincubated with the detergent at 25°C for 30 min, and then ATPase activities were assayed in the reaction mixture omitted the detergent.

As shown in Table I, the ATP hydrolysis by the membranes at the low and high concentraitons of Ca^{2+} were not influenced by treatment with the detergent.

Nucleotide specificity

The nucleotide specificities of the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+}, and Mg^{2+}-ATPase activity were examined.

As shown in Table II, the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in the membranes had broad substrate specificity over ATP, ADP, GTP and CTP. There was hardly any AMP hydrolysis detectable. In addition, Mg^{2+}-dependent ADP hydrolysis was not inhibited by addition of 0.4 mM Ap_5A, a potent inhibitor of adenylate kinase [21].

Effects of several inhibitors

The contribution of alkaline phosphatase on the Ca^{2+}-dependent ATP hydrolysis in the presence of 0.59 and 200 μM free Ca^{2+} were examined using alkaline phosphatase inhibitors such as L-phenylanaline [22] and theophylline [23], because the intestinal brush-border membrane contains alkaline phosphatase activity which can be stimulated by Ca^{2+} [24,25].

As shown in Table III, *p*-nitrophenyl phosphate hydrolysis at pH 7.4 was markedly inhibited by the addition of 10 mM L-phenylalanine or 1 mM theophylline. On the other hand, the Ca^{2+}-ATPase activities were not sensitive to these inhibitors, irrespective of Ca^{2+} concentrations in medium. In addition, *p*-nitrophenyl phosphate hydrolysis by the membranes at pH 7.4 was not enhanced by addition of Ca^{2+} (data not shown).

Next we examined the effect of vanadate on Ca^{2+}-dependent ATP hydrolysis and *p*-nitrophe-

TABLE I

EFFECT OF *n*-HEPTYLTHIOGLUCOSIDE TREATMENT ON Ca^{2+}-ATPase ACTIVITY OF THE MEMBRANES

The values are expressed as menas ± S.E. of triplicate determinations. (A) In the presence of 0.59 μM free Ca^{2+}. (B) In the presence of 200 μM free Ca^{2+}.

Detergent concn. (%)	Ca^{2+}-ATPase (μmol P_i/mg protein per min)	
	A	B
0	0.082 ± 0.003	0.310 ± 0.003
0.05	0.081 ± 0.012	0.308 ± 0.004
0.5	0.076 ± 0.003	0.280 ± 0.002

TABLE II

NUCLEOTIDE SPECIFICITY OF Ca^{2+}-ATPase AND Mg^{2+}-ATPase ACTIVITIES OF THE MEMBRANES

The specific activities of Ca^{2+}-ATPase in the presence of 0.59 and 200 μM were 0.076 and 0.319 μmol P_i/mg protein per min, respectively. The Mg^{2+}-ATPase activity was 1.18 μmol P_i/mg protein per min. The values are expressed as averages of 3–5 determinations. (A) In the presence of 0.59 μM free Ca^{2+}. (B) In the presence of 200 μM free Ca^{2+}.

Nucleotide	Ca^{2+}-ATPase (%)		Mg^{2+}-ATPase (%)
	A	B	
ATP	100	100	100
ADP	88.7	134.3	86.4
AMP	0	0	0.08
GTP	108.3	118.0	85.2
CTP	99.3	99.9	99.8
ADP + Ap_5A	–	–	86.5

nyl phosphate hydrolysis. As can be seen in Table IV, the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+} were almost insensitive to vanadate in the presence and absence of *n*-heptylthioglucoside, whereas *p*-nitrophenyl phosphate hydrolysis was markedly decreased. In addition, vanadate also had no influence on ATP hydrolysis by the membranes in the presence of 0.59 μM free Ca^{2+} and 3 mM $MgCl_2$ as well as on the basal Mg^{2+}-ATPase activity.

The effects of calmodulin antagonists on the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+}, and on the Mg^{2+}-ATPase activity are presented in Fig. 3. As shown in Fig. 3A, the Ca^{2+}-ATPase activities at low and high concentrations of Ca^{2+} were not or little inhibited by the addition of W-7 up to 30 μM, while the Mg^{2+}-ATPase activity was inhibited depending on the concentration over its range from 10 to 50 μM. On the other hand, chlorpromazine (Fig. 3B) and trifluoperazine (Fig. 3C) inhibited both the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities depending on its concentration. In addition, the inhibitory effect of these calmodulin-inhibitors was somewhat stronger on the basal Mg^{2+}-ATPase activity rather than on the Ca^{2+}-ATPase activity, suggesting that the action of these drugs is not specific for the Ca^{2+}-ATPase activity in the membranes.

The effects of sodium azide (20 mM) and ouabain (0.1 mM) on the Ca^{2+}-ATPase activity are shown in Table V. Ouabain was removed from the assay medium of the control. Neither sodium

TABLE III

EFFECTS OF L-PHENYLALANINE AND THEOPHYLLINE ON Ca^{2+}-DEPENDENT ATP HYDROLYSIS AND *p*-NITROPHENYL PHOSPHATE HYDROLYSIS BY THE MEMBRANES AT pH 7.4

The values are expressed as means ± S.E. for triplicate determinations. (A) In the presence of 0.59 μM free Ca^{2+}. (B) In the presence of 200 μ free Ca^{2+}.

Addition	ATP hydrolysis (μmol P_i/mg protein per min)		p-Nitrophenyl phosphate hydrolysis (μmol *p*-nitrophenol/mg protein per min)	
	A	B	A	B
–	0.070 ± 0.010	0.325 ± 0.011	1.72 ± 0.017	1.65 ± 0.023
L-Phenylalanine	0.065 ± 0.010	0.304 ± 0.010	0.70 ± 0.021	0.71 ± 0.029
Theophylline	0.079 ± 0.010	0.284 ± 0.003	0.71 ± 0.018	0.68 ± 0.010

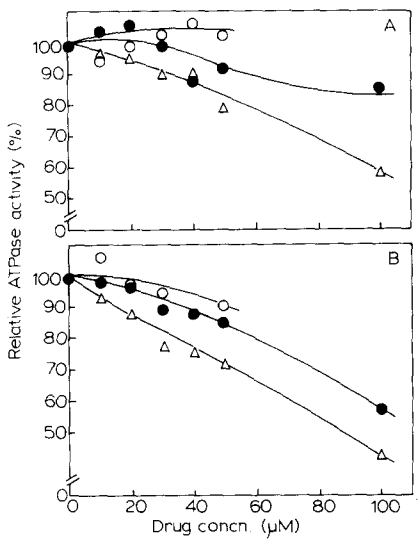
TABLE IV

EFFECT OF VANADATE ON ATP HYDROLYSIS AND *p*-NITROPHENYL PHOSPHATE HYDROLYSIS OF THE MEMBRANES

The vanadate concentration was 0.2 mM. The values were expressed relative to that in the absence of vanadate in each system. (A) In the absence of *n*-heptylthioglucoside. (B) In the presence of 0.5% *n*-heptylthioglucoside.

Cations	Vanadate	ATP hydrolysis (%) [a]		*p*-Nitrophenylphosphate hydrolysis (%) [a]
		A	B	
0.59 μM Ca^{2+}	–	100	100	100
	+	105.7 ± 11.3	91.0 ± 13.1	68.3 ± 0.5
200 μM Ca^{2+}	–	100	100	100
	+	93.8 ± 1.0	113.2 ± 3.5	66.5 ± 0.9
3 mM Mg^{2+}	–	100	100	–
	+	94.9 ± 2.4	97.6 ± 4.7	–
0.59 μM Ca^{2+} + 3 mM Mg^{2+}	–	100	100	–
	+	103.9 ± 1.0	94.5 ± 6.3	–

[a] Mean ($n = 3$) ± S.E.



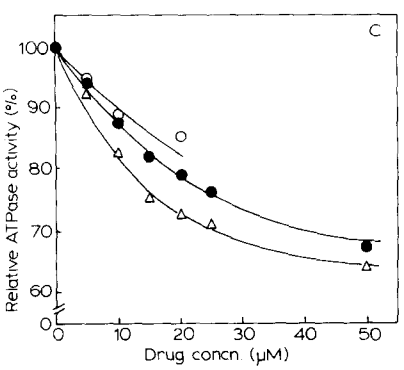


TABLE V

EFFECTS OF SODIUM AZIDE AND OUABAIN ON Ca^{2+}-ATPase ACTIVITY

The concentrations of sodium azide and ouabain were 20 and 0.1 mM, respectively. The data shown are mean of triplicate determinations. The values are represented as relative to those of systems without inhibitors. (A) In the presence of 0.59 μM free Ca^{2+}. (B) In the presence of 200 μM free Ca^{2+}.

Addition	Ca^{2+}-ATPase (%)	
	A	B
–	100	100
Sodium azide	90.9	109.8
Ouabain	106.8	101.8

azide nor ouabain showed an appreciable influence on the reaction in the both low and high concentrations of Ca^{2+}.

Fig. 3. Effects of calmodulin-inhibitors on Ca^{2+}- and Mg^{2+}-ATPase activities. Ca^{2+}-ATPase (○, ●); Mg^{2+}-ATPase (△). The concentrations of W-7 and chlorpromazine were varied from 10 to 100 μM, and trifluoperazine from 5 to 50 μM. The activities are expressed as relative to that in the absence of inhibitor in each system. (A) W-7; (B) chlorpromazine; (C) trifluoperazine. The free Ca^{2+} concentrations were 0.59 (○) and 200 μM (●). The $MgCl_2$ concentration (△) was 3 mM. The values are expressed as means of triplicate determinations.

Discussion

The Ca^{2+} concentration dependence of ATP hydrolysis by the porcine intestinal brush-border membranes showed two distinct phases with a high affinity ($K_m = 0.38$ μM) and a low affinity ($K_m = 98.3$ μM) in respect of Ca^{2+} stimulation (Fig. 1B).

It is well known that alkaline phosphatase is present in the intestinal brush-border membranes. Several investigators proposed that alkaline phosphatase and Ca^{2+}-ATPase are two expressions of the same molecule [7,26,27], while Ghijsen et al. [10] have demonstrated later that these enzymes in the rat duodenal brush-border membranes are distinct molecules. Therefore it is important to distinguish whether Ca^{2+}-dependent ATP hydrolysis examined in the present study is due to alkaline phosphatase or Ca^{2+}-ATPase in the membranes. We reached the conclusion that alkaline phosphatase and Ca^{2+}-ATPase in the membranes are separate entities and that the former enzyme does not contribute to the Ca^{2+}-dependent ATP hydrolysis from the following findings: (a) *p*-nitrophenyl phosphate hydrolysis was markedly inhibited by the addition of L-phenylalanine or theophylline, but the Ca^{2+}-ATPase was not influenced by these inhibitors, irrespective of Ca^{2+} concentration (Table III): (b) *p*-nitrophenyl phosphate hydrolysis at pH 7.4 was not enhanced in the Ca^{2+} concentration range where the Ca^{2+}-ATPase activity was enhanced; and (c) the response of the Ca^{2+}-dependent ATP hydrolysis and *p*-nitrophenyl phosphate hydrolysis for vanadate is different (Table IV).

The Ca^{2+}-ATPase activities in the presence of 0.59 and 200 μM free Ca^{2+} were decreased by addition of Mg^{2+} (Fig. 2) and were not sensitive to vanadate, even in the presence of Mg^{2+} (Table IV). In addition, ouabain and sodium azide also did not influence on the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+} (Table V). From these results, it is concluded that the Ca^{2+}-dependent ATP hydrolysis of the membranes at low and high concentrations of Ca^{2+} are not from the basolateral membranes or mitochondrial membranes, and that the Ca^{2+}-ATPase activities examined in the present study are distinctly different from high-affinity Ca^{2+}-ATPase or $(Ca^{2+} + Mg^{2+})$-ATPase reported in various membrane systems having Ca^{2+} pumping activity. The lack of an effect of specific calmodulin-inhibitors on the Ca^{2+}-ATPase activity, especially the lack of effect of W-7 at low concentrations (below 30 μM) (Fig. 3) also supports this interpretation.

The enzymatic properties of the Ca^{2+}-ATPase activities at 0.59 and 200 μM free Ca^{2+} were almost the same. These results suggest that these ATPase activities reside on the same protein and it seems that a transitional increase of ATP hydrolysis by the membranes at high Ca^{2+} concentration (Fig. 1A) may be due to allosteric stimulation by Ca^{2+}.

Result of sialic acid determination of the membrane vesicle in the presence and absence of 1% Triton X-100 revealed that about 97% of the membrane vesicles are right-side out orientation (21.5 and 20.9 nmol sialic acids/mg protein in the presence and absence of the detergent, respectively). The right-side out orientation of intestinal brush-border membrane vesicle prepared by calcium-precipitation method was also demonstrated by Kessler et al. [28]. Therefore it seems that the Ca^{2+}-ATPase in the membranes is an ecto-type enzyme. Similar ecto-Ca^{2+}-ATPases were also found in Ehrlich ascites tumor cell plasma membranes [29], rat mammary gland cells [30] and rat liver plasma membranes [31].

Recently several investigators have reported the presence of Mg^{2+}-independent Ca^{2+}-ATPases, which have a broad specificity for substrate and no sensitivity for calmodulin and vanadate, in various plasma membranes including Ehrlich ascites tumor cell plasma membranes [29], rat liver plasma membranes [31], rat stomach smooth muscle [32] and rat kidney basolateral membranes [33,34]. In addition, Ilsbroux et al. [35] have also reported that azide-insensitive Ca^{2+}-ATPase presents in the brush-border membranes of pig kidney cortex. This ATPase is able to utilize ATP, GTP, ITP, UTP and CTP as substrates. The enzymatic properties of the Ca^{2+}-ATPase in the porcine intestinal brush-border membranes examined in the present study are very similar to those of Ca^{2+}-ATPase activities in the plasma membranes of other tissues reported previously [29–35].

Recently several studies on Ca^{2+} uptake of

intestinal brush-border membranes have been done in connection with the effect of $1\alpha,25\text{-}(OH)_2D_3$ [36,37] and the membrane lipid fluidity [38], but it is still unclear whether or not Ca^{2+}-ATPase in the membranes is related to the Ca^{2+} uptake activity. Although the physiological function of the intestinal brush-border membrane Ca^{2+}-ATPase remains unknown at present, purification of the Ca^{2+}-ATPase and reconstitution into liposomes could shed additional light on the properties and physiological role of the enzyme, such as rat liver plasma membrane Ca^{2+}-ATPase [31,39].

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