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EFFECT OF CALMODULIN AND FATTY ACIDS ON Ca-DEPENDENT ADENOSINE TRIPHOSPHATASE IN THE MUCOSA OF THE SMALL INTESTINE

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Cells of the mucosa differ from cells of many other tissues in that the area of their cytoplasmic membrane is several times greater than the area of the intracellular membranes. This feature of the morphology of the plasma membrane in the mucosa is connected with its high transport activity. A decisive role in many secretory processes (transport of amino acids and sugars, diffusion of water, active and passive transport of Na^+, K^+, HCO_3^-, Cl^-) through the membrane is played by Ca^{++} ions.

Just as in other tissues, the Ca^{++} concentration in the cytoplasm of mucosal cells is several orders of magnitude lower than in the extracellular medium. There is a continuous passive inflow of Ca^{++} inside the cells, which is considerably increased by the action of various stimulators of secretory processes: hormones and neuromediators, and also many biologically active substances which enter the intestine together with the food [1, 2]. To maintain the Ca^{++} concentration gradient in the plasma membranes of the mucosa, systems of Ca^{++} transport against their concentration gradient must operate. In some tissues transport of this kind is effected by Na–Ca exchange [1]. Active transport of Ca^{++} on account of functioning of a special Ca-ATPase in the plasma membrane also is known. This mechanism of Ca^{++} release from the cell has been demonstrated most convincingly for erythrocytes [10]. The Ca-ATPase activity of erythrocytes can be regulated by calmodulin [5, 6]. This substance is found in many tissues, including the mucosa of the small intestine [3].

Calmodulin evidently activates the Ca-ATPase of the sarcolemma of the heart by increasing the rate of Ca-dependent phosphorylation of membrane proteins [11]. Activation of Ca-ATPase in erythrocytes is achieved by direct interaction between the enzyme and calmodulin [8]. Taking this into account, a one-stage method of isolating homogenous Ca-ATPase from erythrocytes based on affinity chromatography of solubilized membranes on calmodulin-sepharose has been developed.

The mechanism of active transport of Ca^{++} from cells of the mucosa into the intercellular space has not been established. The existence of Ca-ATPase in the cytoplasmic membranes

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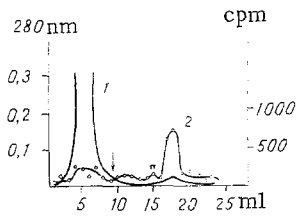

Fig. 1

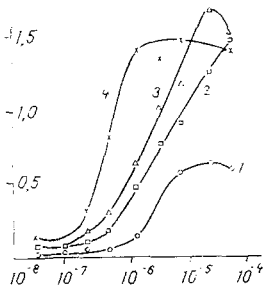

Fig. 2

Fig. 1. Elution profile of protein (1) and Ca-dependent ATPase (2) on column with calmodulin-sepharose. Arrow indicates time of addition of buffer A, two arrows — time of addition of buffer B.

Fig. 2. Dependence of Ca-ATPase activity on Ca^{++} concentration. 1) Control, 2) 10^{-3} M palmitic acid, 3) 10^{-3} M palmitic acid + 10^{-6} M calmodulin, 4) 10^{-6} M calmodulin. Abscissa, Ca^{++} concentration (in M); ordinate, enzyme activity (in μmoles P_i/mg protein/min).

of the mucosa membrane likewise has not been proved. The problem whether Ca^{++} transport in these cells is regulated by calmodulin has not been studied.

In the investigation described below solubilized cytoplasmic membranes of the mucosa were subjected to affinity chromatography on calmodulin-sepharose and the properties of the resulting highly purified Ca-ATPase were studied.

EXPERIMENTAL METHOD

Calmodulin was isolated from bovine brain [14]. To obtain an affinity matrix the preparation of calmodulin (50 mg), its homogeneity confirmed by gel filtration and polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, was incubated with 4 g of CNBr-activated sepharose 4B in 25 ml of a solution containing 0.1 M $NaHCO_3$ and 0.5 M NaCl for 2 h at room temperature, and then for 15 h at 4°C. After washing, the matrix was applied to a column (0.5 × 4.0 cm) and equilibrated with a solution containing 20 mM Tris acetate, pH 7.5, 10 mM mercaptoethanol, 250 mM KCl, 0.1 mM $CaCl_2$, 0.1% Triton X-100, and 0.1% phosphatidylcholine (buffer A).

Plasma membranes were isolated from the mucosa of a rabbit's small intestine by differential centrifugation. The purified membrane preparation (100 mg protein) was solubilized in 5 ml of a solution containing 220 mM Tris acetate, pH 7.5, 1 mM mercaptoethanol, 250 mM KCl, 0.1 mM $CaCl_2$, 0.5% sodium cholate, and 0.2% Triton X-100. The membranes were incubated in this solution for 1 h at 4°C, centrifuged at 150,000g for 1.5 h, after which the supernatant was applied to the column with calmodulin-sepharose. After the protein concentration in the eluting solution had fallen to background values 5 ml of buffer A was applied to the column followed by 10 ml of buffer B, containing 20 mM Tris acetate, 10 mM mercaptoethanol, 250 mM KCl, 1 mM EGTA, 0.02% Triton X-100, and 0.1% phosphatidylcholine.

The yield of protein from the column was measured on an ISCO UA-5 densitometer at 280 nm. Fractions of 1 ml were collected and activity of Ca-ATPase in them was determined. The incubation medium (50 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 0.5 μCi γ-^{32}P-ATP, 1 mM dithiothreitol, and 2 mM Ca-EGTA buffer. The reaction was started by addition of protein (0.2-0.5 μg) and proceeded for 10 min at 37°C. The ATPase reaction was stopped by addition of 1 ml of a cold suspension (150 mg/ml) of activated charcoal in 50 mM phosphate buffer, pH 6.0. Samples were incubated for 30 min at 4°C and then centrifuged at 5000g for 10 min. The content of ^{32}P in the supernatant (0.5 ml) was determined with an Intertechnique SL-4224 scintillation counter.

EXPERIMENTAL RESULTS

The ATPase activity of the membrane preparation from the mucosa of the rabbit small intestine was 0.15 ± 0.08 μmole P/mg protein/min. On the addition of EGTA to the incubation medium the ATPase activity of the membranes was reduced by only 3-7%. Evidently the content of Ca-independent ATPase was high in the mucosal membranes, and against that background it was difficult to detect Ca^{++}-activated ATPase.

TABLE 1. ATPase Activity of Fractions Nos. 16 and 17 Obtained by Affinity Chromatography (Fig. 1) on Column with Calmodulin-Sepharose (M ± m)

Conditions of incubation	nmoles P/mg protein/min	
	without calmodulin	10^{-6} M calmodulin
10^{-5} M Ca^{2+}	548±67	1260±190
10^{-3} M EGTA	32±10	52±18
10^{-5} M Ca^{2+} + 10^{-4} M oleic acid	712±95	812±70
10^{-3} M EGTA + 10^{-4} M oleic acid	50±11	41±14

During chromatography on calmodulin-sepharose practically all the Ca^{++}-insensitive ATPase and small amounts of Ca-activated ATPase were eluted in the overshoot (Fig. 1). Washing the column with buffer A led to replacement of cholate by Triton X-100 and phosphatidylcholine. Under these circumstances no Ca-ATPase was eluted. By the use of buffer B containing EGTA, Ca-ATPase could be removed from the column (Fig. 1). The yield of protein in fractions possessing Ca-ATPase activity was low (30-40 μg), but substantial purification of the enzyme was achieved in this case. The specific activity of Ca-ATPase obtained by affinity chromatography was 0.5-0.6 μmole P/mg protein/min (Table 1), practically equal to the specific activity of the homogeneous Ca-ATPase preparation from human erythrocytes. Purified ATPase from the mucosa was inhibited 10-20 times when EGTAwas added to the incubation medium. This is evidence of a low level of contamination of the preparation by Na,K-ATPase and other ATP-hydrolyzing enzymes.

In the presence of Ca^{++} calmodulin activates ATPase two-threefold. Oleic acid also activates the enzyme, and in this case its activation is abolished by calmodulin (Table 1). In the absence of Ca^{++} the ATPase activity of the preparation was low. It was unchanged both by the action of calmodulin and by the action of oleic acid. ATPase activity detectable in the presence of EGTA perhaps reflects contamination of the preparation with Ca-independent ATPases. However, the possibility likewise cannot be ruled out that this ATPase is "basal" Ca-ATPase activity, i.e., the level of activity of this enzyme which is exhibited in the absence of the activating ion. In that case it can be concluded that Ca^{++} makes the enzyme sensitive not only to calmodulin, but also to fatty acids.

Calmodulin was found to increase the affinity of the enzyme for Ca^{++} (Fig. 2). In the presence of calmodulin the activation constant (K_a) of the enzyme for Ca^{++} was reduced from 2×10^{-6} to 4×10^{-7} M. Calmodulin also increased the maximal reaction velocity (V_{max}). Palmitic (Fig. 2) and oleic acids had no effect on K_a for Ca^{++} but increased V_{max} of Ca-ATPase. Palmitic acid activated the enzyme practically to the same level as calmodulin. No additiveness was found in the action of calmodulin and fatty acids. Moreover, in the presence of palmitic acid the effect of calmodulin on affinity of ATPase for Ca^{++} disappeared (Fig. 2). Fatty acids thus activate the Ca-ATPase of the mucosa of the small intestine, but at the same time they prevent the effect of calmodulin on the enzyme.

This investigation showed that calmodulin may interact also with the Ca-ATPase of the mucosa of the small intestine. Calmodulin-dependent enzymes are characterized by a number of common features. For example, phosphodiesterase and Ca-dependent protein kinase can be activated by limited proteolysis [4, 13], and also under the influence of fatty acids and phospholipids [12, 15]. The action of lipids on these enzymes is highly specific: the acid phospholipids phosphatidylserine and phosphatidylinositol, and arachidonic acid activate them whereas phosphatidylcholine and arachidic acid have no effect [12]. According to our observations for Ca-ATPase of the intestinal mucosa, during activation of the enzyme by lipids calmodulin has no additional effect. This may indicate that during lipid binding the calmodulin-binding sites of the enzyme are modified or binding of calmodulin takes place but the activating effect disappears.

It has been shown that hydrophobic interaction plays a decisive role in the binding of calmodulin with Ca-dependent enzymes [9, 12]. For example, in the presence of Ca^{++}, sites

appear in calmodulin with which such hydrophobic agents as trifluoperazine, 9-anthroylcholine, 8-anilino-1-naphthalene sulfate, etc., interact [7, 9]. On binding with calmodulin these substances prevent interaction of phosphodiesterase with calmodulin-sepharose or interaction of calmodulin with troponin-1-sepharose [9]. The action of such hydrophobic substances as oleic and palmitic acids on regulation of Ca-ATPase by calmodulin can thus be realized both by binding of fatty acids with the enzyme and by their binding with calmodulin.

In the presence of fatty acids the activating effect of calmodulin on Ca-ATPase of the mucosa of the small intestine disappears. Consequently, acceleration of lipolysis may lead to a decrease in the affinity of mucosal Ca-ATPase for Ca^{++} and may thereby evoke a disturbance of the functional and secretory activity of the intestinal cells. We know that cholera enterotoxin causes an increase in cAMP concentration in cells of the mucosa, thereby leading to diarrhea. Since cAMP is one of the main stimulators of lipolysis in the tissues, it can be tentatively suggested that one of the principal ways whereby diarrhea develops in response to an increase in cAMP concentration is by a disturbance of the secretory activity of the mucosal cells, taking place as a result of blocking of calmodulin-dependent regulation of Ca-ATPase by fatty acids.

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