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CALCIUM-STIMULATED ATPASE OF GUINEA PIG PLACENTA

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SUMMARY

1. A Ca^{2+}-activated ATPase of isolated membranes of guinea pig placenta is characterized.

2. This enzyme is preferentially activated by calcium ions. In the presence of 5 mM ATP, maximal enzyme activity being obtained at 3–5 mM Ca^{2+}. The maximal rate of ATP hydrolysis varies between 15 and 22 μmoles P_i per mg protein in 30 min.

3. Mg^{2+} also activates the enzyme, but always to a lesser degree than Ca^{2+}. Mn^{2+}, but not Sr^{2+}, activates the enzyme. At optimal Ca^{2+} concentration, addition of Mg^{2+} is always inhibitory. Ca^{2+} and Mg^{2+} seem to act on the same site.

4. The enzyme does not require Na^+ or K^+ for activation by Ca^{2+}.

5. The optimal pH for Ca^{2+} activation of the enzyme lies between 8.2 and 8.5; at pH 7.1 and 9.5 only 50 % of maximal activation occurs.

6. Addition of increasing amounts of Na_2H_2EDTA leads to progressive decreases of activity, complete inhibition occurring at 5 mM when the incubation fluid contains 5 mM Ca^{2+}.

7. Ethacrynic acid inhibits the enzyme but ouabain does not.

8. Other triphosphates may serve as substrate; but the v_{max} for Na_2ATP is highest.

9. We suggest that this enzyme aids in maintaining the uphill gradient of Ca^{2+} between maternal and fetal circulation.

INTRODUCTION

In many mammalian species transfer of Ca^{2+} across the placenta occurs against a concentration gradient[1–3]. One of the proposed mechanisms by which active transport of calcium occurs between body compartments is by means of Ca^{2+}-Mg^{2+}-ATPases such as the one described in renal cortical plasma membranes[4], and the brush border of intestinal mucosa[5]. This paper deals with a description of the properties of an ATPase located in plasma membranes of guinea pig placenta activated preferentially by Ca^{2+}.

MATERIALS AND METHODS

Pregnant guinea pigs were anaesthetized with sodium phenobarbital (100 mg/kg) around the 60th day of gestation. The abdomen was opened and they were

bled from puncture of the vena cava. The amniotic sac with the fetuses was removed and after opening the sac, blood was taken from the umbilical vein of one or two of the fetuses to establish the maternal–fetal Ca^{2+} gradient. Each placenta was carefully freed from membranes, gross blood vessels and uterine tissue and placed in ice-cold 0.9 % NaCl solution. Using a Thomas tissue grinder, Size C with Teflon pestle, for 10 strokes at 1500 rev./min, the placentas were homogenized in 25 ml of a solution containing 87 g sucrose, 1.169 g NaCl, 1.860 g Na_2H_2EDTA, 0.2 g $MgCl_2 \cdot 6\ H_2O$, and 0.68 g imidazole per l. The tissues were then processed in a manner similar to that described by POST AND SEN[6] for the isolation of renal plasma membranes, omitting the urea stage. The isolated membranes were finally suspended in $5 \cdot 10^{-4}$ M imidazole–histidine, 5 mM Tris-EDTA buffer (pH 7.6) and stored at 4° until assay.

An aliquot from each preparation was examined by electron microscopy to check the purity of the membrane preparation. To test for the various enzymatic properties, 0.1 ml of enzyme suspension containing 10–20 μg of protein, was incubated with 1.0 ml of solutions containing Ca^{2+} or Mg^{2+} in appropriate amounts (0.1 to 10 mM); 20 mM Tris–HCl buffer (pH 8.2) and 70 mM Na^+ (as NaCl); Na_2ATP (Sigma) was added to make 5 mM. Blank specimens did not contain bivalent cations. The samples were incubated for 30 min in a Dubnoff shaking water bath at 37°. The reaction was terminated by plunging the tubes into an ice–water bath and adding 1.0 ml 10 % (w/v) trichloroacetic acid. The rate of ATP hydrolysis was determined by measuring the amount of P_i released from the samples. Using the Auto Analyzer inorganic phosphate was measured by the GOMORI[7] method, total protein by the LOWRY *et al.*[8] procedure. Results are expressed as μmoles P_i released per mg of protein in 30 min, during which time the release is linear.

RESULTS

General

On electron microscopic examination the membrane preparations were shown to consist of plasma membranes. No mitochondria were seen.

In representative plasma samples, Ca^{2+} activity, as measured by ion selective electrode* showed the expected gradient (maternal $[Ca^{2+}]$ 2.2 mequiv/l, fetal $[Ca^{2+}]$ 3.10 mequiv/l).

Activation of the enzyme by bivalent cations

(i) Activation of the enzyme by calcium ions was assessed by varying the Ca^{2+} concentration in the incubation medium from 0.02 to 10 mM. Fig. 1 depicts the rate of P_i production at the various Ca^{2+} concentrations. The apparent K_m for Ca^{2+} of placentas from 5 different guinea pigs was 0.26 $\pm$ 0.01 mM (mean $\pm$ S.E.) (Fig. 2). The rate of P_i release at peak activity ranged from 15.0 to 22.0 μmoles P_i per mg protein in 30 min.

(ii) In the absence of Ca^{2+}, Mg^{2+} also activated the enzyme but always less than Ca^{2+} (Fig. 1). The apparent K_m for Mg^{2+} was 0.56 $\pm$ 0.03 mM (mean $\pm$ S.E.) (Fig. 2). When instead of the single cation we used a 1:1 combination of Ca^{2+} *plus* Mg^{2+}, the

* Orion Research Corporation, Cambridge, Mass. 02139, U.S.A. Ca^{2+}-Selective Flow-Through Electrode No. 99-20; pH meter Model 801.

resultant curve lay between the two curves obtained from incubation with Ca^{2+} or Mg^{2+} alone (Fig. 1).

(iii) The effect of other bivalent cations (Mn^{2+}, Sr^{2+}) on enzyme activation was also tested. Fig. 1 shows that Mn^{2+} activated the enzyme but Sr^{2+} did not. Maximal activation of the enzyme with Mn^{2+} occurred at 2 mM, higher concentrations producing inhibition.

(iv) Activation of enzyme by Ca^{2+} and Mg^{2+} was further tested by using a constant Ca^{2+} concentration (5 mM) and adding increasing amounts of Mg^{2+}, as well as by using a constant Mg^{2+} concentration (5 mM) and adding increasing amounts of Ca^{2+} (up to 8 mM). The addition of Ca^{2+} to 5 mM Mg^{2+} led to increased stimulation,

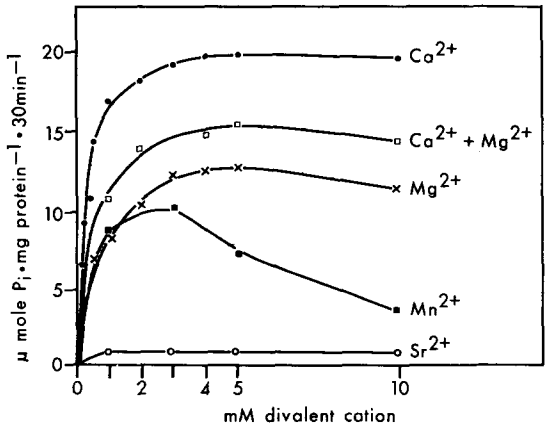


Fig. 1. Stimulation of ATP hydrolysis by bivalent cations. Unless indicated incubation fluids contained 70 mM Na^+ (as NaCl), 20 mM Tris–HCl (pH 8.2) and 5 mM Na_2ATP. ●—●, activation by Ca^{2+}; □—□, Ca^{2+} *plus* Mg^{2+} (in equimolar concentrations); ×—×, Mg^{2+}; ■—■, Mn^{2+}; ○—○, Sr^{2+}.

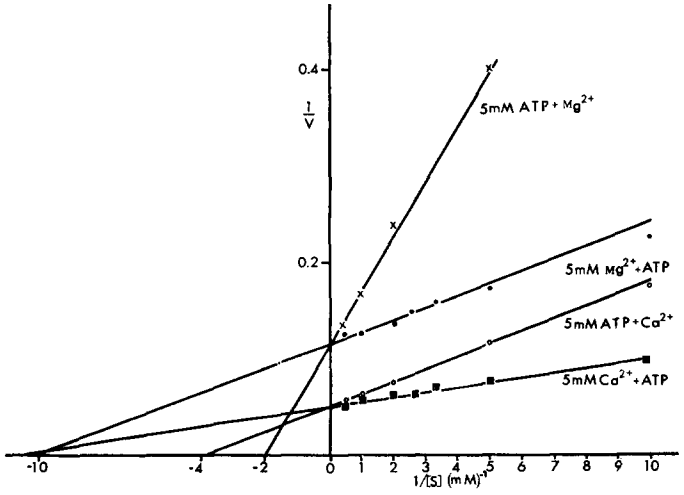


Fig. 2. Variations in Lineweaver–Burk plots of ATPase activity at various concentrations of ATP, Ca^{2+} and Mg^{2+}. v in μmoles P_i formed per mg protein in 30 min. [S] in mM. The incubation mixture contained 20 mM Tris–HCl (pH 8.2), 70 mM Na^+. ×—×, 5 mM ATP + Mg^{2+}; ○—○, 5 mM ATP + Ca^{2+}; ●—●, 5 mM Mg^{2+} + ATP; ■—■, 5 mM Ca^{2+} + ATP.

whereas the addition of Mg^{2+} to 5 mM Ca^{2+} led to inhibition of the enzyme. Fig. 3 gives the curve so obtained. The point of intersection of the two curves was at 5 mM Ca^{2+} *plus* 5 mM Mg^{2+}.

Na^+ independence of the enzyme

The necessity of Na^+ for enzyme activation was tested by incubating the enzyme in 0 or 70 mM Na^+ and adding Tris–ATP instead of Na_2ATP. Specimens incubated without Na^+ showed slightly higher activation than those incubated with Na^+.

The effect of pH on ATP hydrolysis

The Ca^{2+} and Mg^{2+} activation curves of the enzyme were obtained by incubating the enzyme preparation with 0 or 5 mM Ca^{2+} or Mg^{2+} at a pH ranging from 6.5 to 10.7. In each instance the pH was adjusted both in the incubation fluid and Na_2ATP

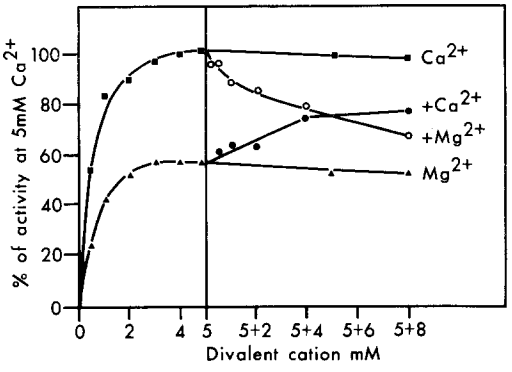


Fig. 3. Enzyme activation by Ca^{2+} and Mg^{2+}. ○—○, constant Ca^{2+} (5 mM) *plus* varying Mg^{2+} concentrations; ●—●, constant Mg^{2+} (5 mM) *plus* varying Ca^{2+} concentrations; ■—■, Ca^{2+} alone; ▲—▲, Mg^{2+} alone. Incubation fluids contained 20 mM Tris–HCl (pH 8.2), 70 mM Na^+ (as NaCl) and 5 mM Na_2ATP.

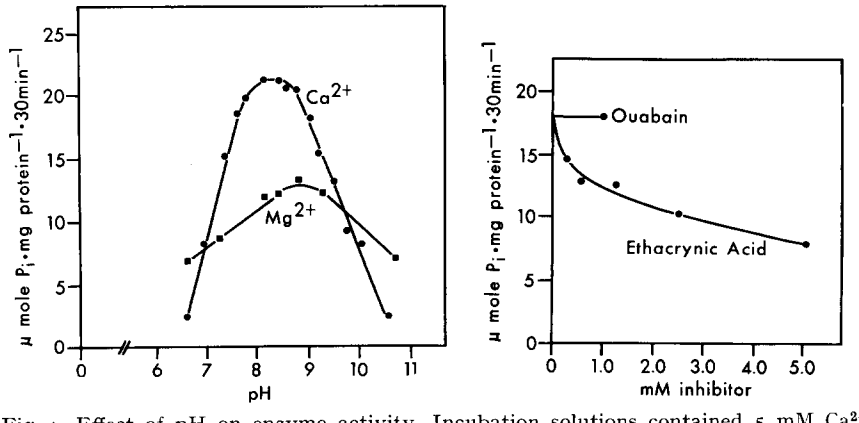


Fig. 4. Effect of pH on enzyme activity. Incubation solutions contained 5 mM Ca^{2+}, 20 mM Tris–HCl, 70 mM Na^+ and 5 mM Na_2ATP. ●—●, pH effect with 5 mM Ca^{2+}; ■—■, pH effect with 5 mM Mg^{2+}.

Fig. 5. Effect of inhibitors. Incubation fluid contained 5 mM Ca^{2+}, 70 mM Na^+, 20 mM Tris–HCl (pH 8.2) and 5 mM Na_2ATP.

solutions and determined before and after incubation. Fig. 4 shows the curves obtained. The pH optimum of Ca^{2+}-ATPase was between 8.2 and 8.5. At pH 7.1 and 9.6 the enzyme was stimulated by Ca^{2+} to only 50 % of peak activity. This experiment was repeated 3 times and a similar curve was obtained each time. The pH optimum of Mg^{2+}-ATPase was between 8.2 and 9.3 with approximately 60 % of the activation by Ca^{2+}. Below pH 7.0 and above 9.7 Mg^{2+} produced higher activation than Ca^{2+}.

Inhibitors

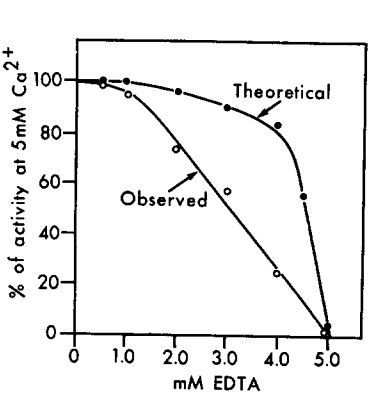
The effects of three different inhibitors on Ca^{2+} activation of the enzyme were tested. Ouabain (1 mM) was added to the solutions containing 0 or 5 mM Ca^{2+}, 20 mM Tris–HCl buffer (pH 8.2), 70 mM Na^+ and 5 mM Na_2ATP. Fig. 5 shows that ouabain did not inhibit the enzyme.

Ethacrynic acid, added to the samples to produce concentrations between 0.15 and 5.0 mM, inhibited enzyme activity by Ca^{2+} (Fig. 5); 5.0 mM ethacrynic acid producing 55 % inhibition.

When Na_2H_2EDTA was added in increasing amounts to the incubation solutions containing 5 mM Ca^{2+}, the Ca^{2+}-sensitive ATPase activity decreased as shown in Fig. 6. The increase in degree of chelation of Ca^{2+} led to decrease in enzyme activity; the enzyme activation by Ca^{2+} could be inhibited completely by the addition of 5 mM Na_2H_2EDTA. However, the inhibition obtained with EDTA was greater than expected from the non-chelated Ca^{2+} activation curve.

Substrate specificity

The substrate specificity of the enzyme was tested by adding increasing amounts (up to 5 mM) of Na_2GTP, Na_2ITP or Na_2ADP to the incubation fluid *plus* enzyme and comparing the P_i release to that produced from Na_2ATP. When the Na_2ATP concentration was varied between 0.1 and 5 mM, maximal Ca^{2+} activation occurred



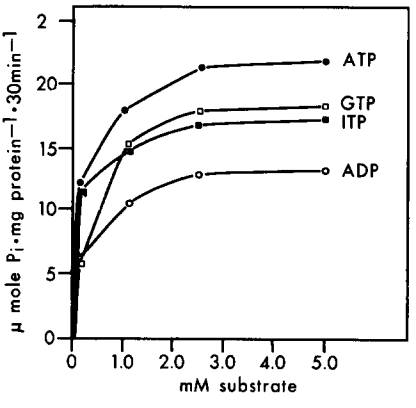


Fig. 6. Effect of EDTA on enzyme activation by Ca^{2+}, ○—○, "observed" curve obtained from incubation with 5 mM Ca^{2+}, 20 mM Tris–HCl (pH 8.2), 70 mM Na^+ and 5 mM Na_2ATP; ●—●, "theoretical" curve obtained with Ca^{2+} concentrations in the incubation fluid corresponding to the non-chelated Ca^{2+} of the "observed" curve.

Fig. 7. Substrate specificity: hydrolysis of ATP compared to that of other high energy tri- and diphosphate nucleotides. ●—●, ATP; □—□, GTP; ■—■, ITP; ○—○, ADP. Incubation fluid contained 5 mM Ca^{2+}, 70 mM Na^+, 20 mM Tris–HCl (pH 8.2).

at 5 mM (Fig. 7) with an apparent K_m of 0.08 mM–0.10 mM. The two other triphosphates and ADP also served as substrates, but GTP and ADP gave a greater K_m (0.38 and 0.15, respectively), whereas the apparent K_m of ITP (0.08 mM) was the same as that for ATP. No change in the K_m for Na_2ATP (0.08–0.1 mM) was obtained when 5 mM Ca^{2+} was replaced by 5 mM Mg^{2+} (Fig. 2) but the v_{max} with ATP was higher.

Temperature dependence

Samples of homogenate were incubated at 23° and 37° in 5 mM Ca^{2+}, 20 mM Tris, 70 mM Na^+ and 5 mM Na_2ATP. The Q_{10} calculated according to the method of GIESE[9] was 1.3.

Stability of the enzyme

No decrease in enzyme activity was noted after 2 months of storage at 4°, whereas freezing the samples destroyed activity more rapidly.

DISCUSSION

A Ca^{2+}-stimulated ATPase is found in several tissues in which active transport of Ca^{2+} occurs, such as in the intestinal mucosa[10–11] and renal tubules[4]. Ca^{2+}-sensitive ATPases have also been described in other tissues in which Ca^{2+} is needed for specific functions, such as sarcoplasmic reticulum[12], brain and nerve tissue[13,14]. Another Ca^{2+}-sensitive ATPase has been characterized in the red cell[15,16]. The present paper describes a further such enzyme present in placental plasma membranes, and stimulated by Ca^{2+} preferentially to other bivalent cations.

The common properties of the various Ca^{2+}-, Mg^{2+}-ATPases are that they do not require Na^+ or K^+ for activation[4,12,13], and that ouabain does not inhibit their activity. However, the requirements for Ca^{2+} and Mg^{2+} differ for each ATPase. In sarcoplasmic reticulum, whereas Mn^{2+} can substitute for both Mg^{2+} and Ca^{2+}, Sr^{2+} can substitute for Ca^{2+} but not for Mg^{2+} (ref. 12). In the erythrocyte[15,17] the enzyme is stimulated by Ca^{2+} and inhibited by Mg^{2+}. In the kidney and intestinal mucosa, Mg^{2+} always stimulates the enzyme more than did Ca^{2+} but either ion could be substituted for the other. Brain Ca^{2+}-ATPase[13,14] can be stimulated equally well by Ca^{2+} and Mg^{2+}

To our knowledge placental Ca^{2+}-ATPase is unique among the Ca^{2+}-sensitive ATPases in that Ca^{2+} are the preferential cations for stimulation. Only 60 % of maximal activity produced by Ca^{2+} can be achieved by substituting 5 mM Mg^{2+} for 5 mM Ca^{2+}. It is not known whether there are different sites for each ion or whether both Ca^{2+} and Mg^{2+} act on the same site. However, we suggest the latter, since there is no essential requirement by the enzyme for either, both producing stimulation. Maximal activation produced by either ion can be modified by the other ion. Thus, we produced inhibition by increasing concentrations of Mg^{2+} to 5 mM Ca^{2+} and stimulation by adding increasing concentrations of Ca^{2+} to 5 mM Mg^{2+}. It seems therefore, that there is competition between Ca^{2+} and Mg^{2+} for the active sites and since the affinity and the v_{max} of Ca^{2+} is higher than that of Mg^{2+}, any combination of these two ions will give lower activity than Ca^{2+} alone but higher than Mg^{2+} alone.

The role of the bivalent cations in this enzyme system is believed to be the production of a bivalent ion ATP complex[5,18] which serves as a substrate for the enzyme. Although the same K_m for Na_2ATP was obtained by replacing 5 mM Ca^{2+}

with 5 mM Mg^{2+}, the v_{max} for Mg^{2+} ATPase was only 60 % of the v_{max} for Ca^{2+} ATPase. This finding indicates that the Ca^{2+}–ATP complex is the preferential substrate. However, the competition of Mg^{2+} with Ca^{2+} in forming the ATP complex was not tested.

The optimal pH for the Ca^{2+}-activated ATPase of the placenta also differs from that for similar enzymes in other tissues. For example it ranges from 7.5 in sarcoplasmic reticulum[12] to pH 9.0 in brain[14]. In other Ca^{2+}-transporting tissues, for example renal cortical plasma membrane, it is at 7.6 and in plasma membranes of intestinal mucosa it is at 8.2 (unpublished observations). We demonstrated a narrow bell-shaped curve of pH dependency in contrast to other Ca^{2+}-ATPases such as kidney[4]. Although we did not isolate the enzyme, the narrow pH curve indicates that we are dealing with only a few enzymes, if not one. Since the optimal pH of our enzyme is 8.2, it is unlikely that an alkaline phosphatase is activated, whose pH optimum is at 10.3 in guinea pig placenta[19].

The absence of substrate specificity makes it likely that this enzyme is a general triphosphatase although ATP is the preferential substrate. We believe that this is the main justification for calling the enzyme a Ca^{2+}-ATPase.

The inhibition obtained with ethacrynic acid suggests that there are SH groups in the active center of the enzyme, since ethacrynic acid is believed to block SH groups[20]. This is similar to its action in the kidney where it inhibits (Na^+, K^+)-ATPase to cause diuresis[20,21]. The essentiality of SH groups for Ca^{2+}-ATPase and Ca^{2+} transport has been demonstrated in sarcoplasmic reticulum[22] by using other SH blockers. In the red blood cell both Ca^{2+}-ATPase and the Ca^{2+} pump are inhibited by ethacrynic acid[23].

The inhibition of Ca^{2+} activation of the enzyme by EDTA was greater than the ability of the latter to chelate Ca^{2+}. The resultant activation curve was linear instead of hyperbolic as would have been expected if the effect of EDTA was solely due to chelation of Ca^{2+} in a 1:1 ratio. We suggest that the Ca^{2+}–EDTA complex has an inhibitory effect by itself but this theory should be tested by adding the Ca^{2+}–EDTA complex to the medium.

The properties of the enzyme described in this paper suggest that it plays a role in Ca^{2+} transport across the placenta. However, the proof will depend on demonstrating correlation between Ca^{2+} transport and enzyme activity.

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