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AN ENDOGENOUS INHIBITOR OF Ca^{++}-ATPase FROM HUMAN PLACENTA

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Intracellular free calcium is regulated by Ca^{++}-ATPase, one form present on the plasma membrane (PM Ca^{++}-ATPase) and the other on sarcoplasmic (endoplasmic) reticulum (SR/ER Ca^{++}-ATPase). An endogenous inhibitor of SR Ca^{++}-ATPase from human placenta was shown to be present in normal placenta and the activity was not detectable in placenta from preeclamptic patients. The inhibitor was distributed in cytosol and microsomes. The inhibition of Ca^{++}-ATPase by this inhibitor was concentration- and time-dependent. The inhibitor neither bound to DEAE- nor CM-sepharose resins at pH 7.5 and 8.5. Furthermore, it was heat stable for 15 min up to 55°C and completely destroyed at 80°C in a few minutes. It was also observed to be stable at room temperature for at least 3 months. The purification and characterization of this inhibitor would be valuable in achieving an understanding of the normal regulation of Ca^{++}-ATPase in the placenta during pregnancy.

Keywords: Inhibitor; Ca^{++}-ATPase; Placenta

INTRODUCTION

Intracellular free calcium has been shown to be an important component for the regulation of many physiological functions. These include muscle contraction, release of signal molecules, enzyme activation and deactivation.[1,2]

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Intracellular free calcium has also been shown to be involved in many pathological conditions.[3,4] The intracellular free calcium is regulated by calcium-dependent ATPase, one form present on the plasma membrane (PM Ca^{++}-ATPase) and the other on sarcoplasmic (endoplasmic) reticulum (SR/ER Ca^{++}-ATPase).[5] Both types of ATPase are distinct from each other in their properties.[5] Modulation of the activity of these Ca^{++}-ATPases by endogenous modulators may play a role in free cytosolic calcium regulation. A number of inhibitor of Ca^{++}-ATPase of SR are known, including sesquiterpene lactones thapsigargin, trilobolide, thapsivillosin A, nonylphenol, H-89 and the hydroquinone 2,5-di-tert-butyl-1,4-benzohydroquinone.[6–8]

Here we show the presence of an endogenous inhibitor of SR Ca^{++}-ATPase isolated from human placenta and which is absent from the placenta of preeclampsia patients.

MATERIALS AND METHODS

Materials

Ca^{++}-ATPase from rabbit skeletal muscle was purified at 4°C as described by Michelangeli and Munkonge.[9] The protein concentration of Ca^{++}-ATPase was about 13 mg/ml and the enzyme was frozen in liquid nitrogen and kept in aliquots at −70°C until required. All chemicals used in this study were of analytical grade and solutions were made in deionized water.

Ca^{++}-ATPase Activity

The activity of Ca^{++}-ATPase was determined at 30°C by a spectrophotometric method.[10] The reaction mixture contained 20 mM MOPS buffer containing 50 μM EGTA, 10 mM PEP, 200 mM KCl, 15 mM $MgCl_2$, 20 units/l of LDH/PK, 10 mM NaN_3, 5 μg/ml purified Ca^{++}-ATPase and 0.5 mM ATP. The reaction rate of non-specific ATPase activity was measured by adding 0.2 mM NADH and monitoring the change in absorbance at 340 nm for about 5–10 min. The Ca^{++}-ATPase activity was then measured[10] for 3 min by adding 50 μM free Ca^{++}. The difference in activity in the absence of Ca^{++} and presence of 50 μM Ca^{++} was defined as the Ca^{++}-ATPase activity, which was considered as 100%. To observe the effect of endogenous placental inhibitor on the purified Ca^{++}-ATPase, the enzyme (15 mg/ml) was diluted to 50 times with 20 mM HEPES buffer (pH 7.4). Fifty μl of this enzyme was incubated with 3 μl of dialyzed (MW cut-off was 14,000 D)

cytosol or membrane fractions for about 60 min at 37°C. Three µl of dialyzed or boiled cytosol or membrane fraction was used as control. Ten µl of this incubated mixture was added to the reaction mixture and the activity for Ca^{++}-ATPase was recorded as described previously. The activity in the control group was considered as 100% activity. Inhibitory activity (effectiveness) of the inhibitor was considered as % inhibition by a fixed concentration of inhibitor under our assay conditions.

Placental Cytosol and Microsomes

The placenta was taken from the labor room of The Aga Khan University Hospital and carried to the laboratory on ice and was used either directly or was frozen at −70°C. All purification procedures were done at 4°C unless otherwise mentioned. Fresh or frozen placenta was divided into small pieces using scissors and washed extensively with cold water to remove blood. The placental pieces were homogenized in a blender in 1 : 5 (w/v) of cold water for about 1–2 min. The extract was passed through cheesecloth and the residues were again homogenized for about 1–2 min and passed through cheesecloth. Both these extracts were mixed and centrifuged for 30 min at 12,000× *g*. The supernatant was further centrifuged for 60 min at 100,000× *g*. The residual microsomes were dissolved/mixed with 20 mM HEPES buffer, pH 7.4 and washed extensively with this buffer, while the cytosol was treated separately. The microsomes were again centrifuged at 100,000× *g* for 1 h. The washed microsomes were mixed in a minimum volume of 20 mM HEPES buffer. These microsomes were then treated with 0.4 M KCl containing 0.01% triton X-100 for 30 min at 4°C, and then centrifuged for 60 min at 100,000× *g*. The supernatant was used as a source of inhibitor.

The original cytosol was precipated with 0–25% ammonium sulfate. The precipitated proteins were dissolved in a minimum volume of 20 mM HEPES buffer, pH 7.4 and dialyzed against 20 mM HEPES buffer overnight. To further purify the proteins, the 0–25% ammonium fraction and detergent isolated proteins from microsomes were passed through DEAE-sepharose or CM-sepharose (at pH 7.4 and pH 8.4) and 1 ml fractions were collected using a fraction collector. After washing the column, the bound proteins were eluted by 0.5 M KCl in 20 mM HEPES buffer, pH 7.4/8.4. Fractions containing proteins (which showed absorbance at 280 nm) were analyzed for inhibitory activity of Ca^{++}-ATPase. Fractions containing inhibitory activity were pooled and passed through a gel filtration column. To check the purity of the proteins, they were analyzed by SDS-PAGE.[11]

RESULTS

Table I shows the effect of 0–25% ammonium sulfate fraction of cytosol and the original crude microsomal extract on Ca^{++}-ATPase activity. There was about 75–88% inhibition. However, the inhibition was variable between different preparations (10–40%). The inhibitory activity was stable even at room temperature for about 3 months. However, the inhibition was temperature-dependent. There was no inhibition at 4°C while there was 10% and 75% inhibition at 25°C and 37°C, respectively (Figure 1). Similarly, the inhibition was also time-dependent and 50% inhibition was obtained in about 60 min at a constant concentration of inhibitor (Figure 2).

TABLE I Effect of various fractions of placenta on Ca^{++}-ATPase activity

Fractions	*Activity (% remaining)*
Control	100
0–25% ammonium sulfate (cytosol)	25
0–25% ammonium sulfate (microsomes)	12

Three µl of dialyzed extract was incubated with 20 µl of Ca^{++}-ATPase for 60 min at 37°C. Three µl bioled and centrifuged extracts of cytosol and microsomes were used as control. The enzyme activity was measured for 3 min as described in Materials and Methods.

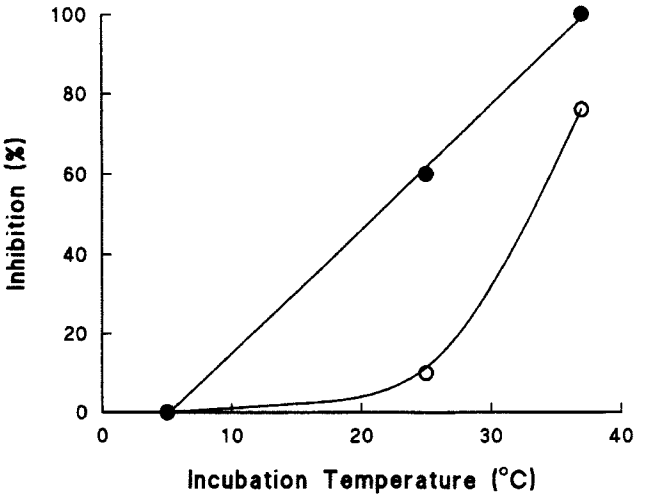

FIGURE 1 Effects of incubation temperature on the effectiveness of inhibition of Ca^{++}-ATPase. A fixed concentration of microsomol inhibitor was incubated with enzyme at 4°C, 25°C and 37°C for 15 min (●—●) and 60 min (○—○). The activity of Ca^{++}-ATPase was measured as described in Materials and Methods.

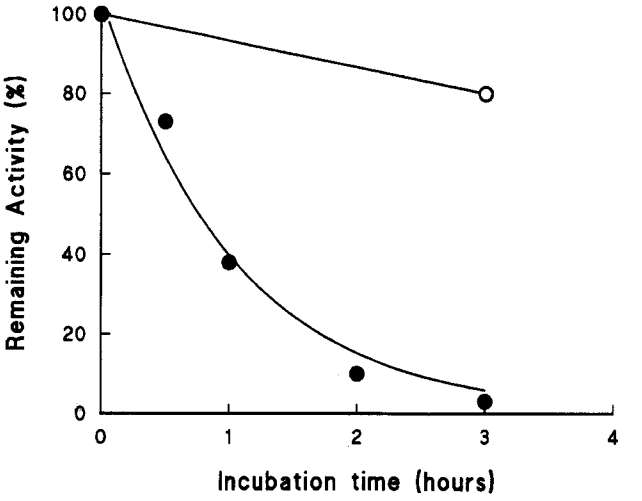

FIGURE 2 Effects of the inhibitor incubation time on the acitivity of Ca^{++}-ATPase. A fixed concentration of microsomal inhibitor was incubated at 37°C with enzymes at various time periods (●—●). An aliquot was taken after a predetermined time period and kept at 4°C in ice. At the end of the experiment Ca^{++}-ATPase activity was measured in all aliquots as described in Materials and Methods. The control experiment (○—○) was done only after 3 h of incubation.

The partially purified inhibitor showed a concentration-dependant inhibition of Ca^{++}-ATPase with an IC_{50} value of about 1.2 mg/ml (Figure 3). Heat-inactivation experiments showed that the inhibitor became about 50% ineffective in about 30 min at 55°C while it was destroyed in 1–2 min at 80°C (Figure 4). The inhibitor was distributed in cytosol and microsomal fractions. In cytosol it was observed in the 0–25% ammonium sulfate fraction but there was no inhibitory activity in the 25–45% and 45–75% ammonium sulfate fractions. In microsomes about 30% inhibitory activity was solubilized by 0.4 M KCl and the remainder (70%) by 0.01% triton X–100. Neither DEAE- or CM-sepharose resins at pH 7.4 and 8.4 retained all three fractions of the inhibitor. The cytosolic fraction after DEAE- and CM-sepharose was applied on gel filtration using sepharose-6B and we obtained a sharp peak corresponding to inhibitor (Figure 5). Due to the unpurified form of the inhibitor from microsomes and cytosol, we were unable to identify the bands corresponding to inhibitor after SDS-PAGE. We have analyzed 8 samples of placenta from preeclamptic patients. In these samples we could detect little or no inhibitor activity in either the cytosol or microsomal fractions.

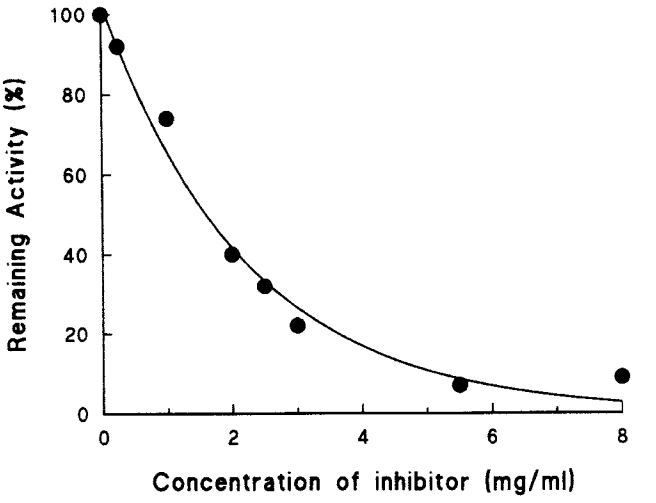

FIGURE 3 Effect of different concentration of partially purified microsomal inhibitor on Ca^{++}-ATPase activity. The inhibitor was incubated with enzyme at 37°C for about 1 h and enzyme activity was then measured as described in Materials and Methods. The values are the average of two different experiments. In this experiment a control was run wtih 10 mg/ml boiled extract.

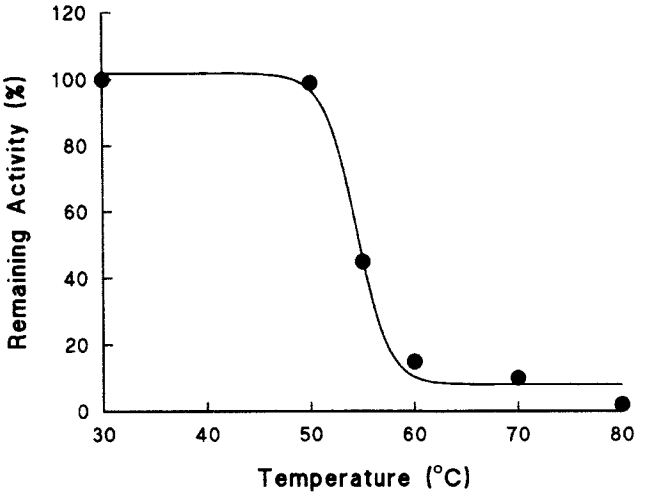

FIGURE 4 Effect of heat-inactivation on the effectiveness of microsomol inhibitor on Ca^{++}-ATPase activity. The incubation was kept at various temperatures for 30 min and then transferred to ice. A fixed concentration of heated inhibitor was incubated with enzyme for 1 h at 37°C and then activity was measured as described in Materials and Methods.

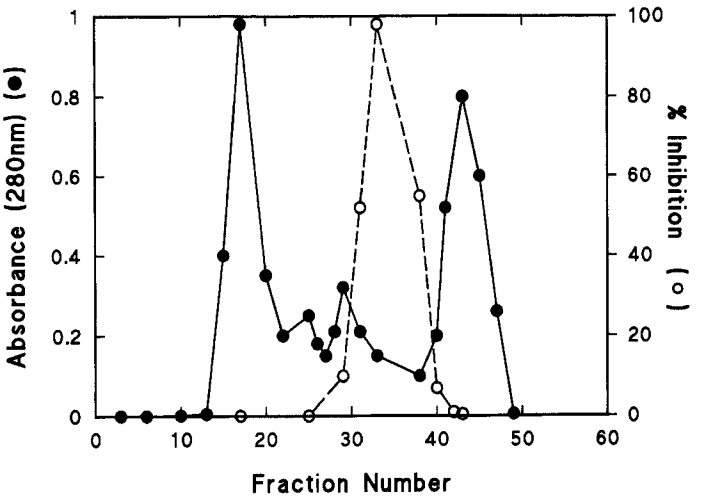

FIGURE 5 Sepharose column chromatography of microsomal inhibitor. A sepharose-6B column (1.5 × 30 cm) was equilibrated with 20 mM HEPES buffer (pH 7.4). A 2 ml sample was applied on the column and fractions were collected. Alternate fractions were analyzed for protein (280 nm) (●—●) and inhibitory activity (○----○) (shown as % inhibition where the fractions which showed maximum inhibition were considered as 100% effective).

DISCUSSION

Intracellular free calcium has been shown to be involved in a number of normal[1,2] and abnormal functions of the cell.[3,4] Therefore the intracellular regulation of free calcium needs to be finely controlled. The most important and extensively studied enzyme that regulates calcium in the cell is the Ca^{++}-ATPase from SR of skeletal muscle.[5] There are a number of modulators of Ca^{++}-ATPase known but all are of plant origin.[6–8]

The inhibitor which we have isolated from normal placenta is a unique type of inhibitor due to the concentration and time-dependence of the inhibition reaction. We have examined this inhibitor against lactate dehydrogenase and pyruvate kinase where it was ineffective against these proteins indicating that Ca^{++}-ATPase was the target. To confirm this specificity additional work needs to be using related ATPases as targets for this inhibitor.

The absence of this inhibitor in placenta from preeclamptic patients may indicate some important role for this inhibitor. It has been suggested that some proteolytic enzymes in placenta be involved in the pathogenesis of this disease.[12] Similarly calcium homeostasis abnormality may be involved in the development of hypertension.[13] It has been shown that increased

intracellular calcium in vascular smooth muscle cells may be responsible for increased muscle tone which can raise systemic blood pressure.[13]

Acknowledgment

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