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ISOLATION OF A THERMOSTABLE MODIFIER OF Ca^{2+}-STIMULATED ATPase FROM HUMAN RED CELLS

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Summary: A fraction was isolated from human red cells capable of decreasing Ca^{2+}-stimulated with a parallel increase of Mg^{2+}-stimulated ATPase. This fraction seems to produce an increase of the apparent affinity for Ca^{2+}. But, when the fraction concentration was increased, there was a decrement of Ca^{2+}-stimulated ATPase up to the point of complete abolishment. At this point, Mg^{2+}-stimulated ATPase reached the initial level of the Ca^{2+}-stimulated ATPase.

Introduction: A Ca^{2+} dependent regulator protein (Calmodulin) has been characterized (1). This thermostable, acidic protein has a molecular weight of about 17,000 daltons and binds Ca^{2+} with high affinity. Calmodulin increases the Ca^{2+}-stimulated ATPase activity of erythrocyte ghosts above the level achieved with addition of Ca^{2+} alone. The active form of this ATPase appears to be an enzyme-calmodulin complex. The complex formation is dependent on Ca^{2+} which binds to calmodulin allowing its interaction with the enzyme (2). Wallace et al (3) described in bovine brain, and Sarkadi et al (4) in red cells a heat labile protein fraction that antagonized calmodulin activation. On the other hand, Mauldin and Roufogalis (5)

Abbreviations: Total ATPase: ATPase activity measured in the presence of Mg^{2+} and Ca^{2+}. Mg^{2+}-stimulated ATPase: ATPase activity measured in the presence of Mg^{2+} and EGTA. Ca^{2+}-stimulated ATPase: Activity remaining after subtracting the Mg^{2+}-stimulated activity from the total activity. EGTA: Ethylene glycol bis (β-aminoethyl ether)-N-N'-tetraacetic acid.

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described the isolation of an activator of Ca^{2+}-stimulated ATPase from red cell membranes apparently different from calmodulin with a molecular weight of 63,000 daltons.

All these proteins act on the Ca^{2+}-stimulated ATPase without any effect on the Mg^{2+}-stimulated ATPase activity (1-5). We present, in this communication, evidence of a factor capable of decreasing Ca^{2+}-stimulated ATPase with a parallel increment in the Mg^{2+}-stimulated ATPase from human red cells. We called it TSM (thermostable modifier).

Materials and Methods:

Human red cells packed in acid-citrate-dextrose were supplied by a blood bank. The red cells were washed thrice in 2-3 volumes of cold 155 mM Tris-HCl pH 8 (5°C) and white cells were removed by aspiration. The erythrocytes were lysed in 10 volumes of 15 mM Tris-HCl pH 8 (5°C) containing 1 mM EGTA. The suspension was kept at 0-5°C for 20 min and then centrifuged at 20,000 x g for 10 min. The supernatant was saved to obtain the modifier. The pellet was washed in the same buffer to remove visible traces of hemoglobin, fractionated and frozen at -40°C. The membranes were frozen and thawed once befor use. ATPase assay: The incubation mixture contained (2 mL): 40 mM Tris-HCl, 100 mM NaCl, 0.1 mM ouabain pH 8 (37°C); 0.4-0.6mg protein as the source of enzyme; 2 mM $ATPNa_2$; 3 mM $MgCl_2$; and 0.1 mM EGTA. The mixture was incubated for 30 min at 37°C. The reaction was stopped by adding either sodium dodecyl sulfate to a final concentration of 0.35% (6) or 0.1 ml of cold 100% trichloroacetic acid. The released inorganic phosphate (Pi) was measured according to Fiske-Subbarow (7). Protein was measured according to Lowry et al (8). All assays were by duplicate and individual experiences were repeated four times. Various controls were made to discover possible alterations produced by the heated-hemolysate when it was added in the assay mixture: a) To avoid any effect upon the ionic strength, NaCl (100 mM) was included in the assay mixture; although similar results were obtained in its absence. b) To discard any contamination by Ca^{2+}, EGTA concentrations up to 5 mM were tried; but the Mg^{2+}-stimulated ATPase activity was the same as in the presence of 0.1 mM EGTA. c) To rule out the possibility that the observed effects were derived from changes on the density of the assay mixture, sucrose or Tris-HCl instead of the heated-hemolysate were added in the assay mixture, In all cases the results were the same as the controls in which only water was added.

Preparation of the thermostable modifier (TSM): The supernatant from the hemolysis (see above) was dialysed for 1 week against

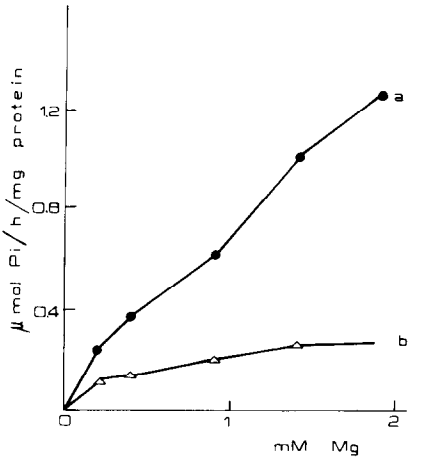

Fig 1: Effect of various concentrations of Mg^{2+} on Mg^{2+}-stimulated ATPase activity in the presence (a) and in the absence (b) of 0.4 ml (0.4 mg protein) of the heated-hemolysate. Experimental conditions were as described in Materials and Methods, except that $MgCl_2$ was added to the reaction mixture as indicated. Similar results were obtained in four experiments.

distilled water. This dialysed hemolysate had no activating effect on Ca^{2+}-stimulated ATPase. Then, it was heated to 85°C and after 1 min the solution was cooled in an ice slurry. The coagulated proteins were removed by centrifugation at 15,000 x g for 30 min. The supernatant was used as the TSM without further purification ("heated-hemolysate").

Molecular weight determination: An aliquot of 2 ml (10 mg protein) of the heated-hemolysate was applied to a Sephadex G-50 column (1.3 x 60 cm) equilibrated with 10 mM Tris-HCl pH 8 (30°C) containing 30 uM $MgCl_2$; 10 uM mercaptoethanol; 1 mM NaCl 0.001% Triton X-100 and 3 uM $CaCl_2$. The column was washed with the same buffer. Fractions of 2 ml/h were collected and monitored for the presence of the modifier. The column was calibrated using proteins of known molecular weight.

Acrylamide gel electrophoresis: Disk electrophoresis was carried out as described by Davies (9).

Materials: Tris, EGTA, ouabain, $ATPNa_2$ (vanadate-free), $MgCl_2$, were from Sigma Chemical Co., St.Louis, MO,USA. All other salts were analytical-reagent grade.

Results:

Fig 1 shows the activity of Mg^{2+}-stimulated ATPase against Mg^{2+} concentrations. The presence of the heated-

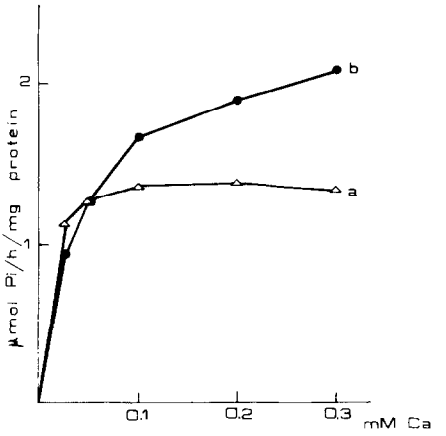

Fig 2: Ca^{2+}-stimulated ATPase activity at different Ca^{2+} concentrations in the presence (a) and in the absence (b) of 0.4 ml of the heated-hemolysate. Experimental conditions were as described in Material and Methods except that $CaCl_2$ was added to the reaction mixture as indicated. Similar results were obtained in four experiments.

hemolysate increased the hydrolytic activity both at saturating and non-saturating $MgCl_2$ concentrations.

Fig 2 shows the effect of the heated-hemolysate on the Ca^{2+}-stimulated ATPase activity at different Ca^{2+} concentrations. In the presence of the TSM the enzyme reached saturation at lower Ca^{2+} concentrations and had a lower Vmax than a control. Thus, the TSM seems to produce an increase of the apparent affinity for Ca^{2+}.

The TSM has different effects on the Mg^{2+}-stimulated and Ca^{2+}-stimulated ATPase activities (Fig 3). When the heated hemolysate concentration was increased, there was an increment in the Mg^{2+}-stimulated activity and a parallel decrease of the Ca^{2+}-stimulated activity. The activation by Ca^{2+} was completely abolished at 0.9 ml of the heated-hemolysate (0.9 ml corresponds to 0.9 mg protein).

When the heated-hemolysate was passed through a Sephadex G-25 column the TSM was eluted in the void volume. The molecular weight determination was made in a Sephadex G-50 column calibrated with proteins of known molecular weights

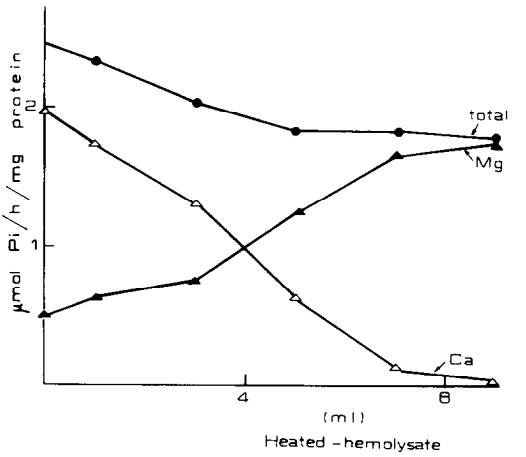

Fig 3: Effect of the TSM concentration on ATPase activities in the presence of Ca^{2+} and Mg^{2+}. Experimental conditions were as indicated in Materials and Methods, except that heated-hemolysate was added as indicated. Total, total ATPase; Mg, Mg^{2+}-stimulated ATPase; Ca, Ca^{2+}-stimulated ATPase.

(Fig 4A). An apparent molecular weight of 6,200 daltons was found (Fig 4B). The fractions with the highest ability to increase Mg^{2+}-stimulated ATPase activity were pooled. These aliquots showed the presence of proteins (by Lowry and A_{280}). Polyacrylamide gel electrophoresis of the peak from the Sephadex G-50 column gave a single band that was stained with amido black.

Discussion:

The preliminary determination of the TSM molecular weight showed a value that differed from calmodulin and the Ca^{2+}-stimulated ATPase activator reported by Mauldin and Roufogalis (5). The cell free hemolysate had calmodulin activity, but it was lost after the dialysis for one week. The possibility cannot be ruled out that the TSM could be either a portion of calmodulin or another molecule produced by the manipulation of the hemolysate.

Soe et al. (10) demonstrated that palmitoleic acid is a factor capable of converting Ca^{2+}-stimulated into Mg^{2+}-

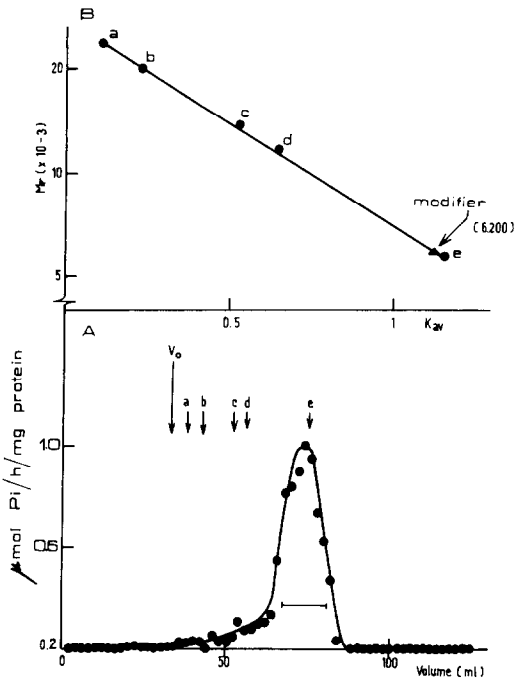

Fig 4: A) Sephadex G-50 column chromatography of the TSM. Heated-hemolysate (10 mg protein) was applied to the column. It was run at room temperature. The Mg^{2+}-stimulated activity was measured as described in Materials and Methods, except that 0.2 ml of the eluate from the column was added to the reaction mixture. Mg^{2+}-stimulated ATPase activity without addition of the eluate was 0.2 umol Pi/h/mg protein. B) Estimation of molecular weight of the TSM. The Sephadex G-50 column was calibrated with a)Trypsin (Mr:23,500), b)Protease (Mr: 20,000), c) RNase (Mr: 13,500), d) Cytochrome C (Mr: 11,700), and e) Insulin (Mr:5,700). Kav is defined as the ratio of (Ve-Vo)/(Vt-Vo) where Ve is the elution volume of the sample, Vo the void volume, and Vt the total volume of the column. The void volume was determined using blue dextran.

stimulated ATPase present in Rhodospirillum rubrum chromatophores. Similarly, Walter and Hasselbach (11) showed that sarcoplasmic reticulum ATPase delipidated with Triton X-100 exhibits a low activity dependent on Mg^{2+} alone. This Ca^{2+}-independent ATPase can be activated to the level of the Ca^{2+} stimulated ATPase by both unsaturated and saturated fatty acids, by anionic phospholipids and various synthetic lipids. This communication shows that the presence of TSM raises the

Mg^{2+}-stimulated activity with a parallel decrease of the Ca^{2+}-stimulated activity. When the activation by Ca^{2+} was completely abolished the Mg^{2+}-stimulated ATPase reached the initial level of the Ca^{2+}-stimulated ATPase. The lower activity of Ca^{2+}-stimulated ATPase is not produced by a lower apparent affinity of the enzyme for Ca^{2+} since the presence of the TSM increase it (Fig 2).

These results could suggest that TSM has the capability of converting Ca^{2+}-stimulated into Mg^{2+}-stimulated ATPase. But this affirmation needs the support of more evidence. Studies upon this aspect are in progress. On the other hand, it cannot affirm that the TSM is a normal component of the erythrocyte or some molecule produced by the treatments of the hemolysate. However, in both cases, the TSM could be a good tool to study the relation between Mg^{2+}-stimulated and Ca^{2+}-stimulated ATPase.

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