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$(Ca^{2+} + Mg^{2+})$-ATPase ACTIVATOR: ITS PRESENCE IN HUMAN RED BLOOD CELLS AND RABBIT SKELETAL MUSCLE

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Summary

We find that both human red blood cells and rabbit skeletal muscle contain a soluble activator which can stimulate $(Ca^{2+} + Mg^{2+})$-ATPase activity. The activator protein from either source can enhance the $(Ca^{2+} + Mg^{2+})$-ATPase of both the red blood cell membrane and the microsomal fraction from skeletal muscle. The data suggest that they are members of the class of Ca^{2+}-binding modulator proteins. A possible physiological role for the skeletal muscle activator protein in the contractile process is discussed.

The human red blood cell membrane contains a pump which actively extrudes Ca^{2+} from the cell and which maintains intracellular Ca^{2+} at micromolar levels [1]. This pump is expressed enzymatically as the $(Ca^{2+} + Mg^{2+})$-ATPase. Bond and Clough [2] reported that this ATPase activity could be enhanced by a soluble protein present in red blood cell hemolysate. Some of the properties of this $(Ca^{2+} + Mg^{2+})$-ATPase activator protein have been characterized in subsequent studies [3—7] including the ability to stimulate Ca^{2+} transport in inside-out vesicles of human red blood cell membranes [8,9]. The purified protein has a molecular weight of 16 700 and its amino acid composition has been determined [7]. It is a member in the class of Ca^{2+}-binding modulator proteins which include troponin C and the modulator proteins of brain, heart and testis which activate adenylate cyclase and 3′,5′-cyclic nucleotide phosphodiesterase in the presence of Ca^{2+} [5—7].

We report here the apparent presence of a $(Ca^{2+} + Mg^{2+})$-ATPase activator protein in the soluble fraction of rabbit skeletal muscle. This activator is able to stimulate $(Ca^{2+} + Mg^{2+})$-ATPase activity in both red blood cell and skeletal muscle preparations. In addition we find that the Ca^{2+} pump activator protein from red blood cell hemolysates is also able to stimulate increased $(Ca^{2+} + Mg^{2+})$

ATPase activity in skeletal muscle microsomes. The possible physiological significance of this finding is dscussed.

Red blood cell membranes were prepared from outdated human blood. For each preparation 2 ml of washed cells were hemolyzed at 5°C in 15 ml 20 mM sucrose/10 mM NaCl/20 mM Tris-HCl (pH 7.45 at 25°C). After 10 min, the cells were homogenized briefly with a Polytron (PT-10) at setting 7 and centrifuged at 50 000 × *g* for 20 min in a Beckman Model L5-50 Ultracentrifuge. The supernatant from this spin was carefully removed and saved. The white pellet, which contained the red blood cell membranes, was washed three times. The first two washes were done in 2 mM sucrose/1 mM NaCl/1 mM KCl/1 mM EDTA/2 mM Tris-HCl (pH 7.7 at 25°C). The last wash was done in this solution minus EDTA. The final pellet was resuspended in 3 ml 17 mM Tris-HCl (pH 7.6 at 5°C).

Skeletal muscle microsomal fractions were prepared from the sartorius muscle of New Zealand rabbits. Typically, 1.5 g muscle were minced and then homogenized with a Polytron model PT-10 for 5 s at setting 7 in 250 mM sucrose/10 mM Tris-HCl (pH 7.8 at 25°C) (10:1, v/w). The homogenate was spun at 5000 × *g* for 10 min. The supernatant fluid was centrifuged at 175 000 × *g* for 1 h. The supernatant from this spin, containing soluble proteins, was saved and the microsomal pellet was resuspended in 30 ml 17 mM Tris-HCl (pH 7.6 at 5°C).

$(Ca^{2+}+Mg^{2+})$—ATPase activity was taken as the activity stimulated by the presence of 0.1 mM Ca^{2+}. The assay medium comprised 3 mM $MgCl_2$, 36 mM imidazole (pH 7.1), 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain, 5 mM NaN_3, 3 mM Na_2ATP (neutralized) and either 0.1 mM EGTA or $CaCl_2$. Assays were run in a total volume of 1 ml for 20 min at 37°C. The reaction was stopped by the addition of 0.5 ml 20% trichloroacetic acid. After centrifugation the supernatant was assayed for inorganic phosphate by the method of Fiske and SubbaRow [10]. Protein content was determined by the method of Lowry et al. [11].

TABLE I

STIMULATION OF $(Ca^{2+}+Mg^{2+})$-ATPase BY RED BLOOD CELL AND SKELETAL MUSCLE ACTIVATOR PROTEINS

Data are expressed as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.02$ computed using Student's paired *t*-test. *n* is the number in parentheses. 0.1 ml red blood cell and skeletal muscle supernatant, typically, contained 2.5 and 0.4 mg protein, respectively.

Membrane preparation	Supernatant added (0.1 ml)	$(Ca^{2+}+Mg^{2+})$-ATPase (μmol P_i/mg protein per h)
Red blood cells (11)	None	0.64 ± 0.10
	Red blood cells	1.15 ± 0.19**
Skeletal muscle (6)	None	39.2 ± 2.5
	Red blood cells	54.0 ± 4.2*
Red blood cells (8)	None	0.58 ± 0.12
	Skeletal muscle	0.91 ± 0.18**
Skeletal muscle (7)	None	31.9 ± 6.2
	Skeletal muscle	38.9 ± 7.3**

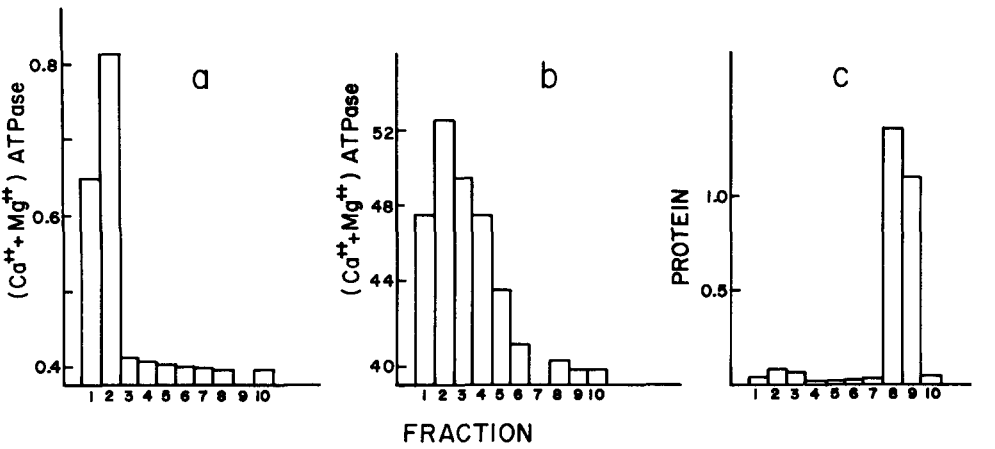

Fig.1. Partial purification of the red blood cell (Ca^{2+} +Mg^{2+})-ATPase activator protein. The column contained 10 ml CM-Sephadex C-50 and was first equilibrated with 20 mM Tris/maleate (pH 6.5 at 25°C). In each experiment 5 ml of red blood cell hemolysate which had been dialyzed against this same buffer was applied to the column. After collecting fraction 1 (all fractions were 5 ml), 10 ml more of 20 mM Tris/maleate (pH 6.5) were passed through the column. Then successive 5-ml aliquots of 20 mM Tris/maleate at pH 6.6, 6.7 and 6.8 were eluted through the column. Finally, 20 ml 90 mM KCl, 50 mM Tris-HCl (pH 8.2 at 25°C) were applied to the column and the last four fractions were collected. a,b. The effect on the (Ca^{2+}+Mg^{2+})-ATPase activity (μmol P_i/mg protein per h) of red blood cell membranes and skeletal muscle microsomes, respectively, of 0.1 ml of each fraction of the red blood cell hemolysate. c. The concentration of protein (mg/0.1 ml) in each eluted fraction of the red blood cell hemolysate. All data are the average of two experiments.

The effects of soluble supernatant from human red blood cells and rabbit skeletal muscle on the (Ca^{2+} +Mg^{2+})-ATPase activities of both red blood cell membranes and skeletal muscle microsomal fraction are shown in Table I. In each case a statistically significant stimulatory effect was observed. The enhancement of red blood cell (Ca^{2+} +Mg^{2+})-ATPase activity by red blood cell hemolysate has been reported previously [2—6]. We find that the red blood cell hemolysate also produces a 38% increase in skeletal muscle microsomal (Ca^{2+} +Mg^{2+})-ATPase. In addition the supernatant from the high speed spin of the skeletal muscle microsomal preparation is able to stimulate the (Ca^{2+} +Mg^{2+})-ATPase activities of both the red blood cell and skeletal muscle membrane preparations by 57 and 22%, respectively. In no case did the supernatant by itself possess any (Ca^{2+} +Mg^{2+})-ATPase activity. Overnight dialysis of the supernatants did not diminish their activator properties. Heating of the supernatants at 85°C for 30 min caused the stimulatory activity to disappear. These last two observations suggest that a protein acts as the activator substance as has been previously established for the activator present in red blood cell hemolysate [3,6,7].

To obtain a partial purification of the (Ca^{2+} +Mg^{2+})-ATPase activator which is present in the red blood cell hemolysate a carboxymethyl-Sephadex C-50 (Pharmacia) column was used [3]. The results of this experiment are shown in Fig.1. As previously described [3], all of the red blood cell membrane (Ca^{2+} +Mg^{2+})-ATPase activator elutes quickly through the column at low pH

(Fig.1a). The bulk of the protein (predominantly hemoglobin) remains bound to the Sephadex until the pH is raised (Fig.1c). In Fig.1b it is seen that the red blood cell activator of skeletal muscle (Ca^{2+} +Mg^{2+})-ATPase behaves in a manner qualitatively similar to that of the red blood cell activator of red blood cell (Ca^{2+} +Mg^{2+})-ATPase, In this case, however, the distribution of activator is somewhat more spread out in the initial fractions.

When the high speed skeletal muscle supernatant was applied to the column the analogous result was obtained. That is the activator of both the skeletal muscle and red blood cell (Ca^{2+} +Mg^{2+})-ATPases eluted in the initial fractions. These results suggest that the activator proteins present in the skeletal muscle and red blood cell supernatants are related proteins.

The response of skeletal muscle and red blood cell (Ca^{2+} +Mg^{2+})-ATPase to varying amounts of the partially purified activator from red blood cells is demonstrated in Fig.2. The two curves are similar and begin to saturate when more than 0.1 mg of fraction 2 protein is added to the incubation medium.

The results indicate that a soluble (Ca^{2+} +Mg^{2+})-ATPase activator protein exists in both human red blood cells and in rabbit skeletal muscle cells. The activator from either type of cell can stimulate both the red blood cell membrane and skeletal muscle microsomal (Ca^{2+} +Mg^{2+})-ATPases. The skeletal muscle activator protein behaves on the carboxymethyl-Sephadex column in

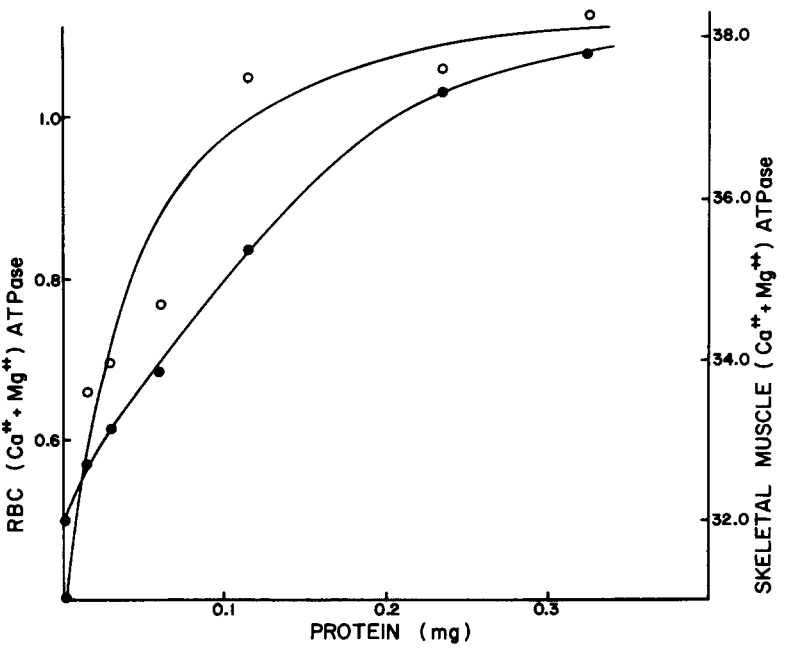

Fig.2. Response of (Ca^{2+} +Mg^{2+})-ATPase to varying amounts of activator protein. Different quantities of fraction 2 (see Fig.1) protein obtained from red blood cell (RBC) hemolysate were added to the assay tubes. Shown are the responses of human red blood cell membrane (•) and rabbit skeletal muscle microsomal (○) (Ca^{2+} +Mg^{2+})-ATPase to this protocol. All data are the average of two experiments. ATPase activities are expressed as μmol P_i released/mg protein per h.

a similar manner to that of the red blood cell. This suggests that, like the red blood cell activator [7], the skeletal muscle (Ca^{2+} +Mg^{2+})-ATPase activator protein is a member of the class of Ca^{2+}-binding modulator proteins. Further purification and characterization is needed to determine this with certainty. Bovine brain phosphodiesterase activator, one of these modulator proteins, is able to stimulate human red blood cell membrane (Ca^{2+} +Mg^{2+})-ATPase [5,6]

The sarcoplasmic reticulum is by far the major source of skeletal muscle (Ca^{2+} +Mg^{2+})-ATPase. This ATPase is distinct from red blood cell (Ca^{2+} +Mg^{2+})-ATPase, having several different properties including molecular weight and Ca^{2+}-pumped/ATP hydrolyzed stoichiometry [1, 12]. It is thus surprising that both these (Ca^{2+} +Mg^{2+})-ATPases could be regulated by the same protein. It has been reported [13, 14] that skeletal muscle also has a (Ca^{2+} +Mg^{2+})-ATPase associated with its plasma membrane and it may be that it is only this ATPase which is being stimulated by the modulator proteins in this study. To distinguish which of the two (Ca^{2+} +Mg^{2+})-ATPases of skeletal muscle is being affected we have undertaken studies to separate skeletal muscle plasma membrane and sarcoplasmic reticulum. Results of two experiments indicate that the sarcoplasmic reticulum (Ca^{2+} +Mg^{2+})-ATPase is stimulated by the partially purified red blood cell activator protein.

If indeed the skeletal muscle (Ca^{2+} +Mg^{2+})-ATPase activator is a Ca^{2+}-binding modulator protein, a possible physiological role becomes apparent. Skeletal muscle relaxation requires the lowering of cytoplasmic Ca^{2+}levels by increased Ca^{2+} pumping by the sarcoplasmic reticulum. Thus at precisely the time when increased (Ca^{2+} +Mg^{2+})-ATPase activity was necessary the modulator protein would be exerting its stimulatory effect due to the increased Ca^{2+} level. It is worth noting that the bovine brain regulator protein has a class of Ca^{2+}-binding site with a dissociation constant of $1 \cdot 10^{-6}$ M [15]. The phosphodiesterase activator from bovine heart requires a Ca^{2+} concentration of 2.3 μM for 50% activation [16]. An analogous skeletal muscle protein would thus be in its active state during muscle contraction when the Ca^{2+} concentration rises to these levels. Although cardiac muscle has also been found to possess a Ca^{2+}-binding regulatory protein [16], it has not been tested for ability to stimulate cardiac (Ca^{2+} +Mg^{2+})-ATPase. Elucidation of the roles of these modulator proteins in muscle contraction could be of major significance.

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