Properties of the Sarcolemmal Calcium Ion-Stimulated Adenosine Triphosphatase of Hamster Skeletal Muscle

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1. A sarcolemmal fraction was isolated from hamster hind-leg skeletal muscles by successive treatment with lithium bromide and potassium chloride. The membranous fraction was observed to contain a highly active Ca^{2+} -stimulated ATPase (adenosine triphosphatase), a Mg^{2+} -stimulated ATPase, and an $\text{Na}^+ + \text{K}^+$ -stimulated Mg^{2+} -dependent ouabain-sensitive ATPase. 2. The Ca^{2+} -stimulated ATPase activity was pH-dependent, the optimum being pH 7.6. 3. Optimum activation of this enzyme was obtained with 3–4 mM- Ca^{2+} when 4 mM-ATP was present as a substrate, and was not influenced by Na^+ , K^+ or ouabain, whereas 2,4-dinitrophenol, sodium azide, oligomycin, sodium fluoride and ethanedioxybis(ethylamine)tetra-acetate were inhibitory. 4. The Ca^{2+} -stimulated ATPase was markedly inhibited by thiol-blocking reagents, and cysteine was able to reverse this inhibition. 5. Various bivalent cations stimulated ATP hydrolysis by the sarcolemmal fraction in the following decreasing order of potency: Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Sr^{2+} , Ba^{2+} , Zn^{2+} , Cu^{2+} .

The intimate involvement of the muscle sarcolemma in the process of excitation-contraction coupling is well recognized (Sandow, 1965, 1970). This cell membrane is considered to play an important role in the regulation of the ionic milieu of the cell interior (Whittam & Wheeler, 1970). Sulakhe, Fedelesova, McNamara & Dhalla (1971) have reported a method for the isolation of skeletal-muscle sarcolemmal fragments and demonstrated the presence in them of a highly active $\text{Na}^+ + \text{K}^+$ -stimulated Mg^{2+} -dependent ouabain-sensitive ATPase* ('transport ATPase'). In the present paper we describe the properties of Ca^{2+} -stimulated ATPase of the skeletal-muscle membranous fraction. The presence of this enzyme system in the sarcolemma of dog heart has been reported (Sulakhe & Dhalla, 1971a).

MATERIALS AND METHODS

Materials. Tris-ATP and oligomycin were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.); ouabain was from Calbiochem (Los Angeles, Calif., U.S.A.); sucrose (special enzyme grade) was from Mann Laboratories (New York, N.Y., U.S.A.); all analytical-grade bivalent-metal salts were from J. T. Baker (Toronto, Ont., Canada) or Fisher Chemicals (Toronto, Ont., Canada).

Isolation of sarcolemmal fraction. After dissection of the skeletal muscle from the hind legs of hamsters, the tissue was immediately placed in ice-cold buffer (0.25 M-sucrose–0.2 mM-EDTA–0.1 M-tris-HCl buffer, pH 7.4–7.6). After removal of the fat and connective tissue, the muscles (10–12 g) were minced, thoroughly washed and then homogenized in the above medium in a Waring Blendor for 1 min in a cold-room to obtain a 10% (w/v) homogenate. The homogenate was filtered through several layers of gauze and centrifuged at 1000g for 10 min in a Sorvall RC-2B refrigerated centrifuge. The residue, after being washed, was extracted with 0.5 M-LiBr–0.05 mM-ethanedioxybis(ethylamine) tetra-acetate–16 mM-tris-HCl buffer, pH 8.5, for 10–15 h in a cold-room. The jelly-like solution was filtered through gauze, spun at 2500g for 10 min, then the residue was discarded and the supernatant further centrifuged at 100 000g for 30 min in an International model B-60 refrigerated ultracentrifuge (rotor no. A-211). The residue thus obtained was suspended in 60 ml of 0.6 M-KCl in 10–15 mM-tris-HCl buffer, pH 8.0–8.2, then allowed to stand for 15 min, and spun at 5000g for 10 min; the resulting supernatant was centrifuged at 100 000g for 30 min. This process of suspension in KCl buffer and centrifugation was repeated and the final sediment was then suspended in distilled deionized water, pH 7.0, at a protein concentration of 1–2 mg/ml as checked by the method of Lowry, Rosebrough, Farr & Randall (1951). This membranous fraction was immediately assayed for enzyme activities and the rest of the suspension was divided and frozen at -20°C in separate vials.

* Abbreviation: ATPase, adenosine triphosphatase.

Assay of enzyme activity. The membranous fraction was incubated in a total volume of 1 ml containing 30 mM-tris-maleate buffer, pH 7.4, 1 mM-EDTA, 4 mM-tris-ATP and various concentrations of Ca^{2+} and/or other bivalent cations, as indicated in the text, at 37°C in a constant-temperature shaking water bath. After incubation for 5 min the reaction was started by the addition of ATP and stopped after a further 10 min by the addition of 1 ml of 12% (w/v) trichloroacetic acid; P_i present in the clear centrifuged supernatant was determined by the method of Taussky & Shorr (1953). The concentration of protein in the incubation medium was in the range 50–100 $\mu\text{g}/\text{ml}$; not more than 10% of the added ATP was hydrolysed. The reaction was linear under the conditions of the assay. The other details for the isolation of the sarcolemmal fraction and the enzyme assays are essentially similar to those published (Sulakhe *et al.* 1971; Sulakhe & Dhall, 1971a).

Isolation of fragments of sarcoplasmic reticulum and determination of Ca^{2+} accumulation. The hind-leg muscles of hamsters were immediately placed in ice-cold buffer (0.3 M-sucrose–1 mM-EDTA–10 mM-histidine buffer, pH 7.0) and freed of fat and connective tissues. The muscle was cut into small pieces with scissors, washed thoroughly and homogenized with 8 vol. of the above buffer in a Waring Blender for 30 s. The homogenate was filtered through several layers of gauze and centrifuged at 1000g for 10 min. The supernatant was again filtered through the gauze and spun at 10000g for 15 min. This residue was discarded and the supernatant was centrifuged at 40000g for 1 h to obtain the crude heavy-microsomal fraction. This sediment was then suspended in 0.6 mM-KCl–20 mM-tris-HCl buffer, pH 6.8, and kept for 30 min with occasional mixing then centrifuged at 40000g for 45 min. This fraction (10000–40000g) was suspended in 0.25 M-sucrose–10 mM-histidine buffer, pH 7.0, at a protein concentration of 2–4 mg/ml and was designated 'fragments of sarcoplasmic reticulum'. All the above procedure was done at 0–4°C. All the fraction was used within 2 h of its isolation. The abilities of this fraction to accumulate Ca^{2+} and to hydrolyse ATP were determined by the procedures described by Sulakhe & Dhall (1970, 1971b). The ATP-linked Ca^{2+} accumulation by the fragment of sarcoplasmic reticulum was not altered by the presence of 5 mM- NaN_3 .

RESULTS

Dependency on Ca^{2+} concentration. The muscle sarcolemmal fraction was incubated in a medium containing 4 mM-ATP in the absence and in the presence of various concentrations of Ca^{2+} . An increase in ATP hydrolysis in the presence of Ca^{2+} was observed (Fig. 1), considered to be caused by Ca^{2+} -stimulated ATPase present in this membranous fraction. Optimum activation was at 3–4 mM- Ca^{2+} ; higher concentrations of Ca^{2+} (10 mM) had a slight but significant ($P < 0.05$) inhibitory effect.

Influence of ATP concentration. The stimulation of ATP hydrolysis by this membranous fraction caused by Ca^{2+} was influenced by the amount of

ATP present in the incubation medium, the optimum being 3–4 mM-ATP. Lineweaver-Burk analysis of these results gave a K_m of 1.03 mM for ATP (Fig. 2). These characteristics suggest the possibility of Ca^{2+} -ATP serving as a substrate for this enzyme. Similar results with Ca^{2+} -stimulated ATPase of the dog heart sarcolemmal fraction have been reported (Sulakhe & Dhall, 1971a).

Influence of pH. The Ca^{2+} -stimulated ATPase had a sharp pH optimum at 7.6 (Fig. 3). All subsequent experiments reported in this study were therefore done at pH 7.6.

Requirement for thiol groups. The Ca^{2+} -stimulated ATPase activity present in this fraction was markedly decreased by the thiol-blocking reagent, *p*-hydroxymercuribenzoate (Table 1). This inhibition was reversed by the addition of 1 mM-cysteine. However, the addition of 1 mM-cysteine alone to the incubation medium did not influence the activity of this enzyme. *N*-Ethylmaleimide (2 mM) was also found to inhibit this enzyme.

Effect of other inhibitors. The Ca^{2+} -stimulated ATPase was inhibited by 2,4-dinitrophenol (1 mM),

Fig. 1. Effects of bivalent ions on ATP hydrolysis by skeletal-muscle membrane. The enzyme (50–100 μg) was incubated at 37°C in 30 mM-tris-maleate buffer, pH 7.4, in the presence of 1 mM-EDTA with various concentrations of Ca^{2+} (○), Mg^{2+} (●), Mn^{2+} (□) and Co^{2+} (▲). After 5 min the reaction was started by the addition of 4 mM-tris-ATP. The total volume of the assay system was 1 ml. After 10 min the reaction was stopped by the addition of 1 ml of 12% (w/v) trichloroacetic acid. Other details are as described in the Materials and Methods section.

Fig. 2. Lineweaver-Burk plot of the effect of ATP concentration on Ca^{2+} -stimulated ATPase activity of skeletal-muscle membrane. The assay system was as indicated in Fig. 1 legend except that the Ca^{2+} concentration was kept at 2.5 mM and that of ATP was varied as indicated.

Fig. 3. Influence of pH on ATP hydrolysis by skeletal-muscle membrane. Ca^{2+} -stimulated ATPase activity was assayed as described in Fig. 1 legend except that the Ca^{2+} concentration was kept at 2.5 mM and the pH of the tris-maleate buffer was varied.

Table 1. *Effect of various inhibitors on Ca^{2+} -stimulated ATPase activity of skeletal-muscle membrane*

Conditions of incubation were as described in Fig. 1 legend except that 2.5 mM- Ca^{2+} was used in the assay medium. Control activity under these conditions was 22 μmol of P_i formed/h per mg of protein.

Treatment	Activity (%)
None (control)	100
NaN_3 , 2.5 mM	90
2,4-Dinitrophenol, 1 mM	75
NaF , 2.5 mM	72
Oligomycin, 1 $\mu\text{g}/\text{min}$	70
Ethanedioxybis(ethylamine)tetra-acetate, 4 mM	15
HgCl_2 , 0.05 mM	2
<i>p</i> -Hydroxymercuribenzoate, 3.75 μM	14
<i>p</i> -Hydroxymercuribenzoate, 7.5 μM	5
<i>p</i> -Hydroxymercuribenzoate, 3.75 μM + cysteine, 1 mM	96
<i>p</i> -Hydroxymercuribenzoate, 7.5 μM + cysteine, 1 mM	95
Cysteine, 1 mM	98

sodium azide (2.5 mM), sodium fluoride (2.5 mM) and oligomycin; mercuric chloride (0.05 mM) almost completely inactivated this enzyme (Table 1). Addition of the chelating agent, ethanedioxybis-

(ethylamine)tetra-acetate, markedly decreased the Ca^{2+} -stimulated ATPase activity.

Influence of other bivalent cations. This fraction

was incubated in the presence of various bivalent cations, all of which were capable of stimulating ATP hydrolysis (Table 2). Relative to Ca^{2+} , Mg^{2+} was slightly more potent whereas Mn^{2+} , Co^{2+} , Sr^{2+} , Ba^{2+} , Zn^{2+} and Cu^{2+} were decreasingly effective in that order. The influence of various concentrations of Mg^{2+} , Mn^{2+} and Co^{2+} on ATP hydrolysis by the skeletal-muscle sarcolemma is shown in Fig. 1. The optimum concentrations for Mg^{2+} and Co^{2+} were about 4 mM and that for Mn^{2+} was 2.5 mM. However, the higher concentrations of Mn^{2+} and Co^{2+} (10 mM) inhibited the ATP hydrolysis considerably in comparison with Mg^{2+} or Ca^{2+} (see Fig. 1).

Influence of Na^+ and K^+ . When these membrane fragments were incubated in the presence of Ca^{2+} (2.5 mM), Mg^{2+} (2.5 mM) or Mn^{2+} (2.5 mM) with 100 mM- Na^+ or 10 mM- K^+ , no significant difference in the ATP hydrolysis was observed in comparison with that in the absence of these univalent cations. However, in the presence of Mg^{2+} or Mn^{2+} the addition of Na^+ (100 mM) and K^+ (10 mM) together markedly increased the ATP hydrolysis. This in-

crease is considered to be caused by a Na^+ + K^+ -stimulated ATPase in these fragments, since it was almost completely blocked by the addition of ouabain (0.4 mM). This stimulatory effect of Na^+ and K^+ together was not observed in the presence of 2.5 mM- Ca^{2+} , and there was no effect of ouabain in the presence or absence of Na^+ or K^+ . These results are shown in Table 3. The properties of Na^+ + K^+ -stimulated ATPase in the skeletal-muscle sarcolemmal fraction have been reported (Sulakhe *et al.* 1971).

Comparison of sarcolemmal fraction with sarcoplasmic reticulum. To determine the amount of contaminating fragments of sarcoplasmic reticulum in the membranous fraction employed in this study, the abilities of both sarcolemmal and sarcoplasmic-reticular fractions to transport Ca^{2+} and to split ATP in the presence of Mg^{2+} were determined. The results in Table 4 show that both sarcoplasmic-reticular and sarcolemmal fractions bound about 10 nmol of Ca^{2+} /min per mg of protein in the absence of ATP; however, only sarcoplasmic reticulum showed an increase in Ca^{2+} binding in the presence of ATP. Further, 5 mM-oxalate markedly augmented Ca^{2+} accumulation in the presence of ATP by sarcoplasmic reticulum but had no effect on sarcolemmal Ca^{2+} binding. Our observation that ATP is unable to augment Ca^{2+} accumulation by sarcolemmal fraction is in agreement with other reports (Thorpe & Seeman, 1971; Manery, Madapallimattam & Chaudry, 1971). It is noteworthy that extra ATP was split in the presence of 0.1 mM- Ca^{2+} with sarcoplasmic reticulum but not with sarcolemmal fraction (Table 4).

DISCUSSION

In the present study we have demonstrated the presence in the skeletal-muscle sarcolemmal fraction of an active ATPase complex that is stimulated

Table 2. *Relative rates of ATP hydrolysis by skeletal-muscle membrane in the presence of different bivalent ions*

Conditions of incubation were as described in Fig. 1 legend, except that 2.5 mM-bivalent ion was substituted for Ca^{2+} in the assay medium.

Ion	Activity (% of that with Ca^{2+})
Mg^{2+}	120
Ca^{2+} (control)	100
Mn^{2+}	70
Co^{2+}	65
Sr^{2+}	58
Ba^{2+}	45
Zn^{2+}	14
Cu^{2+}	12

Table 3. *Requirement for Na^+ + K^+ -stimulated ATPase activity of skeletal-muscle membrane*

The enzyme (50–100 μg) was incubated at 37°C in 30 mM-tris-maleate buffer, pH 7.4, in the presence of 1 mM-EDTA with additions as indicated. After 5 min the reaction was started by the addition of tris-ATP (4 mM). The total volume of the assay system was 1 ml. After 10 min the reaction was stopped by the addition of 1 ml of 12% (w/v) trichloroacetic acid. Other details were as described in the Materials and Methods section.

Addition(s)	ATPase activity (μmol of P_i formed/h per mg of protein)		
	With Ca^{2+} (2.5 mM)	With Mg^{2+} (2.5 mM)	With Mn^{2+} (2.5 mM)
None (control)	23.0	28.0	20.0
Na^+ , 100 mM	22.5	29.5	21.2
K^+ , 10 mM	23.5	30.5	23.0
Na^+ , 100 mM + K^+ , 10 mM	23.0	42.0	31.0
Na^+ , 100 mM + K^+ 10 mM + ouabain, 0.25 mM	22.2	29.0	21.7
Ouabain, 0.25 mM	21.9	27.5	19.5

Table 4. Ca^{2+} accumulation and ATP hydrolysis by skeletal-muscle sarcolemma and fragments of sarcoplasmic reticulum

The fractions were incubated at 37°C. For Ca^{2+} accumulation, the medium contained 100 mM-KCl, 10 mM- MgCl_2 , 20 mM-tris-HCl buffer, pH 6.8, and 0.1 mM- $^{45}\text{CaCl}_2$ in a total volume of 2 ml; the protein concentration of the sarcolemmal fraction was 300–400 $\mu\text{g}/\text{ml}$; the protein concentrations of sarcoplasmic reticulum were 300–400 $\mu\text{g}/\text{ml}$ and 20–40 $\mu\text{g}/\text{ml}$ of reaction medium in the absence and presence of oxalate respectively. For ATP hydrolysis, the reaction medium for sarcolemma is described in the Materials and Methods section whereas the reaction medium for sarcoplasmic reticulum is as described above. The values are means $\pm$ S.E.M. of the number of experiments given in parentheses.

Conditions	Ca ²⁺ accumulation (nmol/min per mg of protein)	
	Sarcolemma	Sarcoplasmic reticulum
Without ATP (6)	12 $\pm$ 2	10 $\pm$ 2
With ATP, 4 mM (6)	14 $\pm$ 3	125 $\pm$ 5
With ATP, 4 mM + oxalate, 5 mM (6)	15 $\pm$ 2	3500 $\pm$ 50
	ATP hydrolysis (μmol of P_i formed/min per mg of protein)	
	Sarcolemma	Sarcoplasmic reticulum
Mg ²⁺ , 5 mM (5)	0.45 $\pm$ 0.03	1.8 $\pm$ 0.4
Mg ²⁺ , 5 mM + Ca ²⁺ , 0.1 mM (5)	0.47 $\pm$ 0.04	3.5 $\pm$ 0.5
Extra ATP split because of Ca ²⁺ (5)	—	1.7 $\pm$ 0.3

by Ca^{2+} or Mg^{2+} . The Ca^{2+} -stimulated ATPase and Mg^{2+} -stimulated ATPase activities in this fraction cannot be attributed to the presence of myosin or myofibrillar contaminants, since extraction with lithium bromide and potassium chloride is believed to remove most of these proteins (Stam, Shelburne, Feldman and Sonnenblick, 1969). The lack of influence of K^+ on the Ca^{2+} -stimulated ATPase of this fraction also supports this view. Under a phase-contrast microscope this fraction appeared as empty sacs of membranes and was devoid of most of the myofibrillar, nuclear or mitochondrial contaminants. The presence of Ca^{2+} -stimulated ATPase in rabbit skeletal-muscle sarcolemma, obtained by a different procedure, has been reported by Ferdman, Himmelreich & Dyadyusha (1969, 1970).

As Ca^{2+} -stimulated ATPase is known to be present in sarcoplasmic reticulum (Hasselbach & Makinose, 1962; Yamamoto & Tonomura, 1967; Inesi, Maring, Murphy & McFarland, 1970), it is possible that the observed Ca^{2+} -stimulated ATPase activity of the fraction used in the present study is caused by contaminating sarcoplasmic-reticulum fragments. However, this possibility seems unlikely because, in contrast with sarcoplasmic reticulum, the fraction used in this study did not show ATP-dependent Ca^{2+} binding, ATP-dependent Ca^{2+} uptake in the presence of oxalate, or extra ATP split because of Ca^{2+} . Purified ATPase of the sarcoplasmic reticulum of skeletal muscle (MacLennan, 1970) requires both Ca^{2+} and Mg^{2+}

for ATP hydrolysis (MacLennan & Wong, 1971), whereas Ca^{2+} -stimulated ATPase of the membrane fraction used in the present study does not require Mg^{2+} for ATP hydrolysis. Further, in contrast with the present results on the insensitivity of Ca^{2+} -stimulated ATPase, Rubin & Katz (1967) have reported a marked stimulation by Na^+ and K^+ in microsomal (sarcoplasmic-reticulum) ATPase activity in the presence of Ca^{2+} .

The presence of an $\text{Na}^+ + \text{K}^+$ -stimulated Mg^{2+} -dependent ATPase in this fraction can also serve as a useful indication of its sarcolemmal origin, as this 'transport ATPase' is believed to be located mainly at the cell surface. In addition, our attempts to detect 'transport ATPase' activity in the myofibrillar, mitochondrial or microsomal fractions obtained by conventional methods have always met with a limited success (D. B. McNamara, P. V. Sulakhe & N. S. Dhalla, unpublished work). Rubin & Katz (1967) have also reported that both Na^+ and K^+ had no demonstrable effect on the basic ATPase activity of the sarcoplasmic reticulum in the presence of Mg^{2+} . Therefore we believe that the membrane fraction investigated in the present study is derived from muscle sarcolemma, and Ca^{2+} -stimulated ATPase and Mg^{2+} -stimulated ATPase are its constituents in addition to $\text{Na}^+ + \text{K}^+$ -stimulated ATPase, which is considered to participate in the active transport of Na^+ and K^+ (Sulakhe *et al.* 1971).

The presence of a Ca^{2+} -stimulated ATPase in the muscle membrane has been long suspected, and its

role in Ca^{2+} efflux has been considered by some investigators (Sonnenblick & Stam, 1969; Sulakhe & Dhalla, 1971a). Since Ca^{2+} efflux from the muscle cell occurs against the concentration gradient, it is possible that there is a 'calcium pump' in the muscle sarcolemma. Thus the demonstration of a Ca^{2+} -stimulated ATPase in hamster skeletal-muscle sarcolemma lends support to the belief that this enzyme is involved in the active efflux of Ca^{2+} across the muscle cell membrane by providing the required energy for the 'calcium pump' through the hydrolysis of ATP.

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