ISOLATION OF SKELETAL MUSCLE MEMBRANE FRAGMENTS CONTAINING ACTIVE Na^+- K^+ STIMULATED ATPase: COMPARISON OF NORMAL AND DYSTROPHIC MUSCLE SARCOLEMMA*

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SUMMARY

A method for the isolation of skeletal muscle membrane is described. These membrane fragments were found to contain a highly active Na^+- K^+ stimulated Mg^{++} dependent ATPase which is sensitive to ouabain. The Na^+- K^+ stimulated ATPase activities of the muscle membranes isolated from normal and genetically dystrophic hamsters (B10 14.6) were about 18 and 46 μmoles ATP hydrolyzed/mg protein/hr respectively.

INTRODUCTION

Na^+- K^+ stimulated ATPase, which is considered to be associated with active transport of sodium and potassium across cell membrane (1), has been reported to be present in skeletal muscle (2 - 6). Attempts to localize Na^+- K^+ stimulated ATPase in skeletal muscle have met with a limited success and its existance at the sarcolemmal membrane is not convincing (2,7). In the present communication we wish to report the isolation of a membranous fraction derived from the sarcolemma of hamster skeletal muscle. These membrane fragments are shown to contain a highly active Na^+- K^+ stimulated ATPase. In addition, the activities of this "transport ATPase" in the sarcolemmal fractions isolated from the skeletal muscles of normal and genetically dystrophic hamsters (B10 14.6) were determined. While this study was in progress, Peter (8) reported the isolation of sarcolemma from rat skeletal muscle containing Na^+- K^+ stimulated ATPase; however, the activity of the enzyme reported by this investigator is much less than that obtained by the present method.

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METHODS

The skeletal muscle (10 to 12 g) from the hind legs of 240 to 270 day old control and genetically dystrophic (B10 strain 14.6) hamsters was dissected out quickly after decapitating the animals. The muscle was placed in an ice cold buffer (0.25 M sucrose, 0.5 mM EDTA, 0.1 M Tris-HCl, pH 7.4 to 7.6), then freed from fat and connective tissues, thoroughly washed and cut into small pieces. The tissue was homogenized in a cold room with 10 volumes of the above medium in a Waring Blendor for 1 min (30 sec each time with an interval of 1 min). The homogenate was filtered through several layers of gauze, centrifuged at 1000 x g for 10 min in a Sorvall RC 2B refrigerated centrifuge (Rotor #SS-34). The sediment (R_1) was washed and extracted with 0.5 M LiBr, 0.05 mM EGTA, 16 mM Tris-HCl, pH 8.5 for 10 to 15 hours at 4°C. The jelly-like suspension was filtered and spun at 2,500 x g for 10 min, the residue (R_2) discarded and the supernatant centrifuged at 100,000 x g for 30 min in an International B-60 centrifuge (Rotor #A211). This sediment (R_3) was suspended in 30 ml of 0.6 M KCl, 10 to 15 mM Tris-HCl, pH 8.0 to 8.2, allowed to stand for 15 min at 4°C, spun at 5,000 x g for 15 min, the residue (R_4) discarded and the supernatant further centrifuged at 100,000 x g for 30 min. After repeating the above 0.6 M KCl buffer suspension and centrifugation procedure 3 times, the final sediment (R_5) was suspended in distilled deionized water (pH 7.0) at a protein concentration of 1 to 2 mg/ml (Lowry's method, 9).

The membranous fraction isolated by the above procedure was immediately assayed for enzyme activities by incubating in a total volume of 1 ml in a medium containing 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 4 mM ATP, 1 mM EDTA, 50 mM Tris-HCl, pH 7.2 at 37°C for 10 min in the absence or presence of 0.4 mM ouabain. The membrane protein concentration in the reaction was 0.02 to 0.08 mg/ml; the reaction was started by the addition of ATP and terminated by addition of 1 ml of 12% trichloracetic acid. The amount of Pi released into the medium due to the hydrolysis of ATP was estimated by method of Taussky and Shorr (10); the values corrected for the nonenzymatic hydrolysis of ATP. The difference between ATP hydrolysis in the absence and that in the presence of ouabain was taken to be due to the activity of Na^+- K^+ stimulated ATPase whereas the values obtained in the presence of ouabain were assumed to be due to Mg^+- ATPase. These values for Mg^{++}- ATPase and Na^+- K^+ stimulated ATPase agreed closely when determined in the absence or presence of 100 mM Na^+ and 20 mM K^+. The reaction was found to be linear with time and protein concentration used in this study.

The enzyme activities of the membranous fraction declined to about 50% of the values for the fresh preparation after 4 to 5 days of storage at -20°C. At each step of

the isolation procedure, the fractions were assayed for the above enzyme activities as well as examined under phase contrast microscope (Zeiss). The fraction (R_5) employed in this study consisted of empty sacs of membranes devoid of apparent myofibrillar, nuclear and mitochondrial contaminants.

RESULTS

Freshly isolated membranes from the skeletal muscle of normal hamsters were observed to hydrolyze 12 to 25 μmoles of ATP/mg protein/hr (average value 18, N = 6) due to Na^+- K^+ stimulated ATPase. This membrane fraction (R_5), which was obtained after three successive treatments with 0.6 M KCl solution, exhibited maximal Na^+- K^+ stimulated ouabain sensitive ATPase activity, whereas other fractions obtained during the isolation procedure did not show such activity. The use of LiBr in the initial extraction procedure was found more effective in yielding active membranous preparation than other salts such as NaI, KI and KCl. Furthermore, the sarcolemmal fraction isolated by various published procedures (3-5,8) invariably resulted in lesser active membranes in comparison to the present method.

The specific activities of Mg^{++} ATPase and Na^+- K^+ stimulated ATPase, as well as

Table 1. The yields and ATP hydrolyzing activities of skeletal muscle sarcolemmal fractions isolated from normal and genetically dystrophic hamsters.

Preparations	Yield mg/g muscle	ATP hydrolyzing activity (μmoles ATP hydrolyzed/mg protein/hr)		
		Total	Mg^{++} ATPase	Na^+- K^+ stimulated
A: Normal				
Prep. I	0.150	56	35	21
Prep. II	0.125	61	50	11
Prep. III	0.140	62	38	24
B: Dystrophic				
Prep. I	0.083	139	88	51
Prep. II	0.100	118	73	45
Prep. III	0.108	120	77	43

The membrane fractions (30 to 80 μg/ml) were incubated in the medium containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM Tris-ATP, 100 mM NaCl, 20 mM KCl with and without ouabain (0.4 mM) for 10 min at 37°C. Total refers to the ATP hydrolysis in the absence of ouabain and Mg^{++} ATPase in the presence of ouabain whereas Na^+- K^+ stimulated refers to the difference between these two activities.

the yields of sarcolemmal membranes from three paired preparations of normal and dystrophic muscles are shown in Table 1. The yield of sarcolemmal membranes from the normal muscle (N = 6) varied from 0.10 to 0.18 mg/g muscle weight whereas that from the dystrophic muscle (N = 6) was in the range of 0.075 to 0.12 mg/g muscle weight. On the other hand, the total ATP hydrolyzing activity of dystrophic membranes was higher than that of the control (Table 1). The Na^+- K^+ stimulated ATPase activity of the freshly prepared muscle sarcolemma from the genetically dystrophic hamsters (N = 6) varied from 40 to 55 μmoles ATP hydrolyzed/mg protein/hr.

The activities of Na^+- K^+ stimulated ATPase in the sarcolemmal membranes of the normal and dystrophic hamsters were also studied in the presence of varying concentrations of Na^+- K^+ without changing the osmolarity of the incubation medium. In both cases the maximal activity was observed when 80 mM Na^+ and 40 mM K^+ were present in the medium

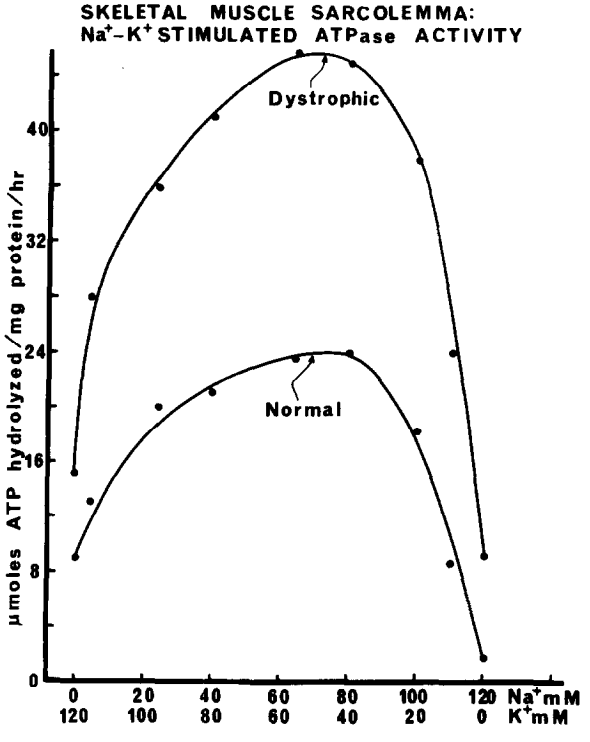

Figure 1. Na^+- K^+ stimulated ATPase activities of the skeletal muscle sarcolemma obtained from normal and genetically dystrophic hamsters in the presence of varying concentrations of Na^+ and K^+ in the incubation medium.

The membranes (40 - 70 μg/ml) were incubated in the medium containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM Tris-ATP in the presence of different concentrations of Na^+ and K^+ with and without ouabain (0.4 mM) for 10 min at 37°C. Na^+- K^+ stimulated ATPase activity was calculated by subtracting the values in the presence of ouabain from those in the absence of ouabain (N = 3).

but the enzyme activity of dystrophic membranes was higher than the normal at each Na:K ratio (Figure 1). The Na^+- K^+ stimulated ATPase activity showed pH optima at 7.2 for both normal and dystrophic membranes and the enzyme activity of dystrophic membranes was higher than the normal at every point (Figure 2). From Table 2 it can be seen that Na^+- K^+ stimulated ATPase activities of both normal and dystrophic membranes were inhibited by ouabain without any effect on Mg^{++} ATPase. On the other hand, oligomycin inhibited both Mg^{++} ATPase and Na^+- K^+ stimulated ATPase of the normal and dystrophic membranes (Table 2).

Table 2. Influence of ouabain and oligomycin on Mg^{++}-ATPase and Na^+- K^+ stimulated ATPase of the skeletal muscle sarcolemmal fractions obtained from normal and genetically dystrophic hamsters.

ATPases	Percent activity			
	Ouabain (0.4 mM)		Oligomycin (1.25 µg/ml)	
	Normal	Dystrophic	Normal	Dystrophic
Mg^{++} ATPase	102	102	35	40
Na^+- K^+ stimulated ATPase	9	7	20	30

The membrane fraction (40 - 75 µg/ml) was incubated in the medium containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 4 mM Tris-ATP for 10 min at 37°C. The activity of Mg^{++} ATPase was determined in the presence of 5 mM $MgCl_2$, whereas Na^+- K^+ stimulated ATPase activity was obtained by subtracting these values from those in the presence of 5 mM $MgCl_2$, 100 mM NaCl and 20 mM KCl. The values for Mg^{++} ATPase and Na^+- K^+ stimulated ATPase in the absence of inhibitors were taken as 100% (N = 4).

N-ethylmaleimide (0.25 mM) inhibited the Na^+- K^+ stimulated ATPase activity of both normal and dystrophic membranes by 50 to 75%, and this inhibition was prevented by the addition of cysteine (1 mM). It was also observed that the presence of cysteine in the incubation medium increased the activity of Na^+- K^+ stimulated ATPase by 20 to 40%. The requirement of SH-groups for the activity of this enzyme from skeletal muscle is in good agreement with our earlier observation with heart sarcolemma (11).

DISCUSSION

In this study we have obtained very active skeletal muscle membrane fragments in terms of their Na^+- K^+ stimulated ouabain sensitive ATPase activity. We consider that this preparation is of sarcolemmal origin since nuclear, myofibrillar and mitochondrial fractions,

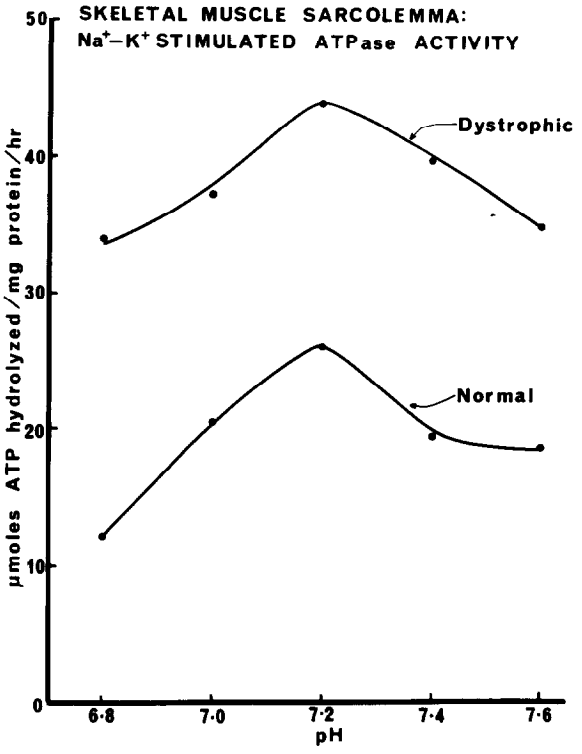

Figure 2. Na^+- K^+ stimulated ATPase activities of the skeletal muscle sarcolemma obtained from normal and genetically dystrophic hamsters at different pH of the incubation medium.

The membranes(40 - 75 µg/ml) were incubated in the medium containing 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM Tris-ATP, 100 mM NaCl, 20 mM KCl, at different pH with and without ouabain (0.4 mM) for 10 min at 37°C. Na^+- K^+ stimulated ATPase activity was calculated by subtracting the values in the presence of ouabain from those in the absence of ouabain (N = 4).

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obtained by conventional techniques, did not show Na^+- K^+ stimulated ATPase activity. The microsomal fraction (10,000 to 105,000 x g) showed very low Na^+- K^+ stimulated ATPase activity occasionally but sarcolemmal contamination in this fraction cannot be ruled out. Although some investigators have shown that Na^+- K^+ stimulated ATPase is present in the microsomal fraction (2,12), the majority of workers using various tissues, including skeletal muscle, are of the view that this "transport ATPase" is a constituent of cell membrane (13). This is further supported by recent observations with cardiac muscle by Stam et al (14) and also from this laboratory (11). The lack of effect of ouabain on the ATPase activities of myofibrillar, mitochondrial and microsomal fractions of skeletal muscle also indicates that the membrane ATPase reported in this study is derived from sarcolemma.

The Na^+- K^+ stimulated ATPase activity of skeletal muscle from different species has been reported in the literature to vary from 3 to 9 μmoles Pi released/mg protein/hr (3-5,8). On the other hand, the activity of the membrane fragments, prepared from the hamster skeletal muscle by the method described here was about 18 μmoles ATP hydrolyzed/ mg protein/hr. The sarcolemma prepared by this method from rat skeletal muscle also gave values for Na^+- K^+ stimulated ATPase activity comparable to that reported here for hamster muscle. Our values for skeletal muscle sarcolemma are even higher that those reported by us for the Na^+- K^+ stimulated ATPase activity (7.4 μmoles Pi released/mg protein/hr) in heart sarcolemma (11).

Since the presence of cysteine in the incubation medium elevated Na^+- K^+ stimulated ATPase activity by 20 to 40%, it is likely that some of the sulfhydryl groups of this enzyme could have been oxidized during this isolation procedure. A study to determine the SH-group content and requirement for the activation of this enzyme by Na^+ and K^+, ouabain sensitivity and allosteric nature of activation kinetics is in progress.

It is interesting to find that the activity of Na^+- K^+ stimulated ATPase in the skeletal muscle membranes of the genetically dystrophic hamsters (B10 14.6) was significantly higher than that of the control. The reasons for the increased specific activities of ATPases of the dystrophic muscle membrane are not clear at present. However, it is not likely to be due to different degrees of purification since both control and dystrophic membranes were prepared concomitantly under similar conditions. The lower yield of sarcolemmal fraction of dystrophic tissue in comparison to control may be due to an overall decrease in the muscle mass in the dystrophic tissue (15).

Genetically dystrophic hamsters (B10 14.6) have been considered to be an excellent model for studying the pathogenesis of muscular dystrophy (16). It may be noted that permeability changes as well as an increase in the intracellular concentration of Na^+ in dystrophic skeletal muscle have been reported (17-20). It is therefore likely that increased specific activity of "transport ATPase" in dystrophic muscle cell membrane may serve as a compensatory mechanism. But this seems to be complicated because the high energy phosphate stores in these dystrophic muscles are decreased (21). A detailed investigation concerning the relation between changes in biochemical activities and development of muscular dystrophy is in progress.

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