*Biochimica et Biophysica Acta*, 298 (1973) 239–245


BBA 76182

# A COMPARISON OF THE OUABAIN-SENSITIVE ($Na^+ + K^+$)-ATPase OF NORMAL AND DYSTROPHIC SKELETAL MUSCLE

GARTH M. BRAY

*Division of Neurology, Department of Medicine, The Montreal General Hospital and McGill University, Montreal, Quebec (Canada)*

(Received September 1st, 1972)

## SUMMARY

An NaI-extraction procedure was modified to prepare muscle fiber segments with $Mg^{2+}$-dependent, ouabain-sensitive ($Na^+ + K^+$)-ATPase activity. This enzyme was assayed in preparations of skeletal muscle from normal and dystrophic mice. The ouabain-sensitive ($Na^+ + K^+$)-ATPase activity of dystrophic muscle preparations was found to be significantly lower than that of control preparations.

## INTRODUCTION

There is indirect evidence for an abnormality of sarcolemmal plasma membranes in dystrophic muscle. Such an abnormality is suggested by observations in dystrophic muscle of increased intracellular $Na^+ : K^+$ ratios (Horvath *et al.*[1]), decreased muscle fiber resting potentials (McComas and Mrozek[2], Harris[3]), and leakage of intracellular enzymes (Zierler[4]).

Resting potentials in muscle fibers, as in other cells, are due to concentration gradients of $Na^+$ and $K^+$ across plasma membranes. These ionic gradients are, in turn, dependent on a plasma membrane ATPase which is characterized by $Mg^{2+}$-dependence, ($Na^+ + K^+$)-enhancement, and ouabain sensitivity (Albers[5]). Since this enzyme has been shown to be a biochemical "marker" of plasma membranes (Kamat and Wallach[6]), the object of the present study was to compare the ouabain-sensitive ($Na^+ + K^+$)-ATPase activity of muscle from Bar Harbour strain 129/ReJ dystrophic mice and their unaffected litter mates.

## METHODS

### *Tissue preparation*

For initial studies of normal muscle, male white mice weighing 20–30 g were used. For studies of dystrophic muscle, tissue from 2–4 dystrophic mice of Bar Harbour strain 129/ReJ (either sex, age 1–2 months) was pooled in order to obtain sufficient material for assay; for comparison, equal amounts of muscle were taken from unaffected litter mates and prepared simultaneously. Animals were killed by

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

stunning and decapitation. Skeletal muscle was dissected free of extraneous tissue, minced and suspended in 70–80 ml of Solution A (250 mM sucrose, 1 mM ethyleneglycol-bis-($\beta$-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA), and 20 mM Tris–maleate buffer, pH 7.5). This suspension was gently homogenized for 30 s in a Waring blender with reversed blades and strained through terylene mesh. Phase-contrast microscopy of portions of this suspension showed that it consisted of individual muscle fiber segments varying in length from 100 to 500 $\mu$m. These muscle fiber segments were processed by a modification of the method reported by Stam *et al.*[7]. After sedimentation by centrifugation for 5 min at $8000 \times g$ the fibers were resuspended in Solution A. This washing procedure was repeated five times. The sedimented muscle fiber segments were then suspended in 50 ml of 1 M NaI (pH 7.0) and extracted for 2 h at 4 °C by gentle tumbling with a vertical rotator. Extracted muscle fiber segments were centrifuged at $3000 \times g$ for 5 min and resuspended in Solution B (Solution A prepared using Tris buffer rather than Tris–maleate); this washing step was repeated twice. The final sediment was suspended in 1–2 ml Solution B and the protein concentration of this mixture determined by the method of Lowry *et al.*[8] after digestion for 30 min with 0.5 M NaOH.

*ATPase assays*

$Mg^{2+}$-ATPase activity of extracted muscle fiber segments was assayed immediately after preparation by incubating aliquots (0.04–0.16 mg protein) in 0.5 ml incubation medium (Solution B to which 3 mM Tris–ATP and 3 mM $MgCl_2$ had been added). $(Mg^{2+}+Na^{+}+K^{+})$-ATPase activity was determined by adding 100 mM NaCl and 10 mM KCl to the incubation medium. Ouabain sensitivity of the $(Mg^{2+}+Na^{+}+K^{+})$-ATPase activity was determined by adding 1 mM ouabain, a concentration well above that required for maximum inhibition of this ATPase (Skou[9]). Reactions were run in duplicate for 30 min at 37 °C and stopped by the addition of 0.15 ml cold trichloroacetic acid (10% w/v). Inorganic phosphate in the protein-free supernatant was measured according to the method of Dryer *et al.*[10]. Tissue and ATP blanks were included with each assay. ATPase activities were calculated as $\mu$moles $P_i$ released per mg protein per h.

Reagent grade chemicals were used; Tris–ATP and ouabain were obtained from Sigma Chemical Company, St. Louis.

To obtain higher specific $(Na^{+}+K^{+})$-ATPase activities, extracted muscle fiber segments were disrupted in a Virtis microhomogenizer (full speed, 2 min) and differentially centrifuged at $1000 \times g$ for 10 min and $8000 \times g$ for 20 min. Aliquots of the original homogenates, the $1000 \times g$ sediments and the $8000 \times g$ sediments and supernatants of each preparation were tested for ATPase activity. Specific $(Na^{+}+K^{+})$-ATPase activities were highest in the $8000 \times g$ supernatant fractions. Therefore, in studies of dystrophic muscle, where limited amounts of tissue were available, only the $8000 \times g$ supernatant fraction was assayed.

*Morphologic studies*

Wet squash mounts of muscle fiber segments at various stages of preparation were examined by phase-contrast microscopy. For electron microscopy, muscle fiber segments or various sediments and supernatants after homogenization were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4), centrifuged

into pellets at 100000 $\times g$ for 30 min, post-fixed in 2% $OsO_4$ in phosphate buffer and embedded in epoxy resin. Ultrathin sections, stained with lead citrate and uranyl acetate, were examined by electron microscopy.

For statistical analysis, Student's $t$ test was used.

## RESULTS

### *Normal muscle*

$Mg^{2+}$-ATPase and ($Mg^{2+}+Na^{+}+K^{+}$)-ATPase activities of ten separate preparations were assayed (Table I). $Mg^{2+}$-ATPase activity in 8 of the 10 preparations

TABLE I

ATPase ACTIVITY OF NaI-EXTRACTED MUSCLE FIBER SEGMENTS FROM NORMAL MICE

Ten preparations of NaI-extracted muscle fiber segments were assayed for ATPase activity in the presence of 20 mM Tris buffer, 1 mM EGTA, 3 mM Tris–ATP and 3 mM $MgCl_2$. Concentrations of NaCl, KCl, and ouabain were 100, 10, and 1 mM, respectively. Values are mean ± S.E.

| *ATPase* | *Specific activity* (*μmoles $P_i$ per mg protein per h*) |
|---|---|
| $Mg^{2+}$-ATPase | 3.42 ± 0.22 |
| ($Mg^{2+}+Na^{+}+K^{+}$)-ATPase | 4.15 ± 0.31 |
| ($Mg^{2+}+Na^{+}+K^{+}$)-ATPase *plus* ouabain | 2.80 ± 0.30* |

* Significantly lower than ($Mg^{2+}+Na^{+}+K^{+}$)-ATPase activity, $P < 0.01$.

TABLE II

ATPase ACTIVITIES OF NaI-EXTRACTED MUSCLE FIBER SEGMENTS AFTER FRAGMENTATION AND DIFFERENTIAL CENTRIFUGATION

NaI-extracted muscle fiber segments from normal mice were homogenized and differentially centrifuged. ATPase activities were measured in the presence of $Mg^{2+}+Na^{+}+K^{+}$ or $Mg^{2+}+Na^{+}+K^{+}$ *plus* 1 mM ouabain, as well as 20 mM Tris buffer, 1 mM EGTA and 3 mM Tris–ATP. Concentrations of $MgCl_2$, NaCl and KCl were 3, 100 and 10 mM, respectively.

| *Preparation* | *ATPase activity* (*μmoles $P_i$ per mg protein per h*) | | | *Ouabain inhibition* (%) |
|---|---|---|---|---|
| | *$Mg^{2+}+Na^{+}+K^{+}$* | *$Mg^{2+}+Na^{+}+K^{+}$ plus ouabain* | *Ouabain-sensitive* | |
| 1. Extracted homogenate | 5.3 | 4.5 | 0.8 | 15 |
| 1000 $\times g$ sediment | 2.6 | 2.4 | 0.2 | 7.5 |
| 8000 $\times g$ sediment | 2.3 | 1.6 | 0.7 | 30 |
| 8000 $\times g$ supernatant | 6.8 | 4.2 | 2.6 | 38 |
| 2. Extracted homogenate | 4.6 | 3.4 | 1.2 | 26 |
| 1000 $\times g$ sediment | 4.9 | 4.6 | 0.3 | 6 |
| 8000 $\times g$ sediment | 3.1 | 2.0 | 1.1 | 36 |
| 8000 $\times g$ supernatant | 3.9 | 1.7 | 2.2 | 56 |

was enhanced by 100 mM NaCl and 10 mM KCl and the mean ATPase activity rose from 3.42 to 4.15 with the addition of these monovalent cations. The $(Mg^{2+}+Na^{+}+K^{+})$-ATPase activity of all ten preparations was inhibited by 1 mM ouabain and the mean ATPase activity fell from 4.15 to 2.80. It was therefore concluded that a ouabain-sensitive $(Na^{+}+K^{+})$-ATPase was present in these preparations.

$(Na^{+}+K^{+})$-ATPase activity of NaI-extracted muscle fiber segments could be partially purified by homogenization and differential centrifugation. As shown in Table II, the ouabain-sensitive portion of the $(Mg^{2+}+Na^{+}+K^{+})$-ATPase activity was highest in the $8000\times g$ supernatant portion of these representative preparations. This ATPase activity in the $8000\times g$ supernatants was completely sedimented by centrifugation at $100000\times g$ for 30 min.

*Dystrophic muscle*

The $8000\times g$ supernatant portions of NaI-extracted muscle fiber segments from 8 paired preparations of dystrophic and non-dystrophic muscle were compared (Table III). The mean $Mg^{2+}$-ATPase activity of dystrophic preparations (8.16) was significantly higher ($P<0.001$) than the mean $Mg^{2+}$-ATPase activity of control preparations (3.16). However, the addition of $Na^{+}$ and $K^{+}$ to the incubation medium produced less enhancement of ATPase activity in dystrophic preparations than in controls; the mean $(Na^{+}+K^{+})$-dependent ATPase activity of dystrophic preparations (0.45) was significantly less ($P<0.001$) than the mean $(Na^{+}+K^{+})$-dependent ATPase activity of non-dystrophic preparations (1.54). Similarily, the $(Mg^{2+}+Na^{+}+K^{+})$-ATPase activity of dystrophic preparations showed less ouabain sensitivity than the control preparations; the mean ouabain-sensitive ATPase activity of dystrophic preparations (1.23) was significantly less ($P<0.05$) than the mean ouabain-sensitive ATPase activity of control preparations (1.94). In addition, ouabain inhibition

TABLE III

ATPase ACTIVITIES OF NaI-EXTRACTED MUSCLE FIBER SEGMENTS FROM DYSTROPHIC AND NON-DYSTROPHIC MICE

ATPase activities were assayed for eight paired preparations of the $8000\times g$ supernatant portions of NaI-extracted muscle fiber segments from dystrophic and non-dystrophic mice of Bar Harbour strain 129/ReJ, as outlined in the text. ATPase activities in $\mu$moles $P_i$ per mg protein per h. Ouabain inhibition in percents. Values are the means ± S.E. of 8 determinations.

| | *Non-dystrophic* | *Dystrophic* | *Statistical significance (P)* |
|---|---|---|---|
| *ATPase activities* | | | |
| $Mg^{2+}$ | 3.16±0.28 | 8.16±1.10 | <0.001 |
| $Mg^{2+}+Na^{+}+K^{+}$ | 4.70±0.28 | 8.61±1.14 | <0.01 |
| $(Na^{+}+K^{+})$-enhanced | 1.54±0.13 | 0.45±0.21 | <0.001 |
| $Mg^{2+}+Na^{+}+K^{+}$ *plus* ouabain | 2.89±0.33 | 7.39±0.99 | <0.01 |
| Ouabain-sensitive | 1.94±0.14 | 1.23±0.24 | <0.05 |
| Ouabain inhibition | 43.0±4.3 | 14.5±2.2 | <0.001 |

expressed as a percentage of the $(Mg^{2+}+Na^{+}+K^{+})$-ATPase activity was significantly less for dystrophic than control preparations ($P<0.001$); the mean ouabain inhibition of dystrophic preparations was 14.5% while that of control preparations was 43%.

The mean yield of membrane protein from non-dystrophic muscle was 0.83 mg/g wet muscle while the mean yield from dystrophic muscle was 0.56 mg protein/g wet muscle. The lower yield for dystrophic muscle may be more apparent than real, however. Since there are increased amounts of fat and connective tissue evident histologically in dystrophic muscle, wet muscle weights do not reflect the actual amounts of muscle tissue and are therefore unsatisfactory reference bases. In the present study, gross extraneous tissue was removed by dissection and straining through mesh. Furthermore, the protein reference base used in the comparison of normal and dystrophic muscle was found to be similar to the "non-collagenous protein" of Lilienthal *et al.*[11]. Thus, the lower yields of membrane protein from dystrophic muscle were undoubtedly due to the decreased proportion of actual muscle fibers within a given weight of whole muscle and were unlikely to be responsible for the differences in ATPase activity observed.

Electron microscopic appearances of $100000\times g$ pellets of the $8000\times g$ supernatant preparation were similar for dystrophic and non-dystrophic muscle (Fig. 1). Both consisted of membranous vesicles varying in size from 35 to 200 nm and were free of identifiable mitochondria, myofilaments and other cellular components. It is presumed that these vesicles were derived from the sarcolemmal plasma membranes and their invaginations — the transverse tubules, as well as the sarcoplasmic reticulum.

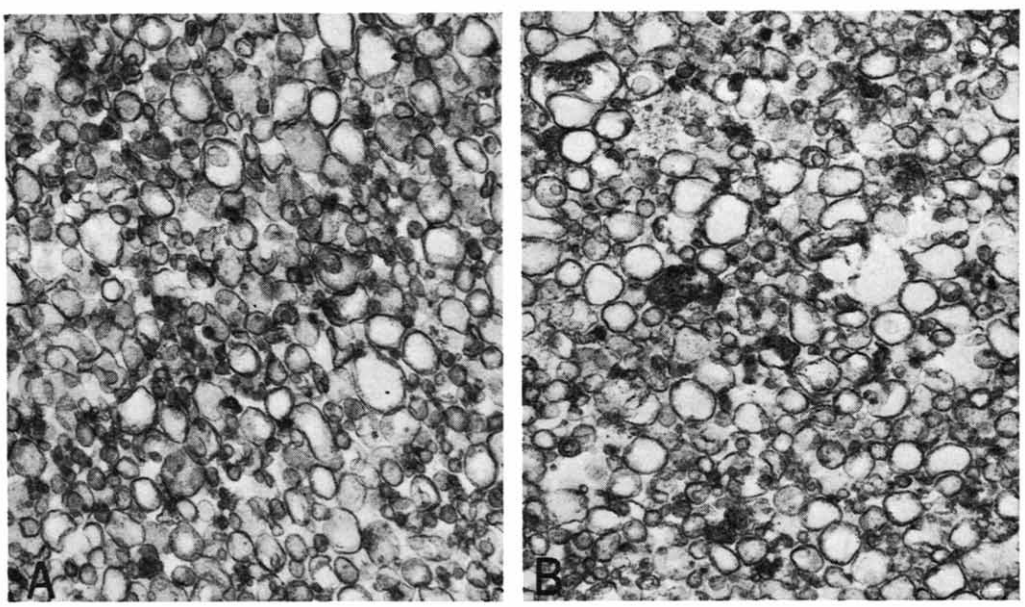


Fig. 1. Membranous vesicles from normal (A) and dystrophic (B) muscle fiber segments extracted with NaI (electron micrographs, ×24 125).

## DISCUSSION

A NaI-extraction method for preparing sarcolemmal membranes from cardiac muscle (Stam *et al.*[7]) was modified so that plasma membranes from dystrophic and non-dystrophic skeletal muscle could be compared. By reducing the NaI-extraction time to 2 h, it was possible to prepare cylindrical segments of sarcolemmal basement membranes which enclosed numerous membranous vesicles. These vesicles were characterized by ATPase activity which was similar to that of plasma membrane from other cells; that is, it was magnesium dependent, $(Na^+ + K^+)$-enhanced and ouabain-sensitive. Although it was not possible to identify the precise origin of the membranous vesicles with which this ATPase activity was associated, it is presumed that at least some were derived from plasma membranes or their invaginations, the transverse tubules, since ATPase activity of the sarcoplasmic reticulum[12], mitochondria[13] or myofilaments[14] are neither $(Na^+ + K^+)$-enhanced nor ouabain-inhibited. The ouabain-sensitive $(Na^+ + K^+)$-ATPase activity observed in microsomal fractions of deoxycholate-treated muscle homogenates (Samaha and Gergely[15], Rogus *et al.*[16]) could also have been due to the presence of plasma membrane fragments (Kamat and Wallach[6]).

ATPase activities of NaI-extracted muscle fiber segments differed for dystrophic and non-dystrophic mice. Preparations of dystrophic muscle showed significantly higher specific $Mg^{2+}$-ATPase activities but lower ouabain-sensitive $(Na^+ + K^+)$-ATPase activities. Elevations of $Mg^{2+}$-ATPase activity have previously been reported for mitochondria[17] and microsomes[18,19] derived from dystrophic mouse muscle. Although diminished ouabain-sensitive $(Na^+ + K^+)$-ATPase activity has not been previously reported for dystrophic mouse muscle, this finding correlates with observations of decreased $K^+$ and increased $Na^+$ concentrations in dystrophic muscle[1], as well as the electrophysiologic evidence of abnormally low membrane potentials in dystrophic muscle[2,3], both of which are dependent on the sarcolemmal ATPase.

The present findings in dystrophic mouse muscle are in agreement with those of Brown *et al.*[20], who reported a lack of ouabain sensitivity in the $(Mg^{2+} + Na^+ + K^+)$-ATPase activity of microsomal preparations from muscle of dystrophic ducks. On the other hand, sarcolemmal membranes prepared from dystrophic hamster muscle by LiBr extraction showed elevated $(Na^+ + K^+)$-ATPase activity[21]. Such divergent results could be due to different methods of preparation or to differences of the dystrophic process in various animal species.

## ACKNOWLEDGEMENTS

The author acknowledges with gratitude the encouragement and advice of Dr Ellen R. Gordon, assistance of Dr Lorna Medd, S. Harrington, A. Liberman and W. Downey and financial support from the Muscular Dystrophy Association of Canada.

## REFERENCES

1 Horvath, B., Berg, L., Cummings, D.J. and Shy, G. M. (1955) *J. Appl. Physiol.* 8, 22
2 McComas, A. J. and Mrozek, K. (1967) *J. Neurol. Neurosurg. Psychiat.* 30, 526
3 Harris, J. B. (1971) *J. Neurol. Sci.* 11, 45

4 Zierler, K. L. (1958) *Bull. Johns Hopkins Hosp.* 102, 17
5 Albers, R. W. (1967) *Annu. Rev. Biochem.* 36, 727
6 Kamat, V. B. and Wallach, D. F. H. (1965) *Science* 148, 1343
7 Stam Jr, A. C., Shelburne, J. W., Feldman, D. and Sonnenblick, E. H. (1969) *Biochim.Biophys. Acta* 189, 304
8 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265
9 Skou, J. C. (1962) *Biochim. Biophys. Acta* 58, 314
10 Dryer, R. L., Tammes, A. R. and Routh, J. I. (1957) *J. Biol. Chem.* 225, 177
11 Lilienthal, Jr., J. L., Zierler, K. L., Folk, B. P., Buka, R. and Riley, M. J. (1950) *J. Biol. Chem.* 182, 501
12 MacLennan, D. H. (1970) *J. Biol. Chem.* 245, 4508
13 Peter, J. B., (1970) *Biochem. Biophys. Res. Commun.* 40, 1362
14 Kielley, W. W. (1967) in *The Enzymes*, (Boyer, P. B., Lardy, H. and Myrbäck, K., eds), p. 149, Academic Press, New York
15 Samaha, F. J. and Gergely, J. (1965) *Arch. Biochem. Biophys.* 109, 76
16 Rogus, E., Price, T. and Zierler, K. L. (1969) *J. Gen. Physiol.* 54, 188
17 Pennington, R. J. (1961) *Biochem. J.* 80, 649
18 Martonosi, A. (1968) *Proc. Soc. Exp. Biol. Med.* 127, 824
19 Sreter, F. A. Ikemoto, N. and Gergely, J. (1967) in *Exploratory Concepts in Muscular Dystrophy and Related Disorders* (Milhorat, A., ed.), p. 289, Excerpta Medica Foundation, Amsterdam
20 Brown, H. D., Chattopadhyay, S. K., Patel, A. B. and Rigdon, R. H. (1967) *Experientia* 23, 522.
21 Sulakhe, P. V., Fedelesova, M., McNamara, D. B. and Dhalla, N. S. (1971) *Biochem. Biophys. Res. Commun.* 42, 793