Ca^{++}-ATPase ACTIVITY AND LIPID COMPOSITION OF SARCOPLASMIC RETICULUM OF THE GASTROCNEMIUS MUSCLE OF DENERVATED FROG

R.V. KRISHNAMOORTHY

Department of Zoology,
University of Agricultural Sciences, Hebbal, Bangalore-560024

Summary: Abnormalities have been noticed in the phospholipid and cholesterol composition of the atrophied gastrocnemius muscle of frog denervated for 1 month. Cholesterol : phospholipid molar ratios in the muscle increased on denervation. Sphingomyelin and cardiolipin fractions increased in contrast to phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine in the sarcoplasmic reticulum (SR) of denervated muscle. Na-azide sensitive Ca^{2+} ATPase activity of the mitochondria did not alter whereas that of SR decreased on denervation. Phospholipase C digestion impaired the organelle Ca^{++}-ATPase activity. The above abnormalities in enzyme activities have been correlated to the changes in the lipid composition of the denervated muscle. On the basis of these changes it is discussed that the primary change in the muscle due to denervation is the change in the permeability of the membrane.

Key words: denervation atrophy, frog-gastrocnemius muscle, cholesterol, phospholipid molar ratios, sphingomyelin and cardiolipin-organelle, Ca^{2+}ATPase activity, mitochondrial azide-sensitive Ca^{2+}ATPase

INTRODUCTION

The phenomena of muscular dystrophy and denervation - atrophy show autosomal impairment to varying degree, but in both, the mitochondria are functional (8,9,30). What happens to the sarcoplasmic reticulum (SR) of the muscle during these phenomena is not clearly known. The denervation-atrophy results in changes in mechanical and electrical activities of the muscle (5, 9). In dystrophic muscles the SR is shown to undergo degenerative changes with reference to Ca^{2+} uptake and phospholipid composition (26).

In anticipation that such changes in SR may also prevail and be related to the quiescence of the denervated-atrophied muscle, similar studies were initiated in frogs, denervated for 1 month.

MATERIALS AND METHODS

Denervation in the common South Indian frog *Rana hexadactyla* was done as described by Krishnamoorthy and Das (12) by surgical deprivation of about 1 cm length of sciatic nerve root for 1 month. Only one leg was denervated while the contralateral innervated (non-operated) leg served as control. Male frogs in a size range 60-65 g were chosen for experiments.

Subcellular fractions were prepared from the bulked gastrocnemii by the differential centrifugation procedure of Martonosi and Feretos (19). The Grana I fraction obtained by centrifuging at 35000 g was washed with 0.6 M KCl, pH 7.0, to extract contaminating actomyosin. Protein was determined (16) following solubilization of the particulate samples with 0.3 *ml* 1% sodium dioxycholate, pH 8.00.

Total lipid extracts were obtained from subcellular suspensions by homogenization in 19 Vol. ice-cold chloroform-methanol (2:1, v/v) containing butylated hydroxy toluene (5 *mg*/100 *ml*). After heating at 50°C for 5 min., the suspensions were cooled to room temperature, washed (7), dried under reduced pressure and stored in chloroform-methanol (2:1, v/v).

Thin layer chromatography of phospholipids was performed on glass plates (20 cm x 20 cm) coated with 0.25 mm layers of silica gel G (E. Merck, AG, Darmstadt, Germany) which had been activated at 110°C for 1 hr. 80 μl aliquots of lipid extract was applied to the plates. Ascending development in unlinked tanks was carried out in chloroform-methanol-aqueous 30% methylamine (65:25:4, v/v/v) solvent (13). Neutral lipid run faster than phospholipid in this system. The individual phospholipids were detected by spraying with 2,7-dichlorofluorescein. Five clearly definable spots containing phosphorus were observed under ultraviolet light. These were identified (15) by comparison with standard phospholipids (Applied Science Laboratories, USA).

The spots were scraped from the plates and the phospholipids were eluted through glass chromatography columns (18 x 1.3 cm) with chloroform, methanol (1:1, v/v) containing 10% water (29). The lipid content of each spot was then estimated following evaporation (2). The recovery of total lipid P using these column procedures was 94.4 ± 1.6% (mean ± S.E.). Phospholipid content was expressed in μg atoms of P per gram protein. The sum of P from all the spots denoted the total phospholipid content of the subcellular fraction.

The cholesterol in the fractions was determined by two dimensional thin-layer chromatography in the presence of $HgCl_2$ (23).

The specific activities of Ca^{2+} stimulated ATPase (18), cytochrome SDH oxidase (22), and Ouabain-sensitive (Na^+qK^+)-stimulated ATPase (26) were used as markers to characterize the mitochondrial and sarcoplasmic reticular preparations immediately following their isolation. ($Na^+ + K^+$)-ATPase was determined (26) by incubation for 20 min. at 30°C in a medium of 120 mM NaCl, 5 mM $MgCl_2$, 4 mM Tris-ATP and 50 mM Tris-HCl (pH 7.4). The results were expressed as the difference in ATPase activities in the presence or absence of 10^{-4}M Ouabain. The reaction was initiated by the addition of the subcellular suspension.

Phospholipase C (3:1:4:3) digestion (17) of the organelle lipids was carried out at 37°C by incubating 0.1 *mg* 1 *ml* enzyme in 1% albumin with a mixture of solution containing 2 *ml* 0.1 M Tris-maleate, pH 7.3 buffer, 0.5 *ml* 0.05M, 0.05M $CaCl_2$, 0.9 *ml* of 1% albumin (Bovine serum albumin: Biochemicals Unit, V.P. Chest Institute, Delhi) and 1.5 *ml* of organelle suspension. After incubation for 30 minutes, the Ca^{2+} ATPase activity in the mixture was estimated as described according to MacLennan (18).

RESULTS

The results of the SDH of the organelles (Table I) provided biochemical evidence that the homogenization had reduced the apparent mitochondrial contamination of sarcoplasmic reticulum. The SR is chosen as the most appropriate organelle for examination in relation to membrane function (26). Using membrane bound enzymes as markers of cross-contamination both normal and denervated muscle SR were noted to contain about 1.5% mitochondrial contamination (Table I).

TABLE I: Specific activities of organelle enzymes from the normal and denervated gastrocnemii of frog. Mean ± S.D. of enzyme units.

Enzyme assay	*No. of observations*	*Sarcoplasmic matter*		*Mitochondria*	
		Normal	*Denervated*	*Normal*	*Denervated*
1. SDH	8	52 ± 8	61±11	3651 ± 126	3462 ± 205
% Contamination		1.4	1.7	100	100
2. Cytochrome coxidase	8	64 ± 9	67 ± 16	4281 ± 182	4196 ± 216
% Contamination		1.5	1.6	100	100

Another possible contamination is the proteins due to disruption of sarcolemma (1). This was tested by the activity of the sarcolemmar marker enzyme, the Ouabain-sensitive ($Na^+ + K^+$) ATPase. In both organelle fractions, the Ouabain-sensitive AT Pase activity was very low (Table II), hence the sarcolemmar contamination is minimum in the isolation process of the organelles.

Table III demonstrates sharp differences between the cholesterol content of SR and mitochondria; in which the SR shows a higher level. Both preparations from the denervated muscle contained significantly ($p<0.001$) higher cholesterol level. Total phospholipid content of the

denervated SR was significantly ($p<0.001$) lower and the content of mitochondria was the same. The C:P molar ratio of the denervated organelles were substantially above normal ($p<0.001$). However, C:P molar ratio of the denervated SR and mitochondria was elevated significantly Table III).

TABLE II: Changes in the ATPase activity of sarcoplasmic reticulum and mitochondria from the normal and denervated gastrocnemius muslces of frog. Mean ± S.D. of 8 observations.

Fraction	*μmoles Pi ± S.D./mg protein/min at 30°C*			
	Ca^{2+} ATPase		*$Na^+ + K^+$ ATPase*	
	Normal	*Denervated*	*Normal*	*Denervated*
1. Mitochondria	2.35 ± 0.18	2.41 ± 0.21	0.07 ± 0.01	0.08 ± 0.01
2. Sarcoplasmic reticulum	3.14 ± 0.11	1.18 ± 0.12	0.36 ± 0.015	0.06 ± 0.02

TABLE III: Phospholipid and cholesterol content of sarcoplasmic reticulum and mitochondria from the normal and denervated gastrocnemius muscles of frog. Mean±S.D. of 8 observatons.

Fraction	*Sarcoplasmic reticulum*		*Mitochondria*	
	Normal	*Denervated*	*Normal*	*Denervated*
1. Phospholipid μatom P/100 *mg* protein	126 ± 14	88 ± 16	48 ± 12	43 ± 15
2. Cholesterol μmoles/100 *mg* protein	14 ± 3.1	21 ± 2.6	5.2 ± 1.2	9.8 ± 0.8

The elevated molar ratios may be related to the impairment of Ca^{2+} binding since the Ca^{2+} binding to mixed monolayers of cholesterol and phosphatidyl choline is dependent upon the molar ratio (31). The total phospholipid content was significantly depressed in the SR, but the same showed small differences in the mitochondria of the denervated muscle. Denervation did not alter the qualitative composition of both organelles (Table IV). Five phospholipid fractions were noticed in both organelles. In mitochondria sphingomyelin and cardiolipin formed the major fraction, the quantitative composition remained unaltered on denervation. In SR, the phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine levels decreased significantly on denervation in contrast to the levels of sphingomyelin and cardiolipin (Table IV). Reduced levels of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine on denervation may therefore be related to the elevated C:P molar ratio as well as the impairment of Ca^{2+} binding of SR.

Further, Fanburg *et al.* (6) related the significance of elevated C:P molar ratio to the impairment of Ca^{2+} ATPase of the SR. Surprisingly, the Na-azide sensitive Ca^{2+} ATPase activity

of the SR decreased on denervation whereas that of mitochondria did not alter (Table II). These results confirm the impairment of Ca^{2+} ATPase of the SR during denervation.

TABLE IV: Phospholipid composition of normal and denervated muscle. Mean ± S.D. of 7 observations.

Lipid	*μg atoms P/g protein*			
	Sarcoplasmic reticulum		*Mitochondria*	
	Normal	*Denervated*	*Normal*	*Denervated*
1. Phosphatidyl choline	861 ± 16	436 ± 22	9 ± 3	8 ± 2
2. Phosphatidyl ethanolamine	262 ± 29	93 ± 11	126 ± 14	131 ± 19
3. Phosphatidyl serine	11 ± 3	4 ± 2	6 ± 1	8 ± 2
4. Sphingomyelin	82 ± 19	176 ± 29	91 ± 15	93 ± 12
5. Cardiolipin	42 ± 14	74 ± 12	167 ± 29	168 ± 24

TABLE V: Effect of phospholipase C digesion and on the Ca^{2+} ATPase activity of muscle organelles. Mean±S.D. of 4 observations.

Fraction	*μmoles Pi/mg protein/min*		
	Undigested control extract	*Digested extract*	*Incidence of change on phospholipase digesion*
1. Mitochondria of normal muscle	2.35 ± 0.18	2.28 ± 0.21	No change
2. Sarcoplasmic fraction of normal muscle	3.14 ± 0.11	0.61 ± 0.11	Decrease
3. Mitochondria of denervated muscle	2.41 ± 0.21	2.31 ± 0.26	No change
4. Sarcoplasmic fraction of denervated muscle	1.18 ± 0.12	0.42 ± 0.14	Decrease

Phospholipase C digestion is known to impair the organelle Ca^{2+} ATPase activity (20). A survey of the effects of phospholipase C digestion on the mitochondrial and SR fractions in the present study (Table V) revealed that on digestion the SR Ca^{2+} ATPase decreased significantly. The decrease was more pronounced (about 50%) in the denervated SR. The mitochondrial enzyme was not affected by the lipase digestion. These observations indicate that the SR-Ca^{2+} ATPase seem to be a lipid dependent enzyme unlike that of mitochondria. Probably decreased phospholipid of the denervated SR could be responsible for lowered Ca^{2+} ATPase activity of the SR.

DISCUSSION

Increase in C:P molar ratios has been recorded in human skeletal muscle dystrophy (14), murine muscle dystrophy (11) and in ventricular hypertrophy of cardiomyopathic hamster (25, 26). In the hereditary murine and chicken dystrophies also a similar situation was noted (10,24). The increase in ratio in denervation atrophy as in the present study means interesting since both the disorders viz., dystrophy and denervation-atrophy show similar abnormalities of phospholipid and cholesterol levels of the SR although the patterns of fat metabolism are dissimilar (33).

The changes in the levels of phospholipid and cholesterol of the SR denervated muscle seem to have influence on the lipid dependent enzyme functions and ion-transport activities of the membrane. The activities of metal ion-ATPase are activated by membrane phospholipids (21) and the cholesterol level influences the ion-transport activities of the membrane (4). In the present study, it is demonstrated (Table II) that Ca^{2+} ATPase activity of the SR is impaired due to denervation. The impairment is a decrease in phospholipid content with a concomitant decrease in Ca^{2+} ATPase activity of the SR.

The differences in lipid composition of SR included the relatively high content of phosphatidyl choline and cholesterol (Tables III and IV). The mitochondria were characterized by a high cardiolipin and low cholesterol. Phosphatidyl serine, a lipid known to be particularly prevalent in sarcolemma (26) was found in very small quantities in both SR and mitochondria. These results corroborate the earlier findings (26) in cardiac myopathy of the hamster.

The SR of *Longissimus dorsi* muscle from the pigs (34) showed five phospholipid fractions, like the frog muscle of present study. These fractions are common to the mitochondria also. The hamster cardiac muscle showed eight fractions (26). Only a few of these phospholipid fractions increased due to denervation. For example, the sphingomyelin and cardiolipin increased in contrast to phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine (Table IV).

The decrease in phosphatidyl choline, together with an increase in spingomyelin content observed here, was also found in the SR from dystrophic skeletal muscle of the mouse (24), the chicken (10) and also in human muscular Duchenne dystrophy (14).

The noteworthy observation is that although the C:P ratios are elevated, the mitochondrial phospholipid content was not altered due to denervation (Table III) and the elevated ratio was only due to the increase in cholesterol content. Consequently, the Ca^{2+} ATPase activity decreased only in SR but not in mitochondria due to denervation. It is evident from the results (Table V) that phospholipase digestion impaired the Ca^{2+} ATPase of SR alone. It is possible

that Ca^{2+} ATPase of mitochondria are different from those of SR, the latter are characterized by the activation of phospholipids.

All these findings illustrate that the SR of denervated muscle showed the degenerative characteristics which involve loss of membrane phospholipids and ion-dependent enzyme activities. There is evidence (9) that the denervation affects the contractile properties of the muscle which involve the SR of the muscle. Although the mitochondria of the denervated muscle are functional (32, 33), the high cholesterol content of them impair the ion-binding and ion transport activity.

Buller (3) suggests that there is a trophic substance secreted by nerve which regulates the metabolism of the muscle (8,9) and which converts newborn red to adult white or mixed muscle (3).

There is unifying evidence (28) to show that the motoneuron regulates the form and function of the muscle. Conceivably, the denervated (atrophied) muscle of the present study, showed abnormalities in SR because trophic material from the motoneuron is unavailable.

A reduced level of Ca^{2+} binding has been demonstrated in human (27) and chicken dystrophies (10), which in turn is dependent on the lipid composition of the muscle.

In the light of reproduced abnormalities here, it could be speculated that the lipid components of the denervated muscle result in a partial loss of Ca^{2+} binding also.

REFERENCES

1. Azzone, G.F. ATP-ase reactions in muscle cells. In Proc. 5th Internat. Congress of Biochemistry. Vol. II : Functional Biochemistry of Cell Structures. Ed by O. Lindberg, Pergamon Press NY, pp 271-285, 1963.
2. Barlett, G.R. Phosphorus assay in column chromatography. *J. biol. Chem.*, **234** : 466-471, 1959.
3. Buller, A.J. Functional differences between mammalian fast twitch and slow twitch skeletal muscle fibers. Research in Muscular Dystrophy. Proc. 3rd Symposium. Pitman Medical Publ. Co. London, 1965.
4. De Gier, J., J.G. Mandersloot and L.L.M. Van Deenan. Lipid composition and permeability of liposomes. *Biochim. Biophys. Acta*, **150** : 666-675, 1968.
5. Eberstein, A. and A. Sandow. Fatigue mechanisms in muscle fibres. pp 516-526 Nakladatelstvi Ceskoslovenghe akademie ved Praha, 1963.
6. Fenburg, B., R.M. Finkel and A. Martonosi. The role of calcium in the mechanism of relation of cardaic muscle. *J. biol. Chem.*, **239** : 2298-2306, 1964.
7. Folch, J., M. Lees and G.H. Sloane-Stanley. A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.*, **226** : 497-509, 1957.
8. Guth, L. 'Trophic' influences of nerve on muscle. *Physiol. Revs.*, **8** : 645-687, 1968.
9. Gutmann, E. The trophic function of the nerve cell. *Scientia*, **104** : 1-20, 1969.
10. Hsu, Q.S. and G. Kaldor. Studies on the lipid composition of the fragmented sarcoplasmic reticulum of normal and dystropic chickens. *Proc. Soc. Exp. Biol. Med.*, **138** : 733-737, 1971.
11. Hughes, B.P. Research in Muscular Dystrophy. Proc. 3rd Symposium pp 187-194. Pitman Medical Publ. Co. Ltd., London, 1965.

12. Krishnamoorthy, R.V. and A.B. Das. Biochemical effects of denervation in skeletal muscle. Part I. Changes in protein synthesis and accumulation in frog sartorius muscle. *Indian J. Expt. Biol.*, **6** : 221-223, 1968.

13. Kuhn, N.J. and F. Lynen. Phosphatidic acid synthesis in yeast. *Biochem. J.*, **94** : 240-246, 1965.

14. Kunze, D., D. Olthoff and K. Schellnack. Veranderangen des Lipidgehaltes der Skelettmuskulatur bei progressivev Muskeldystrophie als Bases einer Hypothese uber den primaren Enzymdefekt. *Acta Biol. Med. Ger.*, **21** : 669-678, 1968.

15. Levine, C. and E. Chargaff. Procedures for the microestimation of mitogenous phosphatide constituents. *J. biol. Chem.*, **192** : 465-479, 1951.

16. Lowry, O.H., N.J. Roseborough, A.L. Farr and R.J. Randall. Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193** : 265-275, 1951.

17. MacFarlane, M.G. and B.C.J.G. Knight. The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchi* toxins. *Biochem. J.*, **35** : 884-902, 1941.

18. MacLennan, D.H. Purification and properties of an adenosine triphosphatase from Sarcoplasmic reticulum. *J. biol. Chem.*, **245** : 4508, 1970.

19. Martonosi, M.A. and F.R. Feretos. Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. *J. biol. Chem.*, **239** : 648-658, 1964.

20. Martonosi, A., J. Donley and R.A. Halpin. Sarcoplasmic reticulum III. The role of phospholipids in the adenosine triphosphatase activity and Ca^{++} transport. *J. biol. Chem.*, **243** : 61-70, 1968.

21. Meissner, G. and S. Fleischer. The role of phospholipid in Ca^{2+} stimulated ATPase activity of Sarcoplasmic reticulum. *Biochim. et. Biophys. Acta*, **255** : 19-23, 1972.

22. Oliviera, M.M., W.B. Weglicki, A. Nason and P.P. Nair. Distribution of α-tocopheron in beef heart mitochondria. *Biochemica. et. Biophysica. Acta*, **180** : 98-113, 1969.

23. Owens, K. A two-dimensional thin-layer chromatographic procedure for the estimation of plasmologens. *Biochem. J.*, **100** : 354-361, 1966.

24. Owens, K. and B.P. Hughes. Lipids of dystrophic and normal mouse muscle : Whole tissue and particulate fractions. *J. Lipid. Res.*, **11** : 486-495, 1970.

25. Owens, K., W.B. Weglicki, E.H. Sonnenblick and E.W. Gertz. Phospholipid and cholesterol content of ventricular tissue from the cardiomyopathic Syrian hamster. *J. Mol. Cell. Cardiol.*, **4** : 229-236, 1972.

26. Owens, K., W.B. Weglicki, R.C. Ruth, A.C. Stam and E.H. Sonnenblick. Lipid composition, Ca^{2+} uptake and Ca^{2+} stimulated ATPase activity of sarcoplasmic reticulum of the cardiomyopathic hamster. *Biochem. et. Biophys. Acta*, (L) **296** : 71-78, 1973.

27. Pearson, C.M. and R.F. Coleman. The basis of muscle dystrophies and certain other myopathies. In the Biological Basis of Medicine. Vol. 3. Ed. by E. E. Bittar and N. Bittar. Academic Press London pp 311-343, 1969.

28. Ramirez, B. and J.V. Luco. Some physiological and biochemical features of striated muscle reinnervated by preganglionic sympathetic fibres. *J. Neurobiology*, **4** : 525-533, 1973.

29. Rouser, G., G. Kritechevsky and A. Yamamoto. Lipid chromatographic analysis. Vol I p 154. Marcel Dekker, New York, 1967.

30. Shafiq, S.A., V. Askanas, S.A. Asiedu and A.T. Milhorat. Structural changes in human and chicken muscular dystrophy. In Muscle Biology Vol. I (ed : R.G. Cassens), pp 255-272, 1972.

31. Shah, D.Q. and J.H. Schulman. The ionic structure of lecithin monolayers. *J. Lipid Res.*, **8** : 227-233, 1967.

32. Singarachari, A. Lipid metabolism in relation to muscle function of the denervated muscle of a selected vertebrate. Doctoral dissertation, Bangalore University, 1973.

33. Singarachari, A. and R.V. Krishnamoorthy. Fatty acid metabolism in denervated muscle of frog *in vitro*. *Life Sciences*, **13** : 1597-1605, 1973.

34. Wood, J.D. and D. Lister. The fatty acid and phospholipid composition of Longissimus dorsi muscle from Pietrain and large white pigs. *J. Sc. Fd. Agric.* **24** : 1449-1456, 1973.