The pH Stability of Myofibrillar Adenosine Triphosphatase of Five Fiber Types in Skeletal Muscles of the Sheep and Cattle

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(Received August 22, 1975)

Abstract The pH stability of the myofibrillar adenosine triphosphatase (ATPase) of muscle fiber types in four different skeletal muscles of the sheep and cattle was histochemically investigated. By incubating frozen sections in acid or alkaline solution before reaction for ATPase, it was found that the myofibrillar ATPase of the type A and B fibers was acid-labile and alkali-stable, whereas the ATPase of the type C, D, and E fibers was acid-stable and alkali-labile in both species. The ATPase activity in the D fibers was more strongly inhibited by preincubation at pH 4.0 than that in the C and E fibers. Few A fibers were acid- and alkali-stable. Such A fibers were found in a few muscles from some animals, but not in any muscles from other animals. With the ATPase reaction after pH 4.3 preincubation at room temperatures (16–18°C), slight differences in pH sensitivity between the sheep and cattle were noted in the B fibers of a certain kind of muscles. The temperature during acid and alkaline preincubation was shown to influence the inhibition of the ATPase activity.

The ATPase reaction demonstrated by the method of PADYKULA and HERMAN¹⁾ is localized in the myofibrils and believed to be due to myosin ATPase in skeletal muscle fibers. With this reaction, the muscle fibers can be distinguished into two kinds: strongly reacting fibers and weakly reacting fibers.^{2,3)} Subsequently, the intensity of reaction in two kinds of fibers was shown to be reversed by immersing cryostat sections in acid solution before incubation for ATPase reaction.^{4,5)} This has been interpreted as indicating that the myofibrillar ATPases in muscle fibers have different pH stability.

Skeletal muscle fibers of the sheep⁶⁻⁸⁾ and cattle⁹⁾ were histochemically classified into five fiber types. The type A and B fibers were strong in the routine ATPase activity demonstrated by the method of PADYKULA and HERMAN,¹⁾ the type C and D fibers weak, and the type E fibers moderate. On the basis of the results of GUTH and SAMAHA⁴⁾ and of BROOKE and KAISER,⁵⁾ the myofibrillar ATPase of the A and B fibers is assumed to be acid-labile and alkali-stable, and the ATPase of the C and D fibers is acid-stable and alkali-labile, but it is difficult to predict the pH stability of the myofibrillar ATPase of the E fibers. The present study was undertaken to investigate the pH sensitivity of the myofibrillar ATPase and to define the pH stability of it in the five fiber types of the sheep and cattle.

Materials and Methods

Muscle samples were obtained from two ewes (Corriedale), three wethers (Suffolk), two steers (Holstein) at the age of 17 months, two steers (Holstein) at the age of 20 months, and

three steers (Japanese Black Cattle) at the age of 30 months. After slaughter, the samples were excised from the *M. serratus ventralis*, *M. semitendinosus*, *M. longissimus thoracis*, and *M. masseter*, and frozen in a mixture of dry ice and acetone. Sections ($8\ \mu$) were serially cut in a cryostat, mounted on clean glass slides, and dried at room temperature for 30 minutes.

The procedure for acid and alkaline preincubation largely followed that of BROOKE and KAISER.^{5,10} Acid preincubation solution was composed of 15 ml of MICHAELIS' buffer stock solution (sodium acetate 1.94 g + sodium barbiturate 2.94 g + distilled water 100 ml), 30 ml of 0.1 M HCl, and 24 ml of distilled water. The pH was adjusted to 3.8, 4.0–4.3, and 4.6 with 0.1 M HCl. The sections were immersed in the acid solutions for 7–8 minutes. Alkaline preincubation solution was composed of 20 ml of 0.1 M sodium barbiturate, 10 ml of 0.18 M CaCl_2 , and 70 ml of distilled water. The pH was adjusted to 9.4, 10.5–10.7, and 11.0 with 0.1 M NaOH. The sections were immersed in the alkaline solutions for 26 minutes. Both the sections immersed in acid or alkaline solution were washed in the alkaline preincubation solution at pH 9.4 described just above for 20 seconds. They were immediately incubated in the incubating mixture for ATPase reaction.¹¹

The sections preincubated in the alkaline solutions were incubated in the incubating mixture for 30 minutes, whereas the sections preincubated in the acid solutions were incubated for 45 minutes. The procedure following the incubation for ATPase reaction was the same as that of PADYKULA and HERMAN.¹¹ The preincubation solution and the incubating mixture were prepared immediately before use.

The acid and alkaline preincubation and the incubation for ATPase reaction were carried out at room temperatures between 16 and 18°C in the sections from the sheep and the 17- and 30-month-old steers, and at room temperatures between 23 and 25°C in the sections from the 20-month-old steers.

Some sections were incubated for the demonstration of the routine ATPase activity¹¹ and used for the classification of fiber types. For the purpose of classification the activity of dihydronicotinamide adenine dinucleotide (NADH) diaphorase was demonstrated by using the method described previously.⁶ Muscle fibers were classified into five types (A, B, C, D, and E) in the same manner as reported in the previous studies.^{6–10}

Results

1. Fiber types.

In the sheep and cattle, the type A fibers reacted strongly for NADH diaphorase and the routine myofibrillar ATPase at pH 9.4 (Figs. 3, 13). The type B fibers reacted weakly for NADH diaphorase and strongly for the myofibrillar ATPase. The type C and D fibers reacted strongly for NADH diaphorase and weakly for the myofibrillar ATPase. The D fibers were larger in size than the C fibers, and showed the distinctive reticular pattern of diformazan in NADH diaphorase reaction (Figs. 3, 13). The type E fibers reacted very strongly for NADH diaphorase and moderately for the myofibrillar ATPase (Fig. 9).

2. The sensitivity of myofibrillar ATPase to the pH of preincubation solution in each fiber type.

Preincubation at room temperatures between 16 and 18°C: The myofibrillar ATPase activity of the A and B fibers in ovine and bovine muscles was very strong after preincubation at the pH between 9.4 and 10.7, and moderate to strong at pH 4.6 (Figs. 1, 3–8, 13–20). After pre-

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Fig. 1. The pH sensitivity of myofibrillar ATPase reaction in muscle fiber types of the sheep and cattle at room temperatures of 16~18°C

Fig. 2. The pH sensitivity of myofibrillar ATPase reaction in bovine muscle fiber types at room temperatures of 23~25°C

incubation at pH 4.3, the ATPase reaction was negative in the A fibers of both species and in the B fibers of ovine muscles and the bovine *M. serratus ventralis* (Figs. 1, 3, 7, 13-18), whereas the B fibers of the bovine *M. longissimus thoracis* and *M. semitendinosus* reacted negatively to weakly for the ATPase (Figs. 1, 19-22). The negatively reacting B fibers were fewer than the weakly reacting B fibers in these muscles and were 8.9-12.5% of the B fibers (0.47-5.0% of the total fibers). The weakly reacting B fibers were completely inhibited by preincubation at a pH below 4.2.

The ATPase activity of the C and D fibers in both species was very strong after preincubation at pH 4.3 and 4.6, moderate to strong at pH 9.4, weak to moderate at pH 10.5, and negative at pH 10.7. At pH 4.0 preincubation the activity was moderate to strong in the C fibers and weak to moderate in the D fibers; the latter were more lightly stained than the former (Figs. 1, 3-8, 13-18). The ATPase activity of the E fibers in both species was very strong after pH 4.0-4.6 preincubation, moderate to strong at pH 9.4, weak to moderate at pH 10.5, and negative to very weak at pH 10.7 (Figs. 1, 9-12).

Some fibers reacted strongly to very strongly for the ATPase after preincubation at the pH between 4.3 and 10.7 and weakly at pH 4.0 (Figs. 1, 16-18). They reacted strongly for both NADH diaphorase and routine ATPase at pH 9.4, so that they fell under the category

of the A fibers and were expressed as the acid- and alkali-stable A fibers for convenience sake.

In one of the sheep, the acid- and alkali-stable A fibers were found in the *M. semitendinosus* (3.1% of the A fibers, 1.0% of the total fibers), but not in the *M. serratus ventralis* nor in the *M. longissimus thoracis*. Nor were these fibers seen in three kinds of muscles from other sheep. Such A fibers were none to very few (0–2.0% of the A fibers, 0–0.4% of the total fibers) in these muscles from the 30-month-old steers. In the 17-month-old steers, they were 18.8–24.1% of the A fibers (4.6–4.9% of the total fibers) in the *M. serratus ventralis*, but none to very few (0–0.7% of the A fibers, 0–0.1% of the total fibers) in the *M. longissimus thoracis* and *M. semitendinosus*.

The myofibrillar ATPase reaction of all the fiber types in both species was completely inhibited by preincubation at pH 3.8 and pH 11.0.

Preincubation at room temperatures between 23 and 25°C: In the 20-month-old steers, the A and B fibers were negative in the ATPase reaction after preincubation at pH 4.3, moderate at pH 4.6, and very strong at pH 9.4 and 10.5 (Fig. 2). With the reaction after pH 4.6 preincubation, the A fibers were stained slightly more lightly than the B fibers in the *M. longissimus thoracis* and *M. semitendinosus*, but no difference in staining intensity between the A and B fibers was seen in the *M. serratus ventralis*. The acid- and alkali-stable A fibers were weak in the reaction after preincubation at pH 4.0, moderate at pH 4.3 and 10.5, and very strong at pH 4.6 and 9.4. They were found only in the *M. serratus ventralis* from one of two steers and 8.6% of the A fibers (1.3% of the total fibers).

The C fibers of the *M. longissimus thoracis* and *M. semitendinosus* and the D fibers were weak in the reaction after preincubation at pH 4.0, whereas the C fibers of the *M. serratus ventralis* were moderate. The C and D fibers reacted very strongly for the ATPase after pH 4.3–4.6 preincubation and moderately at pH 9.4 preincubation. The E fibers were weak to moderate in the reaction after preincubation at pH 4.0, very strong at pH 4.3 and 4.6, and moderate to strong at pH 9.4. The reaction of the C, D, and E fibers was negative after pH 10.5 preincubation. The ATPase reaction of all the fiber types was completely inhibited by preincubation at pH 3.8 and a pH above 10.7.

Discussion

The results of the ATPase reactions after acid and alkaline preincubation revealed that the myofibrillar ATPase of the E fibers as well as the C and D fibers was acid-stable and alkali-labile, whereas the ATPase of the A and B fibers was acid-labile and alkali-stable. In the sheep, the muscle fibers that showed a positive reaction for β -hydroxybutyrate dehydrogenase and were liable to develop fatty change in starvation^{11,12)} proved to be the fibers having the acid-stable and alkali-labile ATPase.

The acid- and alkali-stable A fibers found in bovine and ovine muscles resembled the type IIC fibers of BROOKE and KAISER.¹⁰⁾ BROOKE *et al.* have suggested that the type IIC fibers are the primitive fibers and differentiated into both type IIA fibers and type IIB fibers during development.¹³⁾ The type IIA and IIB fibers resemble the type A and B fibers of bovine and ovine muscles, respectively. It is therefore presumed that the acid- and alkali-stable A fibers have the nature similar to that of the type IIC fibers, and that they may be transformed into the acid-labile and alkali-stable fibers in the animal kept for a longer period, because they were fewer in the 20- and 30-month-old steers than in the 17-month-old steers and rarely observed in the muscles of the adult sheep. Accordingly, the difference in proportion of the acid- and

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alkali-stable A fibers is not assumed to represent the difference between breeds and between species.

With the ATPase reaction after pH 4.3 preincubation at 16–18°C, large numbers of the B fibers in the *M. longissimus thoracis* and *M. semitendinosus* of the cattle were weakly positive. The weakly positive B fibers were not seen in the same muscles of the sheep, nor in the *M. serratus ventralis* of the cattle. These facts indicate that the pH sensitivity of the myofibrillar ATPase slightly differs between the cattle and sheep and among different muscles of the same animal.

During the preincubation in alkaline solution and the incubation in the mixture for ATPase reaction, the sections are liable to loosen and crumple. Although fixation can prevent the sections from loosening or crumpling, myofibrillar ATPase is sensitive to fixatives.^{2,3,14} When the sections from rat muscles are fixed in buffered formalin before alkaline preincubation, the formaldehyde-sensitive and the formaldehyde-resistant fibers were observed.⁴ In addition, the formalin fixation has variable effects on a particular fiber type in different species.¹⁵ In this study, the sections were not fixed with any fixative in order to avoid the influence of fixation on the myofibrillar ATPase reactions. The results obtained may not always be in agreement with the reactivity in the sections fixed with formalin before incubation.

On the other hand, the sections preincubated in acid solution little loosen or crumple during the following incubation. Accordingly, satisfactory results in staining are easier to be obtained. Instead, however, of the reaction after alkaline preincubation or the routine ATPase reaction, the ATPase reaction after acid preincubation is unsuitable to use for the classification of fiber types, because the acid- and alkali-stable A fibers are regarded as the C fibers by mistake.

The ATPase reaction after preincubation at pH 4.0 and pH 10.7 was inhibited more strongly in room temperatures between 23 and 25°C than in those between 16 and 18°C. This indicates that the temperature during acid and alkaline preincubation influences the inhibition of the ATPase reaction. KHAN *et al.* have shown that the specific temperature of acidic and alkaline preincubation bath is a major contributing factor in the histochemical demonstration of myosin ATPase.¹⁶

The pH of preincubation solution and the duration of preincubation very greatly influence the following ATPase reaction.⁴ Moreover, this reactivity in muscle fibers under the same condition differs among different species.^{10,16} If the same pattern or intensity of the staining in the ATPase reactions is to be obtained among various muscles of different species, the pH and duration of preincubation must be modified to some extent. In addition, the sections need to be preincubated at a selected, constant temperature.

This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education.

The author is greatly indebted to Dr. M. OKADA of National Grassland Research Institute and to Mr. S. TANAKA of Chugoku National Agricultural Experimental Station for providing the materials of the cattle, and to Mr. S. OHWADA for assistance in collecting the samples.

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Explanation of Figures

Figs. 3-8. The *M. serratus ventralis* of the sheep. A, B, C, and D indicate the type A, B, C, and D fibers, respectively. X 280. Fig. 3. NADH diaphorase. Fig. 4. ATPase preincubated at pH 9.4. Fig. 5. ATPase preincubated at pH 10.7. Fig. 6. ATPase preincubated at pH 4.6. Fig. 7. ATPase preincubated at pH 4.3. Fig. 8. ATPase preincubated at pH 4.0.

Figs. 9-12. The *M. masseter* of the sheep. E indicates the type E fibers. X 280. Fig. 9. NADH diaphorase. Fig. 10. ATPase preincubated at pH 9.4. Fig. 11. ATPase preincubated at pH 10.7. Fig. 12. ATPase preincubated at pH 4.0.

Figs. 13-15. The *M. serratus ventralis* of the 30-month-old cattle. Fig. 13. NADH diaphorase. Fig. 14. ATPase preincubated at pH 10.7. Fig. 15. ATPase preincubated at pH 4.3.

Figs. 16-18. The *M. serratus ventralis* of the 17-month-old cattle. Arrows indicate the acid- and alkali-stable A fibers. X 280. Fig. 16. NADH diaphorase. Fig. 17. ATPase preincubated at pH 10.7. Fig. 18. ATPase preincubated at pH 4.3.

Figs. 19 and 20. The *M. semitendinosus* of the sheep. X 280. Fig. 19. NADH diaphorase. Fig. 20. ATPase preincubated at pH 4.3.

Figs. 21 and 22. The *M. semitendinosus* of the cattle. X 280. Fig. 21. NADH diaphorase. Fig. 22. ATPase preincubated at pH 4.3.

メソ羊と牛の骨格筋の5種類の筋線維型における myofibrillar adenosine triphosphatase の pH 安定性について

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メソ羊と牛の骨格筋の筋線維型における myofibrillar ATPase の pH 安定性を組織化学的に調べた。クリオスタット切片を ATPase の反応の前に、酸およびアルカリの溶液に浸漬することにより、これらの動物の A 型と B 型筋線維の myofibrillar ATPase は、酸不安定・アルカリ安定性であり、C 型、D 型、E 型筋線維のそれは、酸安定・アルカリ不安定性であることがわかった。pH 4.0 の前処理で、D 型筋線維の ATPase 活性は、C 型

および E 型筋線維よりも強く抑制された。酸およびアルカリ安定性の A 型筋線維が見つかったが、その数は少なく、個々の筋肉および個体ではないものもあった。室温 (16~18°C) で pH 4.3 の前処理後の ATPase 反応では、メソ羊と牛の間で pH に対する感受性のわずかな差がある種の筋肉の B 型筋線維でみられた。酸およびアルカリ前処理における温度は、この ATPase 活性の抑制に影響を及ぼすことが示された。