A ^1H -Nuclear Magnetic Resonance Study on Lactate and Intracellular pH in Frog Muscle

Yoshiteru SEO, Kazuo YOSHIKAZI, and Taketoshi MORIMOTO

*Department of Physiology, Kyoto Prefectural University of
Medicine, Kamigyo-ku, Kyoto, 602 Japan*

Abstract High resolution proton magnetic resonance spectra of intact frog muscle, obtained by the selective saturation of water signal with 15 min accumulation, showed well-resolved signals from lactate, carnosine, and other compounds. This technique was used to monitor the changes in lactate content and in intracellular pH which was estimated from the chemical shift of carnosine in muscle. The intracellular pH was estimated to be 7.1 in fresh muscles. Under an anaerobic condition, resting muscle showed a gradual increase in lactate content with intracellular acidification. When the anaerobic muscle was electrically stimulated for 5 sec, a transient acceleration of the lactate production and the intracellular acidification was observed. The intracellular buffer value was estimated to be 27 mEq/(pH·kg muscle), based on the pH change due to the increase in lactate content.

Key Words: intracellular lactate, intracellular pH, intracellular buffer value, ^1H -NMR.

Phosphorus nuclear magnetic resonance technique has been widely used for the metabolic studies of living organs such as muscle (HOULT *et al.*, 1974; BURT *et al.*, 1976), heart (JACOBUS *et al.*, 1977), liver (COHEN *et al.*, 1978), and brain (NARUSE *et al.*, 1983). The intracellular acidification has been observed under anaerobic conditions such as hypoxia, anoxia, and ischemia by using the chemical shift of inorganic phosphate, and it has generally been attributed to lactate production due to anaerobic glycolysis (YOSHIKAZI, 1978; DAWSON *et al.*, 1978; GADIAN and RADD, 1981).

The selective water saturation technique has made ^1H -nuclear magnetic resonance (^1H -NMR) applicable to obtain the high resolution NMR spectra of intact muscle (YOSHIKAZI *et al.*, 1981).

In this paper, a further ^1H -NMR study on frog muscle will be reported and the increase in lactate content and the corresponding intracellular acidification will be shown from the chemical shift of carnosine for resting state and after

Received for publication May 23, 1983

瀬尾芳輝, 吉崎和男, 森本武利

muscular contraction under anaerobic condition. This is the first report on the direct nondestructive measurement of lactate production caused by muscular contraction on the same muscle.

MATERIALS AND METHODS

Muscle preparation. The femoral biceps muscles of bullfrogs, *Rana catesbeiana*, were used. After pithing the frog, the biceps muscle was removed and rinsed in Ringer's solution. The muscle (wet weight *ca.* 1.0 g) was incubated with Ringer's solution saturated with N₂ gas for about 10 min and then inserted into an NMR tube (10 mm diameter) previously filled with N₂ gas saturated Ringer's solution. The muscle was covered with a tight-fitting Teflon plug and the tube was capped; such conditions are referred to as anaerobic in this article. The Ringer's solution contained 116 mM NaCl, 2.1 mM KCl, 2.0 mM CaCl₂, 2.4 mM NaHCO₃, and 0.37 mM KH₂PO₄ (pH 7.4). When necessary, 1 mM monoiodoacetate was added. The tetanic stimulation with the supramaximal shock (50 Hz, 50 V) for 5 sec was applied to the muscle after transferring the muscle from the NMR tube into a vessel containing the Ringer's solution bubbled with nitrogen gas continuously, and then the muscle was immediately placed again in the NMR tube. Further details have been reported previously (YOSHIKAZAKI *et al.*, 1979).

Muscle homogenates were prepared with an ultra-high-speed homogenizer (Physoctron NS-500, Nihon Seimitu Kogyo, Japan) after the addition of 5 volumes of water. The homogenate was centrifuged at $1,000 \times g$ for 7 min and the supernatant was centrifuged at $100,000 \times g$ repeatedly to obtain the supernatant. The supernatant (cytosol) was lyophilized and dissolved in ²H₂O solution.

¹H-NMR using selective water saturation technique. ¹H-NMR studies were carried out by a PFT-100 NMR (JEOL, Japan) with a Fourier transform operating at 99.54 MHz. The field frequency was controlled by locking on the deuterium signal of ²H₂O in a capillary tube (3 mm diameter). The spinning of the sample tube was performed. For the selective saturation of the water signals in the muscle sample, the homonuclear gated decoupling was performed as reported previously (YOSHIKAZAKI *et al.*, 1981). In brief, a 45° pulse was applied and 100 times accumulation was performed for 15 min. The water signal was pre-saturated for 5 sec with the 20 mG of RF pulse. The sample tube was cooled by air flow and kept at room temperature (24°C). When necessary, the high resolution portion of the spectra was obtained by subtracting the broad component from the original spectra.

For the quantitative analysis of lactate, a capillary (1.5 mm diameter) containing 10% tetramethylsilane in C²HCl₃ was used as an external reference, and the area of the signal was calibrated against the samples of lactate solutions with known concentrations. The areas of signals were measured with a picture analyzing system (MOP AM03, Kontron, West Germany). Because the increase

in irradiation power on water signal caused the decrease in the lactate signals as reported previously, the irradiation power was held constant and the rate of decrease in the signal intensities was examined on the same muscle. The signals except those of lactate and reference were allowed to overflow in the spectra obtained without the irradiation. The ratio of lactate signal intensities without and then with the irradiation to the muscle sample was 2.3. In this experiment, the correction factor of 2.3 was used for the quantitation of lactate content in muscle.

When necessary, ^{31}P -NMR spectra were obtained at 40.29 MHz as reported previously (YOSHIZAKI *et al.*, 1979). In brief, a 30° pulse was applied every 1.5 sec to the muscle sample and a 1,000 times accumulation was performed in 30 min.

Fig. 1. The high resolution proton spectra of frog muscle and the spectral assignment of lactate. a) The high resolution portion of ^1H -NMR spectrum of intact frog muscle after subtraction of broad component. The arrow shows the irradiation of water signal. b) The supernatant (cytosol) of frog muscle homogenate after centrifugation of $100,000 \times g$ for 60 min, lyophilized and dissolved in $^2\text{H}_2\text{O}$. c) The spectrum of 35 mM lactate in $^2\text{H}_2\text{O}$.

RESULTS

¹H-high resolution NMR spectra of frog muscle and lactate measurement

High resolution proton magnetic resonance spectra of intact frog muscle can be obtained by the selective saturation of water signal with 15 min accumulation. The spectra consisted of the broad component and high resolution portion.

The high resolution portion showed seven relatively sharp signals (I–VII) as shown in Fig. 1a. The well-resolved signals could be attributed to those of highly mobile small molecules in the cytosol fraction of frog muscle (Fig. 1b). On the basis of a spectrum of a model solution of muscle, signals I–V obtained in intact muscle seem to be mainly from the protons of creatine phosphate (III, V) and carnosine (I, II, IV) as reported previously (YOSHIZAKI *et al.*, 1981).

Signal VI was in the position corresponding to the aliphatic protons, and would be due to the protons of methylene and methyl groups of the alkyl chain of lipids. However, signal VI was split into two peaks in the cytosol fraction. The pH

Fig. 2. The pH titration of the model solution (14 mM carnosine, 30 mM creatine phosphate, and 10 mM inorganic phosphate) (○); of the second solution (5 mM ATP, 100 mM KCl, and 10 mM NaCl added) (□); of the third solution (20 mM MgCl₂ added to the second one) (●); and of the fourth solution (15 mM CaCl₂ added to the third one) (×).

dependent-chemical shift and spin-spin coupling coincided with those of lactate (Fig. 1c). After a 12-hr incubation with the Ringer's solution gassed with room air, signal VI became very small and usually undetectable. Therefore, we concluded that signal VI originated mainly from lactate in frog muscle.

Intracellular pH measurements in frog muscle using the chemical shift of carnosine

The signals (I, II) of the aromatic protons originate from carnosine. Fortunately, the pK values of aromatic protons of carnosine are around 7 and the pH titration curves show the change of the chemical shifts around the physiological range of pH. The chemical shifts of creatine phosphate and ATP show no pH dependency in the physiological pH range. Thus, it is possible to use the chemical shifts of aromatic protons of carnosine as a pH indicator in muscle cells, as inorganic phosphate can be used in ^{31}P -NMR (HOULT *et al.*, 1974; BURT *et al.*, 1976; YOSHIKAWA *et al.*, 1979).

The effect of ions on the pH-dependent chemical shift was carefully examined. Figure 2 shows the pH titration of chemical shifts of carnosine and ATP in 4 model solutions. The first model solution consists of 14 mM carnosine, 30 mM

Fig. 3. The time course in the first 6 hr of spectral changes of resting muscle under the anaerobic condition, indicating the gradual increase in lactate content (signal VI). The left portion is expanded about 1.5 times, indicating intracellular acidification based on the lower field shifts of carnosine signal (I).

creatine phosphate and 10 mM inorganic phosphate, and for the second one, 5 mM ATP, 100 mM KCl, 10 mM NaCl are added to the first model solution. Twenty mM MgCl_2 is added to the second solution in order to make the third one, while the fourth one is made by having 15 mM CaCl_2 added to the third one. These ionic effects can be observed on the chemical shift of histidine-H4 (signal II) in carnosine but not significantly on that of histidine-H2 (signal I). Thus, signal I is more suitable for the pH measurement because of the large range of chemical shifts and lesser degree of ionic effects.

Using the titration curve obtained in the model solution, the intracellular pH was estimated from the chemical shift of signal I in intact frog muscle. The average and S.D. of the calculated pH value in frog biceps muscles was 7.1 ± 0.1 for 5 samples freshly prepared.

The signal of carnosine in the highest magnetic field (#) observed in the supernatant (Fig. 1b), which corresponds to *N*-methylene protons (MARGOLIS *et al.*, 1979), could not be observed in the muscle spectra. The disappearance of this signal from muscle spectra indicates that carnosine molecules might have an interaction with unknown macromolecules *via* the *N*-methylene residues in muscle cells. Therefore, the chemical shift of histidine of carnosine might be changed by the interaction, and the absolute pH value calculated from the shift

Fig. 4. The gradual increase in lactate content and the corresponding intracellular acidification in resting muscle under the anaerobic condition for 10 hr.

may be influenced. To test this effect, proton spectra and phosphorus spectra were alternatively measured on the same muscle under the anaerobic condition. As a result, we observed a similar magnitude of changes of intracellular pH in frog muscle determined by using the chemical shift of inorganic phosphate and that of carnosine. Therefore, we concluded that the use of chemical shift of carnosine is valid for the estimation of pH change in frog muscle.

Lactate production and intracellular acidification in resting anaerobic frog muscle and by tetanic contraction

Figure 3 shows the spectral changes in the first 6 hr of resting frog muscle under the anaerobic condition, indicating the slow increase in the lactate content (signal VI). The increase in the signal was considered to be the result of the lactate production in the anaerobic muscle, and plotted in Fig. 4. In fact, the increase in the signal was not observed in the muscle poisoned by 1 mM monoiodoacetate. The careful examination of the change of the chemical shifts of aromatic signal (I) of carnosine revealed the intracellular acidification and the calculated intracellular pH was also plotted in Fig. 4.

Resting muscle under the anaerobic condition showed the gradual increment of lactate content accompanied by intracellular acidification for about 5 hr, and

Fig. 5. The effect of tetanic contraction on ^1H -NMR spectra of frog muscle. The left portion is presented as 4 times amplification of the height, indicating the intracellular acidification based on the lower field shifts of carnosine signal (I) after tetanic stimulation. The right portion shows the increase in lactate content in the muscle (signal VI).

Fig. 6. The intracellular pH acidification and the increase in lactate in resting muscle (●), and after muscular contraction (○), under the anaerobic condition. The arrows show the 5-sec tetanic stimulation on muscle.

thereafter the production of lactate and the corresponding intracellular acidification were accelerated (Fig. 4). The average rate and S.D. of lactate production in 4 samples was 0.8 ± 0.2 and 1.8 ± 0.3 mmol/(kg muscle · hr) in the range of 0 to 5 hr and 5 to 10 hr, respectively. The effect of muscular contraction is shown in Fig. 5. The increase in lactate is clearly observed after tetanic stimulation for 5 sec. Simultaneously, the intracellular acidification was observed as the shift of aromatic signal (I) of carnosine to a lower magnetic field. Figure 6 shows the increase in lactate content and the change in intracellular pH in the muscle stimulated for 5 sec three times under the anaerobic condition. The significant increase in lactate production and the corresponding intracellular acidification were observed after the tetanic contraction.

DISCUSSION

Lactate production measured by ^1H -NMR in frog muscle. We previously reported the intracellular acidification of frog muscle under the anaerobic condition using the chemical shift of inorganic phosphate detected by ^{31}P -NMR technique. The acidification was considered to be the result of the lactate production

caused by anaerobic glycolysis because monoiodoacetate suppressed the acidification (YOSHIZAKI *et al.*, 1979). DAWSON *et al.* (1978, 1980) used the pH change for the estimation of lactate production based on the assumption of intracellular buffer value.

By ^1H -NMR technique we succeeded in measuring lactate and other small molecules such as carnosine and creatine (phosphate) using the selective saturation of water signal with 15 min accumulation. BROWN *et al.* (1977) also succeeded in observing lactate in red blood cells by the WEFT (water-elimination Fourier transform) method using high field ^1H -NMR with a superconducting magnet.

However, it is impossible to exclude the possibility of the contribution of lipid to the lactate signal. Therefore it would be safe to deal with the increase in lactate content in muscle. Anaerobic resting muscle showed the gradual increment of lactate content accompanied with intracellular acidification, and muscular contraction caused the transient acceleration of glycolysis leading to the increase of the lactate production and the intracellular acidification.

The quantitation of lactate and the significant increase in lactate content in caffeine-treated muscle has also been observed using ^{13}C -NMR (DOYLE and BÁRÁNY, 1982). However, the low natural abundance of ^{13}C atom does not provide the sufficient time resolution to follow the change of lactate content produced by muscular contraction.

KARPATKIN *et al.* (1964) reported that the rate of lactate production was 0.80 mmol/(kg muscle·hr) in resting anaerobic sartorius muscle of frog at 20°C using the modified Barker-Summerson method. The present result with NMR is in good agreement with their result, which also suggests the accuracy of the present method.

Intracellular pH and the buffer value in frog muscle. The present technique was used to monitor the intracellular pH from the chemical shift of the aromatic proton originating from carnosine. Signal I was suitable for the pH measurement because of the wide range of shift and of lesser degree of ionic effects. The intracellular pH was estimated to be 7.1 in fresh frog muscle, which was slightly lower than the value of 7.2 measured by the chemical shift of inorganic phosphate by ^{31}P -NMR (BURT *et al.*, 1976; YOSHIZAKI *et al.*, 1979; SEO *et al.*, 1983). However, the difference would be considered to be within the experimental error at the present time. On the basis of the careful examination to reveal the accuracy of intracellular pH measurement using ^{31}P -NMR, uncertainties in absolute intracellular pH measurement is of the order of 0.1–0.3 pH unit without the exact knowledge of the intracellular milieu such as ionic strength and concentration of Mg ion (ROBERTS *et al.*, 1981; SEO *et al.*, 1983). Thus, the value of 7 as the intracellular pH in muscle coincides with pH values obtained by using different methods (MATSUMURA *et al.*, 1980; HANNAN and WIGGINS, 1976).

The intracellular acidification can be observed simultaneously with the increase of lactate content in frog muscle. Thus, based on the intracellular pH

Fig. 7. The estimation of intracellular buffer value (β) in frog muscle based on the increase in lactate content and the corresponding intracellular pH change in the same muscle. The value calculated from the slope was 26.7 mEq/(pH·kg muscle).

change accompanying the increase of lactate, we can simply estimate the intracellular buffer value (Fig. 7), whose average and S.D. turn out to be 27 ± 3 mEq/(pH·kg muscle) for 4 samples. The effects of the breakdown of creatine phosphate to creatine and the effluxes of H^+ ion of lactate to extracellular space must be taken into consideration but they remain to be elucidated. However, the estimated value agreed in general with the values of 23–27 mEq/(pH·kg muscle) which were calculated from the reports of CURTIN and WOLEDGE (1978), indicating that these effects would be negligible, as assumed by DAWSON *et al.* (1978, 1980) for their analysis of metabolic aspects of muscular fatigue.

In conclusion, 1H -NMR is quite as useful as ^{31}P -NMR for studying the anaerobic metabolism in frog muscle, based on the changes of lactate content and those of intracellular pH.

The authors thank Prof. H. Masuda (Kyoto Prefectural University of Medicine) for editorial help of the manuscript. This research was partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- BROWN, F. F., CAMPBELL, I. D., KUCHEL, P. W., and RABENSTEIN, D. C. (1977) Human erythrocyte metabolism studies by 1H spin echo NMR. *FEBS Lett.*, **82**: 12–16.
- BURT, C. T., GLONEK, T., and BÁRÁNY, M. (1976) Analysis of phosphate metabolites, the intracellular pH, and the state of adenosine triphosphate in intact muscle by phosphorus nuclear magnetic resonance. *J. Biol. Chem.*, **251**: 2584–2591.

- COHEN, S. M., OGAWA, S., ROTTENBERG, H., GLYNN, P., YAMANE, T., BROWN, T. R., SHULMAN, R. G., and WILLIAMSON, J. R. (1978) ^{31}P nuclear magnetic resonance studies of isolated rat liver cells. *Nature*, **273**: 554–556.
- CURTIN, N. A. and WOLEDGE, R. C. (1978) Energy changes and muscular contraction. *Physiol. Rev.*, **58**: 690–761.
- DAWSON, M. J., GADIAN, D. G., and WILKIE, D. R. (1978) Muscular fatigue investigated by phosphorus nuclear magnetic resonance. *Nature*, **274**: 861–866.
- DAWSON, M. J., GADIAN, D. G., and WILKIE, D. R. (1980) Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance. *J. Physiol. (Lond.)*, **299**: 465–484.
- DOYLE, D. D. and BÁRÁNY, M. (1982) Quantitation of lactic acid in caffeine-contracted and resting frog muscle by high resolution natural abundance ^{13}C NMR. *FEBS lett.*, **140**: 237–240.
- GADIAN, D. G. and RADDA, G. K. (1981) NMR studies of tissue metabolism. *Annu. Rev. Biochem.*, **50**: 69–83.
- HANNAN, S. F. and WIGGINS, P. M. (1976) Intracellular pH of frog sartorius muscle. *Biochim. Biophys. Acta*, **428**: 205–222.
- HOULT, D. I., BUSBY, S. J. W., GADIAN, D. G., RADDA, G. K., RICHARDS, R. E., and SEELEY, P. J. (1974) Observation of tissue metabolites using ^{31}P nuclear magnetic resonance. *Nature*, **252**: 285–287.
- JACOBUS, W. E., TAYLOR IV, G. J., HOLLIS, D. P., and NUNNALLY, R. L. (1977) Phosphorus nuclear magnetic resonance of perfused working rat hearts. *Nature*, **265**: 756–758.
- KARPATKIN, S., HELMREICH, E., and CORI, C. F. (1964) Regulation of glycolysis in muscle. II. Effect of stimulation and epinephrine in isolated frog sartorius muscle. *J. Biol. Chem.*, **239**: 3139–3145.
- MARGOLIS, F. L., GRILLO, M., BROWN, C. E., WILLIAMS, T. H., PITCHER, R. G., and ELGAR, G. J. (1979) Enzymatic and immunological evidence for two forms of carnosinase in the mouse. *Biochim. Biophys. Acta*, **570**: 311–323.
- MATSUMURA, Y., KAJINO, K., and FUJIMOTO, M. (1980) Measurement of intracellular pH of bullfrog skeletal muscle and renal tubular cells with double-barreled antimony microelectrodes. *Membr. Biochem.*, **3**: 99–129.
- NARUSE, S., TAKADA, S., KOIZUKA, I., and WATARI, H. (1983) *In vivo* ^{31}P NMR studies on experimental cerebral infarction. *Jpn. J. Physiol.*, **33**: 19–28.
- ROBERTS, J. K. M., WADE-JARDETZKY, N., and JARDETZKY, O. (1981) Intracellular pH measurements by ^{31}P nuclear magnetic resonance. Influence of factors other than pH on ^{31}P chemical shifts. *Biochemistry*, **20**: 5389–5394.
- SEO, Y., MURAKAMI, M., WATARI, H., IMAI, Y., YOSHIZAKI, K., NISHIKAWA, H., and MORIMOTO, T. (1983) Intracellular pH determination by a ^{31}P -NMR technique. The second dissociation constant of phosphoric acid in a biological system. *J. Biochem.*, **94**: 729–734.
- YOSHIZAKI, K. (1978) Phosphorus nuclear magnetic resonance studies of phosphorus metabolites in frog muscle. *J. Biochem.*, **84**: 11–18.
- YOSHIZAKI, K., NISHIKAWA, H., YAMADA, S., MORIMOTO, T., and WATARI, H. (1979) Intracellular pH measurement in frog muscle by means of ^{31}P -nuclear magnetic resonance. *Jpn. J. Physiol.*, **29**: 211–225.
- YOSHIZAKI, K., SEO, Y., and NISHIKAWA, H. (1981) High-resolution proton magnetic resonance spectra of muscle. *Biochim. Biophys. Acta*, **678**: 283–291.