博士学位論文

脱神経されたカエル再生骨格筋の筋収縮とエネルギー代謝の減少について

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Doctoral Dissertation

Loss of energy consumption during muscular contraction in partially denervated regenerating frog sartorius muscle

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Abstract

1. The dynamics of the response of partially denervated regenerating frog sartorius muscle to muscular contraction were investigated by in vivo ^{31}P-NMR spectroscopy.

2. Morphologically, muscle stump became occupied with muscle fibers and showed no significant differences from controls eight weeks after the operation. The frog skeletal muscle seemed to regenerate not only muscle stumps but denervated and innervated sites of the muscle.

3. Compared with innervated and control muscles, denervated regenerating muscle showed low tetanic tension and small changes in the relative concentrations of PCr to β-ATP, Pi to β-ATP, and in intracellular pH value with direct muscle stimulation.

4. These results suggested that partially denervated regenerating muscle may have reduced consumption of high-energy phosphate compounds resulting in a decrease in muscular contraction, or have decreased muscular contractile activity resulting in a reduction in the consumption of high-energy compounds.

Key words: muscle regeneration, ^{31}P-NMR spectroscopy, energy metabolism, muscular contractile activity

Introduction

It is well known that frog skeletal muscle regenerates from muscle stumps or from remnants of a muscle,[1–4] while denervated frog skeletal muscle shows atrophy[5–8] which parallels the deterioration of muscular contraction,[9] the changes in muscle fiber types,[1, 10, 11] and the changes in sensitivity to neurohumoral transmitters.[12] In frog, peripheral motor axons regenerate after a lesion to reinnervate the original synaptic sites on muscle fibers.[4, 13–15] However, little is known with respect to energy metabolism in partially denervated regenerating muscle. There has been only one previous report which demonstrated that denervated muscle showed almost similar metabolism to control muscle in caffeine contracture.[16] In this study, both denervated and innervated muscles showed similar sequential changes in the relative concentrations of PCr to β-ATP and Pi to β-ATP, but intracellular pH value of denervated muscle was lower than that of control. It is useful to understand the energy metabolism with serial morphological changes of partially denervated regenerating muscle.

^{31}P-NMR spectroscopy is the method of

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choise for studying high-energy phosphate compounds such as phosphate creatine (PCr) and inorganic phosphate (Pi), which are closely associated with the metabolism of ATP, and intracellular pH value under different conditions in various tissues. We investigated the serial changes in intracellular pH value and the state of high-energy phosphate metabolism during muscular contraction in the early stages of regeneration of frog sartorius muscle with ^{31}P-NMR, and evaluated the decreases in muscular contraction in response to the decreases in phosphate metabolism.

Materials and Methods

Biological materials and surgical procedures

Bullfrogs (*Rana catesbeiana*) of either sex, weighing between 250–450 g, were anesthetized by intraperitoneal injection of 1% MS-222® (tricaine methanesulfonate) 1 ml/50 g body weight. To ensure complete denervation of a portion of muscle, the sartorius muscle of the left leg was amputated completely at a site 1 mm distal to its center. The pelvic site of the muscle was then dissected carefully from the body and the stumps were sutured together under a microscope (Fig. 1-A, B). After washing with Ringer's solution including antibiotics, skin closure was performed. The contralateral muscle was left untreated as control.

After surgery, the frogs were kept in water at a constant room temperature (22°C–25°C).[17] At one, two, four and eight weeks after the operation, five specimens in each group were again anesthetized in the same manner and the sartorius muscles were removed bilaterally under a microscope, respectively. The wet weight of each muscle was measured immediately after dissection.

Morphological studies

The dissected sartorius muscles were first fixed with 3.5% formaldehyde for 48 hours as the natural length. Specimens were then dehydrated and embedded in paraffinwax. Sections, 4 μm thick, were made at the positions of 40, 50, and 60% of the whole muscle length from the knee joint (Fig. 1-B), stained by conventional HE staining and examined under a microscope.

For each specimen, the ratio of muscle fiber area to the whole cross-sectional area was expressed as a percentage, and the total number of muscle fibers in each section were calculated using the LUZEX-3 image analyzing system (Nikon Co., Ltd., Tokyo) connected to an NEC PC-98VX computer

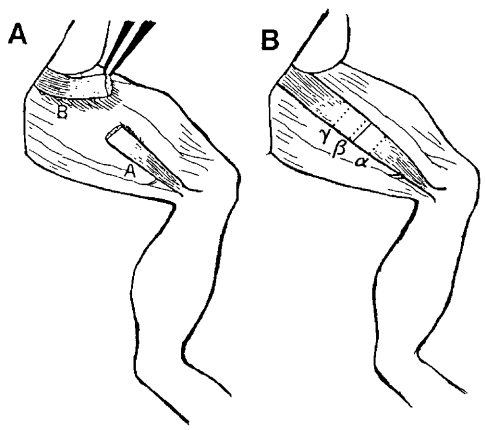

Fig. 1. Schematic sketches of the experimental preparation for morphological measurements, and determination of muscular contractile activities and energy metabolism in partially denervated regenerating frog muscle. Sartorius muscle divided into two parts, the innervated (part A) and the denervated (part B). Part B was dissected carefully to make a perfect denervated site and the stumps were sutured together under a microscope. Sections, 4 μm thick, cut from positions at 40 (α), 50 (β) and 60% (γ) of the whole muscle length from the knee joint, were examined for morphology.

(NEC Corporation, Tokyo) at a final magnification of 1430.

^{31}P-NMR spectroscopy

As shown in Fig. 1-A, the muscle was divided into innervated (A) and denervated (B) parts. For each part of the muscle, tetanic tension and NMR spectra measurements were made. After measuring the wet weight and natural length of each part of the muscle, both ends were fixed vertically to a supporting rod (2 mm in diameter) to maintain the natural length, placed in an NMR sample tube, 10 mm in diameter, and soaked in Ringer's solution through which a mixture of 95% O_2 and 5% CO_2 was constantly bubbled. The tube was placed in a FT-NMR device. Projection was fixed on the rod at a height of 50 mm from the bottom of the NMR tube. The temperature in the device was maintained at 24°C, and the concentration of phosphorus containing metabolites was measured.[13, 18–20] The ^{31}P-NMR spectra of each muscle were recorded with a JNM GX-400 NMR spectrometer (JEOL, Tokyo) operating at 161.7 MHz for ^{31}P nuclei with a 45 degree pulse and sampling 240 seconds (120 scans at 2 seconds intervals). The Pi, PCr, and ATP peaks were integrated to calculate the concentrations, which were then multiplied by saturation factors (PCr: 1.54; ATP: 1.10; Pi: 2.1) which had been determined from measurements at a pulse interval of 20 sec.[16] The intracellular pH of the muscle was determined from the chemical shift difference between PCr and Pi. PCr at 0 ppm was used as the standard for the chemical shift.[21] All measurements were performed in water at 24°C. The intracellular pH values were calculated from the following equation: $pH = 6.74 + \log (a-a1)/(a2-a)$. a: observed chemical shift; a1: −3.24; a2: −5.60. Changes in the concentration of each compound were evaluated from the area under the resonance line of the signals obtained. Immediately after recording the NMR spectra, the muscle was immersed in a glass chamber filled with Ringer's solution. One end of the muscle was fixed to a supporting rod and the other end was tied to a strain gauge in the natural length. Electrical stimulation was applied for 1 minute (50 Hz, 6 msec, 10 V) to stimulate the muscle directly. The tetanic tension was monitored by a strain gauge calibrated for quantitative measurements, and amplified by a carrier amplifier. Six minutes after measurement of tetanic tension, muscle metabolism was again evaluated from the NMR spectra (sampling time: 4 minutes). The stimulation and recording were routinely carried out three times in the same manner.

Statistics

The Student's t-test was used to calculate the significance of differences between experimental groups and controls. $P<0.05$ was considered as significant. All values are expressed as mean and SD.

Results

Wet weight

The ratio of the wet weight of the each part of denervated and innervated muscle to that of contralateral control muscle showed no significant differences in the all experimental groups.

Morphology

In control, the percentage of muscle fiber area to the whole cross-sectional area was about 95% at points α, β, and γ in all specimens. In the experimental groups, the ratio at point α was similar to control

throughout the experimental period. However, muscle fiber area became lower at point β, and the ratio decreased to 65% one week after the operation and was below 30% after two weeks. The quantity of connective tissue became progressively less after this time, and by eight weeks it reached a level comparable to that observed in normal control muscle. Meanwhile, at point γ, in denervated part, connective tissue increased to a level more than that observed in controls after one and two weeks, but filled with muscle fibers to a percentage similar to control muscle after eight weeks (Fig. 2).

On the other hand, as shown in Fig. 3, the number of muscle fibers decreased one week after the operation at point β, but became more abundant than in control after two weeks. These muscle fibers were smaller than those of control and were grouped together. Finally, eight weeks after surgery, the number of muscle fibers exceeded to that of control at each point, and these fibers matured larger in diameter so the percentage of muscle fiber area to the whole cross-sectional area also recovered to that of control. In summary, eight weeks after the operation, muscle regenerated from the stumps to a level not significantly different from control in morphology.

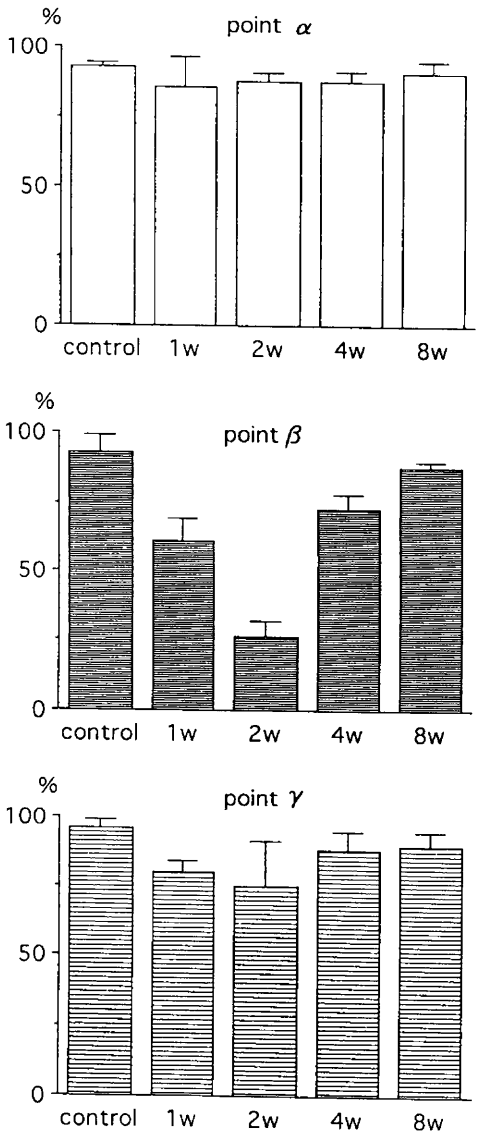

Fig. 2. Percentage of muscle fiber area relative to the whole cross sectional area of regenerating muscle at points α, β and γ. At muscle stumps (point β), loose connective tissue became abundant two weeks after the operation, and returned to near control levels after eight weeks. Values are given as mean and SD.

Tension

The tetanic tension that could be developed by direct stimulation of denervated and innervated muscles as a percentage of that in contralateral muscle is shown in Fig. 4-1, 2, 3 and 4. The tetanic tension in part A (innervated muscle) was nearly equal or more than that in the same region of contralateral control muscle throughout the experimental period. The ratio of the tension in part A to that of contralateral control muscle ranged from 97% to 125%. On the other hand, the tetanic tension in part B (denervated muscle) showed serial decreases and was less at all time than that in part A. Decreases were apparent in tetanic

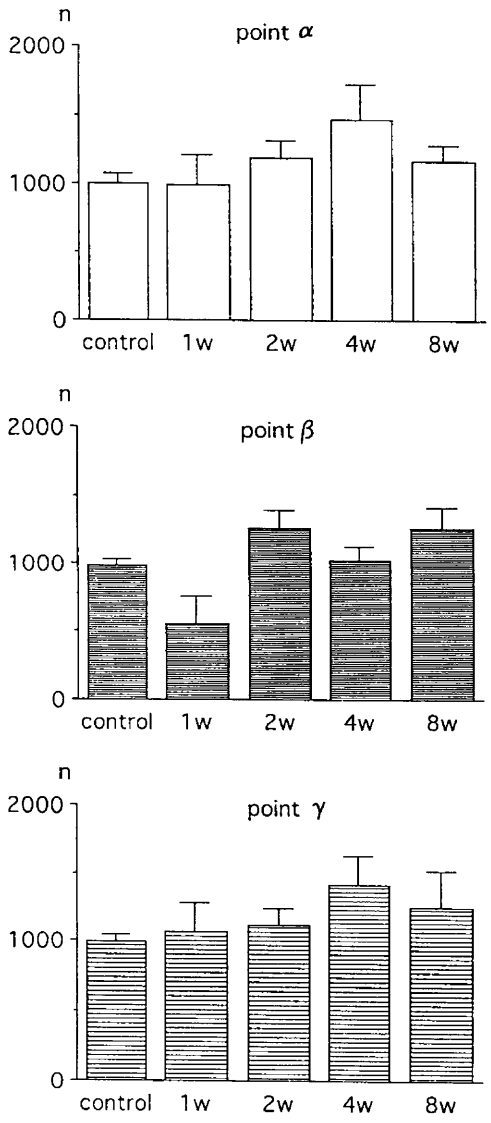

Fig. 3. Number of muscle fibers at points α, β and γ. The number of fibers exceeded to that of control by eight weeks postoperatively. These fibers were much smaller than that of control one and two weeks after the operation and matured larger in cross-sectional area eight weeks after the operation. Values are given as mean and SD.

tension in each part of muscle, especially at the first stimulation.

One week after the operation, the tetanic tension of muscle of part B decreased from 74% to 26% (in the first stimulation, 74% to 32%) of that observed in contralateral control and became marked two weeks after surgery (55% to 15%). After eight weeks, this value increased again (76% to 32%), the maintenance of the tetanic tension showing a recovery. In all experimental periods, the maintenance of tension was least with the last electrical stimulation.

NMR spectroscopy

In vivo ^{31}P-NMR spectrometry of normal frog sartorius muscle showed Pi, PCr and ATP peaks. The resting spectrum is shown as the 0 minute value. Figure 5 illustrates the changes in intracellular pH value observed in all experimental groups. The pH value decreased progressively with each direct stimulation in control muscle. One week after the operation, the final value was the highest in all experimental groups in part A of muscles, and its slope was apparently less than that of control. The pH value decreased with longer postoperative periods. Two and four weeks after the operation, the pH decreased with the same rate and showed no statistically significant differences from that in control for each electrical stimulation. However, after eight weeks, the pH was 6.82 at rest and changed little with each stimulation.

In part B of the muscle, all experimental groups showed little acidification. Eight weeks after surgery, the pH was 6.80 at rest, and little changes were observed as in part A. The differences between all experimental groups at all time points with the exception of eight weeks postoperatively were small in part B as compared with part A.

Compared with part A, the decreases in the relative concentration of PCr to initial β-ATP, and the increases in the relative concentration of Pi to initial β-ATP ob-

served in part B were very small and showed no variations in all experimental periods. (Fig. 6-A, -B; Fig. 7-A, -B) The increases in the ratio of Pi/β-ATP one and two weeks after the operation were slightly greater than those after four and eight

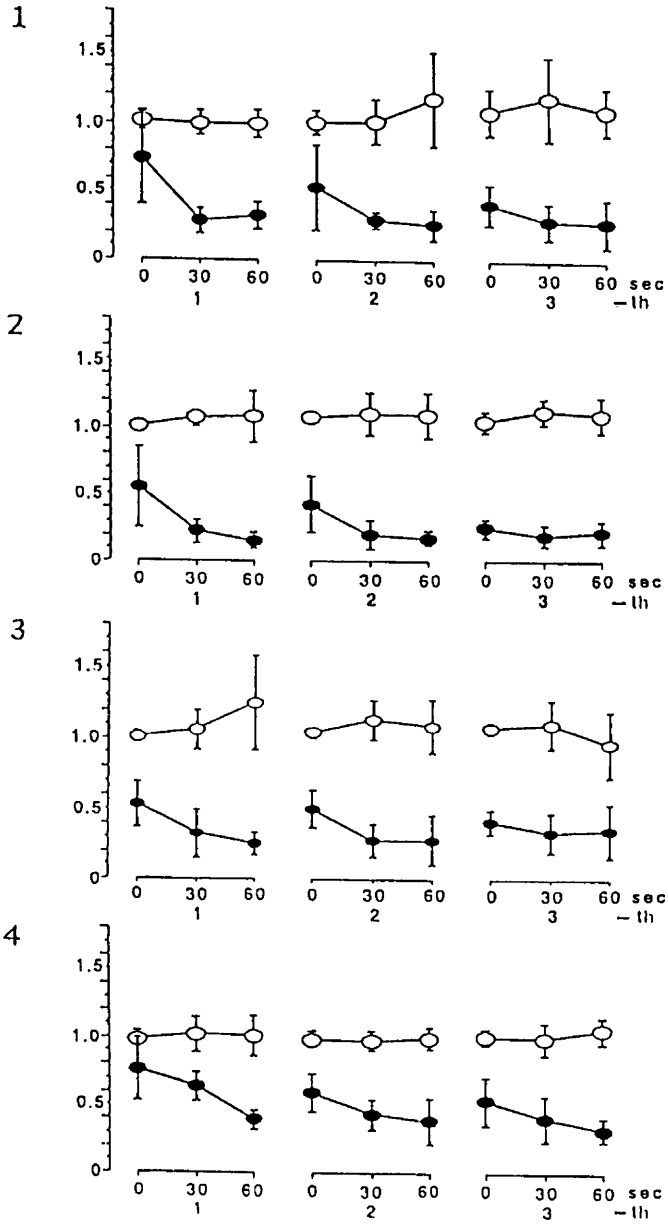

Fig. 4. Serial changes in ratio of relative tetanic tension to the contralateral control muscle (ordinate) evoked by three periods one-minute direct muscle stimulation. The innervated muscle showed similar tension to control, but the denervated part of the muscle showed serial decreases and smaller tetanic tension throughout the experimental period. Open circles (○) indicate the innervated (part A) muscle, closed circles (●) the denervated (part B) muscle. (1: one week after the operation; 2: two weeks after the operation; 3: four weeks after the operation; 4: eight weeks after the operation). Values are given as mean and SD.

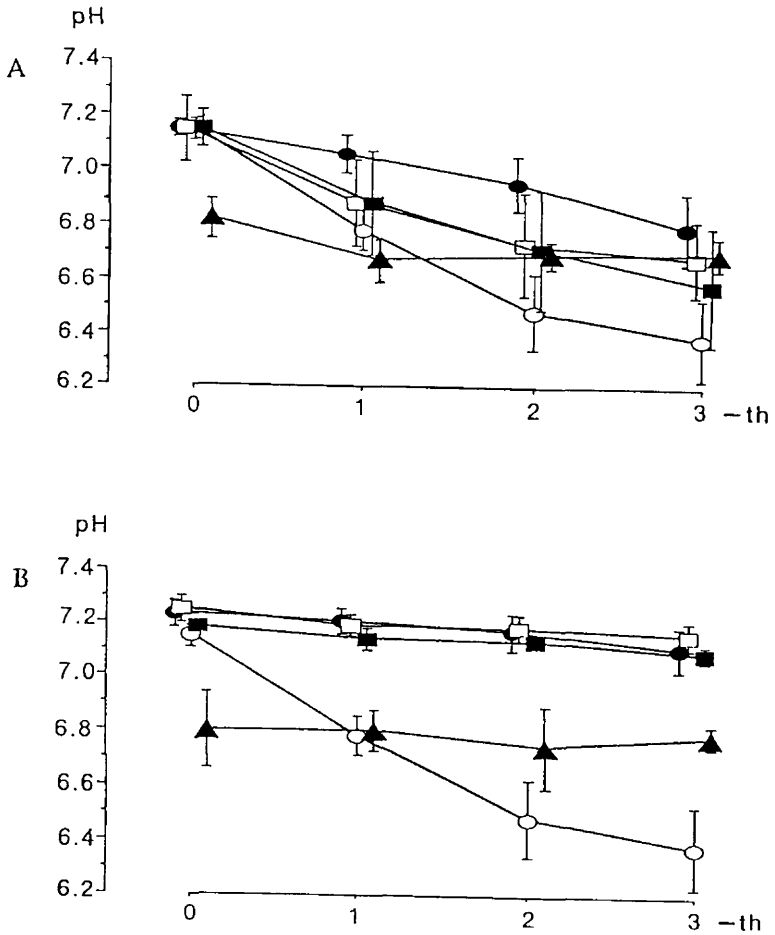

Fig. 5. Serial changes in intracellular pH value with each period of electric stimulation. Compared with innervated (part A) muscle, denervated (part B) muscle showed little acidification after one, two, and four weeks postoperatively. Control muscle showed the greatest decrease in pH in both parts A and B. Open circles (○) represent control, closed circles (●) one week, open squares (□) two weeks, closed squares (■) four weeks, and closed triangles (▲) eight weeks after the operation (A: part A, innervated muscle; B: part B, denervated muscle). Values are given as mean and SD.

weeks. Even in part A, the slopes of the increases of Pi/β-ATP and the decreases of PCr/β-ATP in control was the greatest. The changes in intracellular pH value and the levels of phosphate metabolites related to energy consumption of the muscular contraction were very small in parallel with the low levels of tetanic tension observed in part B.

Discussion

Regeneration of skeletal muscle has been studied extensively,[1, 2, 4, 8, 9, 13, 22, 23] in relation to the sprouting of the motor axon to reinnervate the original muscle in the recovery of denervated and partially denervated muscles.[2, 4, 13, 24, 25, 26] It is well known that denervated muscle shows atrophy,[4, 5, 6, 7, 8, 16] and supersensitivity to Ach and to other chemical agents but not to

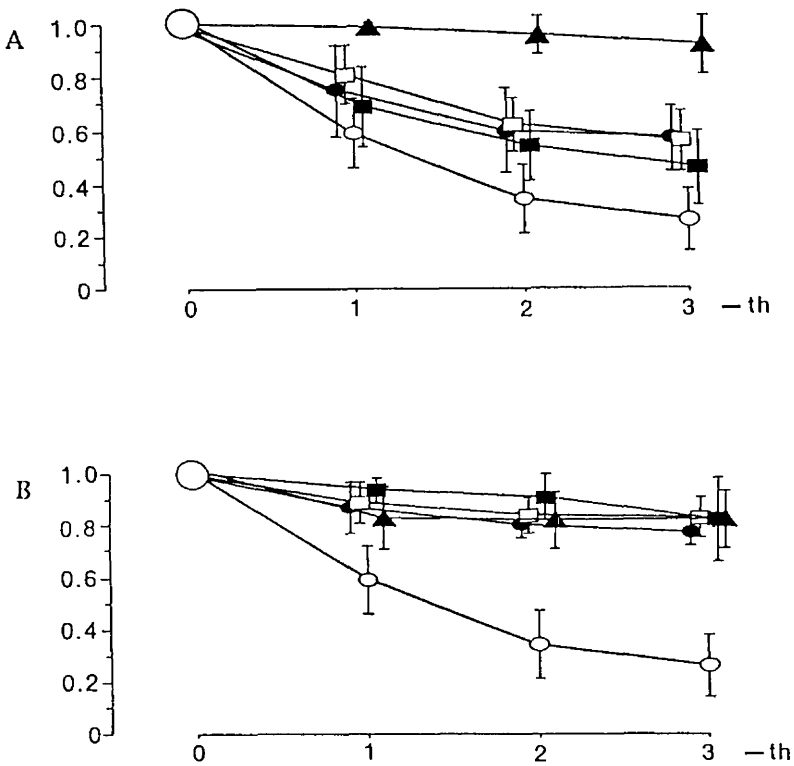

Fig. 6. Serial changes in the ratio of relative concentration of PCr to β-ATP (ordinate) in each tetanus. Denervated muscle showed little decrease in the ratio at all experimental time points, while marked reductions were observed in innervated muscle. Open circles (○) represent control, closed circles (●) one week, open squares (□) two weeks, closed square (■) four weeks, and closed triangles (▲) eight weeks after the operation (A: part A, innervated muscle; B: part B, denervated muscle). Values are given as mean and SD.

electrical stimulation.[12] Recovery produces a population of normally functioning motor neurons and reinnervate muscle fibers. Reduction of the functioning motor axons supplying muscle fibers evokes a compensatory sprouting response from the remaining neurons.[4, 13, 14, 15, 22, 24, 25, 27] Furthermore, inactive muscle fibers are a source of some sprouting factors.[2, 3, 4, 23] Extracts of denervated muscle are more active than those of normally innervated muscle. It has been reported that, in the presence of denervated muscle separated by a filter, regenerating axons turn and grow toward their target cells.[2] In studies of the regeneration process, there have been many reports concerning sprouting, and the electrical and the mechanical characteristics of various portions of regenerating axons. However, little is known about energy metabolism in such muscles.

In the present report, the dynamics of partially denervated regenerating muscle energy metabolism in response to direct muscular stimulation were investigated *in vivo* using ^{31}P-NMR spectroscopy. The time course of changes in phosphocreatine, inorganic phosphate, and ATP peaks and intracellular pH value were also examined.

Frog muscles have been used as models in various studies of muscle physiology and metabolism, and NMR spectroscopy has often been used to investigate metabolism of high-energy compounds in muscles.[29, 30]

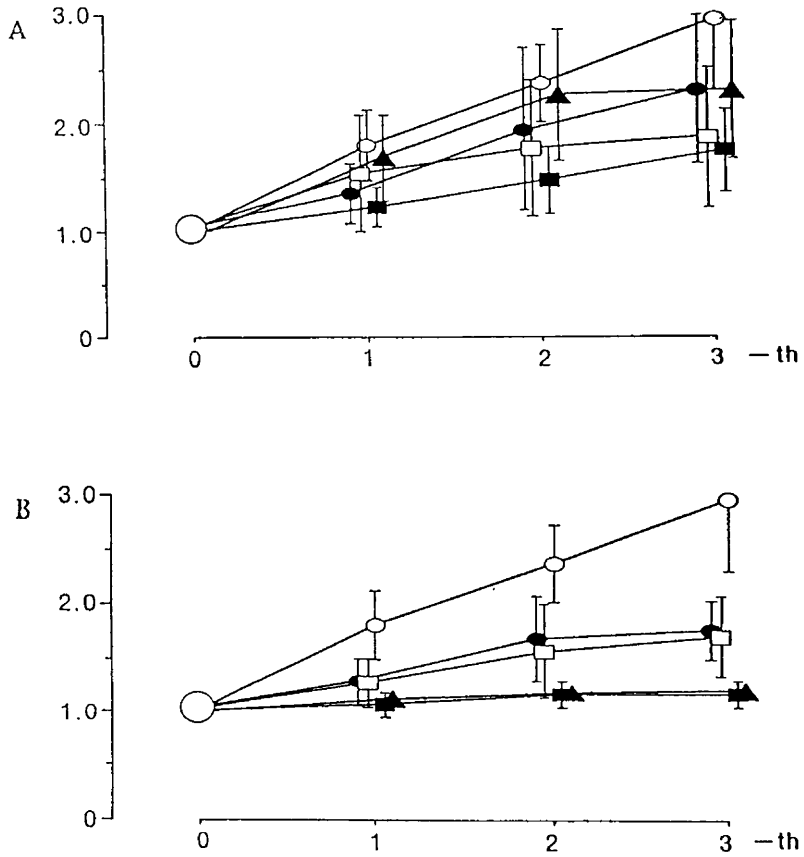

Fig. 7. Serial changes in the ratio of relative concentration of Pi to β-ATP (ordinate) in each tetanus. Similar to the decrease in PCr/β-ATP, the values increased little in denervated muscle. Open circles (○) represent control, closed circles (●) one week, open squares (□) two weeks, closed squares (■) four weeks, and closed triangles (▲) eight weeks after the operation (A: part A, innervated muscle; B: part B, denervated muscle). Values are given as mean and SD.

In the present study, we investigate the frog sartorius muscle, a pure twitch muscle, because the location of the end plate of this muscle has been well characterized,[28] and it shows fast regeneration in the recovery process.

Frog skeletal muscles regenerate from muscle stumps or even from minced muscle tissue. Satellite cells have been proposed to comprise the main source of regenerating muscle fibers, and the basal lamina provides the scaffolding that maintains normal structural morphology during regeneration.[1, 23] In the present study, morphological changes were observed; within the stumps, loose connective tissue became abundant two weeks after the operation, subsequently returning to control levels by eight weeks postoperatively, and the number of muscle fibers increased with time. These regenerating fibers which had been smaller in diameter matured to a level equivalent to normal control twitch fibers, so the precentage of muscle fiber area to the whole

cross-sectional area became similar level to control eight weeks after the operation. At points α and γ, the number of muscle fibers also increased. It has been reported that these regenerating fibers would change their fiber types.[1, 4, 7, 8, 9, 10, 11]

There is general agreement that regenerated muscle fibers have essentially normal contractile properties because the twitch and tetanic tension of such muscle have been shown to correspond roughly to their reduced wet weights.[1, 7, 16] However, our present results suggest that the wet weights of the regenerating muscles were not significantly different from those of similar parts of contralateral normal control muscle in contrast to the observed decreases in tetanic tension. The tetanic tension of regenerating muscle was reduced by 26% to 74% of that of contralateral control muscle one week after the operation, and this reduction became more marked after two weeks (15 to 55%). Eight weeks after the operation, this value increased again by 32% to 76%. The decreases in maintenance of tension were most apparent with the first stimulation because twitch muscle has poor characteristics in maintenance of tension.[10, 30, 32] The denervated regenerating muscles were tetanized three times for one minute each, with an interval of 10 minutes because the level of PCr continues to decrease for a few minutes after muscle relaxation from brief tetanus.[30, 32, 33] The PCr/β-ATP ratio of these muscles decreased very little (77%; control = 26%) with a corresponding small increase in the Pi/β-ATP ratio (176%; control = 297%) and little acidification of pH (7.09; control = 6.38) after one, two and four weeks postoperatively. After eight weeks, the mechanisms what both A and B parts of muscles showed low pH and little acidification were not clear. The decrease in tetanic tension is expected to correspond roughly to the reduced energy consumption.[34] The consumed ATP is replenished at first by the Lohmann reaction, which is reversible and fast. Glycogenolysis, which changes the pH in the acidic direction due to lactic acid production and dissociation, occurs only when the total amount of contractile activity exceeds a critical value[34] (that is when the Pi concentration increases to 8 mM). This may indicate that the regenerating muscle fibers had some abnormalities in their contractile properties and energy metabolism, or in their sensitivities to direct stimulation.

On the other hand, the muscles of innervated sites showed slightly greater tension than control muscle (101 to 125%) and variety in their energy consumption throughout the experimental period. A previous study suggested that denervated and innervated muscles showed similar levels of contraction in response to stimulation with caffeine, and similar energy metabolism, but differed in changes in intracellular pH value.[16] However, in the present study, partially denervated, regenerating muscle showed low contractile activity, small energy metabolism and small acidification. The reason why the results of this previous study differed from those of our present study is still not clear. Many previous studies have reported morphological, histological, and electrophysiological observations of regenerating muscle but little exists in the literature concerning energy metabolism in such tissue. Further studies are required at a variety of levels to further elucidate the mechanism of muscle regeneration.

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