J Physiol Sci (2009) 59:439–446
DOI 10.1007/s12576-009-0054-6

ORIGINAL PAPER

Effects of adrenaline on glycogenolysis in resting anaerobic frog muscles studied by ^{31}P-NMR

Kimio Kikuchi · Takenori Yamada · Haruo Sugi

Received: 17 May 2009 / Accepted: 6 July 2009 / Published online: 8 August 2009

Abstract The effects of adrenaline (also called epinephrine) on glycogenolysis in living anaerobic muscles were examined based on time-dependent changes of ^{31}P-NMR spectra of resting frog skeletal muscles with and without iodoacetate treatments. The phosphate-metabolite concentration and the intracellular pH determined from the NMR spectra changed with time, reflecting the advancement of various phosphate metabolic reactions coupled with residual ATPase reactions to keep the ATP concentration constant. The results could be explained semi-qualitatively as the ATP regenerative reactions, creatine kinase reaction and glycogenolysis, advanced with time showing the characteristic two phases. Thus, it was clarified for living muscles that adrenaline activates the phosphorylase step of glycogenolysis, and the adrenaline-activated glycogenolysis is further regulated at the phosphofructokinase step by PCr and also possibly by AMP. Associated with the adrenaline-activated glycogenolysis in the examined muscles, the P_i concentration and the intracellular pH, factors affecting the muscle force, changed significantly, suggesting complicated effects of adrenaline on the muscle contractility.

Keywords ^{31}P-NMR · Skeletal muscle · Glycogenolysis · Adrenaline · Phosphate metabolism · Intracellular pH

K. Kikuchi · T. Yamada · H. Sugi
Department of Physiology, School of Medicine, Teikyo University, Tokyo 173-8605, Japan

T. Yamada (✉)
Department of Physics (Biophysics Section), Faculty of Science, Tokyo University of Science, Tokyo, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan
e-mail: yamada@rs.kagu.tus.ac.jp

Introduction

In anaerobic muscles, glycogenolysis, the degradation of glycogen, regenerates ATP consumed in the cytosol and ends up in lactate production. The generalized scheme of glycogenolysis and its regulation has been documented in the literature [1] (see a simplified version in Fig. 1) and has been established based mainly on in vitro experiments. Glycogenolysis is thought to be regulated sophisticatedly at the phosphorylase and phosphofructokinase (PFK) steps, the key regulatory steps, by various factors related to cellular activity. As for glycogenolysis for muscles, however, whether or not the general scheme of glycogenolysis can be applied and which factors are directly involved in its regulation still need to be clarified.

^{31}P-NMR studies clarified that glycogenolysis in living muscle is activated by Ca^{2+} released for contraction [2, 3] and further regulated by a factor(s) related with the energy state of muscle [4]. It is thought that adrenaline (also called epinephrine) [5] activates the phosphorylase step of glycogenolysis via cAMP production [1]. However, as far as we know, limited studies have been made on how adrenaline affects the phosphate metabolism of muscle. In the present studies, therefore, the effects of adrenaline on the phosphate metabolism and the intracellular pH (pH_i) of living frog muscles were examined based on improved ^{31}P-NMR measurements. NMR spectra of muscles thus obtained had well-resolved resonance signals for essential phosphate metabolites related to glycogenolysis and made it possible to analyze the advancement of glycogenolysis and its regulation in muscles. To facilitate the analysis, comparative studies were made for resting anaerobic muscles with and without iodoacetate (IAA) treatments, considering that IAA is known specifically to inactivate glyceraldehyde 3-phosphate dehydrogenase, one of the key

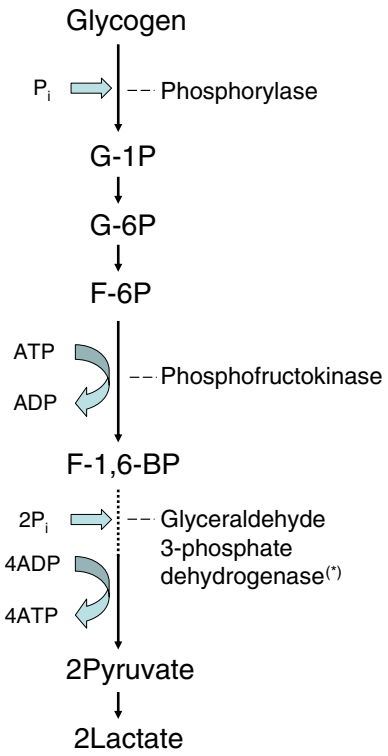

Fig. 1 A simplified glycogenolysis scheme. *Inactivated by IAA treatment

enzymes of glycogenolysis, but not significantly to affect other phosphate metabolic reactions in muscle [6].

Materials and methods

Materials

The care of the animals and the experimental protocol were approved by the Animal Care and Use Committee of Teikyo University School of Medicine. Semitendinosus muscles were freshly dissected from the summer bullfrog (*Rana catesbeiana*) for each experiment. Isolated muscles (about 35 mm in length, about 0.3 g in wet weight) were kept for about 1 h in a frog Ringer's solution with the following composition (in mM): NaCl, 95; KCl, 2.5; $MgCl_2$, 2.1; $CaCl_2$, 1.0; $NaHCO_3$, 20 (pH 7.2), and continuously bubbled with 95% O_2/5% CO_2 at 0°C. To put muscles in an anaerobic state, they were transferred to a frog Ringer's solution containing 2 mM NaCN and continuously bubbled with 95% N_2/5% CO_2 at 0°C for 1 h. These muscles are referred to as CN-treated muscles. The IAA treatment of muscle was made as described previously [3, 4] by incubating muscles for 45 min in a frog Ringer's solution containing 1 mM IAA and then treated with NaCN for 15 min as above. These muscles are referred to as (IAA, CN)-treated muscles. After the above treatments, muscles were immediately used for NMR measurements.

Phosphocreatine (PCr), ATP, ADP, AMP, IMP, glucose 1-phosphate (G-1P), glucose 6-phosphate (G-6P), fructose 6-phosphate (F-6P), fructose 1,6-bisphosphate (F-1,6-BP), and adrenaline were purchased from Sigma Chemicals (St Louis, MO). IAA was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytical grade and purchased from Wako Chemicals (Osaka, Japan).

NMR measurements

^{31}P-NMR spectra of muscles were obtained as described previously [3, 4] using an NMR instrument [161.8 MHz for ^{31}P; GX-400, JEOL (Tokyo, Japan)] with samplings from usually 256 scans with a 45° pulse at 3-s intervals. A muscle preparation was fixed at a slack length alongside a glass tube (1 mm in diameter) by tying each tendon to the glass tube with a cotton string. Then it was put in an NMR glass tube (5 mm in diameter) filled with a frog Ringer's solution containing 2 mM NaCN pre-bubbled with 95% N_2/5% CO_2. The NMR tube was put in the NMR instrument after capping its top end. Muscle preparations were kept at 20–22°C during NMR measurements by continuously purging temperature-controlled air around the NMR glass tube. Time-dependent NMR spectra of muscle were obtained consecutively at 30-min intervals. Adrenaline was applied to muscle preparations in between NMR measurements by transferring them to a separate NMR tube filled with a frog Ringer's solution containing 1.1 μM adrenaline [7] and 2 mM NaCN pre-bubbled with 95% N_2/5% CO_2. Then it was put back in the NMR instrument as described above to resume measurements.

NMR spectra of muscles thus obtained had well-resolved resonance signals for phosphate metabolites, some of which previously could only be identified as sugar phosphates [2–4]. The resonance peaks in NMR spectra of muscles were assigned based on those similarly obtained for standard phosphate compounds dissolved in 10 mM K^+-phosphate solution (pH 7.0). The concentration of phosphate metabolites in muscle was determined as described previously [3, 4] by integrating corresponding resonance signals in NMR spectra, correcting for the saturation factors and assuming the PCr concentration of fresh intact muscles to be 27 mM. The pH_i of muscles was determined based on the pH-dependent chemical shift of inorganic phosphate (P_i) [8, 9].

Analysis of phosphate metabolic reactions

The time-dependent changes of the phosphate-metabolite concentration and pH_i of muscles were analyzed by assuming the advancement of major phosphate metabolic

reactions following [10], i.e.: (1) ATPase reaction, ATP → ADP + P_i + 0.30H^+; (2) creatine kinase reaction (also called the Lohmann reaction), PCr + ADP ↔ Cr + ATP − 0.74H^+; (3) complete glycogenolysis, glycogen (*n* residues) + 3ADP + 3P_i → glycogen [(*n*-1) residues] + 3ATP + 2lactate + 1.11H^+; (4) incomplete glycogenolysis (I), glycogen (*n* residues) + P_i → glycogen [(*n*-1) residues] + G-1P (or G-6P or F-6P) + 0.33H^+; (5) incomplete glycogenolysis (II), glycogen (*n* residues) + ATP + P_i → glycogen [(*n*-1) residues] + F-1,6-BP + ADP + 0.96H^+. The H^+ changes of reactions were calculated as previously [4] by assuming that the pH_i was 7.0 and relevant ionization constants of metabolites were as follows: ATP, 6.5; ADP, 7.0; P_i, 6.9; G-1P, G-6P and F-1,6-BP, 6.1; lactic acid, 3.9; PCr, 4.6. The buffering power of muscles was assumed to be 35 mM/pH [4, 11].

It should be remembered that the Lohmann reaction is reversible, and the incomplete glycogenolysis (I and II) cannot regenerate ATP (Fig. 1). Thus, under the above assumptions, (1) ATP is consumed by residual ATPase reactions and by PFK; (2) ATP is regenerated by the Lohmann reaction and/or glycogenolysis in CN-treated muscles, and it is regenerated only by the Lohmann reaction in (IAA, CN)-treated muscles; (3) P_i is released only by ATPase reactions while it is taken up only by the phosphorylase reaction in the incomplete glycogenolysis (I and II) and by the phosphorylase reaction as well as by the glyceraldehyde 3-phosphate dehydrogenase reaction in complete glycogenolysis; (4) PCr is consumed and produced associated with the forward and backward Lohmann reactions, respectively.

Statistics

All data are expressed by the mean ± the standard error of mean (SEM) ($n = 7$–9). The analysis of data was made by using the Student's *t* test, and $P < 0.05$ was considered significant.

Results

Effects of adrenaline on the phosphate metabolism of resting CN-treated muscles

Figure 2 shows a typical series of time-dependent NMR spectra of resting CN-treated muscles with adrenaline applied as indicated. Fresh CN-treated muscles had characteristic NMR spectra, which were essentially the same as those of intact fresh muscles and composed of PCr, ATP and P_i peaks with no peaks for other phosphate metabolites. Gradual changes of PCr and P_i peaks with time indicate the Lohmann reaction and residual ATPase reactions advanced

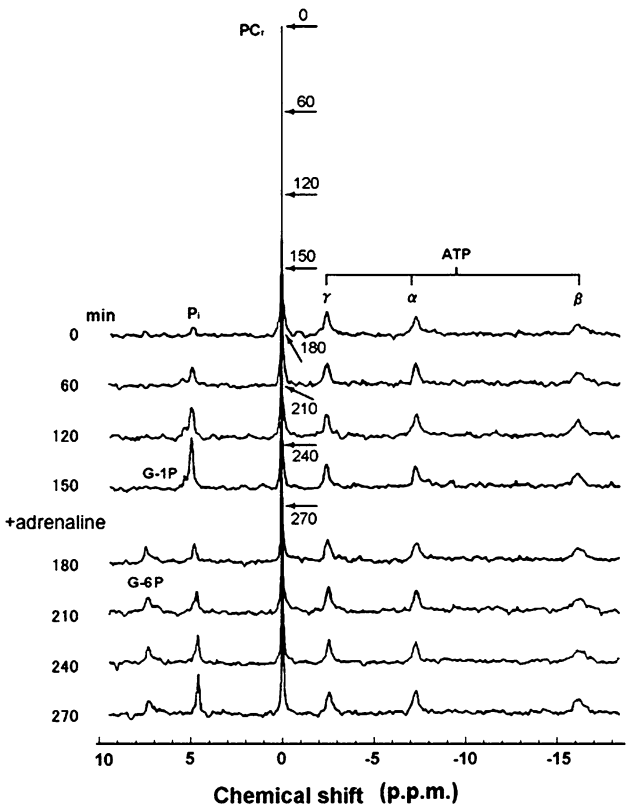

Fig. 2 A time-dependent series of ^{31}P-NMR spectra of a resting CN-treated muscle. Spectra are vertically shifted in the order of the time of NMR measurement. The peak height of PCr signal for each spectrum is indicated by an *arrow* with the time of NMR measurement. Adrenaline was applied to muscle in between NMR measurements as indicated

in muscles [4]. The G-6P peak appeared after the application of adrenaline, clearly due to the degradation of glycogen (Fig. 1). The time-dependent changes of phosphate-metabolite concentration and pH_i determined from NMR spectra similarly obtained are summarized in Figs. 3 and 4, in which adrenaline was applied to muscles at two characteristic timings after the start of NMR measurements. The concentration of ATP was about 4 mM for all the CN-treated muscles examined with and without adrenaline applications, indicating that ATP consumed in muscles was completely regenerated by the Lohmann reaction and/or complete glycogenolysis.

Without adrenaline application, the pH_i changed with time, apparently showing two phases [see the control data in Fig. 3 ($n = 9$)], consistent with the results of our previous study [4]. Initially the pH_i shifted to an alkaline direction at 0.034 ± 0.004 pH units/h, and later at about 2.5 h after the start of NMR measurements, it gradually reversed to an acidic shift up to 0.023 ± 0.006 pH units/h, so that the two phases are called the early and the late phases. Like the pH_i changes, the concentrations of PCr and P_i changed, showing two similar phases, namely the

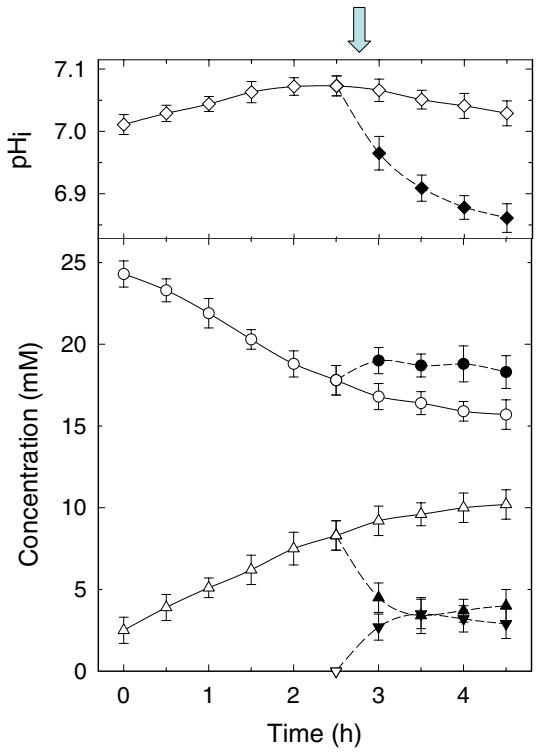

Fig. 3 Time-dependent changes of the phosphate-metabolite concentration and the pH_i in resting CN-treated muscles. (*Open circle*, *closed circle*) PCr; (*open triangle*, *closed triangle*) P_i; (*open inverted triangle*, *closed inverted triangle*) G-6P; (*open diamond*, *closed diamond*) pH_i. The *open* and the *solid symbols* represent the data without and with adrenaline applications, respectively, and the *error bars* SEM. Adrenaline was applied to muscles at the time indicated by an *arrow* at the *top* (near the start of the late phase). The concentration of ATP was all about 4 mM and omitted for clarity. See details in the text

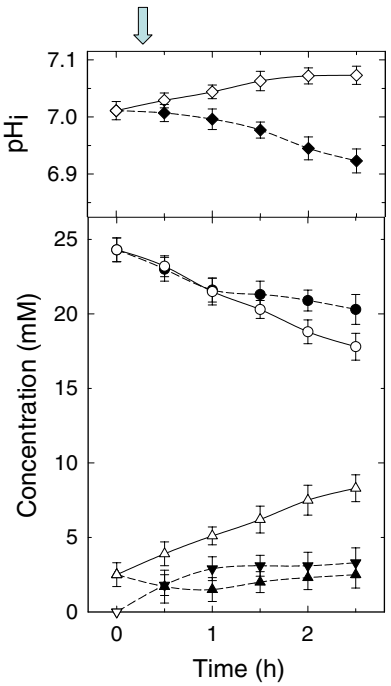

Fig. 4 Time-dependent changes of the phosphate-metabolite concentration and the pH_i in resting CN-treated muscles. (*Open circle*, *closed circle*) PCr; (*open triangle*, *closed triangle*) P_i; (*open inverted triangle*, *closed inverted triangle*) G-6P; (*open diamond*, *closed diamond*) pH_i. The *open* and the *solid symbols* represent the data without and with adrenaline applications, respectively, and the *error bars* SEM. Adrenaline was applied to muscles at the time indicated by an *arrow* at the *top* (at the start of the early phase). The concentration of ATP was all about 4 mM and omitted for clarity. See details in the text

PCr decrease and the P_i increase, which were fast in the early phase (2.7 ± 0.2 and 2.5 ± 0.2 mM/h respectively) and slow in the late phase (0.8 ± 0.1 and 0.8 ± 0.2 mM/h, respectively). The changes of phosphate-metabolite concentration and pH_i are qualitatively explained as ATP is steadily hydrolyzed in muscle cytosol at about 2.6 mM/h, and it is completely regenerated by the ATP regenerative reactions advancing with two phases, i.e., in the early phase, ATP is exclusively regenerated by the Lohmann reaction advancing at about 2.6 mM/h and in the late phase it is partly regenerated by the Lohmann reaction advancing at about 0.8 mM/h and partly by the complete glycogenolysis advancing at about 0.6 mM glucose/h [(2.6–0.8)/3] ([4] and see below). Taking these results into consideration, attention was focused in the following experiments on how adrenaline affects the phosphate metabolism of anaerobic muscles in the early and late phases.

Figure 3 shows the metabolic changes when adrenaline was applied to CN-treated muscles near the start of the late phase ($n = 8$). Shortly after adrenaline application, P_i was decreased by 4.7 ± 0.9 mM ($P < 0.005$) and G-6P produced by 2.7 ± 0.8 mM ($P < 0.025$), compared with those without adrenaline application, clearly indicating that the glycogenolysis was advanced. The pH_i decrease by 0.101 ± 0.023 pH units ($P < 0.01$) and the PCr increase by 2.2 ± 0.8 mM ($P < 0.05$) suggest that glycogenolysis was advanced up to lactate production, and ATP was regenerated. After the transient period, the concentrations of PCr, G-6P and P_i stayed nearly unchanged, while the pH_i decrease was slowed down to 0.048 ± 0.009 pH units/h. These results suggest that ATP was exclusively regenerated by the complete glycogenolysis.

Figure 4 shows the metabolic changes when adrenaline was applied to CN-treated muscles at the start of the early phase ($n = 7$). Shortly after adrenaline application, G-6P was gradually increased up to about 3 mM ($P < 0.025$), and P_i decreased slightly to about 2 mM, although not significantly, while the pH_i was not changed significantly. These results indicate that the glycogenolysis was advanced, and P_i produced by ATPase reactions was completely incorporated into G-6P. Notably, during the transient period, PCr continued to decrease as in muscles

without adrenaline application, suggesting that ATP was not significantly regenerated by glycogenolysis, but almost regenerated by the Lohmann reaction. After the transient period, the concentrations of G-6P and P_i stayed nearly unchanged, while the PCr decrease became slow, and the pH_i increasingly shifted to an acidic direction up to 0.055 ± 0.009 pH units/h. These results suggest that the Lohmann reaction was gradually replaced by complete glycogenolysis in the regeneration of ATP.

The results for CN-treated muscles can be roughly summarized as (1) without adrenaline application, the degradation of glycogen is suppressed in the early phase and advances up to lactate production in the late phase, (2) adrenaline activates the phosphorylase step, and (3) the adrenaline-activated glycogenolysis is interrupted after the G-6P production without regenerating ATP in the early phase and advances up to lactate production in the late phase. The two-phase advancement of glycogenolysis is in accord with stepwise accumulations of lactate in resting anaerobic frog muscle examined by ^{1}H-NMR [12].

Effects of adrenaline on the phosphate metabolism of resting (IAA, CN)-treated muscles

Figure 5 shows a typical series of time-dependent NMR spectra of resting (IAA, CN)-treated muscles with adrenaline applied as indicated. Fresh (IAA, CN)-treated muscles had essentially the same NMR spectra as those of fresh CN-treated muscles. The gradual increase of F-1,6-BP, having characteristic double peaks, clearly indicates that the glycogenolysis was advanced and blocked at the glyceraldehyde 3-phosphate dehydrogenase step because of the treatment of muscles with IAA [6]. The time-dependent changes of phosphate-metabolite concentration and pH_i determined from NMR spectra similarly obtained are summarized in Fig. 6b. The results without adrenaline application are summarized in Fig. 6a as the control results. The concentration of ATP was again about 4 mM in all the (IAA, CN)-treated muscles examined with and without adrenaline applications, suggesting that ATP consumed in muscles was completely regenerated by the Lohmann reaction (Fig. 1).

Without adrenaline application, the accumulation of F-1,6-BP advanced with time showing two phases, an early and a late phases, as can be seen in Fig. 6a ($n = 7$). In the early phase, in which F-1,6-BP was not produced, the changing pattern of the phosphate-metabolite concentration and the pH_i was essentially the same as that in the early phase of CN-treated muscles, indicating that ATP was exclusively regenerated by the Lohmann reaction. In the late phase, in which F-1,6-BP was increasingly accumulated (up to about 4 mM/h), G-6P also started to be accumulated almost in parallel with, or slightly prior to, the

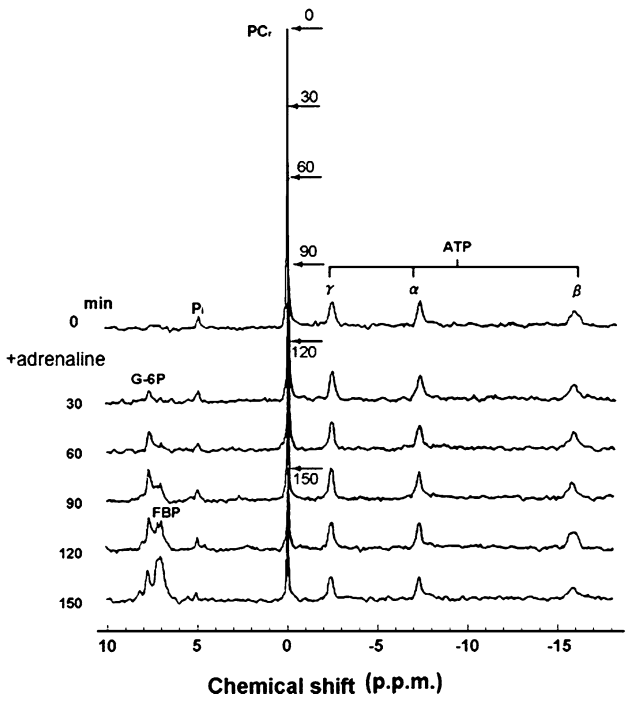

Fig. 5 A time-dependent series of ^{31}P-NMR spectra of a resting (IAA, CN)-treated muscle. Spectra are vertically shifted in the order of the time of NMR measurement. The peak height of the PCr signal for each spectrum is indicated by an *arrow* with the time of NMR measurement. Adrenaline was applied to muscle in between NMR measurements as indicated

accumulation of F-1,6-BP, and gradually leveled off at about 3 mM. On the other hand, the PCr decrease became accelerated (up to about 6 mM/h), while the P_i concentration was slowly decreased, and the alkaline shift of pH_i became slow. The changing pattern of the PCr and F-1,6-BP concentrations suggests that the glycogenolysis was advanced up to the F-1,6-BP production, and ATP consumed at the PFK step was fully regenerated by the Lohmann reaction.

Figure 6b shows the metabolic changes when adrenaline was applied to (IAA, CN)-treated muscles at the start of the early phase ($n = 7$). During the early phase, the concentrations of PCr, G-6P and P_i, and the pH_i changed nearly in the same fashion as in CN-treated muscles with adrenaline similarly applied. Notably, G-6P was accumulated up to about 4 mM ($P < 0.025$), but F-1,6-BP was not produced significantly, clearly indicating that the degradation of glycogen was advanced and interrupted before the PFK step. After the late phase started, F-1,6-BP increasingly accumulated (up to about 7 mM/h), and the PCr decrease became accelerated (up to about 10 mM/h) nearly in parallel while the P_i concentration gradually decreased, the pH_i stayed almost unchanged, and the concentration of G-6P gradually decreased after a slight increase. These results indicate that incomplete glycogenolysis (II) was increasingly advanced.

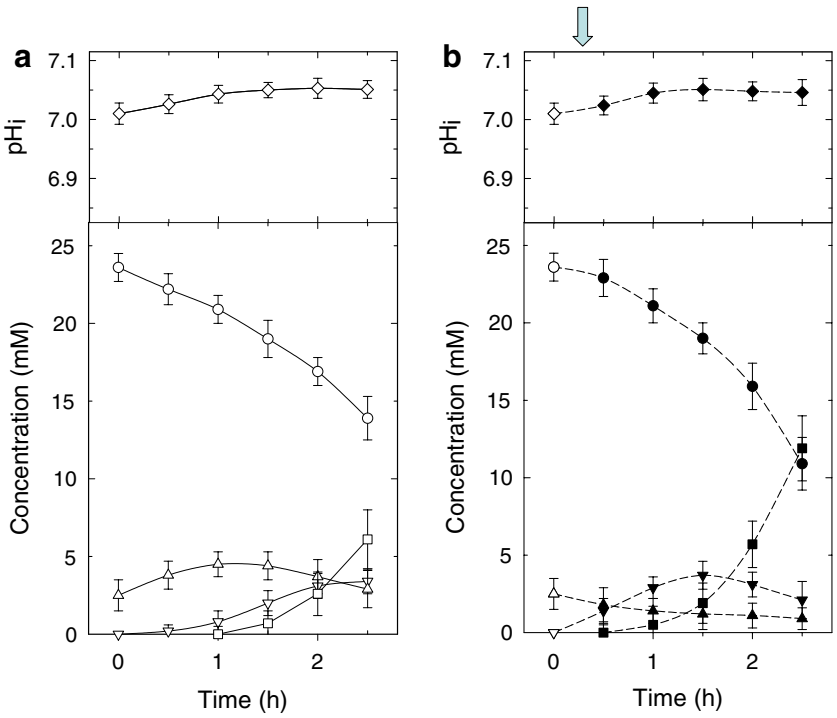

Fig. 6 Time-dependent changes of the phosphate-metabolite concentration and the pH_i in resting (IAA, CN)-treated muscles **a** without and **b** with adrenaline applications. (*Open circle*, *closed circle*) PCr; (*open triangle*, *closed triangle*) P_i; (*open inverted triangle*, *closed inverted triangle*) G-6P; (*open square*, *closed square*) F-1,6-BP; (*open diamond*, *closed diamond*) pH_i. The *open* and *solid symbols* are the data without and with adrenaline applications, respectively, and the *error bars* SEM. Adrenaline was applied to muscles at the time indicated by an *arrow* at the *top* (at the start of the early phase). The concentration of F-1,6-BP is doubled for clarity. The concentration of ATP was all about 4 mM and omitted for clarity. See details in the text

The results for (IAA, CN)-treated muscles can be roughly summarized as (1) without adrenaline application, the degradation of glycogen is suppressed in the early phase and advanced over the PFK step in the late phase, (2) adrenaline activates the phosphorylase step, and (3) the adrenaline-activated glycogenolysis does not significantly advance over the PFK step in the early phase and increasingly advances over the PFK step in the late phase.

Analysis of the advancement of phosphate metabolic reactions in CN-treated and (IAA, CN)-treated muscles

Thus, in CN-treated and (IAA, CN)-treated muscles, ATP is steadily hydrolyzed by residual ATPase reactions at about 2.6 mM/h, and it is fully regenerated by the ATP regenerative reactions advancing with two characteristic phases. Notably, the early and the late phases for CN-treated muscle and those for (IAA, CN)-treated muscle advance with a similar time scale, suggesting that the two phases for these two muscles are in comparable metabolic states. Apparently the late phase of (IAA, CN)-treated muscles started slightly earlier than that of CN-treated muscles, possibly due to artifacts associated with the treatment of muscles with IAA. Thus, we may assume that, in resting anaerobic muscle, (1) both the phosphorylase and PFK steps are suppressed in the early phase, and they are activated in the late phase, (2) adrenaline specifically activates the phosphorylase step, and (3) the adrenaline-activated glycogenolysis is interrupted at the PFK step in the early phase and advances up to lactate production in the late phase. Based on these assumptions, the pH_i changes were calculated by assuming the release of protons associated with the advancement of relevant metabolic reactions, and the calculated values are compared with the experimental results as follows.

In the early phase without adrenaline application (see Fig. 4), the Lohmann reaction advances at 2.6 mM/h to completely regenerate ATP consumed by residual ATPase reactions. The net reaction is $PCr \rightarrow Cr + P_i - 0.44H^+$, which makes the pH_i alkaline at 0.033 pH units/h [$2.6 \times (0.44)/35$]. This value is consistent with the experimental result of the alkaline shift of pH_i at about 0.034 pH units/h. When adrenaline is applied, the incomplete glycogenolysis (I) advances at 2.6 mM/h with completely incorporated P_i produced by residual ATPase reactions into G-6P. As the Lohmann reaction advances in parallel to regenerate ATP, the net reaction is glycogen (n residues) + PCr $\rightarrow$ glycogen [(n-1) residues] + Cr + G-6P $- 0.11H^+$, making the pH_i alkaline at 0.008 pH units/h ($2.6 \times 0.11/35$). This is in accord with the experimental result of almost no pH_i changes. After the transient period, the complete glycogenolysis gradually and then completely replaces the Lohmann reaction in the regeneration of ATP. Thus, the net reaction is glycogen (n residues) $\rightarrow$ glycogen [(n-1) residues] + 2lactate + $2.01H^+$ advancing at 0.9 mM glucose/h [$2.6 \times (1/3)$], making the pH_i acidic at 0.050 pH units/h [$2.6 \times (1/3) \times 2.01/35$]. This value is comparable to the experimental result of the increasing acidic shift of pH_i up to about 0.055 pH units/h.

In the late phase without adrenaline application (see Fig. 3), ATP is regenerated by the Lohmann reaction advancing at 0.8 mM/h and also by the complete glycogenolysis advancing at about 0.6 mM glucose/h. The net former reaction, the Lohmann reaction coupled with ATPase reactions, makes the pH_i alkaline at 0.010 pH units/h ($0.8 \times 0.44/35$). The net latter reaction, the complete glycogenolysis coupled with ATPase reactions, makes the pH_i acidic at 0.034 pH units/h ($0.6 \times 2.01/35$). The net pH_i change is an acidic shift at 0.024 pH units/h, which is comparable to the experimental value of an acidic pH_i shift at about 0.023 pH units/h. Shortly after adrenaline is applied, the incomplete glycogenolysis (I) advances to produce G-6P by 2.7 mM, which makes the pH_i acidic by 0.025 pH units ($2.7 \times 0.33/35$). The PCr increase of 2.2 mM is due to the backward Lohmann reaction, coupled with the complete glycogenolysis [$2.2 \times (1/3)$ mM glucose]. The net reaction is glycogen (n residues) + $3P_i$ + 3Cr → glycogen [(n-1) residues] + 3PCr + 2lactate + $3.33H^+$, which makes the pH_i acidic by 0.070 pH units. The net pH_i change is an acidic shift by 0.095 pH units, consistent with the experimental result of an acidic shift of pH_i by about 0.101 pH units. After the transient period, ATP is regenerated exclusively by the complete glycogenolysis, which shifts the pH_i to being acidic at 0.050 pH units/h. This is comparable to the experimental value of the acidic shift of pH_i at about 0.048 pH units/h.

Thus, the time-dependent pH_i changes in CN-treated muscles with and without adrenaline applications can semi-qualitatively be explained based on major phosphate metabolic reactions by assuming the two-phase advancement of the ATP regenerative reactions. The time-dependent pH_i changes in (IAA, CN)-treated muscles with and without adrenaline applications (Fig. 6a, b) can similarly be explained semi-quantitatively (the results not included).

Discussion

In ^{31}P-NMR spectra of CN-treated and (IAA, CN)-treated muscles obtained in the present studies, essential phosphate metabolites related to glycogenolysis were well resolved, with occasional trace signals of G-1P (at a shoulder of P_i peak) and of F-6P (as overlapped to F-1,6-BP peaks). As detailed above, the time-dependent changes of the phosphate-metabolite concentration and the pH_i clearly showed that the phosphorylase step of glycogenolysis was activated by adrenaline application [1], and the PFK step was further regulated, producing the two-phase advancement of ATP regenerative reactions.

It is generally documented in the literature [1] that glycogenolysis is sophisticatedly regulated at the phosphorylase and the PFK steps, the key regulatory steps of glycogenolysis, by many factors, such as ATP, pH_i and so on. For living muscles, previous NMR studies [2, 3] confirmed that the phosphorylase step is activated by Ca^{2+} released for muscle contraction, in accord with the result of in vitro study [13]. Based on the results obtained above, other factors can further be examined concerning how they are involved in the glycogenolysis regulation observed in muscles examined. Thus, ATP may not be a regulatory factor as its concentration stayed unchanged. Similarly, neither P_i nor pH_i may significantly be involved in the regulation as the magnitude of their changes was relatively small compared with that expected from in vitro experiments [14]. Remarkably, it could consistently be noted that the late phase of the ATP regenerative reactions was started, or the PFK step was significantly activated, after the PCr concentration was decreased below around 20 mM (Figs. 3, 6a, b). As it has been documented that PCr significantly suppresses PFK in this concentration range [15, 16], this strongly suggests that PCr may be involved directly at the PFK step in the regulation of glycogenolysis in muscle. On the other hand, in CN-treated muscles with adrenaline applied at the start of the early phase (Fig. 4), glycogenolysis significantly advanced over the PFK step, apparently making the start of the late phase early. This may be due to the acceleration of the PFK step by its substrate F-6P, which would be converted by phosphoglucose isomerase from G-6P accumulated by adrenaline application [1].

Other further factor(s), having low concentrations and undetectable by the present NMR measurements, could be involved in the regulation of the glycogenolysis observed above. AMP is one such candidate [1] and significantly activates both phosphorylase and PFK at the concentration of less than 1 mM [14, 17, 18]. The concentration of AMP is indirectly coupled with the PCr concentration via the Lohmann reaction plus the myokinase reaction, 2ADP ↔ ATP + AMP [10]. Therefore, as the concentration of PCr was decreased with time as observed, the AMP concentration (about 30 μM in fresh muscles [19]) would be increased and eventually activate both phosphorylase and PFK. Separately, in the late phase of (IAA, CN)-treated muscles (Fig. 6a, b), F-1,6-BP was increasingly accumulated. This could partly be due to the activation of PFK by fructose 2,6-bisphosphate (F-2,6-BP), which is converted from F-1,6-BP by PFK 2 and strongly activates PFK in the concentration range of 0.1–1 μM [1, 18].

In the present NMR studies of living muscles, the P_i concentration and the pH_i, factors affecting the force production of muscle [20–23], could be determined. As the effects of adrenaline on the muscle contractility are still not clear [24–26], it would be worth noting how these factors were affected by adrenaline application in the muscles examined above. When the adrenaline-activated glycogenolysis advanced up to the lactate production, P_i was

decreased and the pH_i acidified with the ATP concentration unchanged as shown above. As the former factor enhances the muscle contractility and the latter factor suppresses it [20–23], adrenaline would have a dual and opposite effect on the force production of muscle. As Ca^{2+} released for contraction activates the actomyosin ATPase [10] as well as glycogenolysis [2, 3], the situation would be greatly complicated in contracting muscles. Further, as the excitability of muscle fibers is also affected by various metabolites [27, 28], elaborated studies are required to clarify the effects of adrenaline on the contractility of muscle in situ.

Acknowledgments The authors would like to thank Professor M. Endo, Saitama Medical College, for his kindly informing us about important studies on the actions of adrenaline on skeletal muscle. We are also grateful to the members of the NMR facility, Teikyo University School of Medicine, for their kind cooperation.

References

1. Berg JM, Tymoczko JL, Stryer L (2002) Biochemistry. Freeman, New York, pp 577–600
2. Dawson MJ, Gadian DG, Wilkie DR (1980) Studies of the biochemistry of contracting and relaxing muscle by the use of ^{31}P-NMR in conjunction with other techniques. Philos Trans R Soc London B289:445–455
3. Yamada T, Sugi H (1987) ^{31}P-NMR study of the regulation of glycogenolysis in living skeletal muscle. Biochim Biophys Acta 931:170–174
4. Yamada T, Kikuchi K, Sugi H (1993) ^{31}P nuclear magnetic resonance studies on the glycogenolysis regulation in resting and contracting frog skeletal muscle. J Physiol 460:273–286
5. Bowman WC, Nott MW (1969) Actions of sympathomimetic amines and their antagonists on skeletal muscle. Pharmacol Rev 21:27–72
6. Padieu P, Mommaerts WFHM (1960) Creatine phosphoryl transferase and phosphoglyceraldehyde dehydrogenase in iodoacetate poisoned muscle. Biochim Biophys Acta 37:72–77
7. Danforth WH, Helmreich E, Cori CF (1962) The effect of contraction and of adrenaline on the phosphorylase activity of frog sartorius muscle. Proc Natl Acad Sci 48:1191–1199
8. Dawson MJ, Gadian DG, Wilkie DR (1977) Contraction and recovery of living muscles studied by ^{31}P nuclear magnetic resonance. J Physiol 267:703–735
9. Seo Y, Murakami M, Watari H, Imai Y, Yoshizaki K, Nishikawa H, 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
10. Woledge RC, Curtin NA, Homsher E (1985) Energetic aspects of muscle contraction. Academic Press, London, pp 167–171
11. Curtin NA (1986) Buffering power and intracellular pH of frog sartorius muscle. Biophys J 50:837–841
12. Seo Y, Yoshizaki K, Morimoto T (1983) A ^{1}H-nuclear magnetic resonance study on lactate and intracellular pH in frog muscle. Jpn J Physiol 33:721–731
13. Ozawa E, Hosoi K, Ebashi S (1967) Reversible stimulation of muscle phosphorylase b kinase by low concentrations of calcium ions. J Biochem 61:531–533
14. Reinhart GD, Lardy HA (1980) Rat liver phosphofructokinase: kinetic activity under near-physiological conditions. Biochemistry 19:1477–1484
15. Krzanowski J, Matshinsky FM (1969) Regulation of phosphofructokinase by phosphocreatine and phosphorylated glycolytic intermediates. Biochem Biophys Res Commun 34:816–823
16. Walker JB (1979) Creatine: biosynthesis, regulation, and function. In: Meister A (ed) Advances in enzymology, vol 50. Wiley, New York, pp 177–242
17. Helmreich E, Cori CF (1964) The role of adenylic acid in the activation of phosphorylase. Proc Natl Acad Sci 51:131–138
18. Uyeda K, Furuya E, Luby LJ (1981) The effect of natural and synthetic D-fructose 2, 6-bisphosphate on the regulatory kinetic properties of liver and muscle phosphofructokinases. J Biol Chem 256:8394–8399
19. Mommaerts WFHM, Wallner A (1967) The break-down of adenosine triphosphate in the contraction cycle of the frog sartorius muscle. J Physiol 193:343–357
20. Dawson MJ, Gadian DG, Wilkie DR (1978) Muscle fatigue investigated by phosphorus nuclear magnetic resonance. Nature 274:861–866
21. Dawson MJ, Gadian DG, Wilkie DR (1980) Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance. J Physiol 299:465–484
22. Cooke R, Franks K, Luciani GB, Pate E (1988) The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. J Physiol 395:77–97
23. Chase PB, Kushmerick MJ (1988) Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. Biophys J 53:935–946
24. Richter EA, Ruderman NB, Gavras H, Belur ER, Galbo H (1982) Muscle glycogenolysis during exercise: dual control by adrenaline and contractions. Am J Physiol 242:E25–E32
25. Spriet LL, Ren JM, Hultman E (1988) Adrenaline infusion enhances muscle glycogenolysis during prolonged electrical stimulation. J Appl Physiol 64(4):1439–1444
26. Ren JM, Hultman E (1989) Regulation of glycogenolysis in human skeletal muscle. J Appl Physiol 67(6):2243–2248
27. Allen D, Westerblad H (2004) Lactic acid—the latest performance-enhancing drug. Science 305:1112–1113
28. Pedersen TH, Nielsen OB, Lamb GD, Stephenson DG (2004) Intracellular acidosis enhances the excitability of working muscle. Science 305:1144–1147