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^{31}P NUCLEAR MAGNETIC RESONANCE STUDIES ON THE GLYCOGENOLYSIS REGULATION IN RESTING AND CONTRACTING FROG SKELETAL MUSCLE

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SUMMARY

1. Regulation of glycogenolysis in frog skeletal muscle at rest and following contraction was studied by measuring the concentration of phosphate-containing metabolites and the intracellular pH (pH_i) in CN-treated muscles, in which oxidative phosphorylation was inhibited by NaCN, using the ^{31}P nuclear magnetic resonance (NMR) technique.

2. When CN-treated muscles were kept at rest, the phosphocreatine (PCr) concentration very slowly decreased with time with a corresponding increase of the inorganic phosphate (P_i) concentration, while the ATP concentration remained unchanged. The pH_i changed in the alkaline direction for the first 3 h, and then started to change in the acidic direction.

3. When CN-treated muscles were tetanized for 10 s, the PCr concentration decreased with a corresponding increase of the P_i concentration and acidification of pH_i, while the ATP concentration remained unchanged.

4. When CN-treated muscles were tetanized repeatedly (each for 2 s) at constant intervals, the pH_i changed in the alkaline direction following the first and the second tetani, and then changed in the acidic direction following the subsequent tetani, indicating that the consumed ATP is first replenished by the Lohmann reaction, while glycogenolysis starts only when the total amount of contractile activity exceeds a critical value.

5. Irrespective of whether CN-treated muscles were kept at rest or tetanized repeatedly, the P_i concentration increased to about 8 mM (mmol/kg wet muscle) when glycogenolysis started, suggesting that the onset of glycogenolysis in CN-treated muscles is regulated by the P_i concentration.

6. The 'internal' buffering power of muscle cytosol was estimated to be 35 mM H^+/pH unit in anaerobic muscles and 25 mM H^+/pH unit in CN- and iodoacetic acid (IAA)-treated muscles. The 'internal' buffering power contains a contribution due to flux of carbon dioxide and lactic acid across the cell membrane. Evidence indicated that lactic acid flux is small.

INTRODUCTION

In CN-treated muscles, in which oxidative phosphorylation in mitochondria is inhibited by NaCN, the ATP consumed by contractile activities is regenerated by the Lohmann reaction, the equilibrium reaction between ATP and phosphocreatine (PCr) catalysed by creatine kinase, and glycogenolysis. The mechanism of regulation of glycogenolysis in living muscle still remains to be investigated, though *in vitro* biochemical studies have shown that there are two regulatory mechanisms: Ca^{2+} regulation at the phosphorylase step and phosphate-product regulation at the phosphofructokinase step (Newsholme & Start, 1973; Lehninger, 1982).

The technique of ^{31}P nuclear magnetic resonance (NMR) spectroscopy provides information about the concentrations of phosphate-containing metabolites and the intracellular pH (pH_i) in living muscle (Gadian, 1982). Using this technique, Dawson, Gadian & Wilkie (1980) studied glycogenolysis in CN-treated frog skeletal muscles by measuring the pH_i changes resulting from lactate production, and suggested that glycogenolysis is regulated by cytosolic Ca^{2+} released from the sarcoplasmic reticulum (SR). Yamada & Sugi (1987) also performed ^{31}P NMR studies on CN-treated frog muscles, which were treated with iodoacetic acid (IAA) to interrupt glycolysis at the glyceraldehydephosphate dehydrogenase step (Padieu & Mommaerts, 1960), and by measuring the resulting increase of sugar phosphate (SP) concentrations presented evidence that glycogenolysis is initially regulated by the inorganic phosphate (P_i) concentration produced by contractile activities before its Ca^{2+} regulation becomes apparent.

Since IAA is known to react with sulfhydryl, imidazole, thioether and amino groups of enzymes (Means & Feeney, 1971), it may be argued that, in IAA-treated muscles, IAA might react with enzymes other than glyceraldehydephosphate dehydrogenase to modify the regulatory mechanism of glycogenolysis. It may also be argued that accumulation of sugar phosphates in IAA-treated muscles might change the regulatory mechanism of glycogenolysis. To exclude the above possible side-effects of IAA on glycogenolysis, we performed ^{31}P NMR measurements of the phosphate metabolite concentrations in CN-treated frog skeletal muscles without IAA treatment, the onset of glycogenolysis being examined by measuring the pH_i, which changed in the acidic direction due to accumulation of lactic acid which dissociates to form H^+ and lactate. It will be shown that, in agreement with our previous report (Yamada & Sugi, 1987), the onset of glycogenolysis in resting as well as in contracting muscles may be regulated by the P_i concentration.

We also estimated the buffering power of muscle cytosol from the relation between the pH_i and the amount of proton (H^+) produced by the metabolic reactions in CN-treated muscles with and without IAA treatment.

METHODS

Muscle preparation

Bullfrogs (*Rana catesbeiana*) were kept at 10 °C for 1 week or more prior to experiments. After the animals were pithed, sartorius muscles (slack length, 45–50 mm) were carefully dissected from the animals and kept for 1 h in Ringer solution oxygenated with 95 % O_2–5 % CO_2 at 0 °C (pH, 7·2), which had the following composition (mM): NaCl, 95; KCl, 2·5; $CaCl_2$, 1; $MgCl_2$, 1; $NaHCO_3$, 20. In some experiments, the muscles were treated with 1 mM IAA for 1 h at 0 °C to interrupt glycolysis by inhibiting glyceraldehydephosphate dehydrogenase (Padieu & Mommaerts, 1960).

NMR measurements

The experimental set-up for the NMR measurements was the same as that described previously (Yamada & Sugi, 1988). Three muscles (total wet weight, 1·3–1·7 g) were placed vertically in a glass tube (diameter, 10 mm) fitted to an NMR probe; their pelvic ends were clamped while the tibial ends connected to a strain gauge to record isometric force. The muscles were maximally tetanized with trains of 5 ms current pulses (20 Hz) through a pair of Pt wire electrodes in contact with the muscles. During the course of the NMR measurements, the muscles were continuously superfused with Ringer solution, which contained 2 mM NaCN to inhibit oxidative phosphorylation at a flow rate of about 100 ml/min with a peristaltic pump (Cole-Parmer Inst. Co., USA). The temperature of the Ringer solution in the NMR glass tube was kept at 10 °C with a thermoelectric device unless otherwise stated. ^{31}P NMR spectra of muscles were obtained with a JEOL GX-400 NMR spectrometer (JEOL, Japan) operating at 161·8 MHz for ^{31}P nuclei with a 40 deg pulse with spectral width of 8 kHz and sampling time of 768 s (256 scans at 3 s intervals), unless otherwise stated. In order to obtain the NMR spectra after metabolic reactions resulting from contractile activities were complete, the accumulation of the spectra was usually started 7 min after each tetanus. The concentration of each phosphate-containing metabolite was determined by integrating the corresponding peak in the NMR spectra, assuming the concentration of PCr in fresh resting muscles to be 27 mM (mmol/kg wet muscle) (Dawson, Gadian & Wilkie, 1977). The chemical shift was expressed in p.p.m. with reference to that of PCr.

Saturation factors for PCr, P_i and SP were determined for CN-treated muscles (either with or without IAA treatment), in which these phosphates compounds were stably produced by contractile activities. The factors thus obtained were 1·03, 1·17 and 1·12 for PCr, P_i and SP respectively, and these values were used for data correction.

Estimation of pH_i is based on the pH-dependent chemical shift of the P_i peak in ^{31}P NMR spectra (Dawson *et al.* 1977). As the P_i peaks were broad and often not symmetric, we defined the P_i peak position as that where the P_i peak was divided into two equal areas.

Calculation of the amount of H^+ produced by major metabolic reactions

In order to study the progress of metabolic reactions in muscles based on the pH_i changes, we have to know the amount of H^+ produced. We therefore calculated the amount of H^+ produced by major metabolic reactions in anaerobic muscles, assuming the values of the ionization constant for relevant metabolites to be as follows: P_i, 6·88; ATP, 6·5; ADP, 7·0; PCr, 4·6; lactic acid, 3·9; dihydroxyacetone phosphate (dihydroxyacetone-P) and glyceraldehyde-3-phosphate (glyceraldehyde-P), 6·5; glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and fructose-1,6-diphosphate (FDP), 6·1 (Sober & Harte, 1968; Davson, Elliott, Elliott & Jones, 1969; Dawson *et al.* 1977). For the sake of convenience, the pH_i of CN-treated muscles was taken to be 7·08 although the pH_i of muscles actually ranged from 7·0 to 7·2 during the experiments. The results of the calculations are as follows:

ATP-splitting reaction,

$$ATP \rightarrow ADP + P_i + 0{\cdot}37\ H^+; \quad (1)$$

Lohmann reaction,

$$PCr + ADP \leftrightarrow ATP + Cr - 0{\cdot}75\ H^+, \quad (2)$$

where Cr represents creatine;

glycogen degradation reactions (up to lactate production),

$$1/3n(C_6H_{10}O_5)_n + ADP + P_i \rightarrow ATP + 2/3\,(\text{lactate}) + 0{\cdot}30\ H^+; \quad (3)$$

glycogen degradation reactions (after IAA treatment),

$$1/n(C_6H_{10}O_5)_n + P_i \rightarrow \text{G-1-P (or G-6-P or F-6-P)} + 0{\cdot}29\ H^+, \quad (4)$$

or

$$1/n(C_6H_{10}O_5)_n + ATP + P_i \rightarrow FDP + ADP + 0{\cdot}05\ H^+, \quad (5)$$

or

$$1/n(C_6H_{10}O_5)_n + ATP + P_i \rightarrow \text{glyceraldehyde-P (or dihydroxyacetone-P)} + ADP - 0{\cdot}07\ H^+. \quad (6)$$

Buffering power

Buffering power is defined as the ratio of the amount of H^+ produced in muscle cytosol to the pH_i change (in mM per pH unit) (Curtin, 1986).

RESULTS

Time-dependent changes of ^{31}P NMR spectra of CN-treated muscles at rest

A typical ^{31}P NMR spectrum of fresh CN-treated muscles at rest is shown in Fig. 1*A*. As indicated by a barely noticeable P_i peak, the concentration of P_i was low,

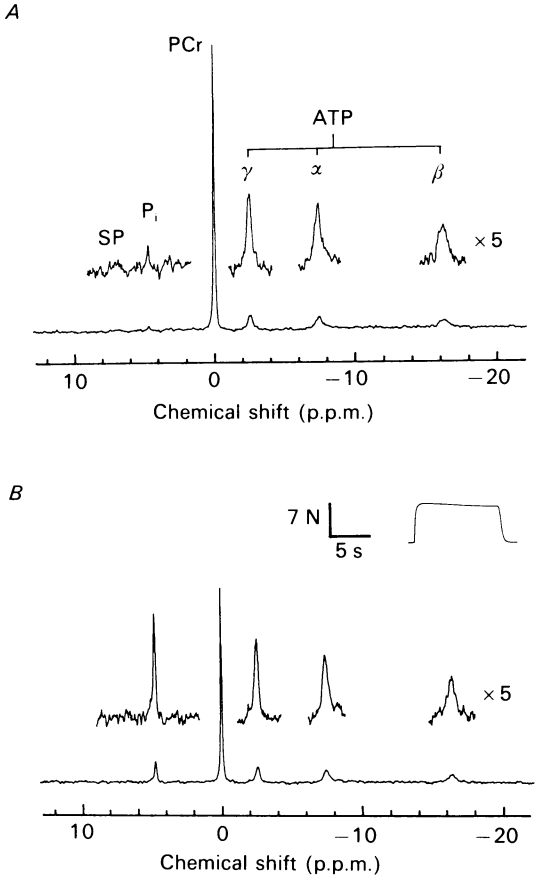

Fig. 1. Typical ^{31}P NMR spectra from fresh CN-treated skeletal muscles at rest (*A*) and after a 10 s tetanus (*B*). Inset spectra are vertically enlarged five times to show clearly the P_i peak and the three ATP peaks (α, β and γ). A tension record of a 10 s tetanus is also shown. Note a marked increase of the P_i peak with a corresponding decrease of the PCr peak after the tetanus, while the ATP peaks remain virtually unchanged. SP, sugar phosphate. Number of scans, 384 at 3 s intervals. Temperature, 10 °C.

$1{\cdot}2 \pm 0{\cdot}3$ mM (mean $\pm$ S.E.M.; $n = 17$), and the pH_i was $7{\cdot}08 \pm 0{\cdot}02$ ($n = 17$). The P_i concentration is in accord with that determined by direct chemical analysis (Seraydarian, Mommaerts, Wallner & Guillory, 1961). The pH_i also agrees well with that measured by ^{31}P NMR (Dawson *et al.* 1977; Tanokura & Yamada, 1984). No significant peaks for SP were observed in fresh CN-treated muscles at rest.

When CN-treated muscles were kept standing at rest over several hours, the P_i concentration was found to exhibit a very slow increase with time with a

corresponding decrease of PCr concentration, indicating the occurrence of ATP-splitting reactions coupled with processes other than contractile activity. To measure the time-dependent P_i and PCr concentration changes more accurately, the temperature of Ringer solution was raised from 10 to 20 °C. The changes of ^{31}P NMR

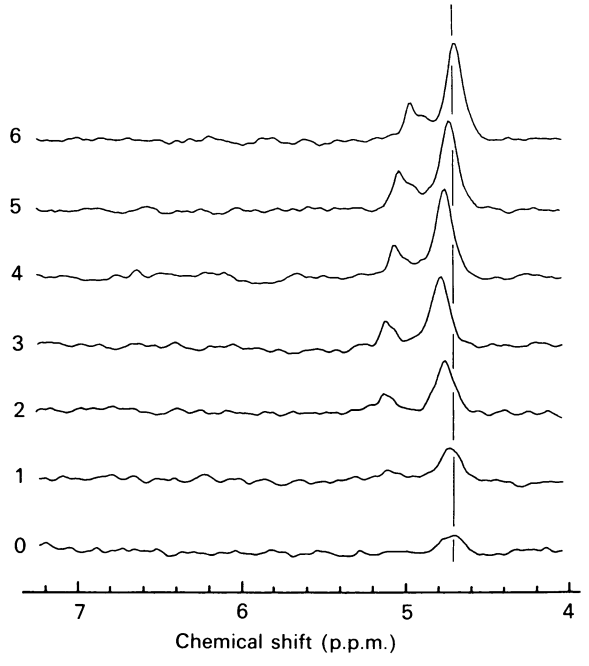

Fig. 2. Time-dependent changes of ^{31}P NMR spectra of CN-treated muscles at rest in the range of 4–7 p.p.m. The time of recording of the spectra (hours) is shown alongside each spectrum. Vertical line shows the initial position of the P_i peak. Temperature, 20 °C.

spectra (4–7 p.p.m.) in a typical experiment are shown in Fig. 2 and the results obtained from seven different muscles are summarized in Fig. 3. The P_i concentration increased at a rate of about 0·04 mM/min with a corresponding decrease of the PCr concentration, while the ATP concentration remained unchanged. The pH_i first shifted in the alkaline direction, but after about 3 h when the P_i concentration increased to about 8 mM, the pH_i turned to shift in the acidic direction. The rate of both the decrease in the PCr concentration and the increase in the P_i concentration decreased after about 3 h when the direction of the pH_i change turned from alkaline to acidic. A small G-1-P peak started to appear after about 2–3 h and slowly increased. No peaks appeared in the spectral range of 6–7 p.p.m. where the other SP peaks are located. None of the muscles studied showed tension development during the course of NMR measurements.

Changes in ^{31}P NMR spectra of CN-treated muscles following contractile activities

When CN-treated muscles were tetanized for 10 s, ^{31}P NMR spectra changed as shown in Fig. 1*B*. The P_i peak became much more prominent, reflecting a marked increase in the P_i concentration, while the PCr concentration showed a corresponding

decrease. The ATP concentration was 4·3 ± 0·5 mM (mean ± S.E.M.; $n = 17$), and did not change appreciably after a 10 s tetanus nor after any of the kinds of contractile activity used in the present study.

In Fig. 4 are shown the PCr concentration and pH_i changes in a typical experiment in which CN-treated muscles were tetanized two times (10 s for each) at an interval

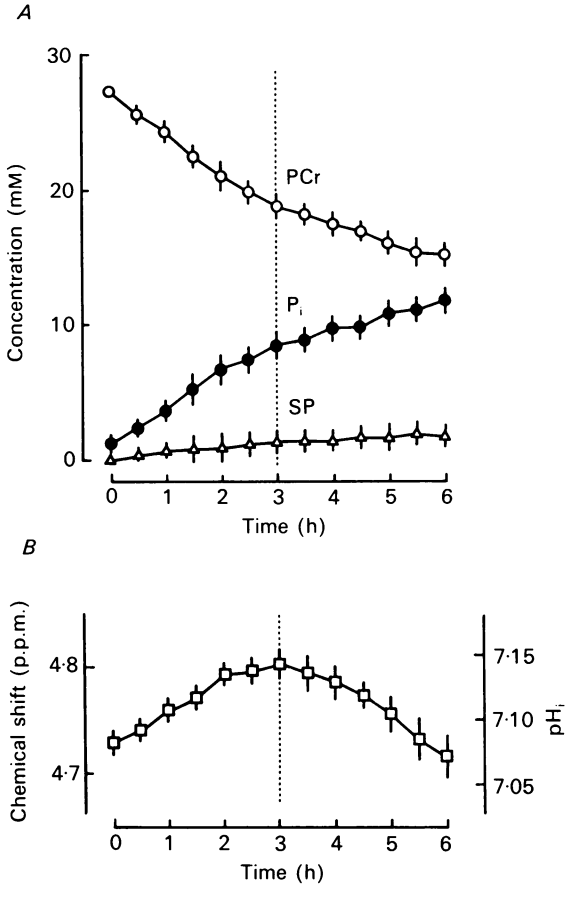

Fig. 3. Changes of PCr (○), P_i (●) and SP (△) concentration (*A*) and of pH_i (*B*) in CN-treated muscles at rest as a function of time obtained from the experiments as shown in Fig. 2. Each data point with vertical bar represents mean ± S.E.M. ($n = 7$). Temperature, 20 °C.

of 150 min. Each tetanus caused an increase in the P_i concentration and a corresponding decrease in the PCr concentration, both of which were nearly completed within the time resolution of the measurements (5–10 min) after the end of each tetanus. Following the first tetanus, the pH_i showed a rapid transient shift in the alkaline direction (by about 0·03 pH units) before changing in the acidic direction. Following the second tetanus, however, the pH_i changed only in the acidic direction. In both cases, the acidified pH_i gradually returned to the original level with a half-time of about 70 min. This may be due to a leaking out of the produced

lactic acid through the muscle cell membrane (Mainwood & Worsley-Brown, 1975; Mason & Thomas, 1988). Similar results were obtained in five other experiments with different muscles. The above results indicate that the energy state of CN-treated muscles can be changed in a stepwise fashion by appropriately tetanizing them repeatedly.

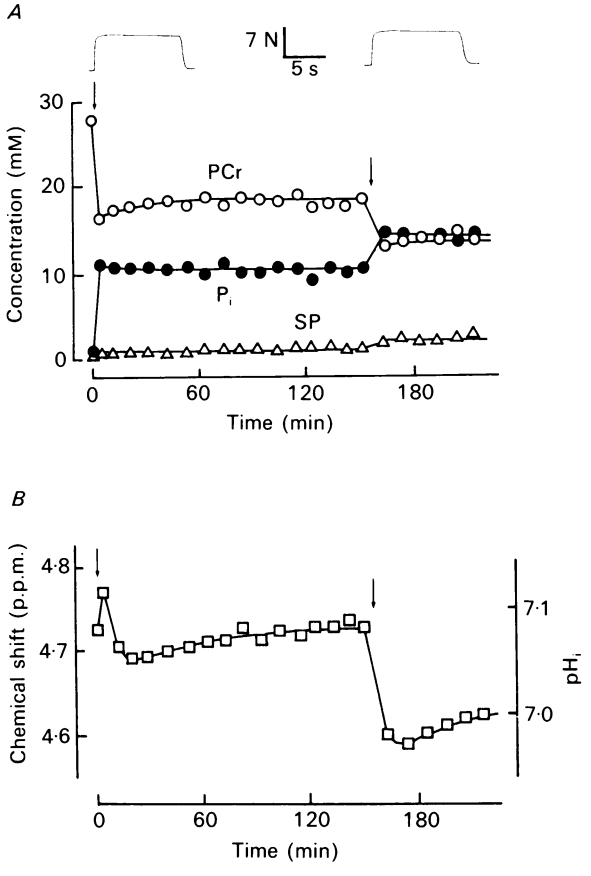

Fig. 4. Changes in the PCr (○), P_i (●) and SP (△) concentrations (*A*) and in pH_i (*B*) in CN-treated muscles following two successive 10 s tetani at an interval of 150 min. Each arrow indicates time of onset of tetanic stimulation. The records shown in *A* and *B* were obtained from the same experiment (128 scans at 3 s intervals). Isometric force records of the first and second 10 s tetani are shown at the top of the figure. Temperature, 10 °C.

To study how glycogenolysis in CN-treated muscles is affected by the concentration of metabolites produced by contractile activities in detail, the muscles were tetanized repeatedly (2 s for each) at intervals of 20 min. Figure 5 is a typical example showing the changes of ^{31}P NMR spectra (4–7 p.p.m.) following each 2 s tetanus. The P_i peak increased in magnitude as tetani were repeated, while its chemical shift changed in the alkaline direction following the first and the second tetanus, and then continued to change in the acidic direction following the third to the sixth tetani. A trace shoulder of G-1-P started to appear after the fifth tetanus and slightly increased after

the sixth tetanus. No peaks appeared in the spectral range of 6–7 p.p.m. where the other SP peaks are located. These features are similar to those observed in the NMR spectra of CN-treated muscles at rest except for the difference in time scale. The magnitude of peak tetanic tension developed in the second to the sixth tetani

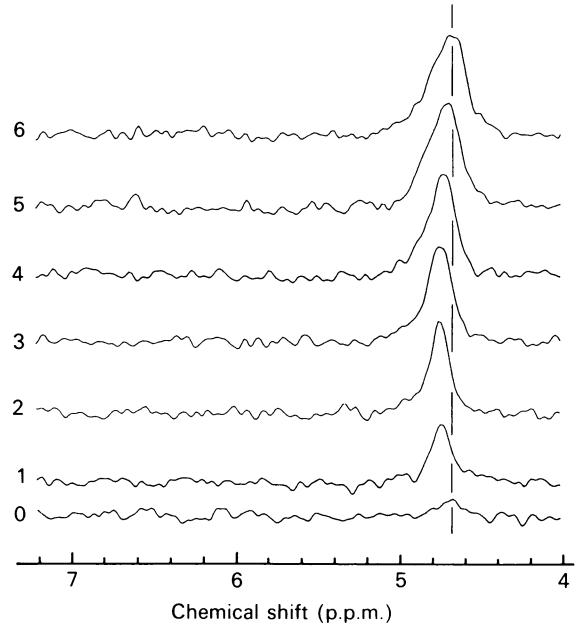

Fig. 5. Changes in ^{31}P NMR spectra of CN-treated muscles in the range of 4–7 p.p.m. following consecutive 2 s tetani at intervals of 20 min. The sequence of recording the spectra after each tetanus is shown alongside each spectrum. Temperature, 10 °C.

(relative to that of the first) was $93 \pm 0{\cdot}8\%$, $89 \pm 1{\cdot}9\%$, $87 \pm 2{\cdot}2\%$, $84 \pm 2{\cdot}5\%$, and $83 \pm 3{\cdot}0\%$ (means $\pm$ S.E.M.; $n = 9$) respectively.

In Fig. 6 are summarized the changes of the PCr, P_i and SP concentrations and of the pH_i in CN-treated muscles as a function of the number of consecutive 2 s tetani. As the muscles were tetanized repeatedly, the rates of both the decrease in the PCr concentration and the increase in the P_i concentration decreased after the third tetanus, and at the same time the direction of the pH_i change turned from alkaline to acidic.

DISCUSSION

Relative contribution to the replenishment of ATP of the Lohmann reaction and glycogenolysis depending on contractile activities

The present ^{31}P NMR studies on CN-treated frog skeletal muscles, in which oxidative phosphorylation is inhibited by NaCN, have demonstrated how the complete replenishment of ATP following contractile activities is made by the Lohmann reaction and glycogenolysis, depending on the amount of contractile activity. The Lohmann reaction (2) is reversible and fast (about 1·6 mM/s) (Gadian,

Radda, Brown, Chance, Dawson & Wilkie, 1981) so that it quickly buffers the ATP concentration changes in muscle cytosol. Thus, following contractile activities, the consumed ATP is immediately replenished by the Lohmann reaction. When the ATP concentration remains constant, the net result of the ATP-splitting reaction and the

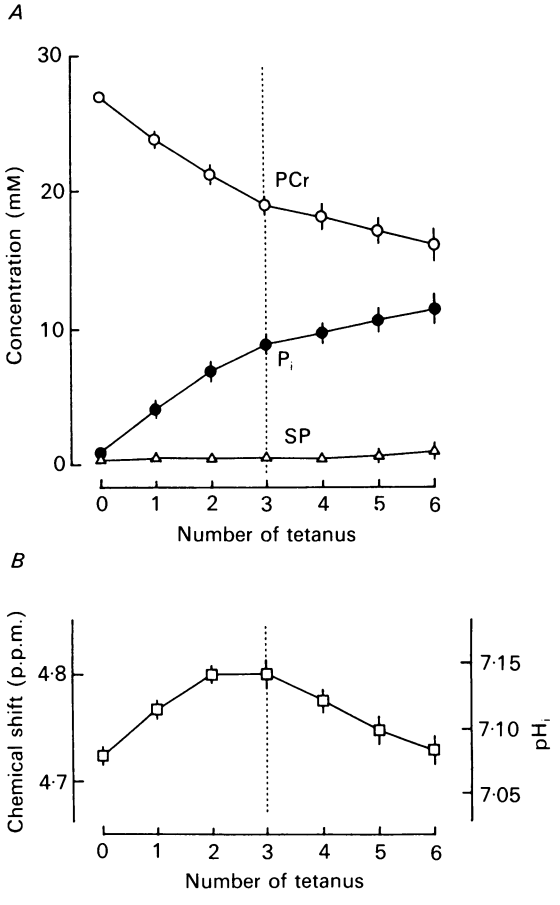

Fig. 6. Changes in PCr (○), P_i (●) and SP (△) concentrations (*A*) and in pH_i (*B*) in CN-treated skeletal muscles as a function of the number of consecutive 2 s tetani given at intervals of 20 min obtained from the experiments as shown in Fig. 5. Bars represent the S.E.M. of nine experiments with different muscles. Temperature, 10 °C.

forward Lohmann reaction (reactions (1) and (2) respectively, in Methods) can be written as

$$PCr \rightarrow Cr + P_i - 0{\cdot}38\ H^+, \tag{7}$$

thus changing the pH_i in the alkaline direction. Glycogenolysis, on the other hand, changes the pH_i in the acidic direction due to lactic acid production and dissociation. On this basis, the result that the initial transient pH_i shift in the alkaline direction was observed following the first 10 s tetanus but not following the second one (Fig. 4*B*) indicates that the relative contribution of the Lohmann reaction to the

replenishment of ATP decreases as the total amount of contractile activity is increased. This idea is consistent with the result that both the decrease in the PCr concentration and the corresponding increase in the P_i concentration are less following the second tetanus than the first (Fig. 4*A*).

The relative contribution of the Lohmann reaction and glycogenolysis to the ATP replenishment could be studied in more detail by repeatedly tetanizing CN-treated muscles (2 s for each) at appropriate intervals. Following the first and second 2 s tetani, the pH_i changed in the alkaline direction, while it changed in the acidic direction following the third to the sixth tetani (Figs 5 and 6). These results clearly indicate that, in agreement with our previous report (Yamada & Sugi, 1987), glycogenolysis starts only when the total amount of contractile activity exceeds a critical value; with moderate contractile activity, the consumed ATP is replenished only by the Lohmann reaction.

Regulation of glycogenolysis by the P_i concentration

An interesting finding brought about in the present study is that, when CN-treated muscles were kept at rest for many hours, the PCr and P_i concentrations and the pH_i exhibited time-dependent changes (Figs 2 and 3) similar to those observed in CN-treated muscles following repeated tetani (Figs 5 and 6), except for the differences in time scale; in both cases, the pH_i started to change in the acidic direction when the P_i concentration increased to about 8 mM. The results indicate that, irrespective of whether CN-treated muscles are kept at rest or tetanized repeatedly, glycogenolysis starts when the P_i concentration increases to about 8 mM. Since the intracellular Ca^{2+} concentration in resting skeletal muscle is kept below 10^{-7} M (Ebashi & Endo, 1968), the regulation of glycogenolysis by the elevated intracellular Ca^{2+} concentration during contractile activities (Dawson *et al.* 1980) may not work at the onset of glycogenolysis. It has been known that P_i activates phosphorylase with a Michaelis–Menten constant (K_m) of about 7·5 mM (Morgan & Parmeggiani, 1964). On this basis, the present results indicate that P_i may regulate the phosphorylase step of glycogenolysis in both resting and contracting CN-treated frog skeletal muscles. In conclusion, the onset of glycogenolysis in frog skeletal muscle is regulated by the P_i concentration; Ca^{2+} regulation of glycogenolysis may function after contractile activities exceed a critical amount.

Buffering power of muscle cytosol estimated from the pH_i changes

When glycogenolysis starts to replenish the consumed ATP, the net reaction (7) first takes place and the glycogenolytic reactions follow (Fig. 4). When the ATP concentration is constant, the net reaction of glycogenolysis (reaction (3)) to produce ATP and lactate and backward Lohmann reaction (2) can be written as,

$$1/3n(C_6H_{10}O_5)_n + Cr + P_i \rightarrow PCr + 2/3\,(\text{lactate}) + 1{\cdot}05\ H^+. \quad (8)$$

The result that the increase in the P_i concentration became less steep and the pH_i change turned from the alkaline to the acidic direction after about 3 h at rest or following the third to the sixth 2 s tetani (Figs 4 and 6) can be accounted for by the above reactions (although the former is attributable partly to the fatigue which is manifested by the gradual decrease in the magnitude of the tetanic tension). When

glycogenolysis is interrupted by IAA at the glyceraldehydephosphate dehydrogenase step, the net reactions of glycogenolysis, in which the ATP concentration is also constant, can be expressed either as reaction (4),

$$1/n(C_6H_{10}O_5)_n + P_i \rightarrow \text{G-1-P (or G-6-P or F-6-P)} + 0{\cdot}29\ H^+, \quad (4)$$

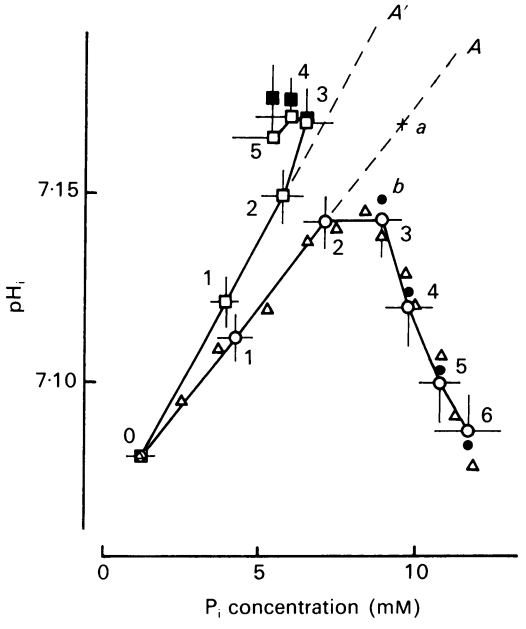

Fig. 7. Changes in pH_i as a function of P_i concentration in resting and repeatedly contracting (2 s tetani) CN-treated skeletal muscles. ○ and □ respectively represent data for muscles without and with IAA treatment, which were re-plotted from the data of Fig. 6 and similar data for IAA-treated muscles. The sequence of the tetanus is shown alongside each data point. The values of pH_i for muscles with IAA treatment were shifted by about 0·01 pH units so that the original pH_i value is equal to that of muscles without IAA treatment. △ represent data for muscles at rest, which were re-plotted from the data of Fig. 3 from which error bars were removed for clarity. ● and ■ respectively represent calculated points based on glycogenolytic reactions assumed for muscles without and with IAA treatment. For further explanation, see text.

or the sum of reaction (5) and forward Lohmann reaction (2),

$$1/n(C_6H_{10}O_5)_n + PCr + P_i \rightarrow FDP + Cr - 0{\cdot}70\ H^+; \quad (9)$$

or the sum of reaction (6) and forward Lohmann reaction (2),

$$1/n(C_6H_{10}O_5)_n + PCr + P_i \rightarrow \text{glyceraldehyde-P (or dihydroxyacetone-P)} + Cr - 0{\cdot}82\ H^+. \quad (10)$$

The above analyses indicate that the progress of the metabolic reactions relevant to the ATP replenishment can be estimated from the P_i concentration changes. Thus in Fig. 7 the pH_i changes as a function of the P_i concentration are summarized for both resting and contracting CN-treated muscles and for contracting CN- and IAA-

treated muscles. It should be noted that the plot for resting muscles almost overlaps with that for contracting CN-treated muscles. When glycogenolysis does not yet take place following ATP-splitting activities, net reaction (7) occurs, where 0·38 mM H^+ is absorbed per mole of P_i produced. The alkalinization of muscle cytosol following stimulation was first reported by Tanokura & Yamada (1984) using ^{31}P NMR. This situation should correspond to the straight line *A* obtained by extrapolating the P_i concentration *versus* pH_i plot following the first and second 2 s tetani of CN-treated muscles in which glycogenolysis does not take place as discussed above. Each 2 s tetanus increases the P_i concentration by 3·0 mM with the pH_i increase of 0·033 pH units. Thus, we obtain the buffering power of muscle cytosol of 35 $(0{\cdot}38 \times 3{\cdot}0/0{\cdot}033) \pm 4$ mM/pH unit. Similarly, we obtain a value of about 25 ± 4 mM/pH unit for the buffering power of CN- and IAA-treated muscles from the corresponding straight line *A'*.

As the bicarbonate–CO_2 buffering system was used in the present experiments and the membrane of muscle cells is permeable to CO_2, the buffering power obtained above is not the intrinsic buffering power of muscle cytosol but includes that of the bicarbonate–CO_2 buffering system. Therefore we refer to the buffering power obtained in the present studies as the 'internal' buffering power of muscle cytosol.

Using the above internal buffering powers of muscle cytosol, the pH_i changes during the progress of glycogenolysis can be estimated. Following the second 2 s tetanus in CN-treated muscle, the P_i concentration and the pH_i are located at a point represented by ○ and 2 in Fig. 7. The third 2 s tetanus produces 2·7 mM P_i if we assume that the amount of ATP split is proportional to the magnitude of peak tetanic tension (i.e. $3{\cdot}0 \times 0{\cdot}89 = 2{\cdot}7$). If glycogenolysis does not take place, the P_i concentration and the pH_i is expected to shift along the line *A* to a point represented by + and *a* following the third tetanus; i.e. P_i, 9·7 (7·0+2·7) mM; pH_i, 7·171 $(7{\cdot}142 + 0{\cdot}033 \times 0{\cdot}89)$. As the actual concentration of P_i (○ and 3) is 9·0 mM, 0·7 (9·7−9·0) mM of P_i is expected to have been incorporated into the glycogenolytic pathway where the net reaction (8) produces 1·05 mM of H^+ per mole of incorporated P_i. As the internal buffering power of muscle cytosol is 35 mM/pH unit, this results in the pH_i decrease by 0·021 $(0{\cdot}7 \times 1{\cdot}05/35)$ units to a point represented by ● and *b*. Similar calculations made for the subsequent 2 s tetani produce a series of points (●) closely located to corresponding experimental results (○). In CN- and IAA-treated muscles, the production of SP is accelerated following the third to the fifth 2 s tetanus (Yamada & Sugi, 1987), and we have found that the SP mainly consists of F-6-P. Therefore, the calculations based on reactions (4) and (7) and the internal buffering power of muscle cytosol of 25 mM/pH unit produce a series of points (■) which also fit well with the experimental results.

It is also known that lactic acid leaks out of the muscle cells. Although the leakage rate is rapid from the surface fibres of a whole muscle (Mason & Thomas, 1988), it is relatively slow from the whole muscle (Fig. 4*B* of the present paper; Mainwood & Worsley-Brown, 1975). The correction made for the leaking out of lactic acid with a half-time of 70 min (Fig. 4*B*) does not produce substantial changes in the above discussion. Anyway the quantitative agreement of the calculated results with experimental data suggests the validity of the present analysis for major metabolic reactions taking place in resting and contracting CN-treated bullfrog sartorius

muscles. In any case, the near overlap of the plot for resting and contracting muscles in Fig. 7 strongly suggests that glycogenolysis is regulated by a common major mechanism in the two muscles; i.e. it may be regulated by the P_i concentration as discussed above.

By the use of the pH-sensitive microelectrode, Bolton & Vaughan-Jones (1977) and Curtin (1986) determined the internal buffering power of the cytosol of frog skeletal muscle to be about 35 and 38 mM/pH unit respectively, a value similar to that in CN-treated frog muscles obtained in the present study. Curtin (1986) further estimated the contribution to the buffering power of various metabolites to be about 20 mM/pH unit, predicting that the remaining buffering power comes from intracellular organelles. The results presented here show that the buffering power of muscle cytosol was significantly reduced by IAA treatment. As IAA is known to modify sulfhydryl, imidazole, thioether and amino groups of proteins in various organelles (Means & Feeney, 1971), the result is consistent with the idea that amino acid residues in intracellular proteins and organelles may contribute to the buffering power of muscle cytosol. Tanokura & Yamada (1984), on the other hand, estimated the internal buffering power of frog skeletal muscle cytosol to be 18 mM/pH unit by tetanizing muscles repeatedly and analysing the transient pH-dependent chemical shift of P_i in ^{31}P NMR spectra. A reason for the discrepancy between their and our values might be due to the difference in the experimental conditions; i.e. they used unpoisoned muscles in aerobic conditions.

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