A gated ^{31}P -n.m.r. study of bioenergetic recovery in rat skeletal muscle after tetanic contraction

R. A. John CHALLISS,*† Martin J. BLACKLEDGE,* Eric A. SHOUBRIDGE*§ and George K. RADDA*

*Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, and †Department of Pharmacology, University of Leicester, University Road, Leicester LE1 9HN, U.K.

1. Gated ^{31}P -n.m.r. spectra were obtained from the ankle flexor muscles of the rat at various times after 3 s isometric tetanic contraction. This allowed the time course of changes in phosphocreatine (PCr), P_i and free ADP concentrations and intracellular pH to be monitored in skeletal muscle *in vivo* with 1 s time resolution. 2. ATP concentration did not change significantly, either during the recovery from a 3 s tetanus or during the overall protocol. 3. The calculated rate of recovery of ADP towards pre-stimulation levels was very rapid ($t_{1/2} < 5$ s). The rate of P_i disappearance ($t_{1/2} = 14$ s) was more rapid than the rate of PCr synthesis ($t_{1/2} = 24$ s), resulting in a significant transient decrease in n.m.r.-visible PCr + P_i between 25 and 45 s after tetanic contraction. 4. The rates of PCr, P_i and ADP recovery are higher than those previously reported for recovery from steady-state exercise in humans or twitch isometric contraction in animals.

INTRODUCTION

The use of ^{31}P -n.m.r. spectroscopy allows continuous non-invasive monitoring of phosphorus-containing metabolites and intracellular pH in skeletal muscle of living animals at rest, during muscle contraction or in the recovery phase (Meyer *et al.*, 1982; Taylor *et al.*, 1983). The spectral acquisition time generally reported in such studies is > 30 s and is governed by a number of factors, including the mass of muscle interrogated (which in turn is determined by the dimensions and positioning of the radiofrequency transmitter/receiver coil), the magnetic-field strength and the signal/noise requirement of the experiment to allow confident quantification of spectra obtained (Taylor *et al.*, 1983). Although the achieved time resolution is adequate for studying metabolism in resting muscle or during steady-state exercise, it is not optimal for investigating metabolic transitions that occur upon initiation of, or on recovery from, exercise.

In this respect there is disagreement as to the time courses of the recovery of phosphocreatine (PCr), P_i , ADP concentration and intracellular pH in skeletal muscle after contraction. It has been reported that the rate of PCr resynthesis occurs at a slower rate than the rate of increase in P_i in flexor digitorum superficialis muscle of the human forearm (Taylor *et al.*, 1983; Arnold *et al.*, 1984; Keller *et al.*, 1985; Molé *et al.*, 1985; Caton *et al.*, 1987), and in the rat ankle flexor muscles (Shoubridge & Radda, 1984), whereas other workers have reported a precise stoichiometric relationship between changes in P_i and PCr concentrations (Kushmerick & Meyer, 1985).

A system has recently been reported for quantifying changes in phosphorus-containing metabolites and intracellular pH in rat muscles during tetanic contraction by gated ^{31}P -n.m.r. spectral acquisition (Shoubridge & Radda, 1987). In the present study this method has been used to monitor the time course of changes in PCr, P_i ,

ATP, ADP concentration and intracellular pH in the ankle flexor muscles of the rat *in vivo*, immediately after tetanic contraction.

MATERIALS AND METHODS

Male Wistar rats (200–220 g) were obtained from sources given previously and were anaesthetized and prepared for sciatic-nerve stimulation as described by Challiss *et al.* (1986, 1987). A Helmholtz coil, used as transmitter and receiver, was placed around the ankle flexor muscles of the prepared limb and was tuned to the ^{31}P resonance frequency. The animal in the n.m.r. probe was placed in the vertical bore of a wide-bore 4.3T magnet. Anaesthesia was maintained by delivering 1% halothane in $\text{N}_2\text{O}/\text{O}_2$ (1:1) via a face mask, and body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ by blowing warm air through the magnet bore. Spectra were collected as 1024 data points using a sweep-width of ± 1500 Hz and were zero-filled to 2048 data points. All spectra were collected under non-saturating conditions using a nominal 90° tip angle (24 μs pulse). All spectra were referenced to the proton resonance of tissue water, which was set at zero, after adjusting the magnetic-field homogeneity. Spectra were quantified by integration, and the pH was determined from the chemical shift of P_i (Gadian *et al.*, 1979).

The acquisition trigger of the spectrometer was gated to specific time intervals after the application of the muscle stimulation pulse. Two series of experiments were performed: in the first, spectral acquisition was gated to occur 5, 25, 45 and 90 s after each 3 s tetanus (Series I); in the second, two sets of acquisitions were interleaved such that acquisition occurred after 2.9 s of tetanic contraction (taken as zero-time) or 1, 15, 30, 45 and 90 s after each 3 s tetanus (Series II). For each experiment performed, fully relaxed spectra were obtained before

Abbreviations used: PCr, phosphocreatine; PME, phosphomonoester.

‡ To whom correspondence and reprint requests should be sent, at the University of Leicester address.

§ Permanent address: Neuromuscular Research Group, Montreal Neurological Institute and Hospital, 3801 University, Montreal, Quebec, Canada H3A 2B4.

Fig. 1. Gated acquisition of ^{31}P -n.m.r. spectra from ankle flexor muscles during recovery from a 3 s isometric tetanus

Tetanic contraction of the ankle flexor muscles was induced by sciatic-nerve stimulation (100 Hz, 100 μs pulse-width, 15 V supramaximal voltage). Gated spectral acquisition occurred 5, 25, 45 and 90 s after 3 s tetani. The experiment shown consisted of 16 tetani separated by 2 min with spectral acquisition after the subsequent 48 tetani. Spectral parameters: 24 μs pulse-width, 1024 data points, zero-filled to 2048, Lorentzian line-broadening of 10 Hz, 3000 Hz spectral window.

initiating muscle stimulation and 30 min after the end of the experimental procedure. Between each 3 s tetanus, the muscle was allowed to recover for > 120 s (120 s + acquisition storage time); this was sufficient to allow full PCr recovery, but clearly not full recovery of the muscle to the resting state. Each experiment consisted of either 64 (Series I) or 80 (Series II) tetani; after 16 tetani the tension developed during each tetanus was constant, and gated ^{31}P -n.m.r. spectral acquisition was initiated [i.e. 48 (Series I) or 2×32 (Series II) scan data blocks were accumulated].

At the end of each experiment the gastrocnemius/plantar muscles from both the stimulated and contralateral limbs were freeze-clamped in aluminium tongs pre-cooled in liquid nitrogen. Perchloric acid extracts were prepared as described previously (Challiss *et al.*, 1986, 1987) and adenine nucleotides, creatine and PCr concentrations were determined by h.p.l.c. analysis

(Harmsen *et al.*, 1982). Resonance intensities were determined by cutting the peaks from the plotted spectra and weighing them; the weight of the β -ATP peak was assumed to be equal to the ATP concentration determined by h.p.l.c. (Taylor *et al.*, 1983). Free ADP concentrations were calculated by assuming the near-equilibrium nature of the creatine phosphokinase reaction (Lawson & Veech, 1979).

RESULTS AND DISCUSSION

Fundamental to the understanding of muscle bioenergetics is the coupling of the rate of ATP resynthesis to utilization during the contraction/relaxation cycle. ^{31}P -n.m.r. spectroscopy provides a unique opportunity for obtaining quantitative information *in vivo* on the changes in PCr, P_i , ATP and intracellular pH during this process.

Representative ^{31}P -n.m.r. spectra collected during recovery from a 3 s tetanus are shown in Fig. 1. In this series of experiments, spectra were acquired 5, 25, 45 and 90 s after each of 48 consecutive 3 s tetani. For either series of acquisitions, the minimum delay between excitation pulses was 14 s, and therefore all spectra were collected under essentially non-saturating conditions (Taylor *et al.*, 1983).

Quantification of the β -ATP peak showed that no change in ATP concentrations occurred during the recovery period or between the start and end of the experimental protocol. This latter finding was confirmed by determination of ATP concentrations in extracts of stimulated and contralateral gastrocnemius/plantar muscles [control: 6.57 ± 0.13 (9); stimulated: 6.42 ± 0.24 (9) μmol of ATP/g of tissue].

In all spectra acquired 90 s after tetanus, the intracellular P_i resonance was partially overlapped by a downfield resonance; the two resonances could be resolved by reducing the line broadening during spectral processing. The pH of this overlapping peak was 7.38 ± 0.02 (5), and we tentatively assign this resonance as extracellular P_i . The presence of this resonance is probably due to the hyperaemic response in the post-tetanic phase (Mackie & Terjung, 1983). In all experiments the composite P_i resonance was well resolved from the small phosphomonoester (PME) peak (chemical shift relative to PCr: PME, 6.83–6.88 p.p.m.; extracellular P_i , 5.22–5.26 p.p.m.). At acquisition time > 25 s after the tetanus this downfield resonance could be adequately resolved from the intracellular P_i resonance. However, immediately after tetanic contraction (< 25 s) the elevated intracellular P_i concentration prevented separation of the two peaks during spectral processing. To allow changes in intracellular P_i concentration to be calculated (see below), it was assumed that the extracellular P_i concentration did not change significantly during the immediate post-tetanic recovery period, and therefore the extracellular P_i component was obtained from spectra acquired 45 and 90 s after tetanus and this value subtracted from all spectra.

The rates of recovery of pH, PCr and P_i are shown in Figs. 2(a), 2(b) and 2(c) respectively. At the end of a 3 s tetanus intracellular pH had risen to 7.05 ± 0.02 (6), in agreement with the finding of Shoubridge & Radda (1987). In the initial recovery phase pH decreased by 0.09 ± 0.03 (5) units in the first 5 s (Fig. 2a), followed by a slower decline to 6.87 ± 0.02 (9) after 45 s recovery.

Fig. 2. Changes in intracellular pH (a), PCr (b) and P_i (c) during recovery after 3 s tetanic contraction of the ankle flexor muscles

Two series of experiments were performed (see the Materials and methods section). F/R is a data-point acquired upon full recovery after the protocol.

Between 45 s and 90 s, pH increased and had risen to 6.99 ± 0.01 (9) after 90 s recovery.

PCr and P_i recovery data are shown in Figs. 2(b) and 2(c). At the end of a 3 s tetanus PCr concentration had decreased to $33 \pm 4\%$ of pre-stimulation levels, with a stoichiometric increase in P_i concentration. Rates of PCr and P_i recovery could be accurately approximated to first-order processes. First-order rate constants deter-

Table 1. Time-course of changes in calculated ADP and PCr + P_i concentration during recovery after 3 s tetanic contraction of ankle flexor muscles

Values are presented as means $\pm$ S.E.M. for n experiments. Statistical significance is indicated as * $P < 0.05$ (Student's t test) for PCr + P_i values during recovery compared with the value at the end of the 3 s tetanus. Abbreviation: F/R, full recovery.

Time after tetanus (s)	n	[ADP] (nmol/g)	[PCr + P_i] (μ mol/g)
0	5	83.5 ± 3.6	30.5 ± 1.1
1	5	51.9 ± 4.7	30.4 ± 2.0
5	6	36.8 ± 3.7	30.1 ± 2.3
15	5	28.6 ± 2.4	26.8 ± 2.7
25	5	16.5 ± 3.2	$25.9 \pm 1.5^*$
30	4	16.4 ± 3.9	$24.4 \pm 2.1^*$
45	8	8.9 ± 1.3	$26.3 \pm 1.6^*$
90	7	3.6 ± 0.7	29.5 ± 2.1
F/R	6	3.0 ± 0.5	29.4 ± 1.4

mined for experiments where spectra were acquired at 1, 15, 30, 45 and 90 s were 0.0203 ± 0.0044 (5) s^{-1} for PCr and 0.0442 ± 0.0091 (4) s^{-1} for P_i . Thus the rate of P_i disappearance was higher than the rate of PCr resynthesis ($P < 0.05$). The apparent disappearance of n.m.r.-visible phosphate during the initial recovery phase is shown in Table 1. After 25 s recovery, PCr + P_i concentration, determined by spectral analysis, had decreased significantly, by 17%, representing an apparent phosphate loss of 5.2μ mol/g of tissue. By 90 s after tetanic contraction the n.m.r.-visible PCr + P_i concentration had returned to levels similar to those observed either at rest or at the end of a 3 s tetanus (Table 1).

The muscle-fibre composition of the ankle flexor muscle mass interrogated is approx. 55% IIB, 53% IIA and 12% I (Armstrong & Phelps, 1984). This may affect the observed responses due to fibre-specific metabolic heterogeneity (Challiss *et al.*, 1988); however, fibre-type differences are unlikely to account for the observed discrepancy between recovery rates of PCr and P_i . The more rapid disappearance of P_i from the n.m.r.-visible pool has been observed previously (Taylor *et al.*, 1983, 1986; Arnold *et al.*, 1984). Although efflux of P_i from exercising skeletal muscle has been reported (Bockman *et al.*, 1976), the transient nature of the change in n.m.r.-visible P_i and the magnitude of the change are unlikely to be due to loss of P_i from the muscle mass (Caton *et al.*, 1987). Some evidence has been presented to suggest that the transient loss of P_i may be due to sequestration into an intracellular n.m.r.-visible pool; this may occur by organellar P_i uptake (e.g. into mitochondria, sarcoplasmic reticulum, etc.) or by microcompartmentation by association of P_i with cytosolic proteins during the immediate post-exercise period (Taylor *et al.*, 1986; Caton *et al.*, 1987).

In addition, the rates of recovery observed after tetanic contraction are higher than those previously reported. The half-recovery times for PCr (24 s) and P_i (14 s) determined in the present study are shorter than those reproducibly obtained in human exercise tests (PCr, 50–70 s; P_i , 30–50 s; Taylor *et al.*, 1983, 1986; Arnold

et al., 1984); this may be because the mass-specific metabolic rate is less in humans compared with rats. The data of Shoubridge & Radda (1984) for the changes in PCr concentration during recovery after twitch stimulation of rat gastrocnemius muscle at 4 Hz yield a half-recovery time of approx. 45 s; although this is likely to be an overestimate, because of the poor time resolution obtained in these experiments (36 s spectral acquisition time), it is conceivable that there may be differences in recovery rates after tetanic and twitch stimulation. This may be related to the modest post-tetanic acidosis (Fig. 2a) and the maximal perfusion of the recovering muscle mass during the post-tetanic period due to the maximal hyperaemic response elicited (Mackie & Terjung, 1983; Wesche, 1986).

The time course of ADP recovery is shown in Table 1. The initial recovery rate is very high, such that 50% recovery towards resting values is achieved within the first 5 s after termination of tetanic contraction. This initial phase is followed by a lower rate of recovery over the subsequent period (Table 1). In this respect, ADP recovery is correlated with changes in intracellular pH (Fig. 2a) during the recovery phase. Whether such a correlation has a bearing on respiratory control during the recovery period is at present unknown.

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