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^{31}P NMR Magnetization-Transfer Measurements of ATP Turnover during Steady-State Isometric Muscle Contraction in the Rat Hind Limb in Vivo†

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ABSTRACT: ^{31}P NMR magnetization-transfer measurements have been used to measure the flux between ATP and inorganic phosphate during steady-state isometric muscle contraction in the rat hind limb in vivo. Steady-state contraction was obtained by supramaximal sciatic nerve stimulation. Increasing the stimulation pulse width from 10 to 90 ms, at a pulse frequency of 1 Hz, or increasing the frequency of a 10-ms pulse from 0.5 to 2 Hz resulted in an increase in the flux which was an approximately linear function of the increase in the tension–time integral. The flux showed an approximately linear dependence on the calculated free cytosolic ADP concentration up to an ADP concentration of about 90 μM. The data are consistent with control of mitochondrial ATP synthesis by the cytosolic ADP concentration and indicate that the apparent K_m of the mitochondria for ADP is at least 30 μM.

^{31}P NMR has been used extensively to monitor the concentrations of ATP, ADP, P_i, phosphocreatine, and H^+ in skeletal muscle in vivo and the changes in these concentrations in response to muscle contraction (Radda, 1986). The energy cost of contraction, in terms of ATP utilization, can be determined by gating the NMR acquisition to various time points after the initiation of tetanic contraction (Shoubridge & Radda, 1987). Since the ATP concentration during a tetanus is maintained at the expense of phosphocreatine, the rate of ATP utilization can be determined from the equal rates of phosphocreatine breakdown or P_i production. In this experiment, the rate of ATP utilization is determined by monitoring the transient response of the muscle as it is stimulated to contract from rest. A powerful feature of the NMR experiment, however, is the facility, using magnetization-transfer

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techniques (Alger & Shulman, 1984; Brindle & Campbell, 1987; Brindle, 1988), to measure metabolite turnover during a metabolic steady state. Magnetization-transfer measurements have been used to measure rates of phosphocreatine turnover in heart and skeletal muscle and ATP turnover in yeast, maize root tips, kidney, and heart muscle [reviewed in Brindle and Campbell (1987) and Brindle (1988a)]. We have used this technique here to measure ATP turnover during steady-state isometric muscle contraction in the rat hind limb in vivo over a range of tetanic contraction frequencies and durations.

Early magnetization-transfer studies of ATP turnover suggested that the measured $P_i \rightarrow$ ATP flux was due solely to the activity of the mitochondrial F_1F_0 ATP synthase (Matthews et al., 1981; Freeman et al., 1984). Simultaneous measurements of O_2 consumption indicated that this flux was unidirectional; i.e., ATP synthesized in the mitochondria was hydrolyzed in the cytoplasm, and there was no reversal of the ATP synthase reaction in the mitochondrion. Under these conditions, the measured $P_i \rightarrow$ ATP flux can be equated with the net rate of ATP turnover in the tissue. Subsequent work, however, has shown that in some systems and under certain metabolic conditions, the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] and phosphoglycerate kinase (PGK) can contribute significantly to the measured $P_i \rightarrow$ ATP flux (Brindle & Radda, 1987; Campbell-Burk et al., 1987; Kingsley-Hickman et al., 1987; Brindle, 1988b). Furthermore, these enzymes can catalyze a coupled reaction which is near to equilibrium and can therefore catalyze an exchange reaction (Brindle & Radda, 1987). Under these circumstances, the measured $P_i \rightarrow$ ATP flux will exceed the rates of net ATP synthesis and utilization. The possible involvement of this exchange in skeletal muscle, and therefore the usefulness of the technique for measuring muscle ATP turnover, has been assessed here by comparing the measured rates with rates estimated from previous measurements of oxygen consumption in the perfused hind limb (Hood et al., 1986) and gated ^{31}P NMR measurements of phosphocreatine breakdown during a tetanus (Shoubridge & Radda, 1987). This comparison indicates that the contribution of the glycolytic enzymes is relatively small.

Increasing the stimulation pulse length or frequency is shown to produce an increase in the flux between P_i and ATP which is a linear function of the tension–time integral. The flux shows an approximately linear dependence on the calculated free cytosolic ADP concentration up to an ADP concentration of about 90 μM. Previous work has suggested that in skeletal muscle, ATP generation can be controlled by the availability of ADP to the mitochondria (Jacobus et al., 1982; Constable et al., 1987; Dudley et al., 1987; Radda, 1986). The data presented here are consistent with this proposal and indicate that muscle mitochondria in vivo display an apparent K_m for ADP of at least 30 μM.

Materials and Methods

Muscle Preparation. Male Wistar rats (170–200 g) were obtained from Harlan-OLAC 1976 Ltd., Bicester, Oxon, U.K. Animals were anesthetized and prepared for sciatic nerve stimulation as described previously (Shoubridge et al., 1984; Challiss et al., 1986, 1988). A Helmholtz coil was positioned around the muscles of the prepared limb, tuned, and placed in the vertical bore of a wide-bore 4.3-T magnet. Anesthesia was maintained by delivering 1% halothane in N_2O/O_2 (1:1) via a face mask, and body temperature was maintained at 37 ± 1 °C by blowing warm air through the magnet bore. The distal tendon of the gastrocnemius–plantaris–soleus complex was attached to an isometric force transducer (Shoubridge et al., 1984), the output of which was displayed on an Electromed chart recorder for continuous recording of tension development. The resting length of the muscle was adjusted for maximal isometric contraction, and contraction of the muscles was caused by applying a supramaximal voltage (20–25 V) to the sciatic nerve for variable periods (10–90 ms; 100-μs pulse width, 100-Hz square-wave) at a frequency of 0.5, 1, or 2 Hz. At the start of each experiment, the duration of each tetanus was set (2 discrete 100-μs pulses at 100 Hz gave a 10-ms tetanus; 10 discrete 100-μs pulses at 100 Hz gave a 90-ms tetanus). For the steady-state saturation-transfer measurements, the tetani were gated by the spectrometer to allow synchronization of spectral acquisition with a particular time point in the contraction–relaxation cycle (see below). At the start of each experiment, stimulation was commenced 20 min before the start of the NMR experiment so that a metabolic and mechanical steady state could be established (see Results). Tension development was monitored continuously during the NMR experiment. The mean tension development per unit cross-sectional area of the muscle (newtons per centimeter squared) was calculated by using this information. The cross-sectional area was calculated as mass per length assuming the muscle to be a cylinder of unit specific gravity. To account for the variation in tetanic duration and frequency (10–90 ms, 0.5–2 Hz), the results have been expressed as newtons per second per centimeter squared (see Figure 1).

NMR Measurements. ^{31}P NMR measurements were made at a frequency of 73.84 MHz using an Oxford Instruments 4.3-T vertical-bore magnet interfaced to a Bruker Biospec I console. Saturation-transfer measurements of flux between P_i and the γ-phosphate of ATP and between phosphocreatine and the γ-phosphate of ATP were made as described previously (Brindle, 1988b; Brindle & Radda, 1985). The steady-state P_i and phosphocreatine z magnetizations (M_z) were measured in the presence of selective saturation of the γ-phosphate resonance of ATP. The ratio of M_z to the equilibrium magnetizations (M_0) measured in the absence of selective saturation of the ATP resonance is given by (Forsen & Hoffman, 1963)

$$M_z/M_0 = 1/(1 + kT_{1p})$$

where k is a first-order rate constant describing loss of z magnetization in P_i or phosphocreatine and T_{1p} is the spin–lattice relaxation time for the phosphorus nucleus in P_i or phosphocreatine, respectively. Selective saturation of the ATP resonance was achieved either by using an auxiliary frequency source and amplifier or by lowering the power of the f1 frequency source. The lowest power compatible with complete saturation of the ATP γ-phosphate resonance was used, and the irradiation was applied continuously except during acquisition. The spectra used to obtain values for M_z and M_0 were acquired with a 90° pulse and were collected concurrently in interleaved blocks of a single scan to give a total of 25, 50, or 100 scans per spectrum. In addition, both spectra were acquired at 5 and 200 ms after the stimulation pulse. The muscle stimulation pulse was controlled by the spectrometer for these experiments. Acquisition of the single-scan ATP-irradiated and control spectra at 5 and 200 ms was interleaved to give a total of 50, 100, or 200 scans per spectrum. The total number of scans per experiment were, therefore, 100, 200, or

[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PGK, phosphoglycerate kinase (EC 2.7.2.3); T_1, spin–lattice relaxation time; M_z, observed z magnetization; M_0, equilibrium z magnetization.

Table I: Metabolite Concentrations and Intracellular pH in the Hind Limb Muscle at Rest and during Steady-State Isometric Contraction[a]

stimulation protocol [pulse length (ms) at 1 Hz]	no. of expt	metabolite concn in cell water (μmol/g dry wt)				
		P_i	PCr	ATP	ADP	pH
rest	1	15.8	106.1	25.1	0.039	7.04
10 (0.5 Hz)	3	22.1 ± 3.0	95.7 ± 3.3	29.2 ± 0.5	0.076 ± 0.021	7.06 ± 0.06
10	8	31.2 ± 5.2	87.7 ± 6.5	28.1 ± 2.5	0.088 ± 0.026	7.00 ± 0.04
10 (2 Hz)	4	40.0 ± 3.9	75.9 ± 3.3	31.1 ± 1.1	0.137 ± 0.009	6.99 ± 0.02
30	1	52.4	68.6	26.0	0.116	6.91
40	3	46.3 ± 14.7	69.5 ± 14.1	30.3 ± 1.3	0.146 ± 0.047	6.94 ± 0.06
50	6	61.6 ± 8.6	56.7 ± 9.4	28.7 ± 2.0	0.177 ± 0.058	6.87 ± 0.02
70	2	70.6	47.1	29.3	0.248 ± 6.89	
90	8	66.9 ± 13.2	54.5 ± 10.1	26.7 ± 2.4	0.188 ± 0.066	6.89 ± 0.04

[a] The metabolite concentrations and the intracellular pH were determined as described under Materials and Methods. All values are means ± standard deviations. Muscle stimulation was obtained by using the pulse width shown, at a frequency of 1 Hz, unless otherwise indicated.

400 scans, respectively. The total delay between each scan was 20 s. The spectra were initially stored in the computer memory in order to avoid the long and variable time required for disk storage during the experiment which affected the spectrometer-controlled muscle stimulation. The relaxation rates of the P_i or phosphocreatine z magnetizations to their equilibrium values following inversion, in the presence of selective saturation of the γ-phosphate resonance of ATP, is given by $1/T_{1p} + k$ (Mann, 1977). These were determined by measuring the P_i and phosphocreatine z magnetizations at different delay times following their inversion. The spectra obtained at each different delay were collected in blocks of eight scans. Four cycles through the delay list gave a total of 32 scans for each spectrum. For the T_1 measurements, muscle stimulation was controlled by an external signal generator which was not synchronized with the NMR spectrometer.

Combination of the T_1 measurements for P_i and phosphocreatine with determinations of the M_z/M_0 values gives the exchange rate constants. Multiplication of these rate constants by the steady-state P_i and phosphocreatine concentrations gives the fluxes between P_i and ATP and between phosphocreatine and ATP, respectively. The concentrations of P_i, phosphocreatine, and ATP were calculated by assuming that the total phosphate pool corresponded to 205 μmol/g dry weight (Shoubridge & Radda, 1987). The free ADP concentrations were calculated from the measured equilibrium concentrations of the creatine kinase substrates, ATP, phosphocreatine, creatine, and H^+, assuming an equilibrium constant for the enzyme-catalyzed reaction of 1.66×10^9 M^{-1} (Lawson & Veech, 1979). The creatine concentration was calculated by subtracting the measured phosphocreatine concentration from the total creatine plus phosphocreatine concentration, which was assumed to be 131 μmol/g dry weight. This figure was calculated from the data given in Shoubridge and Radda (1984) and Shoubridge et al. (1984). The free cytosolic ADP concentration was calculated by assuming a wet weight:dry weight ratio of 4.35 and that 0.67 of the wet weight represents intracellular water (Shoubridge et al., 1984). Resonance intensities were determined by cutting the peaks from the plotted spectra and weighing them or by using the integration routine in the computer software. Intracellular pH was calculated from the chemical shift difference between the P_i and phosphocreatine resonances (Taylor et al., 1986).

Results

Increasing the frequency of a 10-ms stimulation pulse from 0.5 to 2 Hz or increasing the length of the stimulation pulse, at a pulse frequency of 1 Hz, produced an approximately linear increase in the tension–time integral (Figure 1). The NMR data have been grouped, therefore, according to the stimulation protocol employed, i.e., pulse duration at 1 Hz or frequency

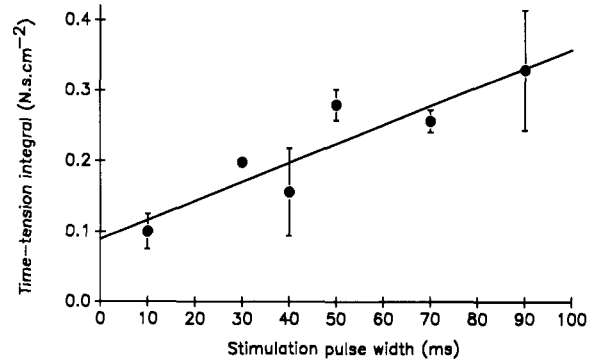

FIGURE 1: Plot of time–tension integral versus the duration of the muscle stimulation pulse at a frequency of 1 Hz. The error bars represent the standard deviations from the mean. The solid line was obtained by least-squares linear regression.

of a 10-ms pulse. Increasing the tension–time integral produced the expected decreases in phosphocreatine concentration and intracellular pH and increases in the P_i concentration and calculated free ADP concentration (Table I) (Shoubridge et al., 1984). The increase in P_i concentration paralleled the decrease in phosphocreatine concentration.

Typical saturation-transfer spectra are shown in Figure 2. Selective saturation of the γ-phosphate resonance of ATP (Figure 2B) resulted in a decrease in intensity of the P_i, phosphocreatine, α-ATP, and β-ATP resonances when compared to the control spectrum (Figure 2A). In the latter, the irradiation was applied at a frequency downfield from the P_i resonance, equal to the frequency difference between the resonances of P_i and the γ-phosphate of ATP. These decreases are readily observable in the difference spectrum (Figure 2A–B). The control irradiation and irradiations at frequencies further downfield from the P_i resonance demonstrated that the decrease in the P_i resonance intensity was due to exchange and was not the result of nonspecific saturation. Further control irradiations at the appropriate frequencies showed that the decreases in the resonance intensities of phosphocreatine and the α- and β-phosphates of ATP were also not the result of nonspecific irradiation (data not shown).

The flux between P_i and ATP can be catalyzed both by the mitochondrial F_1F_0 ATP synthase and by the glycolytic enzymes GAPDH and PGK (Brindle & Radda, 1987; Kingsley-Hickman et al., 1987). The exchange between phosphocreatine and ATP is catalyzed by creatine kinase (Shoubridge et al., 1984). The decrease in intensity of the β-phosphate resonance of ATP is due to exchange between the β-phosphate of ATP and the β-phosphate of ADP, an exchange which is catalyzed by all enzymes that interconvert ATP and ADP. The exchange is observed since saturation of the γ-phosphate resonance of ATP also results in saturation of the β-phosphate resonance of ADP, which is less than 1 ppm upfield (Brindle

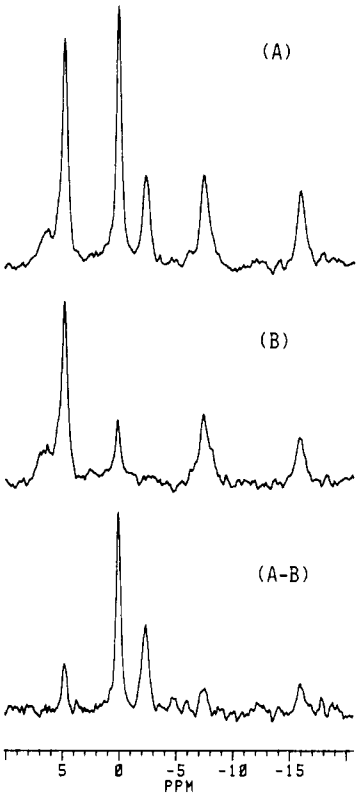

FIGURE 2: Steady-state saturation-transfer measurements of $P_i \rightarrow$ ATP flux in a muscle stimulated with a 90-ms sciatic nerve stimulation pulse at a frequency of 1 Hz. The spectra are the sum of 50 scans, collected in interleaved blocks of a single scan (see Materials and Methods), with an interpulse delay of 20.171 s and a sweep width of 3000 Hz. The chemical shift scale is referenced to the phosphocreatine resonance at 0.0 ppm. An exponential line broadening of 15 Hz was applied. The resonance assignments are, from the downfield end of the spectrum, intracellular P_i, phosphocreatine, γ-phosphate of ATP, α-phosphate of ATP, NAD(H), and the β-phosphate of ATP. Spectrum B was acquired with selective saturation of the γ-phosphate resonance of ATP. Spectrum A is a control spectrum in which the irradiating field was applied at a frequency downfield of the P_i resonance equal to the frequency difference between the resonances of P_i and the γ-phosphate of ATP.

& Radda, 1985). The free ADP concentration is too low for this resonance to be observed directly (Table I). The decrease in intensity of the α-phosphate resonance of ATP, following saturation of the γ-phosphate resonance, has been observed previously in brain and heart in vivo (Shoubridge et al., 1982; Brindle et al., 1988). The origin of this effect, however, is obscure.

Spectra collected with irradiation of the γ-phosphate resonance of ATP or with a control irradiation were acquired at 5 and 200 ms after the stimulation pulse, to give a total of 100–400 scans for the entire experiment (see Materials and Methods). Both the ATP-irradiated and control spectra collected at the two different delay times after the stimulation pulse were identical, showing that steady-state metabolic conditions were maintained during the stimulation protocol. For this reason, the spectra acquired at 5 and 200 ms after the stimulation pulse were routinely added. For stimulation pulse widths up to 40 ms, spectra (100 scans per spectrum) obtained from the beginning and end of the experiment were similar, demonstrating that the metabolic steady state was maintained throughout the experiment. This was consistent with maintenance of mechanical performance during this period (135 min). However, with longer stimulation pulses up to 90 ms, steady-state metabolic conditions could not be maintained for this length of time. There was an increase in

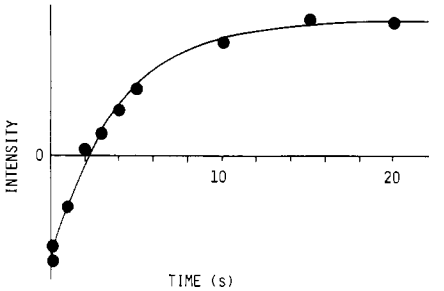

FIGURE 3: Inversion–recovery T_1 measurement on the intracellular P_i resonance in the presence of selective saturation of the γ-phosphate resonance of ATP. Plot of peak intensity versus the time between the 180° pulse and the 90° acquisition pulse in an inversion–recovery T_1 measurement. The spectra obtained at each different delay following inversion were collected in blocks of eight scans. Four cycles through the delay list gave a total of 32 scans for each spectrum. The delay between the end of the acquisition period and the next 180° pulse was 20 s. Peak intensities were determined by measuring peak heights. The solid line is a least-squares fit of the data to the function $y = A_1[1 - A_2e^{-(1/T_{1P}+k)t}]$, where y is the P_i z magnetization, t is the delay between the 180° and 90° pulses, T_{1P} is the intrinsic T_1 of the P_i resonance, the k is the exchange rate constant (see Materials and Methods).

phosphocreatine and decrease in P_i concentrations toward resting levels. These changes coincided with a decline in the tension–time integral. Therefore, for stimulation pulse lengths greater than 40 ms, only 25 scans were acquired per spectrum to give a total of 100 scans per experiment. Over this period (34 min), there were only relatively small changes in the concentrations of P_i and phosphocreatine and in the developed tension. For example, in two consecutive experiments, each lasting 34 min, employing a 40-ms stimulation pulse at a frequency of 1 Hz, there was a 15% decline in P_i concentration and a 16% increase in the phosphocreatine concentration. The calculated free ADP concentration decreased by 33% and the $P_i \rightarrow$ ATP flux by 17%. In a similar experiment employing a 90-ms stimulation pulse, the P_i concentration decreased by 23%, the phosphocreatine concentration increased by 30%, the calculated free ADP concentration decreased by 23%, and the $P_i \rightarrow$ ATP flux increased by 11%. The developed tension declined by only 5% between the start of the NMR experiment and the end.

The ratios of the steady-state magnetizations of P_i and phosphocreatine to their equilibrium magnetizations (see Materials and Methods), under different stimulation conditions, are shown in Table II. Also shown are the T_1's measured in the presence of saturation of the γ-phosphate resonance of ATP. Data from a typical P_i T_1 measurement are shown in Figure 3. Combination of the measured steady-state to equilibrium ratio (M_z/M_0) and the T_1 allows calculation of the exchange rate constant and the intrinsic T_1, i.e., the T_1 which would be observed in the absence of exchange (see Materials and Methods). The average intrinsic T_1's shown in this table were used with each individual determination of M_z/M_0 to calculate an exchange rate constant. Measurement of the equilibrium concentration of the exchanging metabolite then allows calculation of the unidirectional flux (Table I, see Materials and Methods). Determination of the equilibrium concentrations in the case of phosphocreatine and ATP is straightforward since they have well-resolved resonances (Figure 2). The intracellular P_i resonance, however, is partially overlapped by a downfield resonance which is assigned to extracellular P_i. With less line broadening during spectral processing, the extracellular resonance can be shown to constitute less than 20% of the total P_i resonance intensity. The contribution of the extracellular P_i has, therefore, been ignored

Table II: Saturation-Transfer Measurements of Phosphocreatine and P_i Turnover in the Hind Limb Muscle at Rest and during Steady-State Isometric Contraction[a]

stimulation protocol (pulse length at 1 Hz)	no. of expt	ratio of steady-state to equilibrium magnetization (M_z/M_0)		measured T_1 (s)		calcd intrinsic T_1 (s)	
		P_i	PCr	P_i	PCr	P_i	PCr
rest	1	0.81	0.29				
10 (0.5 Hz)	3	0.80 ± 0.03	0.34 ± 0.10				
10	8	0.82 ± 0.04	0.29 ± 0.04	4.12 (n = 2)	1.94 (n = 2)	5.02	6.69
10 (2 Hz)	4	0.85 ± 0.02	0.30 ± 0.09				
30	1	0.85	0.27	4.25 ± 0.75 (n = 3)	2.10 ± 0.11 (n = 3)	5.00	7.78
40	3	0.85 ± 0.01	0.29 ± 0.02				
50	6	0.86 ± 0.04	0.31 ± 0.08	3.91	2.08	4.55	6.71
70	2	0.86	0.41				
90	8	0.85 ± 0.04	0.31 ± 0.08				

stimulation protocol (pulse length at 1 Hz)	no. of expt	exchange rate constants (s^{-1})		fluxes [μmol (g dry wt)$^{-1}$ s^{-1}]	
		$P_i \rightarrow$ ATP	PCr $\rightarrow$ ATP	$P_i \rightarrow$ ATP	PCr $\rightarrow$ ATP
rest	1	0.051	0.33	0.8	35.1
10 (0.5 Hz)	3	0.038 ± 0.020	0.29 ± 0.14	0.9 ± 0.5	27.5 ± 12.4
10	8	0.045 ± 0.016	0.34 ± 0.06	1.4 ± 0.6	30.0 ± 4.6
10 (2 Hz)	4	0.039 ± 0.007	0.35 ± 0.12	1.6 ± 0.4	26.1 ± 8.2
30	1	0.038	0.36	2.0	24.8
40	3	0.037 ± 0.002	0.34 ± 0.03	1.7 ± 0.5	23.7 ± 5.4
50	6	0.037 ± 0.012	0.38 ± 0.16	2.2 ± 0.6	21.3 ± 8.0
70	2	0.036	0.20	2.6	9.2
90	8	0.039 ± 0.011	0.33 ± 0.12	2.5 ± 0.5	18.0 ± 8.5

[a] The muscle was stimulated to contract by using the stimulation pulse widths shown, at a frequency of 1 Hz, unless otherwise indicated. The magnetization-transfer experiment is described under Materials and Methods. The ratios M_z/M_0 represent the ratios of the steady-state z magnetizations of the P_i and phosphocreatine resonances, in the presence of saturation of the γ-phosphate resonance of ATP, to their equilibrium z magnetizations. The T_1 measurement is described under Materials and Methods and in the legend to Figure 3. The measured T_1's were the T_1's measured in the presence of saturation of the γ-phosphate resonance of ATP. Calculations of the intrinsic T_1's and the fluxes are described under Materials and Methods. All values shown are means ± standard deviations. The numbers in parentheses represent the number of T_1 measurements under a given set of stimulation conditions.

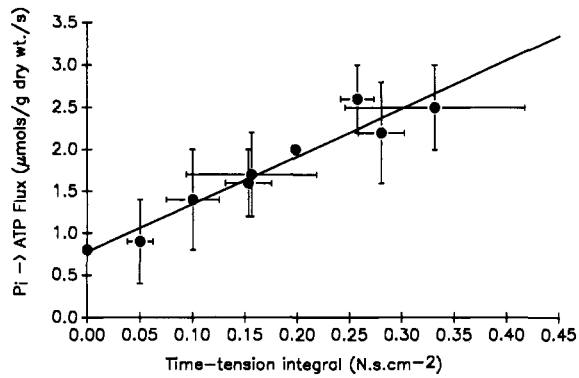

FIGURE 4: Dependence of the flux between P_i and ATP on the mean tension–time integral. The error bars represent standard deviations from the mean. The solid line was obtained by least-squares linear regression.

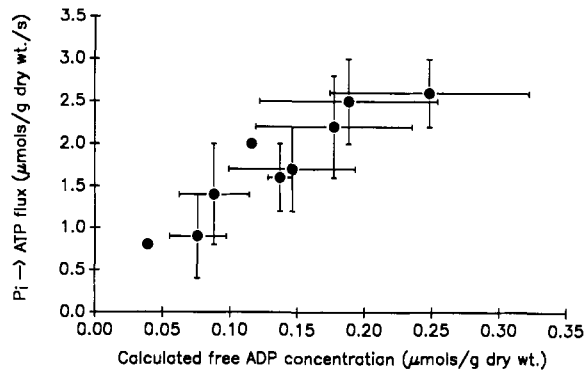

FIGURE 5: Dependence of the flux between P_i and ATP on the free ADP concentration in the muscle. The free ADP concentration was calculated from the near-equilibrium concentrations of the creatine kinase substrates, as described under Materials and Methods.

in estimates of the ratio M_z/M_0 and the equilibrium intracellular P_i concentration.

The steady-state flux between P_i and ATP measured by magnetization transfer was a linear function of the mean tension–time integral (Figure 4). The points in this plot have been grouped according to the stimulation protocol employed, i.e., pulse length at 1-Hz stimulation frequency or frequency of a 10-ms pulse. A single experiment was also performed at rest. The flux between P_i and ATP shows a linear dependence on the calculated free ADP concentration, the flux increasing with increases in free ADP concentration up to a cytosolic concentration of approximately 90 μM (Figure 5). The creatine kinase catalyzed flux between phosphocreatine and ATP, in contrast, is slightly reduced at the higher ADP concentrations (Table II), as has been observed previously in this laboratory (Shoubridge et al., 1984). The creatine kinase fluxes measured here, however, are significantly lower than those measured previously using a surface coil. Although the steady-state ratios, M_z/M_0, are similar, the T_1's measured in this study are nearly twice as long as those measured previously (Shoubridge et al., 1984).

The exchanges between the β-phosphate of ATP and the β-phosphate of ADP and the apparent exchange between the γ- and α-phosphates of ATP showed no correlation with changes in muscle stimulation. The flux between ATP and ADP, however, may have been underestimated due to incomplete saturation of the ADP resonance (Brindle & Radda, 1985).

Discussion

The observed exchange between P_i and ATP could be catalyzed both by the mitochondrial F_1F_0 ATP synthase and by the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) (Brindle & Radda, 1987; Kingsley-Hickman et al., 1987). Although the net glycolytic flux under these stimulation

conditions is expected to be low (Hood et al., 1986), these enzymes can catalyze a coupled near-equilibrium reaction and can, therefore, catalyze an exchange between P_i and ATP which may greatly exceed the net glycolytic flux (Brindle & Radda, 1987). The possible contribution of these enzymes can be evaluated by comparing the $P_i \rightarrow$ ATP fluxes measured here with fluxes calculated from previous measurements of oxygen consumption in the perfused hind limb (Hood et al., 1986) and from ^{31}P NMR measurements of phosphocreatine breakdown during tetanic contraction in vivo (Shoubridge & Radda, 1987). In the perfused hind limb, a tetanic contraction of 100 ms, in the absence of fatigue, gives an oxygen consumption of 0.26 μmol of O_2 (g of muscle)$^{-1}$. Since the tension–time integral shows an approximately linear relationship with pulse length (Figure 1) and since it has been shown previously that there is a linear correlation between oxygen consumption and tension development (Hood et al., 1986), then a 70-ms tetanus at a frequency of 1 Hz should give an oxygen consumption of 0.79 μmol of O_2 (g dry weight)$^{-1}$ s^{-1}, assuming a wet:dry ratio of 4.35 (Shoubridge et al., 1984). Assuming a P:O ratio of 3 (Ferguson, 1986), this oxygen consumption figure gives a calculated ATP turnover rate of 4.7 μmol (g dry weight)$^{-1}$ s^{-1} under these stimulation conditions. The experiments shown here gave a value of 2.6 ± 0.4 μmol (g dry weight)$^{-1}$ s^{-1}. If this is corrected for the 50% loss of developed tension between the start of the experiment and the steady-state period, when the NMR measurements were made, then a value of 5.2 μmol (g dry weight)$^{-1}$ s^{-1} is obtained. The similarity of this value to the ATP turnover rate calculated from O_2 consumption measurements in the perfused hind limb indicates that the contribution of a glycolytic exchange reaction to the observed $P_i \rightarrow$ ATP flux is relatively small. ^{31}P NMR measurements of phosphocreatine breakdown during a tetanus gave an ATP utilization rate during the tetanus of 48.4 μmol (g dry weight)$^{-1}$ s^{-1}. Therefore, a 70-ms tetanus at a frequency of 1 Hz should give an ATP turnover rate of 3.4 μmol (g dry weight)$^{-1}$ s^{-1}. This value is 35% less than the value calculated here. However, given the uncertainties inherent in these calculations, this comparison again provides no evidence for a significant contribution of the glycolytic exchange reaction to the observed flux. This conclusion is further supported by the approximately linear relationship between the tension–time integral and the $P_i \rightarrow$ ATP flux since previous work has shown a linear correlation between oxygen consumption and tension, development, which is maintained during both twitch and tetanic contractions (Hood et al., 1986). However, a contribution from the glycolytic enzymes can not altogether be ruled out. For example, the oxygen consumption at rest in the perfused hind limb is 0.37 μmol min^{-1} (g of muscle)$^{-1}$ (Hood et al., 1986). This corresponds to an ATP turnover rate of 0.2 μmol (g dry weight)$^{-1}$ s^{-1}, which is at the lower end of the range of fluxes measured with a 10-ms stimulation pulse at a frequency of 0.5 Hz (Table II and Figure 4). Furthermore, the glycolytic contribution to the flux may also change with stimulation pulse width and frequency due to changes in the substrate concentrations of GAPDH and PGK, e.g., ADP (Brindle & Radda, 1987).

In the analysis of the NMR measurements, the hind limb has been treated as a homogeneous muscle mass. Although the hind limb muscle is heterogeneous with respect to fiber type (Armstrong & Phelps, 1984), containing 66% fast-twitch white and 27% fast-twitch red fibers, these fibers are expected to have similar energy costs of contraction (Hood et al., 1986). Furthermore, under resting conditions, these fibers have been shown to have similar ATP and phosphocreatine concentrations (Hintz et al., 1982). However, during tetanic stimulation, enzymatic assays of metabolite concentrations in individual muscle fibers have shown significant heterogeneity in the response of the muscle (Hintz et al., 1982). Stimulation for 15 min with a stimulation pulse of 100-ms duration at a frequency of 1 Hz resulted in a 59% loss of ATP and a 97% loss of phosphocreatine in the fast-twitch white fibers compared to a 3% loss of ATP and 17% loss of phosphocreatine in the fast-twitch red fibers (Hintz et al., 1982). The loss of ATP in the fast-twitch white fibers is thought to correlate with the decrease in developed tension under these conditions (Hood et al., 1986). Muscle stimulation with pulses of 90-ms duration at a frequency of 1 Hz in the study shown here resulted in a steady-state-developed tension which was 40 ± 11% of the initial developed tension. However, this was well maintained during the course of the NMR experiment, declining on average by only 5%. With shorter stimulation pulses, the steady-state-developed tension declined to only 86–88% of initial. NMR measurements of total ATP at rest and following stimulation for 20 min (90-ms pulse, 1 Hz) showed a negligible decline in muscle ATP concentration. The enzymatic measurements of ATP concentrations in single fibers described above suggest that there should be an approximately 40% decline in the ATP concentration measured by our coil. Phase-modulated rotating frame imaging experiments also showed a degree of heterogeneity in fiber type response which was less than that expected from the enzymatic assay data on single fibers (Challiss et al., 1988). However, there will be some heterogeneity in the metabolic response of the muscle, and it is worth considering the possible consequences of this for the magnetization-transfer measurements of $P_i \rightarrow$ ATP flux. If loss of developed tension is due to drop out of the fast-twitch white fibers, then presumably the ATP utilization rate in these fibers declines to nearly resting levels (Figure 4). If the metabolite concentrations in these fibers return to resting levels (Table I), where the P_i concentration is very low, then the observed muscle P_i will come largely from those fibers which are still working. The magnetization-transfer measurements will measure flux, therefore, predominantly in these fibers, and the correlation between developed tension and measured ATP turnover will be maintained. Alternatively, those type IIb (fast-twitch white fibers) which have dropped out could be maintained with very low phosphocreatine and ATP concentrations and correspondingly high P_i concentrations. If this is the case, then at the longest stimulation pulse used in this study (90 ms), the total muscle P_i concentration, estimated from the enzymatic assay data (Hintz et al., 1982), would be nearly 3 times greater than the phosphocreatine concentration. This is not observed in the experiments shown here and is consistent with the much less marked heterogeneity observed in the ^{31}P NMR imaging experiment (Challiss et al., 1988). In conclusion, the heterogeneity of fiber type response does not appear to be as extreme as that expected from enzymatic assays of metabolite concentrations in individual fibers. Nevertheless, depending on the metabolic consequences of fiber "drop out", heterogeneity may affect the measured $P_i \rightarrow$ ATP flux.

The flux between phosphocreatine and ATP, catalyzed by creatine kinase, is decreased as the developed tension and ADP concentrations are increased. This decrease, which has been observed previously (Shoubridge et al., 1984), is inconsistent with the observed solution properties of the enyzme and may reflect heterogeneity in the exercising muscle. The creatine kinase fluxes measured here, however, are significantly less than those measured previously in this laboratory (Shoubridge

et al., 1984). Although the measured steady-state magnetizations (M_z/M_o) are similar, the T_1's measured in this study are appreciably longer. This may reflect the use of a transmitter coil which has a much more homogeneous B_1 field than the surface coil used in the previous study. Shoubridge and Radda (1987) noted that the rate of phosphocreatine hydrolysis during a tetanus was very similar to the flux between phosphocreatine and ATP measured by magnetization transfer in the steady state. This implies that creatine kinase is rate limiting for ATP generation during the tetanus, a point which is emphasized by the creatine kinase fluxes and ATP turnover rates measured here. This observation further implies that there must be coordinate control of ATP synthesis and utilization since if the creatine kinase flux is rate limiting for ATP synthesis during a tetanus than we would expect, in the absence of any control over ATP utilization, a decrease in the ATP concentration during the tetanus. Loss of ATP during a tetanus is not observed in normal muscle although it is observed in the muscles of rats fed a diet containing the creating analogue, β-guanidinopropionic acid, in which the muscles are depleted of phosphocreatine (Shoubridge & Radda, 1987).

The flux between P_i and ATP shows an approximately linear dependence on the calculated free ADP concentration in the muscle up to an ADP concentration of about 90 μM. If ATP production is controlled by the availability of ADP to the mitochondria, as has been suggested by previous work (Jacobus et al., 1982; Constable et al., 1987; Dudley et al., 1987; Radda, 1986), then the data presented here indicate that muscle mitochondria in vivo display an apparent K_m for ADP of at least 30 μM. The dependence of the flux on the free ADP concentration at higher concentrations of ADP could not be determined since the hind limb muscle fatigued rapidly at tetanic pulse durations greater than 90 ms. This may reflect saturation of the mitochondria with ADP such that the mitochondria are unable to increase ATP production in response to the increased demand. This is consistent with the observations that in the perfused hind limb, oxygen delivery to the fast-twitch red fibers does not appear to be limiting (Hood et al., 1986) and that in trained muscle the increase in ADP concentration on exercise is less than in controls. In the trained muscle, the higher mitochondrial content means that a smaller increase in ADP concentration is required in order to achieve the same level of whole muscle mitochondrial ATP generation.

In conclusion, we have measured ATP turnover in the rat hind limb during steady-state isometric contraction using the technique of NMR magnetization transfer. Comparison with the fluxes calculated from previous measurements of oxygen consumption or phosphocreatine breakdown during a tetanus indicated that the measured flux is due predominantly to the activity of the mitochondrial F_1F_0 ATP synthase. The ATP synthesis rate shows an approximately linear dependence on the free ADP concentration in the muscle, up to an ADP concentration of about 90 μM, implying that mitochondrial ATP generation in the muscle is controlled by the free ADP concentration with an apparent K_m of at least 30 μM. Although heterogeneity in the metabolic response of the muscle to exercise could affect these measurements, the results presented here and in a previous ^{31}P NMR imaging experiment (Challiss et al., 1988) indicate that this heterogeneity is not as great as expected from enzymatic assays of metabolite concentrations in individual fibers (Hintz et al., 1982). The influence of heterogeneity, however, on the flux measurements can be investigated by combining the magnetization-transfer experiment with the imaging experiment described previously.

Registry No. ATP, 56-65-5; ADP, 58-64-0; P_i, 14265-44-2; phosphocreatine, 67-07-2.

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