Use of superfused rat skeletal muscle for metabolic studies: assessment of pH by ^{31}P n.m.r.

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We developed a muscle superfusion system suitable for metabolic studies of small isolated rat muscle *ex vivo* in real time and in a non-destructive manner by n.m.r. spectroscopy. In order to determine biochemical stability of superfused extensor digitorum longus (EDL) muscle (from fasted 45 and 100 g rats), the energy state and the pH of muscle were continuously monitored by ^{31}P n.m.r. spectroscopy. ATP and phosphocreatine remained stable during 2 h whatever the muscle size (20 or 45 mg). Neither metabolite was a sensitive probe of possible metabolic compart-

mentation within muscle under our experimental conditions. By contrast, the chemical shift of P_i by its sensitivity to pH was a discriminant factor in the assessment of muscle stability. Indeed, heterogeneity of pH was observed only in the 45 mg EDL muscle resulting from a core region with loss of glycogen. Together, these observations suggest deviations of energy metabolism to supply ATP. Consequently, pH may be considered as a new real-time criterion for monitoring a metabolic heterogeneity due to changes in energy metabolism of muscle preparations *ex vivo*.

INTRODUCTION

Isolated skeletal muscle preparations [soleus, extensor digitorum longus (EDL), diaphragm, epitrochlearis] have been widely used in studies of the hormonal and nutritional regulation of protein turnover and amino acid metabolism [1–6] and transport [7,8]. These models are meaningful and provide a better understanding of the situation *in vivo* if their biochemical and physiological stability is not compromised. The major concern with the integrity of these muscle preparations is the adequacy of nutrient exchange by diffusion in the absence of a circulatory system. The diffusion is dependent on the tissue thickness, the metabolic rate of the donor animal and the temperature of the buffer solution, which can influence substrate and O_2 diffusion into muscle cells and induce a core formation with a loss of glycogen [9–11]. The stability of isolated skeletal muscle has been assessed up to now by different criteria: (1) biochemical parameters such as the stability of phosphorylated metabolite levels, glycogenolysis rate and glucose transport, [3,9], (2) physiological parameters, such as the non-impairment of contractile properties during incubation [12] and the protein synthesis rate [10,13], and (3) morphological parameters as defined by histochemistry [10,14,15]. However, to obtain these parameters involves analysis of deproteinized homogenates, except for histochemical studies. By contrast, there have been no published results monitoring the metabolic state of rat small muscle in a non-destructive manner and in real time throughout an experiment.

^{31}P n.m.r. investigation of muscle has rendered continuous monitoring of phosphorylated metabolites [ATP, phosphocreatine (PCr), P_i] and pH possible. However, no investigations have been done until up to now [16] on rat muscle *ex vivo* by spectroscopy, probably because of the low sensitivity of n.m.r. making studies of small quantities of tissue difficult. Since the pH is an important parameter in control of cell metabolism [17], it can be very informative about the integrity of isolated muscle.

Moreover, according to the ^{31}P n.m.r. work of Busby et al. [18], showing compartmentation of P_i in resting muscle and its relation to varying pH in tissue volume, pH might be a very useful parameter when considering possible regional variations within the muscle. Because energy metabolism in muscle is in relation to the release or buffering of protons, i.e. glycolysis, the creatine kinase (CK) reaction [19,20], the measurement of pH in muscle could allow us to show the contribution of these metabolic fates to the ATP homeostasis of muscle preparations *ex vivo*.

Therefore, the aim of this study was (1) to achieve a superfusion system suitable for further studies of amino acid metabolism in small isolated rat muscle by n.m.r., (2) to assess stability in terms of regional homogeneity in the intact superfused muscle *ex vivo* by accurate measurement of intramuscular pH by ^{31}P n.m.r., and (3) to evaluate how the different metabolic fates of production and consumption of energy can explain the intramuscular pH in our preparations of superfused EDL muscle.

MATERIALS AND METHODS

Muscle superfusion

EDL muscles were dissected from 48 h-starved female Wistar rats (Iffa Credo, L'Arbresle, France). Both EDL muscles from four different animals were attached by proximal and distal tendons at resting length on a plastic support which was specifically designed for a 10 mm n.m.r. tube to keep the muscles separate from one another and to prevent them touching the inner side of the n.m.r. tube. In each experiment, the use of eight EDL muscles was necessary to have a sufficient quantity of tissue for n.m.r. detection. Two groups of animals with different body weights were used: about 45 g in group 1 and about 100 g in group 2. EDL masses were respectively 20 mg and 45 mg for groups 1 and 2. All experiments were performed between 11:00 and 13:00 h. Rats were anaesthetized with Pentobarbital (45 $\mu\text{g/g}$ body wt., intraperitoneally). Experiments were timed

Abbreviations used: EDL, extensor digitorum longus; FOG, fast-twitch oxidative glycolytic; FG, fast-twitch glycolytic; SO, slow-twitch oxidative; pH_{in} , intracellular pH; pH_{ex} , extracellular pH; PCr, phosphocreatine; ECP, Energy Charge Potential; MEM, Maximum Entropy Method; CK, creatine kinase.

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beginning at the dissection of the first muscle. Immediately after dissection, EDL muscles were preincubated for about 30 min in Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃), pH 7.4 (37 °C) saturated with O₂/CO₂ (19:1). EDL muscles were then placed in the n.m.r. tube, which was introduced into the n.m.r. spectrometer and constantly superfused with Krebs–Henseleit buffer containing BSA (0.13 %), glucose (10 mM), leucine (3 mM) and α -oxoglutarate (3 mM). These additions of leucine and α -oxoglutarate were included in our experimental conditions to allow further studies of amino acid metabolism. The perfusate (total volume 70 ml) was maintained under constant conditions at 37 °C with O₂/CO₂ in a Silastic tubing oxygenator and was recycled at a flow rate of 5 ml/min, resulting in a changeover of medium in the n.m.r. tube (3 ml) approximately once per min.

Biochemical assessment

Lactate, ATP, ADP, AMP, PCr and glycogen were determined enzymically [21–24] in freeze-dried tissue after muscle deproteinization with HClO₄ (0.4 %) and neutralization with KOH. Measurements were conducted on both EDL muscles freeze-clamped *in situ* and after 2 h superfusion.

Histochemical assessment of glycogen

EDL muscles from eight 45 and 100 g rats were specifically used for histochemical studies. One muscle of each animal was used as a test muscle (after 2 h superfusion) and the contralateral muscle was freeze-clamped *in situ* to be used as a control. The muscles were frozen in isopentane cooled with liquid N₂ and stored in liquid N₂ until analysis. Transverse sections (10 μ m) in the mid-belly region of muscle, made with a cryostat at –25 °C, were stained with the periodic acid/Schiff reagent for glycogen [25].

³¹P n.m.r. techniques

All experiments were performed at 162 MHz in a Bruker AM400 spectrometer using a 10 mm n.m.r. probe. Magnetic-field homogeneity was adjusted by observing the proton signal of water (line width about 15 Hz) in order to obtain a half-width of the PCr signal of 5 Hz. Spectra were collected as 8 K data points by using a sweep width of ± 3250 Hz (quadrature detection), a pulse width of 8.5 μ s (nominal tip angle 60°) and a recycle time of 5 s. Spectra were the result of 120 transients. Chemical shifts were expressed in relation to PCr (insensitive to pH variation), which was assigned a chemical shift of –2.45 p.p.m. with respect to 85 % H₃PO₄ [26]. The β -phosphate peak of ATP was used to determine ATP levels. Relative concentrations of ATP and PCr were calculated from their resonance areas as determined after computerized curve-fitting analysis (trade mark NMRi) of ³¹P spectra.

A calibration curve of P_i chemical shift as a function of pH [27] was established in our experimental conditions (temperature, ionic strength). From these data, the best fit for pK_a value and chemical shifts of the acidic and basic forms of P_i was:

$$\text{pH} = \text{pK}_a - \log[(\delta - 0.922)/(3.451 - \delta)]$$

where δ is the chemical shift of P_i. The curve-fitting and calculation were done with a non-linear regression program (SAS statistical package, 1988). In order to obtain maximum

accuracy in pH measurement, the n.m.r. resolution was enhanced by the Maximum Entropy Method (MEM) [28].

Experiment design and statistical analysis

Data shown are representative experiments (Figures 1–3) in which every type of experiment was repeated 4 times (8 times for histochemistry). Values are means $\pm$ S.E.M. for $n = 4$ or 8 experiments. Means were compared by the unpaired *t* test and differences are considered significant for $P < 0.05$. The mean data are plotted ($n = 3$) in Figure 4. Data obtained before and after anoxia for PCr, P_i and pH are compared by the paired *t* test; differences are considered significant for $P < 0.05$.

RESULTS

Assessment of the muscle superfusion system by biochemical and histochemical methods

To establish that our muscle preparation was appropriate for further metabolic studies, it was necessary to determine whether superfused EDL muscles remain stable during the time of the experiment. We initially compared ATP, ADP, AMP, PCr and lactate levels biochemically in EDL muscles freeze-clamped *in situ* and after 2 h of superfusion (Table 1). Whatever the muscle size (20 or 45 mg), no significant differences in these metabolites were observed between control and superfused EDL, except for lactate, which was twice as great in the 45 mg EDL muscles as in the 20 mg EDL muscles. Moreover, the same results (not shown) were obtained if 20 mg EDL muscle were superfused for up to 4 h. From the biochemical assays, we concluded that the only difference between 20 mg and 45 mg EDL muscles was the lactate level. To estimate quantitatively the energy state of the cell, which has been described as a very sensitive indicator, we have calculated a biochemical criterion reported by Atkinson [29], the Energy Charge Potential (ECP):

$$\text{ECP} = ([\text{ATP}] + \frac{1}{2}[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$$

The ECP ratio was not significantly different between 0 and 2 h of superfusion (0.95 ± 0.05 and 0.91 ± 0.01 respectively) and was not dependent on the muscle size ($n = 8$). These calculated ECP values confirmed that the biochemical parameters did not discriminate between the two preparations.

Table 1 Metabolite contents in 2 h-superfused and in freeze-clamped EDL muscle determined biochemically

EDL muscles (20 and 45 mg) from fasted rats were freeze-clamped *in situ* or superfused for 2 h. ATP, ADP, AMP, PCr and lactate were measured enzymically on HClO₄ extracts as described in the Materials and methods section. Pooled results of 8 experiments on muscles of 20 mg and 45 mg are reported (means $\pm$ S.E.M., $n = 8$), except for lactate in superfused muscles, because the averaged level was different for 20 mg and 45 mg EDL muscles (means $\pm$ S.E.M., $n = 4$): *significantly different ($P < 0.05$) from superfused 20 mg EDL muscles.

	Content (μ mol/g wet wt.)	
	Muscle ... Freeze-clamped	Superfused
ATP	5.81 $\pm$ 0.35	5.61 $\pm$ 0.40
ADP	1.05 $\pm$ 0.49	1.09 $\pm$ 0.05
AMP	0.16 $\pm$ 0.07	0.05 $\pm$ 0.02
PCr	21.9 $\pm$ 1.1	21.3 $\pm$ 2.1
Lactate: 20 mg muscles	1.74 $\pm$ 0.60	1.54 $\pm$ 0.30
45 mg muscles	1.59 $\pm$ 0.53	3.10 $\pm$ 0.25*

Figure 1 Transverse sections through 20 mg EDL muscle *in situ* (a), 45 mg EDL muscle *in situ* (b), 2 h superfused 20 mg EDL muscle (c), 2 h-superfused 45 mg EDL muscle (d), and 45 mg EDL muscle after 35 min of superfusion (e) stained for glycogen

Magnification $\times 42$ for (a) and (c) and $\times 32$ for (b), (d) and (e).

In order to demonstrate possible regional variations within the muscle, as previously reported [10,11], the non-superfused and 2 h-superfused 20 mg and 45 mg EDL muscles were stained histochemically for glycogen (Figures 1a–1d). A central core with loss of histochemically demonstrable glycogen was observed in superfused 45 mg muscles after 2 h (Figure 1d), in contrast with the non-superfused 45 mg EDL muscles (Figure 1b). There were no obvious differences in the size of the central core between

the 8 muscles in any experiment (results not shown), which suggested an intramuscular and not intermuscular heterogeneity within the muscle in our preparation. By contrast, in Figures 1(a) and 1(c), no core devoid of glycogen was found in 20 mg EDL muscles whatever the experimental conditions (superfused or not). We have also measured glycogen levels in these muscles enzymically. Our results for muscles *in situ* from starved rats were 2.8 ± 0.7 mg of glycogen/g wet wt. of muscle, in the same

Figure 2 Typical ^{31}P -n.m.r. spectra obtained with 2 h-superfused 20 mg EDL muscles from fasted 40 g rats

N.m.r. parameters: pulse width 8.5 μs , 120 scans, 8 K data points, line-broadening 15 Hz, ± 3250 Hz spectral window and recycle time of 5 s. Extracellular P_i buffer resonates at 2.8 p.p.m. α , β , γ indicate the resonances of the corresponding phosphate groups of ATP.

range as those of Sugden et al. [30]. For superfused EDL muscles, the glycogen levels were 6.6 ± 0.5 and 4.1 ± 0.4 mg of glycogen/g wet wt. of muscle for 20 and 45 mg EDL muscles respectively ($P < 0.05$). These results showed there was an increase in glycogen level in both preparations superfused with glucose-enriched buffer.

Assessment of the muscle superfusion system by ^{31}P n.m.r.

Typical ^{31}P n.m.r. spectra of superfused 20 mg EDL muscles were recorded (Figure 2). All phosphorylated metabolites (ATP, PCr, intramuscular P_i) remained constant throughout the 2 h experiment. Spectra of phosphorus metabolites for superfused 45 mg EDL muscles demonstrated stability of ATP and PCr throughout the experiment (results not shown), whereas the resonance from intramuscular P_i broadened with the time-course of superfusion, indicating a P_i distribution in this preparation (Figure 3b). By contrast, in both 20 mg and 45 mg muscles, the extracellular P_i , i.e. buffer P_i (1.2 mM), resonated as a sharp peak at about 2.8 p.p.m., which corresponded to pH 7.3–7.4; this value remained stable throughout the experiment. From the P_i

region in the spectra (Figures 3a and 3b), we can accurately separate extracellular P_i [$\text{P}_i(\text{ex})$] and intracellular P_i [$\text{P}_i(\text{in})$] by the MEM (examples of results obtained in Figures 3c and 3d), and thus evaluate the kinetics of pH in muscles of both sizes. During the first 32 min of study, in 20 mg EDL muscles, the intramuscular P_i component was distinguishable from extracellular P_i , and the pH of the muscle was 6.95–7. After 32 min, the muscle P_i component had shifted under the extracellular P_i resonance and the pH ranged between 7 and 7.35. In 45 mg EDL muscles, two different periods were determined in the kinetics of pH: (i) during the first 50 min, a single pH value (about 6.8) was measured; (ii) from 60 min of superfusion until the end of the experiment, two components of intracellular pH were always obtained, pH_{in1} at about 6.95 and pH_{in2} in the range 6.5–6.8, suggesting a heterogeneity of pH within the muscle. Consequently, we carried out an additional histochemical staining of glycogen of the 45 mg muscles before the $\text{P}_i(\text{in})$ peaks resolved into two components (Figure 1e): a slight and diffuse glycogenolysis began to appear without appearance of a central core after 35 min of superfusion.

To assess the importance of a continuous oxygenated buffer supply in our preparation, we disrupted the O_2 supply for 40 min and replaced it by N_2 flux. In this experiment, we chose 45 mg EDL muscles, which seemed to us to be most likely to be influenced by this treatment. As a result, PCr level decreased dramatically (Figure 4a), but increased gradually when the O_2 supply restarted. However, 1 h later, the initial PCr level was not completely restored. The ATP level was not significantly altered during the same period. By contrast, the P_i region was considerably modified after interruption of O_2 supply. Intramuscular P_i was shifted to high field, suggesting acidosis of muscle due to the O_2 deprivation, whereas extracellular P_i was shifted to downfield. The kinetics of both extracellular and intramuscular pH are shown in Figure 4(b). The time course of buffer pH showed a recovery of pH as soon as the O_2 was re-started. The profile of intramuscular pH was of the same type as those of standard superfused 45 mg muscles, except that the two main components of muscle pH were more acidic (pH_{in1} at 6.6–6.8 and pH_{in2} at 6.4–6.5 instead of pH_{in1} about 6.95 and pH_{in2} in the range 6.5–6.8) and were not improved when the O_2 supply re-started. We have also made experiments with no flow of substrates and disruption of O_2 supply. The results obtained in these experiments were similar to those with the single effect of oxygen deprivation (results not shown).

DISCUSSION

Although isolated skeletal muscle has been widely used in metabolic research [1–8], few results have been reported up to now on the possibilities of achieving a continuous non-destructive monitoring of metabolism in small superfused muscle in real time by n.m.r. The EDL muscle used in these experiments consists of mixed fast-twitch fibres [31], with about 60% of fast-twitch oxidative glycolytic (FOG) fibres, 30% of fast-twitch glycolytic (FG) fibres and about 10% of slow-twitch oxidative (SO) fibres in our fasted animals (results not shown). We have verified that the different fibre types are distributed at random within the EDL muscle of our animals. The high pools of PCr and ATP (Table 1) determined in these muscles, whatever the size (20 or 45 mg), are consistent with a mixed-fibre muscle with a high proportion of FOG and FG fibres. Indeed, although the FOG fibres differ from FG fibres in their oxidative capacity, these fibres were not distinguishable on the basis of the PCr, ATP and P_i contents, according to Kushmerick et al. [16]. Therefore, in contrast with Challiss et al. [32], who reported a heterogeneity of

Figure 3 Effect of muscle weight on P_i peak (between 2.8 p.p.m. and 1.9 p.p.m.) in ^{31}P n.m.r. spectra during 2 h superfusion

All details are as described in Figure 2. Abbreviations: $\text{P}_i(\text{ex})$, extracellular P_i or buffer P_i ; $\text{P}_i(\text{in})$, intracellular P_i . (a) 20 mg EDL from 40 g fasted rats. (b) 45 mg EDL from 100 g fasted rats. (c) and (d) Representative spectra after MEM treatment for 20 mg and 45 mg muscles respectively (see the Materials and methods section).

PCr in spatially resolved muscles containing different proportions of fibre types (SO, FOG, FG) in rat hindlimb *in vivo*, we cannot show such a distribution between fibres in the isolated EDL muscle by n.m.r. spectroscopy. The EDL muscle being both very oxidative and very glycolytic due to the presence of FOG fibres [33], we hypothesized that it is a suitable model to demonstrate possible deviations of energy metabolism during the superfusion experiment.

The major observation in this study is that the intramuscular pH measured by ^{31}P n.m.r. is a discriminant criterion for the assessment of muscle preparations *ex vivo*. The most important question in the use of muscle *ex vivo* is the biological stability

during the experiment. The impairment of muscle integrity is mainly dependent on its thickness: anoxia can appear, due to the failure of O_2 to penetrate to the core of tissue [10,11]. The pH criterion allows us to monitor directly regional variations within the muscle, as described by others, with histochemical studies at the end of the experiment [10]. Indeed, the pH of 20 mg EDL muscles was greater than 7 and in good agreement with those determined by Meyer et al. [33] in a muscle with a mixed-fibre composition, the cat biceps. By contrast, the pH of 45 mg EDL muscles was more acidic and did not remain stable throughout the experiment. In the 45 mg EDL muscles, the observed regional heterogeneity of pH could result from a regional glycogen

Figure 4 Effect of disruption of oxygenation (replacement of O_2 by N_2) on ATP, PCr and P_i levels and pH monitored by ^{31}P n.m.r. in superfused EDL muscles

Oxygenation of buffer was disrupted for 40 min (a bar represents the period of disruption). (a) Time course of ATP, PCr and P_i levels expressed in arbitrary units. PCr values were significantly different from the value before anoxia between 70 and 140 min ($P < 0.05$). P_i values were significantly different from the value before anoxia between 80 min and 120 min ($P < 0.05$). (b) Time course of extracellular buffer (pH_{ex}) and intramuscular pH consisting of two components (pH_{in1} and pH_{in2}). All the pH_{in1} and pH_{in2} values were significantly different from each other at any given time ($P < 0.05$). pH_{in1} values during the anoxia period were significantly different from the pH_{in} before anoxia ($P < 0.05$). At 20 min after the anoxia period, pH_{in2} values became significantly different from the pH_{in} before anoxia ($P < 0.05$).

depletion caused by anaerobic glycolysis; lactate may be produced mainly in the core of the muscles, and may thus explain a gradient of acidic pH (6.5–6.8) between the well-oxygenated region and the anoxic region. Moreover, at the beginning of superfusion (35 min), the single pH value (about 6.8) in 45 mg EDL muscle could be consistent with the beginning of glycolysis resulting in a few clear spots depleted of glycogen (Figure 1e) and in a slight acidosis within the muscle. Increased lactate levels and decreased glycogen levels in 2 h-superfused 45 mg versus 20 mg muscles would suggest differences in preparations of different size. By contrast, the assays of phosphorylated metabolites obtained in deproteinized homogenates (ATP, ADP, AMP, PCr) or the ECP ratio did not suggest a poor oxygen diffusion in superfused 45 mg EDL muscles. Therefore, only the pH criterion suggests directly that the physiological state of the superfused muscles (during the experiment *ex vivo*) depends on the muscle weight or thickness. However, in fasted animals, our data do not support the previous hypothesis [9–11] suggesting that the development of an anoxic core depends on the metabolic rate of the animals: the stability of superfused muscles appeared only to correlate with the muscle size and not with the metabolic rate of starved rats.

A second major finding in the present experiments is that there is no detectable change in PCr level (kinetically measured from areas of n.m.r. signals and enzymically measured in $HClO_4$ -treated assays at the end of the experiment), whereas a spread of intracellular pH values appeared during the superfusion of 45 mg EDL muscle. Because of the impairment of oxygen delivery in the anoxic region within muscle, ATP cannot be produced by

both mitochondrial respiration and glycolysis in the EDL muscle, but only by glycolysis, which induces acidosis; the CK reaction could also participate in ATP production from the pool of PCr. Indeed, there is evidence that PCr is in equilibrium with the H^+ concentration in the reaction catalysed by (CK):

Consequently, an acidification of muscle would drive this reaction towards PCr hydrolysis in order to buffer protons [19]. Such a phenomenon was not observed in our experiments. To try to explain this unexpected result, we have calculated the consequences of pH variations on the PCr level from the CK equilibrium, assuming that there is no change in the free ADP:

$$[PCr] = \frac{[TCr][ATP]}{K_{eq, CK}[ADP][H^+] + [ATP]}$$

where TCr is the total creatine and the other metabolites concentrations expressed in intracellular concentrations by reference to intracellular water: TCr = 39 mM and free ADP = 8 μ M in fibres of type IIA and IIB [16]. For ATP, the value in Table 1 was corrected for intracellular water by using a factor of 0.71 (wet-/dry-wt. ratio 4.69 in superfused muscle plus 10 % extracellular space [34]); ATP = 7.9 mM (result in good agreement with the value of Kushmerick et al. [16] in IIA and IIB fibres). The equilibrium constant of the CK reaction, $K_{eq, CK}$, is $1.66 \times 10^9 M^{-1}$ [35]. Depending on whether the whole muscle has a pH of 6.95 or 6.5, the PCr level was 28.4 mM or 23.9 mM respectively. Consequently, if the entire muscle decreased in pH from 6.95 to 6.5, the PCr level would actually only decrease by 16 %. As the anoxia in the 45 mg EDL muscle is only partial (the core area being about 25 % of the transverse section in the midbelly region of muscle, we assumed that the anoxic core could represent approx. 25 % of the muscle volume), the PCr variation in our preparation should be less than 16 %. However, the accuracy of the n.m.r. measurements is about 5 % for a sharp peak such as PCr. For enzymic measurements of PCr, the error can be greater, due to the possible PCr hydrolysis in $HClO_4$ extracts [16]: it is about ± 28 % in Table 1. So it is possible that the local decrease in pH was not great enough to give rise to a detectable decrease in the PCr level in the whole muscle. Consequently, the pH criterion may be evidence of possible and minor deviations of energy metabolism (predominance of glycolysis over the oxidative phosphorylation in a given area of muscle to supply ATP) that PCr measurements cannot reveal.

Because the partial anoxia in the 45 mg EDL muscle due to failure of O_2 to penetrate to the core of tissue impaired the energy metabolism of the muscle, we evaluated the effect of a more pronounced anoxia on this muscle by disruption of O_2 supply. The impact of hypoxia/anoxia on muscle metabolism has been studied previously *in vivo* and *in vitro* [36,37]. In anoxic muscles, pH is a function of anaerobic glycolysis to lactate, which produces protons, and of the creatine kinase reaction, which buffers ATP levels and consumes protons [19]. This coupling is highly developed in some fish species which are unusually resistant to anoxia [38]. Upon reoxygenation, there is no recovery of pH, probably because of an accumulation of products of anaerobic metabolism and because PCr resynthesis produces protons [19]. During complete anoxia, energy metabolism is also impaired at the muscle periphery. Consequently, both PCr and pH are sensitive indexes of recovery from advanced anoxia in a muscle preparation. By contrast, only the muscle pH *ex vivo* can reveal local anoxia.

In summary, the present results are consistent with a role for pH as clear evidence of the changes in energy metabolism that

may appear in a resting muscle in conditions *ex vivo*. Indeed, the controlled use of energy to maintain the cellular energy homeostasis in muscle must be modified if the integrity of muscle is not preserved, as in the 45 mg EDL muscles. Moreover, our experiments showed that ^{31}P n.m.r. allows the assessment of biochemical stability of muscles in conditions *ex vivo*. Observation of pH heterogeneity in muscles by P_i resonances provides a non-destructive approach to the characterization of metabolic heterogeneity in rat muscle *ex vivo* with far greater sensitivity than any comparable histochemical or biochemical measurement.

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REFERENCES

- Stirewalt, W. S. and Low, R. B. (1983) *Biochem. J.* **210**, 323–330
- Stirewalt, W. S., Low, R. B. and Slaiby, J. M. (1985) *Biochem. J.* **227**, 355–362
- Baracos, V., Langman, M. and Mak, A. (1989) *Comp. Biochem. Physiol.* **92A**, 555–563
- Garber, A. J. (1980) in *Glutamine: Metabolism, Enzymology and Regulation* (Mora, J. and Palacios, R., eds.), pp. 259–284, Academic Press, New York
- Parry-Billings, M., Leighton, B., Dimitriadis, G. D., Bond, J. and Newsholme, E. A. (1990) *Biochem. Pharmacol.* **40**, 1145–1148
- Meynial-Denis, D., Prugnaud, J., Arnal, M., Prunet, M., Mignon, M., Bayle, G., Vincent, C. and Chavaroux, A. (1990) in *Stable Isotopes in Paediatric Nutritional and Metabolic Research* (Chapman, T. E., Berger, R. and Okken, A., eds.), pp. 205–211, Intercept, Andover
- Gulve, E. A., Cartee, G. D., Youn, J. H. and Holloszy, J. O. (1991) *Am. J. Physiol.* **260**, C88–C95
- Henriksen, E. J. (1991) *Am. J. Physiol.* **261**, C608–C613
- Maltin, C. A. and Harris, C. I. (1986) *Biochem. J.* **238**, 622
- Maltin, C. A. and Harris, C. I. (1985) *Biochem. J.* **232**, 927–930
- Van Breda, E., Keizer, H. A., Glatz, J. F. C. and Geurten, P. (1990) *Biochem. J.* **267**, 257–260
- Segal, S. S. and Faulkner, J. A. (1985) *Am. J. Physiol.* **248**, C265–C270
- Essig, D. A., Segal, S. S. and White, T. P. (1985) *Am. J. Physiol.* **249**, C464–C470
- Dohm, G. L., Tapscott, E. B., Pories, W. J., Dabbs, D. J., Flickinger, E. J., Meelheim, D., Fushiki, T., Atkinson, S. M., Elton, C. W. and Caro, J. F. (1988) *J. Clin. Invest.* **82**, 486–494
- Newsholme, E. A., Leighton, B., Challiss, R. A. J. and Lozeman, F. J. (1986) *Biochem. J.* **238**, 621
- Kushmerick, M. J., Moerland, T. S. & Wiseman, R. W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7521–7525
- Sugden, P. H. and Fuller, S. J. (1991) *Biochem. J.* **273**, 21–37
- Busby, S. J. W., Gadian, D. G., Radda, G. K., Richards, R. E. and Seeley, P. J. (1978) *Biochem. J.* **170**, 103–114
- Walliman, T., Wyss, M., Brdiczka, D. and Nicolay, K. (1992) *Biochem. J.* **281**, 21–40
- Brown, G. C. (1992) *Biochem. J.* **284**, 1–13
- Gutman, I. and Wahlefeld, A. W. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 1464–1468, Verlag Chemie, Weinheim, and Academic Press, London
- Jaworek, D., Gruber, W. and Bergmeyer, H. U. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2127–2131, Verlag Chemie, Weinheim, and Academic Press, London
- Lamprecht, W., Stein, P., Heintz, F. and Weisser, H. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 1777–1781, Verlag Chemie, Weinheim, and Academic Press, London
- Keppler, D. and Decker, K. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 3, pp. 1127–1131, Verlag Chemie, Weinheim, and Academic Press, London
- Pearse, A. G. E. (1985) *Histochemistry, Theoretical and Applied*, vol. 2, pp. 675–753, Churchill-Livingstone, Edinburgh
- Roberts, J. K. M., Wade-Jardetzky, N. and Jardetzky, O. (1981) *Biochemistry* **20**, 5390–5394
- Moon, R. B. and Richards, J. H. (1973) *J. Biol. Chem.* **248**, 7276–7278
- Mazzeo, A. R., Delsuc, M. A., Kumar, A. and Levy, G. C. (1989) *J. Magn. Reson.* **81**, 512–519
- Atkinson, D. E. (1968) *Biochemistry* **7**, 4030–4034
- Sugden, M. C., Liu, Y. L. and Holness, M. J. (1990) *Biochem. J.* **272**, 133–137
- Smith, D., Green, H., Thompson, J. and Sharratt, M. (1988) *Am. J. Physiol.* **254**, C661–C668
- Challiss, R. A. J., Blackledge, M. J. and Radda, G. K. (1988) *Am. J. Physiol.* **254**, C417–C422
- Meyer, R. A., Brown, T. R. and Kushmerick, M. J. (1985) *Am. J. Physiol.* **248**, C279–C287
- Shoubridge, E. A., Bland, J. L. and Radda, G. K. (1984) *Biochim. Biophys. Acta* **805**, 72–78
- Veech, R. L., Lawson, J. W. R., Cornell, N. W. and Krebs, H. A. (1979) *J. Biol. Chem.* **254**, 6538–6547
- Challiss, R. A., Hayes, D. J., Petty, R. F. H. and Radda, G. K. (1986) *Biochem. J.* **236**, 461–467
- Fagan, J. M. and Tischler, M. (1989) *Life Sci.* **44**, 677–681
- Van Waarde, A., Van Den Thillart, G., Erkelens, C., Addink, A. and Lugtenburg, J. (1990) *J. Biol. Chem.* **265**, 914–923