Temperature and pH dependence of energy balance by $^{31}$P- and $^1$H-MRS in anaerobic frog muscle

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Abstract

The temperature ($T$)-dependence of energy consumption of resting anaerobic frog gastrocnemii exposed to different, changing electrochemical gradients was assessed. To this aim, the rate of ATP resynthesis ($\Delta f P/\Delta t$) was determined by $^{31}$P- and $^1$H-MRS as the sum of the rates of PCr hydrolysis ($\Delta [PCr]/\Delta t$) and of anaerobic glycolysis ($\Delta [La]/\Delta t$, based on a $f_P/\!\!/[La]$ ratio of 1.5). The investigated $T$ levels were 15, 20 and 25 $^\circ$C, whereas initial extracellular pH (pHe) values were 7.9, 7.3 and 7.0, i.e. higher, equal or lower, respectively, than intracellular pH (pHi). The latter was changing with $T$ according to the neutrality point ($dpH/dT = 0.0165$ pH units/$^\circ$C).

Both rates of PCr hydrolysis and of lactate accumulation and that of their sum, expressed as $\Delta f P/\Delta t$, were highly $T$-dependent. By contrast, the pH-dependent rate of muscle energy balance was nil or extremely limited at 15 and 20 $^\circ$C, respectively, but remarkable at 25 $^\circ$C (with a depression of the ATP resynthesis rate up to 25% with a decrease of pHe from 7.9 to 7.0). The pH-dependent reduction of metabolic rate was associated with a down-regulation of anaerobic glycolysis due to reduced activity of ion-transporters controlling acid–base balance and/or to a shift from Na$^-$/H$^+$ to a more efficient Na$^-$/HCO$_3$$^-$/H$^+$ ion exchange.

The described metabolic slowdown observed in isolated muscle preparations subjected to the combined regimes of anoxia/acidosis implies that the mechanism determining survival time at the cellular level is mediated by exchange transport systems. A similar mechanism might affect muscle metabolism of homeotherms during chronic hypoxia and/or ischemia.

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1. Introduction

In aerobic skeletal muscle, intracellular pH (pHi) is more alkaline than predicted if protons were passively distributed across the cell membrane according to electrochemical forces. Indeed, the persistent passive influx of protons driven by the latter is counteracted by active ion pumping aimed at maintaining homeostasis around optimal pHi functional levels. When changing environmental conditions (e.g. in anoxia) in a tissue generally regarded as the most hypoxia-tolerant as skeletal muscle [1], equilibrium between H$^+$ influx and efflux may be altered as a consequence of an adaptive decrease in membrane permeability (the so-called “channel arrest” [2]) and of increasing anaerobic glycolysis accompanied by lactic acid formation. Also changes of temperature, $T$, as is well known, have important consequences on intra- and extracellular muscle pH [3–5]. Thus, primary, as well as $T$-dependent, pHi changes may greatly affect energy consumption as well as metabolite fluxes in most biochemical pathways. However, to our knowledge, the effects of the interaction of $T$ and tissue intra- and extracellular pH on resting anaerobic muscle energetics have not been assessed systematically.

The aim of the present study was to investigate the $T$-dependence of the main determinants of energy consumption of resting anaerobic muscle exposed to different absolute and relative membrane electrochemical gradients. This is the companion study of a previous work in which the effects of
the same physiochemical variables (T and pH) were investigated on proton buffering [7].

Nuclear Magnetic Resonance Spectroscopy (MRS) is the technique of choice for investigating metabolism as a function of acid–base balance and temperature in anaerobic muscle preparations. In fact, by means of joint phosphorus ($^{31}$P) and proton ($^1$H) MRS techniques, high energy phosphates (≈ P = PCr + ATP), inorganic (Pi) and monoester (PME) phosphates as well as lactate (La) concentrations can be monitored, together with intracellular and extracellular pH values. This makes it possible to establish steady-state and transient changes of energy balance [6].

The anoxic isolated frog muscle is a particularly suitable experimental model. In fact, it reproduces conditions occasionally encountered also in animals and man in physiological and paraphysiological conditions when skeletal muscle is deprived of conductive and diffusive oxygen flux. This can occur because of blood flow arrest such as during isometric contractions or as an effect of peripheral vasoconstriction (“diving reflex”), conditions in which also temperature and acid–base state undergo wide changes.

2. Materials and methods

The methods and the experimental protocols adopted in this study were described in detail in Ref. [7]. Shortly, MRS recordings on muscles were carried out on a 4.7-T Bruker AM WB superconducting magnet (Bruker, Karlsruhe, Germany) with a 9-cm vertical bore, equipped

Fig. 1. Stacked plots of $^{31}$P and $^1$H MRS spectra at 4.7 T from a resting frog gastrocnemius perfused with Ringer solution at 20 °C and pH e = 7.3. The changes of the phosphate metabolites and lactate in anoxic conditions are shown by the sequence of the spectra. For the $^{31}$P spectra (a), the acquisition parameters were: spectral width: SW = 5000 Hz; data points: SI = 4 K; excitation hard pulse: $\pi/4 = 15$ µs; recycle time: TR = 7 s, to prevent partial saturation effects; acquisition number NS = 128; total acquisition time TE $\approx 15$ min. $^1$H spectra (b) were acquired by using a zero and double quantum filter sequence for monitoring lactate accumulation. Acquisition parameters were: spectral width: SW = 2200 Hz; SI = 8 K, recycle time TR = 1 s; acquisition number NS = 128; total acquisition time TE $\approx 10$ min. A line broadening, LB = 3 Hz, was applied before Fourier transformation.
with a probehead double tuneable at the $^{31}$P-(81 MHz) and $^1$H-(200.13 MHz) MRS resonance frequencies, respectively, and a with microimaging unit. All experiments were performed using a 15-mm insert and introducing the sample in a non-spinning 10 mm NMR tube. $^{31}$P and $^1$H 1D spectra were alternately recorded (Fig. 1a and b, respectively). $^1$H spectra were acquired by using a zero and double quantum filter sequence for monitoring lactate accumulation. By means of gradient pulses, the sequence, described in detail elsewhere [8], resolved the zero and double quantum lactate coherences from the overlapping lipid multiplet and suppressed the broad water signal. The lactate methyl resonance at 1.33 ppm was referred to sodium-3-trimethylsilylpropionate (TSP) used as internal reference. The areas under the phosphates and lactate peaks were calculated using a numerical integration routine supplied by Bruker and running on the spectrometer. No $T_1$ correction factor was applied to calculate metabolite concentrations. Details on the acquisition parameters appear in the caption of Fig. 1 and in Ref. [7].

The experiments were carried out on the frog (R. esculenta) gastrocnemius muscles during the spring season. Animals from two identical batches were acclimatized to $25^\circ$C in tap water, fed with mealworms over 8–12 weeks and then sacrificed. The experimental muscles were dissected out and reoxygenated in Ringer solution (NaCl 70 mM, NaHCO3 25 mM, KCl 5.1 mM, CaCl2 2.3 mM, MgCl2 1.6 mM equilibrated with 2% CO2 in O2) at three experimental temperatures (15, 20 or $25^\circ$C) and at constant extracellular pH of 7.7 which is the average natural value for the selected $T$ experimental range. The frog muscles investigated at 15 and $25^\circ$C ($n = 12$) are those appearing in Ref. [7] to which additional muscles ($n = 6$; wet weight = $0.38 \pm 0.04$ g) were added for the measurements at $20^\circ$C.

pHe was set initially at 7.0, 7.3 and 7.9 respectively, whereas the initial pHi of each preparation was dictated by the $T$ equilibrium level determined also on a separate set of muscles. pHi was found to be negatively related to $T$, according to the linear function:

$$pHi = 7.59 - 0.0165T \quad (^\circ C) \quad r^2 = 0.96 \quad n = 13$$

The experimental muscle was introduced in the NMR tube soaked in 1.5 ml of a $^2$H2O/H2O (4:1, v/v) deoxygenated Ringer solution, containing 5 mM sodium-3-trimethylsilylpropionate (TSP). Sample tubes were then inserted into the spectrometer and the planned MRS measurements were carried out at the chosen thermostated experimental temperatures (15, 20, $25^\circ$C). $^1$H and $^{31}$P spectra were recorded alternatively at 30-min intervals, and the monitoring proceeded until the $\beta$ ATP peak could be clearly distinguished (see Fig. 1a).

In order to calculate absolute phosphate concentrations, at the onset of each experiment, a known amount (10 mM) of phenylphosphonic acid (PPA) was added as a reference to the perifusion solution. The addition of PPA, a $^{31}$P-MRS-detectable pH-sensitive marker to the solution, allowed the measurement over time of pHe [9] too. On the other hand, $^{31}$P-MRS is known to be a reliable method for determining pHi. The latter is based on the chemical shift of the Pi resonance from titration curves obtained in appropriate conditions. For the present study, the pHi and pHe values were calculated using the calibration curves previously determined on standard solutions at the chosen experimental

![Fig. 2. Concentration versus time plots of the phosphocreatine ([PCr]) and lactate ([La]) of anoxic frog gastrocnemius at the experimental temperature of $20^\circ$C. Data corresponding to different experimental extracellular pH levels (●, black, 7.9; □, white, 7.3; △, grey, 7.0) are shown. Concentrations are expressed in $\mu$mol·g$^{-1}$ of muscle wet weight. Time zero corresponds to the beginning of anoxia (which was set arbitrarily when the PCr peak starts decreasing sharply).](image-url)
temperatures. The obtained titration data were fit to the equation:

\[ \text{pH} = \text{p}K_a + \log(\frac{\delta a}{\delta O})/\log(\frac{\delta b}{\delta O}) \]  

(2)

where \( \text{p}K_a \) is the apparent proton association constant and \( \delta a, \delta b \) and \( \delta O \) are the observed and limiting chemical shifts at acid and basic pH, respectively, of PPA and Pi.

The pH of the perifusion solution was also checked at the beginning and at the end of the MRS measurements by a pH-sensitive glass electrode (Hanna Instruments, Italy).

Lactate concentrations ([La]) were calculated taking into account also the lactate diffused from the muscle to the perifusion solution by a previously described method [6].

| Table 1 |
|-------------------------|---------------------|---------------------|
| Rates of PCr hydrolysis, lactate accumulation and total ATP resynthesis (\( \Delta - P/\Delta t \)) (in \( \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \)) in resting frog muscles calculated over the first 5 h from the beginning of anoxia as a function of \( T \) and pHe. |
| pHe | \( T = 15 \degree C \) | \( T = 20 \degree C \) | \( T = 25 \degree C \) |
| PCr splitting (\( \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \)) | | | |
| 7.9 | 2.26 | 3.86 | 5.18 |
| 7.3 | 2.59 | 3.90 | 5.36 |
| 7.0 | 2.33 | 3.56 | 5.22 |
| Lactate accumulation (\( \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \)) | | | |
| 7.9 | 0.15 | 1.05 | 2.16 |
| 7.3 | 0.13 | 0.64 | 1.20 |
| 7.0 | 0.15 | 0.72 | 0.80 |
| Total ATP resynthesis (\( \Delta - P/\Delta t \)) (\( \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \)) | | | |
| 7.9 | 2.49 | 5.44 | 8.42 |
| 7.3 | 2.79 | 4.86 | 7.16 |
| 7.0 | 2.56 | 4.64 | 6.42 |

3. Results

At 20 °C, [PCr] appears to decrease exponentially at a rate (\( \Delta[\text{PCr}] / \Delta t \)) that is not affected by the acidity of the extracellular medium (Fig. 2a). By contrast, the rate of La production (\( \Delta[\text{La}] / \Delta t \)) appears to be pH-dependent, the greater La accumulation rate being associated with a higher, closer to normal (7.9), pH level (Fig. 2b). The rates of PCr hydrolysis and La accumulation obtained at 15 and 25 °C were calculated from previous measurements [7] and, together with the data appearing in Fig. 2 (20 °C), were used to determine the corresponding muscle energy balance (calculated as the overall rate of ATP resynthesis, \( \Delta - P/\Delta t \)). To this aim, the rate of resynthesis of ATP at each investigated \( T \) value was calculated as the sum of the rate of PCr hydrolysis through the Lohmann reaction and the rate of lactate accumulation, assuming a \( f_{P/La} \) ratio of 1.5 [6]. Average data of energy consumption (\( \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \)) during the first 5 h of anoxia are sum-
The rate of ATP resynthesis in the investigated conditions appears to be greatly affected by $T$ and to a lesser extent by pH. In fact, the overall energy turnover is not affected by pH at 15 °C but tends to decrease substantially with decreasing pH at 20 and 25 °C. Thus, the $Q_{10}$ values (i.e. $\left(R_2/R_1\right)^{\frac{10}{(T_2-T_1)}}$, in which $R_1$ and $R_2$ are reaction rates at temperatures $T_1$ and $T_2$, respectively, and $T_2>T_1$) calculated for the $T$ range 25 to 15 °C is 3.4 when the initial pH was 7.9 and 2.5 at pH of 7.0. Beyond 5 h from the onset of the measurements, the overall rate of energy turnover ($\Delta \sim P/\Delta t$) appears to decrease, particularly at higher temperatures (Fig. 3). After 15 h of incubation, a constant, similar energy consumption ($0.68 \pm 0.22 \, \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) was attained by all preparations that is independent of $T$ and pH (Fig. 3).

### 4. Discussion

The frog muscle is a standard model for the study of muscle energetics, anoxia-tolerant and functionally stable over hours in ex vivo conditions. The systemic effects of blood buffer capacity and chemical composition changes are abolished whereas the extracellular acid–base state can be easily set and controlled.

As is well known, when temperature increases, muscle pH decreases changing in parallel with the neutrality point (pN) [3,4,10]. The pH prevailing at the investigated $T$ values chosen for the present study as well as the slope of the pH–temperature relationship were similar to those reported for the frog muscle by Reeves and Malan [11] and by Marjanovic et al. [12].

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**Fig. 4.** [La] in muscle as function of [PCr] and [Pi] at pH=7.9 and different temperatures (a and c, respectively) and at pH ranging between 7.9 and 7.0 at $T=25$ °C (b and d, respectively).
4.1. Thermal and P-metabolite dependence of the anaerobic energy balance

The well-known fact that ion-motive ATPases are highly temperature-sensitive is reflected by the higher rate of ATP utilization at 25 °C than at 20 and at 15 °C. The thermal dependence of ATP resynthesis in the present study appears to be higher than that calculated earlier by Binzoni et al. [13]. Such discrepancy might depend on the assumptions made by the above authors for calculating La concentration.

Creatine phosphate hydrolysis is the greatest contributor to ATP resynthesis in the muscle from the onset of anoxia, independent of T and pHe (see Fig. 3, upper part, striped boxes). By contrast, despite the large muscle glycogen stores, the energy supplied by anaerobic glycolysis appears to be qualitatively limited and tightly modulated by variables such as T, pHe [7] and P-metabolite concentrations. This is shown in Fig. 4 where the metabolite data appearing in Fig. 2 (at 20 °C) and reported in Ref. [7] (at 15 and 25 °C) are plotted independent of time: a striking dissociation between the amount of glycolysis (assessed from [La]) and [PCr] or [Pi] is shown as a function of T (panels a and c) and of pHe (b and d), respectively. The present results confirm and extend previous observations of Hsu and Dawson [14] in frog sartorius at 4 °C and pHe = 7.0 of uncoupling of glycologenolysis from P-metabolite concentration. A possible partial explanation of the above results could be found in the temperature dependence of phosphofructokinase activity [15]. On the other hand, a slight rise of Pi, a consequence of the slow PCr splitting rate at lower T levels, might impair the activity of phosphorylase and phosphofructokinase.

4.2. Energy cost of pH regulation

As indicated before, living cells regulate proton ion concentration at a level far removed from electrochemical equilibrium and a considerable fraction of the basal metabolic rate fuels ion pumping in order to maintain appropriate ion gradients across the membrane. Indeed, the latter plays important roles in “facilitated” diffusion and secondary active transport of metabolites. In resting muscle fibres, both the cellular production of acid and the negative membrane potential tend to accumulate H⁺. This trend is counteracted by the membrane transporter systems promoting H⁺ efflux. The main fraction (~ 80%) of the latter is carried out by the Na⁺/H⁺ exchange system, whereas only ~ 20% is mediated by bicarbonate-dependent exchange [16]. Reipschläger and Pörtner [17] observed that the rate of oxygen consumption of a perifused muscle preparation of Sipunculus nudus was significantly depressed when reducing pH. The authors suggested that such lower energy demand could be associated to a change of the H⁺-regulatory processes. Since the Na⁺/H⁺exchanger was demonstrated to be inhibited by high extracellular H⁺ concentrations, a shift of the proton transport activity to a Na⁺ dependent Cl⁻/HCO₃⁻ exchanger could have taken place leading to a sizeable reduction of the ATP cost of pHi regulation, whence the metabolic depression [17,18]. Indeed, this result could be reached by a shift from a less to a more ATP-efficient ion transporter whereby the electroneutral Na⁺-dependent Cl/HCO₃⁻ exchanger requires (indirectly) only 0.17 ATP per proton extruded from the cell against 0.33 of the Na⁺/H⁺ exchanger (Fig. 5).

Under anoxia, such as in the present experimental conditions, the ion gradients across the membrane are known to be preserved even in the face of severely depressed metabolic rate and production of lactic acid [1,2,19]. From Fig. 2 and Table 1, it appears that within 5 h from the onset of the experiments, lower pHe values are associated, for given levels of PCr hydrolysis, with a decreased rate of anaerobic glycolysis, particularly at the higher T levels. Thus, an energy-saving mechanism like the one described by Reipschläger and Pörtner [17] for the oxygenated muscle seems to operate also in anaerobic conditions as those prevailing in the present experiments. However, the above conclusion can be drawn only if in the investigated muscle lactate is the only accumulated metabolite, and no other energy-yielding glycolytic intermediates such as phosphoenolpyruvate and phosphodiester upstream from pyruvate and lactate are stored. This was shown to be the case in the companion study [7] and appears also from Fig. 1. As to the pHe-dependent mechanism controlling the rate of glycolysis, the hypothesis can be put forward that the activity of the lactate transporters is differentially modulated by the proton gradient across the membrane [7] (Fig. 5). Although the signalling mechanism remains obscure, it could be hypoth-

Fig. 5. A schematic model of muscle pH regulation at rest during anaerobiosis. On the left: passive flux of undissociated lactic acid. On the right: transport systems of importance for pH regulation. Extracellular protons inhibit the lactate/H⁺ co-transport and Na⁺/H⁺ exchanger. As a consequence, the Na⁺/H⁺/Cl⁻/HCO₃⁻ exchanger becomes increasingly involved with a reduction of energy demand per proton extruded (redrawn and modified after Ref. [17]).
esized that transmembrane proteins could be affected by interstitial or cellular surface [H+] depending on the buffering capacity of the tissue. In support of this hypothesis, changes in cell membrane permeability and in ion transport systems were observed also throughout adaptation of man to chronic hypoxia [20]. Particularly, the system mediating HCO3− transport across the membrane of the erythrocyte was found to be up-regulated at altitude where [HCO3−] is lowered and alterations in H+ and lactate transport systems are probably involved in the changes of dynamic buffer capacity. A relationship between energy expenditure and ion channel density in anoxia-resistant and-sensitive animals was also found in previous studies [21]. Thus, it would appear that the critical difference between anoxia-tolerant and anoxia-sensitive tissues is the reduction in the former of the absolute ATP demand for maintaining ion gradients in response to oxygen lack. This is obtained by blockade or down-regulation of activity of given ion transporters and/or by different choice of the latter.

4.3. Time dependence of ATP resynthesis

At rest, it would be reasonable to expect a constant ATP demand by the muscle throughout the experiment for any given temperature and membrane H+ gradient. Obviously, this does not appear to be the case (see Fig. 3), particularly at high T levels. The time-dependent decrease of metabolic rate cannot be compensated for the known increase of the thermodynamic efficiency of ATP hydrolysis with decreasing PCr concentration [22]. However, the found results are those expected for anoxia-tolerant preparations according to the theory of channel arrest [1]. As appears from Fig. 3 (lower panels), where the experimental data were normalized to an initial ATP resynthesis rate made equal to 100% of the latter.

5. Conclusions

In conclusion, the assessment of the muscle anaerobic energy balance in maintaining the proton gradient across the cell membrane, within a large range of environmental temperatures and extracellular pH levels, allows to draw the following conclusions:

1. Ion-motive ATPases are highly temperature sensitive, particularly at pH values close to normal for the investigated T levels;
2. At any given temperature in the range 15 to 25 °C, the rate of ATP resynthesis is depressed when reducing pH, particularly at T ≥ 20 °C as was the case for the rate of O2 consumption of the muscle of Sipunculus nudus [17];
3. Uncoupling of glycolysis from P-metabolites concentration both as a function of T (≥ 20 °C) and of pH (≤ 7.3) seems to occur, presumably because of the temperature dependence of glycolytic enzyme activity and/or ion transport mechanisms.

How the above described changes in muscle metabolism may interfere with the energetic maintenance of the demands of ionic and osmotic equilibria of muscles in homeotherms when deprived of normal O2 supply is still matter for investigation.

References


