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Effects of temperature and extracellular pH on metabolites: kinetics of anaerobic metabolism in resting muscle by ^{31}P- and ^{1}H-NMR spectroscopy

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Summary

Environmental stress, such as low temperature, extracellular acidosis and anoxia, is known to play a key role in metabolic regulation. The aim of the present study was to gain insight into the combined temperature–pH regulation of metabolic rate in frog muscle, i.e. an anoxia-tolerant tissue. The rate of exergonic metabolic processes occurring in resting isolated muscles was determined at 15°C and 25°C as well as at extracellular pH values higher (7.9), similar (7.3) and lower (7.0) than the physiological intracellular pH. ^{31}P and ^{1}H nuclear magnetic resonance spectroscopy high-resolution measurements were carried out at 4.7 T in isolated frog (*Rana esculenta*) gastrocnemius muscle during anoxia to assess, by means of reference compounds, the concentration of all phosphate metabolites and lactate. Intra- and extracellular pH was also determined.

In the range of examined temperatures (15–25°C), the temperature dependence of anaerobic glycolysis was found to be higher than that of PCr depletion (Q_{10}=2.3). High-energy phosphate metabolism was confirmed to be the initial and preferential energy source. The rate of phosphocreatine hydrolysis did not appear to be affected by extracellular pH changes. By contrast, independent of the intracellular pH value, at the higher temperature (25°C) a lowering of the extracellular pH from 7.9 to 7.0 caused a depression in lactate accumulation. This mechanism was ascribed to the transmembrane proton concentration gradient. This parameter was demonstrated to regulate glycolysis, probably through a reduced lactate efflux, depending on the activity of the lactate–H^+ co-transporter.

The calculated intracellular buffer capacity was related to intra- and extracellular pH and temperature. At the experimental extracellular pH of 7.9 and at a temperature of 15°C and 25°C, calculated intracellular buffering capacity was 29.50 μmol g^{-1} pH $unit^{-1}$ and 69.98 μmol g^{-1} pH $unit^{-1}$, respectively.

Key words: NMR spectroscopy, temperature, pH, anaerobic metabolism, frog, *Rana esculenta*, gastrocnemius muscle.

Introduction

Muscle metabolism is susceptible to a variety of stressing agents affecting particularly the overall rate of energy turnover. As is well known, temperature is a key environmental determinant of the activity of cellular multienzyme systems, such as muscle. In fact, this variable determines the kinetics and flux throughout biochemical pathways, particularly metabolic rates. Another key environmental determinant of cellular multienzyme system activity is intracellular pH. In general, owing to the presence of pH-sensitive ionizing groups, enzymes are active only over a limited range of pH with a definite optimum. Moreover, extracellular acidosis has been shown to play a key role in metabolic processes (Hood et al., 1988). Also, severe hypoxia and anoxia may modulate the ATP demand by the cell (Hochachka, 1986a,b). Skeletal muscle is known to be one of the most hypoxia-tolerant tissues and, in O_2-limiting conditions, can survive by means of a metabolic depression, thereby saving substrates and reducing deleterious end-product formation.

Ectothermic vertebrates, such as the frog, that normally experience rapid and large variations in body temperature (T) and consequent acid–base state changes, based on physico-chemical adaptations (Boutilier, 2001), appear to tolerate such changes with minimal metabolic disturbances. The effects of temperature variations on acid–base balance regulation mechanisms have been well documented (Reeves, 1969). The '*in vivo*' blood pH of most studied ectothermic species decreases as temperature increases with a slope close to –0.018 pH units $°C^{-1}$ (Malan et al., 1976). It has been suggested that $\Delta pH/\Delta T$ changes of such an extent can keep the histidine groups of proteins at an almost constant dissociation state (alphastat hypothesis; Reeves, 1972). Therefore, ectothermic vertebrates offer a suitable model for studying the

influence of changes in both physical (temperature) and chemical (pH) variables on the metabolism.

In the present study, the anaerobic condition was chosen because in this simplified experimental model, owing to the lack of oxygen, the only active energy-yielding mechanisms are the hydrolysis of high-energy phosphates, mainly phosphocreatine (PCr), and glycogenolysis, the end product of which is lactate.

Nowadays, nuclear magnetic resonance spectroscopy is the technique of choice for investigating muscle energetics in O_2-deprived preparations. In fact, by means of joint phosphorus (^{31}P) and proton (^{1}H) NMR spectroscopy techniques, high-energy phosphates (~P; PCr and ATP), inorganic (P_i) and monoester (PME) phosphates and lactate (La) concentrations can be monitored, as well as intracellular and extracellular pH. Moreover, transient changes can be assessed (Vezzoli et. al, 1997).

In the present paper, through the analysis of NMR spectroscopy data on frog gastrocnemius muscles, quantitative estimates of the metabolites' kinetics in experimental conditions simulating ischemia (no re-circulation, no oxygen) have been obtained. The aim of the study was to gain insight into the control of muscle anaerobic metabolism by extra- and/or intracellular pH and temperature. In order to investigate the mechanisms involved in compensatory reactions as well as to identify possible synergic combinations, the effects of these physico-chemical variables were separately studied.

The main results of the present study concerning both temperature and pH variables can be summarized as follows: (1) in the examined range of 15–25°C, the temperature dependence of anaerobic glycolysis was found to be higher than that of PCr depletion. In addition, high-energy phosphate metabolism was confirmed to be the initial and preferential energetic source; (2) the extracellular pH or, more specifically, the transmembrane proton concentration gradient was found to regulate the lactate accumulation rate in isolated muscle. Moreover, the influence of extracellular pH on glycolysis was demonstrated to be the local regulatory mechanism, capable of maintaining the cellular acid–base equilibrium during an anoxic period. In fact, anoxia induces downregulation of ion channels and main energy-consuming processes (active processes, e.g. ion exchange). This mechanism has been established to be independent of the intracellular pH value.

Materials and methods

NMR spectroscopy apparatus and measuring procedure

High-resolution NMR spectroscopy measurements were carried out on a 4.7 T Bruker AM WB spectrometer (Karlsruhe, Germany), with 9 cm vertical bore, equipped with a microimaging unit. This accessory includes a gradient drive unit and a double tuneable probe head at the ^{31}P- (81 MHz) and ^{1}H- (200.13 MHz) NMR spectroscopy resonance frequencies. Three orthogonal self-shielded gradient coils capable of achieving gradient strength up to 50 G cm^{-1} are built into the probe head. Since, in this configuration, the instrument was not equipped with a field-frequency stabilization (lock channel), at the beginning of the experiment the field frequency was set on the H_2O frequency. All experiments were carried out with a 15 mm insert, introducing the sample in a non-spinning 10 mm NMR tube. ^{31}P and ^{1}H one-dimensional spectra were recorded. ^{31}P spectra (spectral width 5000 Hz; 4096 data points) were acquired by applying an excitation hard pulse of 45° (15 μs) and a recycle time of 7 s, which was long enough to prevent partial saturation effects. Each spectrum was the mean of 128 transients, thus requiring approximately 15 min acquisition time. The PCr peak was used as an internal reference (δ=0 p.p.m.). Indeed, when PCr concentration went below a detectable level, so that its signal was lost, all ^{31}P peaks were referred to PCr absolute frequency resonance. As is well known, the PCr NMR signal is not affected by pH in the range of our study owing to its low pKa (about 4.6). Therefore, its resonance stays at the same frequency for the duration of the experiment. On the other hand, in order to obtain absolute metabolite concentrations, the use of sealed capillary external references is avoided to eliminate possible susceptibility effects (Gadian, 1982).

A line broadening of 10 Hz was applied before Fourier transformation. ^{1}H spectra (spectral width 2200 Hz; 8192 data points) were acquired at regular intervals for monitoring lactate accumulation. 100% of the lactate methyl signal was recovered, and both the overlapping lipid multiplet and the broad water signal were suppressed by means of a zero and double quantum filter sequence, the coherences being selected by gradient pulses. The sequence has been described in detail previously (Gussoni et al., 1994b).

The lactate methyl chemical shift (1.33 p.p.m.), representing sodium 3-trimethylsilylpropionate (TSP), was used as internal reference. Each spectrum was the mean of 128 transients, with a recycle time of 1 s and approximately 10 min acquisition time. A line broadening of 3 Hz was applied before Fourier transformation. The areas under the phosphate and lactate peaks were calculated using a numerical integration routine on the Bruker spectrometer. No T_1 correction factor was applied to calculate the metabolite concentrations.

Muscle preparation

All experiments were carried out on frog (*Rana esculenta* L.) gastrocnemius muscles. The frogs were acclimatized at 25°C in tap water, fed with mealworms over 8–12 weeks and then sacrificed. The experiments were carried out during the spring season. Each muscle, after dissection, was reoxygenated in Ringer solution (70 mmol l^{-1} NaCl, 25 mmol l^{-1} $NaHCO_3$, 5.1 mmol l^{-1} KCl, 2.3 mmol l^{-1} $CaCl_2$, 1.6 mmol l^{-1} $MgCl_2$ equilibrated with 2% CO_2 in O_2) at the temperature chosen for the experimental design (T=15°C or 25°C) and at pH=7.7. Under these conditions, the restoration of the basal metabolite levels and the spontaneous equilibration of intracellular pH (pHi), which is known to be T dependent, were achieved. Then, the sample was carefully introduced into a 10 mm NMR tube and soaked in 1.5 ml of a $^{2}H_2O/H_2O$ (4:1 v/v) Ringer solution, containing 5 mmol l^{-1} TSP. The deoxygenated incubation

medium in the sample NMR tube was previously equilibrated to the chosen experimental temperature, and its pH was alternately set to 7.0, 7.3 and 7.9. The sample tube was then inserted into the spectrometer at the chosen thermostatted temperature, and all sets of NMR spectroscopy measurements were performed. ^{1}H and ^{31}P spectra were recorded alternately at 30 min intervals, until the ATP peak could be clearly distinguished.

Experimental design

In '*ex vivo*' experiments performed on whole isolated muscles, the pHi level will depend upon the temperature as well as upon the pH value of the perifusion medium [extracellular pH (pHe)]. The aim of the present study was to compare the temperature dependence of the anaerobic metabolic rate of muscles exposed to different initial pHe values, while the pHi level was spontaneously determined by temperature.

The experiments were carried out on 12 frog gastrocnemii (muscle wet mass, 0.356±0.062 g) at the imposed constant *T* values of 15°C and 25°C, while the pH of the deoxygenated perifusion Ringer solution (pHe) was alternatively set to 7.0, 7.3 and 7.9, thus simulating a positive, null and negative intracellular–extracellular gradient, respectively. Each experimental condition was tested on two different muscle preparations. Since the difference between the repetitions was not statistically significant ($P>0.10$), the data were reported as a single preparation.

In order to analyze the combined role of pHe and pHi, an additional set of experiments was carried out at 25°C on two different preparations (wet mass, 0.368±0.034 g). As before, the transmembrane proton gradient was varied, but, in contrast to the procedure previously described, the initial pHi level was the variable parameter. For both muscle sample preparations, an initial pHe value of 7.9 was adopted whereas the initial pHi was variable.

Quantitative calibration

In order to calculate absolute phosphate concentrations (PCr, ATP, P_i and PME), at the beginning of the experiment a known amount (10 mmol l^{-1}) of sodium phenylphosphonate (PPA) was added as a reference to the perifusion solution. The addition of PPA, a ^{31}P-NMR spectroscopy-detectable pH sensitive marker, to the perifusion solution also allowed measurement of pHe over time (Meyer et al., 1985). In addition, ^{31}P-NMR spectroscopy is known to be a reliable method for determining the pHi in intact muscles, based on the chemical shift of the P_i resonance from a titration curve, obtained in appropriate conditions. In the present study, the pHi values were calculated using the calibration curves previously determined on model solutions at the experimental temperatures (Vezzoli et al., 1997).

By means of the quantitative method, which has been widely described previously (Gussoni et al., 1994a), lactate concentrations were calculated, also taking into account the lactate diffusion from the muscle to the perifusion solution.

Results

Figs 1 and 2 show changes in the absolute concentrations (μmol g^{-1} wet mass) of PCr (Figs 1A, 2A), Pi (Figs 1B, 2B), ATP (Figs 1C, 2C), PME (Figs 1D, 2D) and lactate (Figs 1F, 2F) as well as pHi values (Figs 1E, 2E) as a function of time (h) in anoxic frog gastrocnemii at 15°C and 25°C, respectively. The data were collected at pHe of 7.9 (black symbols), 7.3 (white symbols) and 7.0 (grey symbols).

At all investigated temperatures and pHe levels, the initial PCr (Figs 1A, 2A) and lactate (Figs 1F, 2F) concentrations were not significantly different: 29.35±1.43μmol g^{-1} wet mass and 1.54±0.90μmol g^{-1} wet mass, respectively. During the anaerobic incubation, the PCr concentration value fell exponentially, with a concomitant increase in P_i concentration (Figs 1B, 2B) that could be described by a power function. The kinetics of PCr hydrolysis and the correspondent P_i accumulation were independent of pHe in the investigated range (7.0–7.9).

At all examined experimental conditions, no significant changes in ATP concentration (5.91±0.79 μmol g^{-1} wet mass) were observed (Figs 1C, 2C) until the PCr concentration fell below ~10% of its initial level.

At all pHe levels tested at T=15°C, the concentration of PME remained nearly constant at 3 μmol g^{-1} wet mass (Fig. 1D). By contrast, at 25°C, PME accumulated at a constant rate, which increased according to the decrease of pHe (Fig. 2D).

At all experimental conditions, before the anaerobic glycolysis became noticeable a net alkalinization was detected, the amplitude of which seemed to be dependent on both pHe and *T* (Figs 1E, 2E). The further acidification down to different pHi levels depended on the experimental *T*. However, in spite of different extracellular pH, no difference was detectable among the three different conditions.

La concentration increased almost linearly. Independent of pHe, at 15°C the rate of lactate accumulation was 0.14±0.01 μmol g^{-1} h^{-1} (Fig. 1F). By contrast, at 25°C, La accumulation was found to be related to pHe. In fact, lactate accumulation rate decreased from 2.16 μmol g^{-1} h^{-1} to 0.80 μmol g^{-1} h^{-1} (Fig. 2F) as pHe decreased from 7.9 to 7.0.

By comparing the corresponding data shown in Figs 1 and 2, it was possible to understand the effects of temperature on the kinetics of all the examined metabolites. As expected, the exhaustion of the PCr stores and the rate of the glycolytic process were found to be *T* dependent. A pHe value of 7.9 corresponded to the highest rate of lactate accumulation. The temperature dependence, calculated as temperature coefficient (Q_{10} and TD_{10}), for PCr hydrolysis and La accumulation in the range where the rate of the processes kept constant (i.e. within the first 5 h for PCr hydrolysis and 10 h for lactate accumulation) was 2.3 and 14.4, respectively. As is well known, Q_{10} value $\{(R_2/R_1)^{[10/(T2-T1)]}$, where $R1$ and $R2$ are reaction rates at temperatures $T1$ and $T2$, respectively, and $T2>T1\}$ defines only reaction rates, so it would probably be incorrect to calculate Q_{10} values for biological processes, such

as glycolysis, that assemble a lot of reactions. However, we think that the use of a parameter similar to Q_{10} would help in the interpretation of biological messages on thermal dependence of metabolic processes. Therefore, analogous to Q_{10}, the thermal dependence term TD_{10} is defined here: $TD_{10}=(P_2/P_1)^{[10/(T2-T1)]}$, where P_1 and P_2 are rate processes at temperatures $T1$ and $T2$, respectively, and $T2>T1$.

According to the metabolic request, which is related to the experimental temperature, a decrease in ATP concentration was observed only at 25°C, when PCr concentration fell below 10% of its initial level (see Fig. 2A,C). From this level onwards, the energy re-generated by PCr hydrolysis and by anaerobic glycolysis became insufficient to cope with the energy demand.

During this final part of the experiments, differences could be observed in the kinetics of P_i levels obtained at 15°C (Fig. 1B) and 25°C (Fig. 2B). At 15°C, P_i concentration reached a plateau at a molar level nearly corresponding to the amount of PCr hydrolysed from the beginning of the experiment. On the contrary, at 25°C no plateau was reached. This phenomenon may be attributed to the simultaneous ATP drop: in fact, P_i concentration seems to roughly correspond to the sum of two components (PCr and ATP hydrolysed).

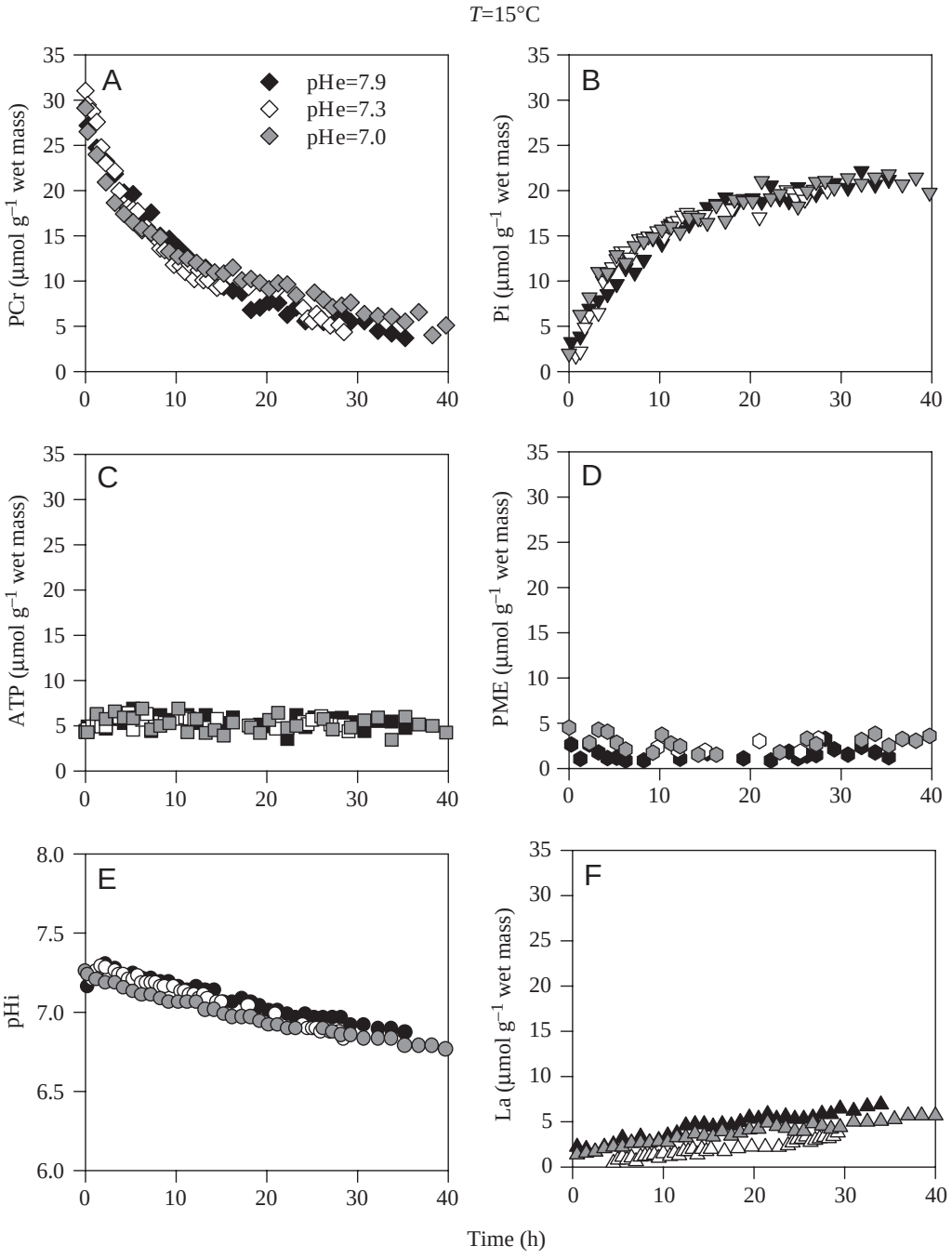

Fig. 1. Concentration *versus* time plots of (A) phosphocreatine (PCr), (B) inorganic phosphate (Pi), (C) adenosine triphosphate (ATP), (D) phosphomonoesters (PME) and (F) lactate (La) at the experimental temperature of 15°C. Concentration levels are expressed in µmol g^{-1} muscle wet mass. (E) Intracellular pH (pHi) *versus* time plots are also shown. All data corresponding to the different experimental extracellular pH (black, 7.9; white, 7.3; grey, 7.0) are shown. Time zero corresponds to the beginning of anaerobiosis (arbitrarily set when the PCr peak started decreasing sharply).

Fig. 3, obtained from the data collected on the two different muscle preparations at T=25°C, shows time-dependent changes of PCr and La concentration (Fig. 3A) and pHi and pHe (Fig. 3B). All the data were obtained from the additional set of experiments where the initial pHe value was 7.9 and pHi was at either a high (filled symbols) or a low level (open symbols). In Fig. 3B, the brackets indicate the corresponding pHi and pHe values. It can be observed that, according to a higher proton gradient (Fig. 3B), La accumulation in preparation 2 was much more extensive (Fig. 3A).

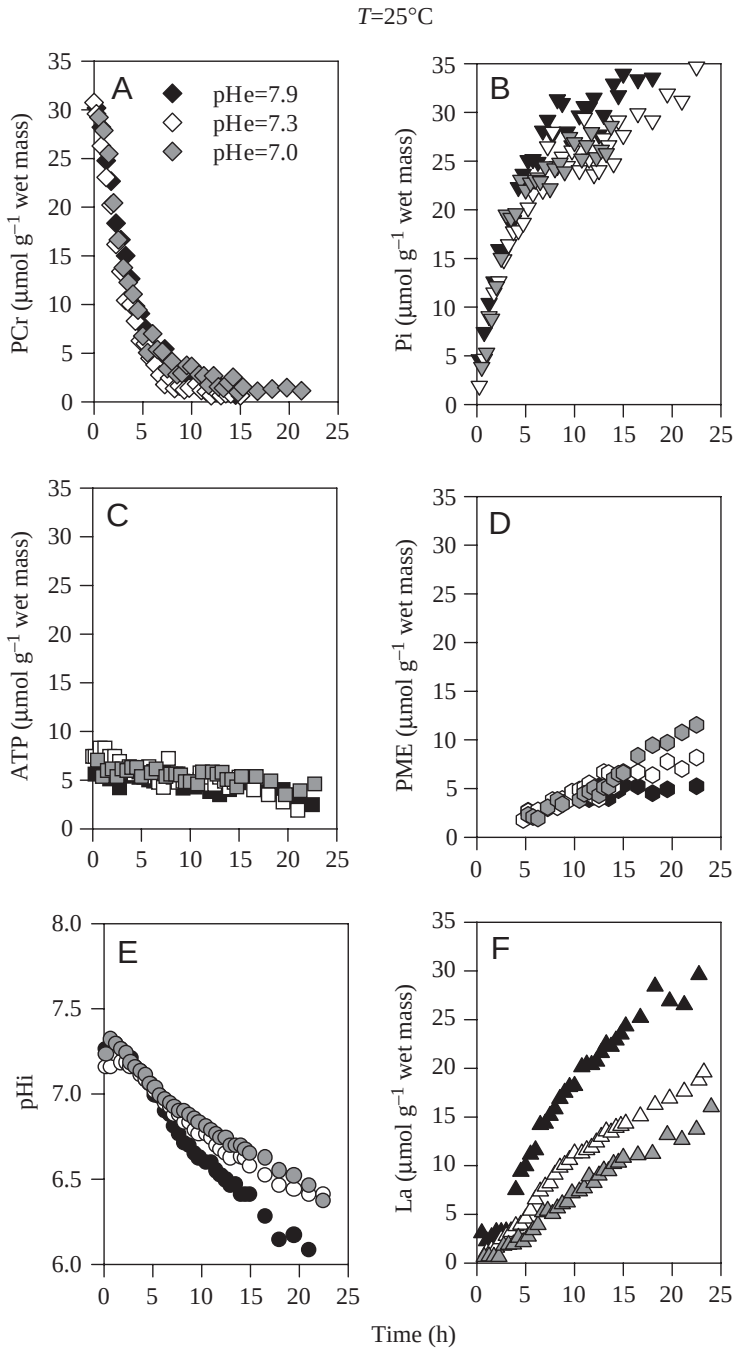

Fig. 2. Concentration *versus* time plots of (A) phosphocreatine (PCr), (B) inorganic phosphate (Pi), (C) adenosine triphosphate (ATP), (D) phosphomonoesters (PME) and (F) lactate (La) at the experimental temperature of 25°C. Concentrations are expressed in μmol g^{-1} muscle wet mass. (E) Intracellular pH (pHi) *versus* time plots are also shown. All data corresponding to the different experimental extracellular pH (black, 7.9; white, 7.3; grey, 7.0) are shown. Time zero corresponds to the beginning of anaerobiosis.

Buffer capacity

According to the well-known creatine kinase equilibrium:

$$[PCr] + [ADP] + [H^+] \leftrightarrow [ATP] + [Cr] , \qquad (1)$$

at the onset of experiments carried out on muscles in anaerobic condition an imbalance between ATP supply and demand causes PCr concentration to decrease. This PCr depletion results in an H^+-consuming reaction, which determines the initial alkalinization (see Figs 1E, 2E). In this phase, the plot of the cumulative changes in pH (ΔpH) against the cumulative consumption of protons (ΔP), resulting from changes in [PCr], would be a straight line passing through the origin with a slope equal to $-1/\beta$ (assuming the buffer capacity, β, to be independent of pH; Kemp et al., 1993). Fig. 4 shows ΔP and ΔpH, both measured with respect to the starting point of the experiment (time=0), determined at 15°C (Fig. 4A) and 25°C (Fig. 4B) and for all pHe levels. For the experimental T of 15°C and 25°C, the values of β, calculated at pHe 7.9, were 29.50 μmol g^{-1} pH $unit^{-1}$ and 69.98 μmol g^{-1} pH $unit^{-1}$, respectively.

Discussion

Thermal dependence of the metabolic processes utilizing anaerobic (alactic and lactic) energy sources

From our data, it is evident that when T was low the energy for the anaerobic metabolism came essentially from PCr splitting. By contrast, the higher temperature was characterized by an enhancement of glycolysis. A possible explanation of this phenomenon could be found in the slow rise of P_i levels as a consequence of the slow PCr splitting observed at the lower T. In fact, P_i is known to regulate the activity of the phosphofructokinase enzyme (Trivedi and Danforth, 1966) and, through it, the flow rate of glycolysis.

However, in a recent study on human quadricep muscles (Starkie et al., 1999), by adopting a protocol that involved heating one leg and cooling the other, an augmented rate of glycogen use, according to muscle temperature, but no differences in high-energy phosphagen metabolism were observed. Therefore, the authors suggested that temperature *per se* might play a regulatory role in intramuscular glycolytic utilization.

Proton loading and efflux

Intracellular pH is known to be kept at its set-point, near to the pKa of the intracellular buffers but far

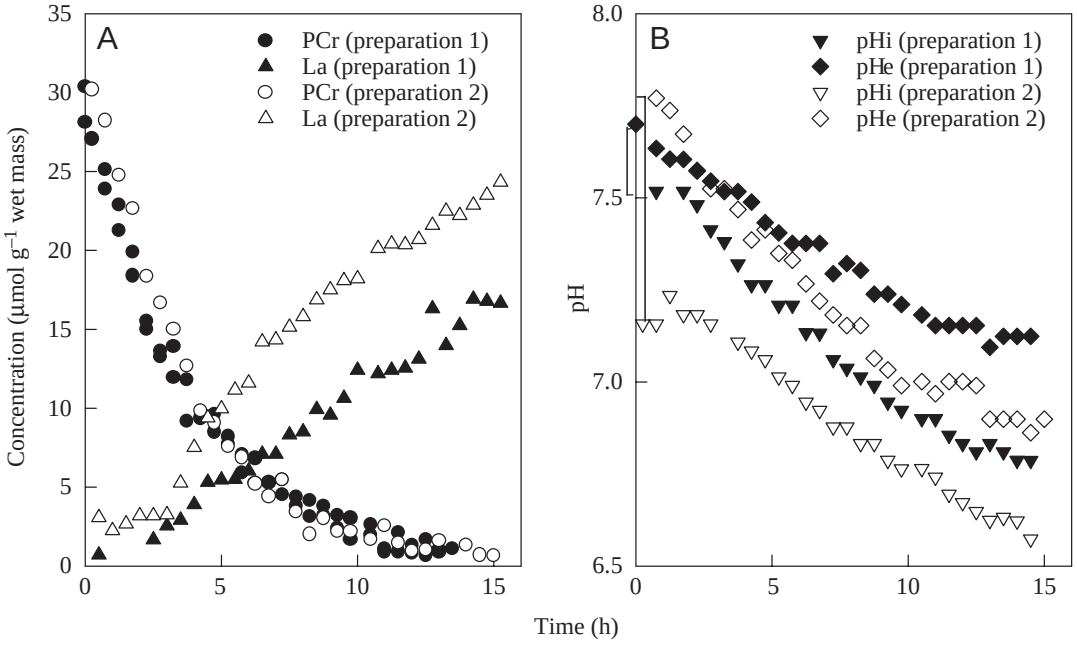

Fig. 3. Time-dependent changes of PCr (circles) and La (squares) concentrations (A) and pHi (inverted triangles) and pHe (diamonds) values (B) are shown. All the data are from the additional set of experiments performed at 25°C where initial pHe value was 7.9 and the muscle pHi initial values were varied: high (filled symbols; preparation 1) and low (open symbols; preparation 2). The brackets in B indicate the corresponding pHi and pHe values (i.e. the proton gradient).

removed from transcellular electrochemical equilibrium, by the mechanisms that remove H^+ from the cytosol. The constancy of pHi, when the tissue is in a physiological steady state, comes from the balance between the rate of H^+ removal and the rate at which H^+ (1) enters the cell down its electrochemical gradient and (2) is intracellularly produced by metabolism.

In all tissues, the maintenance of ion gradients across cell membranes constitutes an important fraction of the cellular energy consumption. Energy supply, under anaerobic conditions, is provided by both PCr hydrolysis and anaerobic glycolysis, the latter resulting in lactate acidosis. In isolated muscle, the external acidosis has been shown to decrease, and alkalosis to increase, the net lactic acid production (Hood et al., 1988). These findings have been mainly ascribed to the pHi dependence of the phosphofructokinase enzyme (Trivedi and Danforth, 1966). Actually, the data in Fig. 2F for 5–10 h after the onset of the experiment show that, at T=25°C, a decrease in the extracellular pH induces a depression of anaerobic glycolysis. However, in our case, pHi can be excluded as the regulatory signal causing the metabolic depression. In fact, at all the examined pHe in the phase corresponding to the induction of the lactate metabolic inhibition (i.e. <5 h; see Fig. 2F), pHi values are similar.

In Fig. 5, obtained from the data collected from the first set of experiments at T=25°C, both pH gradient (Fig. 5A) and lactate concentration (Fig. 5B) are plotted as a function of time. Indeed, the effects of the proton gradient are emphasized. In fact, by the comparative analysis of the two panels the direct

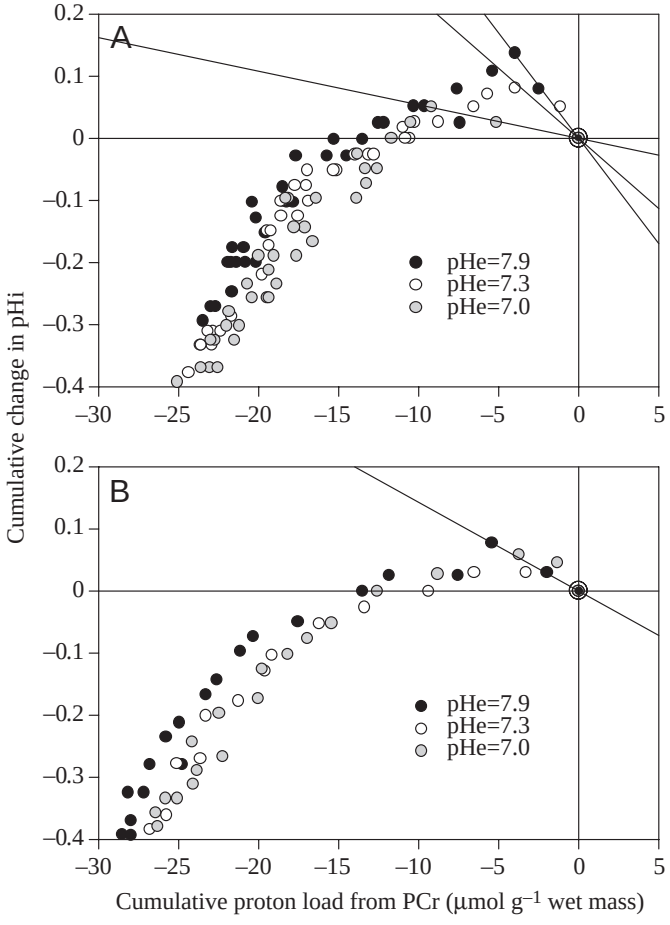

Fig. 4. Cumulative changes in pHi (ΔpH) and in proton load (ΔP) due to changes in [PCr] (expressed in μmol g^{-1} wet mass) at (A) 15°C and (B) 25°C. The data are related to the different experimental pHe (black, 7.9; white, 7.3; grey, 7.0). Point zero (the origin of the axis) corresponds to the values registered at the beginning of the experiments. The diagonal lines through the origin, calculated in the range where the change in pH was due solely to changes in [PCr], have a slope equal to $-1/\beta$ (see text).

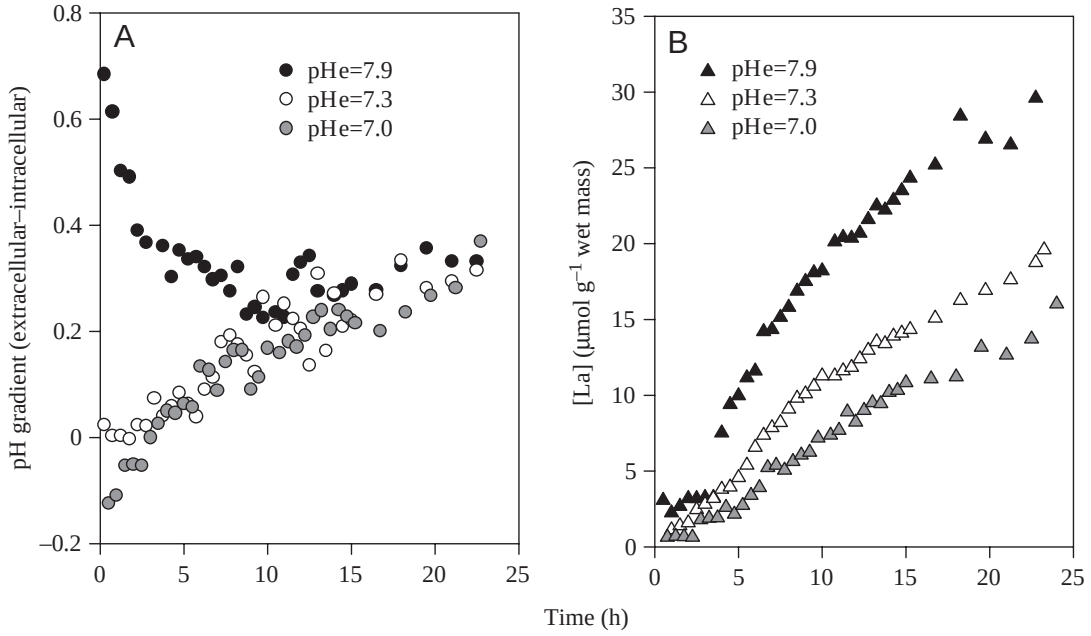

Fig. 5. pH gradient (A) and lactate accumulation (B) plotted as a function of time. The data, corresponding to the different experimental extracellular pH (7.9, black; 7.3, white; 7.0, grey), are the same as in Fig. 2.

correlation between transmembrane gradient and lactate production is evident.

By analysing the time-dependent concentration values of phosphomonoesters, reported in Fig. 2D, it can be observed that PME accumulated at a constant rate that was higher at lower pHe levels. It is well known that PME concentration increases as a consequence of the blockade of glycolysis (Argov et al., 1987a,b; Duboc et al., 1987), which results in an accumulation of the glycolytic intermediates upstream of the inhibition. The reduced lactate production by inhibited glycolysis might be ascribed to an impaired lactate transport out of the muscle. The lactate output is influenced by both lactate generation and extrusion rate.

Lactic acid efflux can occur by two main mechanisms: diffusion and specific transport (Juel, 1997). Undissociated lactic acid can easily diffuse across biological membranes, but, owing to its low pKa (3.86), most of this metabolite was present in the dissociated form, i.e. as lactate and hydrogen ions, at the experimental pH examined here. Lactate transport mostly occurs *via* a specific lactate–H^+ co-transporter system. In fact, experiments on model systems have shown that carrier-mediated transport accounts for 50–90% of lactate transport, depending on both lactate concentration and pH level (Juel, 1991; Juel et al., 1994; McDermott and Bonen, 1993; Roth and Brooks, 1990). This transport mechanism involves an ordered sequential process, whereby a proton first binds to the transporter and then to the lactate anion. This step is followed by the translocation of the transporter with both lactate and proton across the membrane and the sequential release of the lactate and proton on the other side of the membrane. The process is freely reversible. The rate-limiting step for net lactic acid flux appears to be the return of the free carrier across the membrane, which is required to complete the translocation cycle. In the pH range of ~6–8, lactate transport is stimulated not only by a lowering of the pH on the side where lactate is produced but also by a raising of the pH on the opposite side of the membrane (Juel, 1996). Previously, Juel (1995) observed that lactate–H^+ co-transporter activity is mainly driven by the lactate gradient. It is only slightly sensitive to internal pH but is strongly dependent on the external pH. Some authors have observed that alkalosis enhances (Hirche et al., 1975) and acidosis inhibits (Hirche et al., 1975; Spriet et al., 1985) the rate of lactate exit from the cell.

Analysis of the data in Figs 3 and 5, both obtained at T=25°C, shows that when the proton gradient was higher, La accumulation was much more extensive. This demonstrates that lactic acid production is influenced by the intracellular–extracellular proton gradient, almost independently from pHi and pHe absolute levels. In fact, when the proton gradient was depressed by pHe (Fig. 5) as well as by pHi (Fig. 3) changes, the lactate output decreased by an amount proportional to the gradient itself. Thus, we suggest that an unfavourable proton gradient across the cell membrane could decrease the activity of the lactate–H^+ co-transporter. In fact, the intermediate form of the carrier, when only H^+ is bound, keeps immobile or reorients slowly. At the same time, a high H^+ concentration on the outside of the muscle membrane favours the formation of the immobile protonated carrier (Juel, 1997). The depression of lactate release results in an inhibition of the glycolytic enzyme activity through a feedback mechanism, which increases the concentration of the glycolytic intermediates, thus causing a decrease in overall lactate production. In the present experiments, the rate-limiting enzymes are probably hexokinase and/or phosphofructokinase. In fact, in a typical ^{31}P spectrum obtained 20 h from the onset of data acquisition at pHe 7.0 and 25°C (Fig. 6), no peaks are visible at ~3 p.p.m. (i.e. where

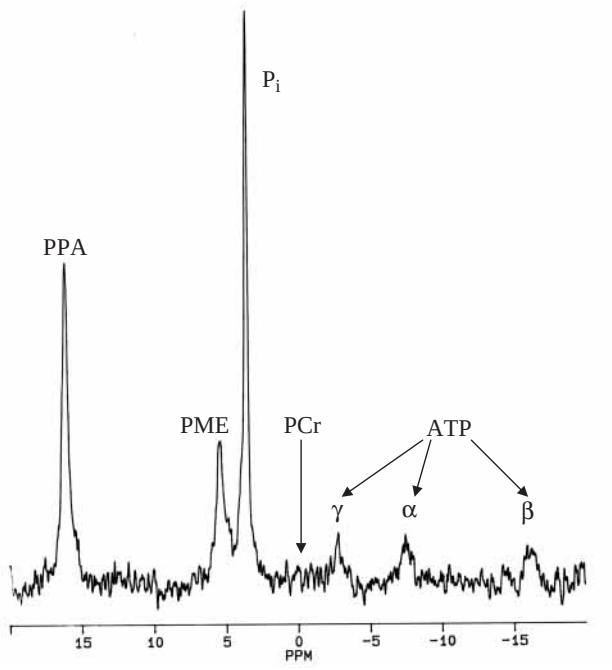

Fig. 6. ^{31}P-NMR spectrum of an isolated gastrocnemius muscle at *T*=25°C and an initial pHe of 7.0 registered at 20 h from the beginning of the experiment. Peak assignment: phenylphosphonate (PPA); phosphomonoesters (PME); inorganic phosphate (P_i); phosphocreatine (PCr); γ, α and β ATP groups.

phosphoenolpyruvate and phosphodiesters are expected), whereas a large peak due to phosphomonoesters (i.e. glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate; grouped as PME) is observed. In conclusion, our findings support the assumption that a change in lactate output may induce a difference in muscle lactic acid generation by an inhibition of the first part of the glycolytic chain.

Incidentally, Fig. 3 demonstrates that changes in pHi should not necessarily be considered as an index of lactic acid production by the muscle. In fact, paradoxically, a higher ΔpHi [0.73 pH units (preparation 1) *vs* 0.61 pH units (preparation 2)] corresponds to a lower lactate accumulation rate [1.22 μmol g^{-1} h^{-1} (preparation 1) *vs* 1.75 μmol g^{-1} h^{-1} (preparation 2)].

It is well known that estimated lactate production rates, based on changes in pHi, are complicated by the dependence on many factors (e.g. creatine kinase reaction rate, muscle protein buffering of protons, bicarbonate concentration, lactate efflux and increase in metabolites characterized by acid dissociation constant values within the physiological range). In particular, we would point out that by using the formula generally adopted for the assessment of net lactate accumulation:

$$\text{Lactate accumulation} = \beta(\Delta\text{pH}) + \varphi\Delta\text{PCr}\,, \qquad (2)$$

where β is the buffer capacity and φ is the number of protons consumed per mole of PCr hydrolysed, starting from data shown in Fig. 3, we would overestimate the La accumulation level under the experimental conditions characterized by a lower proton gradient.

The wrong result might be ascribed to the presence of a misleading proton uptake. In fact, under certain conditions (e.g. when the muscle is exposed to an acidic solution), the proton exchange regulating systems seem to operate in reverse, mediating a fall in pHi.

Buffer capacity

Lactate formation depends on the physicochemical buffering capacity of the myoplasm. PCr breakdown (see equation 1) and proteins seem to assume a most important role in determining intracellular buffer capacity.

The calculated β values are related to pHi and *T*, since both pH (Curtin, 1986) and temperature affect the equilibrium constants of the buffer reactions. Higher values of β correspond to higher *T* and lower pHi values. Similar results were obtained by other authors (Kemp et al., 1993).

The physicochemical buffering, obtained by dynamic measure (Kemp et al., 1993), adopted in the present study is influenced by variations of ion concentrations depending on metabolism as well as by fluxes of the ions across the membrane. In our calculation, no additional generation or flux of protons was assumed. However, the slope of the relationship ΔP/ΔpH depends only on these parameters, with the exception of proton consumption by PCr hydrolysis and passive buffering. As a consequence of a change in intracellular–extracellular pH gradient, where pHe is lower than pHi, the free energy of the proton gradient becomes more favourable to proton influx: this overestimates the β value, giving a higher result. In fact, the presence of a proton influx would shift the relationship to the left (shallower slope, thus a higher β), since ΔP values would underestimate the total proton load to the cytosol by an amount equal to the proton intake (see Fig. 4A). On the contrary, the presence of a proton efflux would shift the relationship to the right (steeper slope, thus a lower β), since ΔP would overestimate the proton load by an amount equal to the protons extruded.

This phenomenon was not so evident at *T*=25°C (see Fig. 4B), probably because of a more relevant buffering effect of the PCr breakdown.

Conclusions

In conclusion, by means of the non-invasive NMR spectroscopy technique, we performed, on isolated muscle, simultaneous measurements of pHi, pHe and all metabolite levels under anaerobic conditions, assessing the effects of changes in *T* and pHe. The results mainly showed that PCr splitting is the principal and primary metabolic process contributing to the energy supply in anaerobic conditions as well as its temperature dependence. In fact, phosphagens are rapidly available energy reserves that will buffer any drastic change in energy demand. By contrast, the shift towards increased glycolytic utilization seems to be driven by heat stress.

In addition, muscle PCr splitting is virtually unaffected by

pHe levels. At higher *T*, a dominant direct effect of pHe on lactate production can be detected whereas a reduction of pHi plays minor importance. In particular, proton gradient across the cellular membrane plays an important direct or indirect role in regulating metabolic processes.

Lower muscle lactate concentrations, observed when the proton gradient was negative or low, could not be explained by a decrease in glycolytic flux rates but rather by an inhibition of the process. This fact is evident in Fig. 2, where a decrease in the rate of La accumulation (Fig. 2F) is associated with a complementary increase in the rate of PME accumulation (Fig. 2D). The inhibition operates through a feedback control mechanism on phosphofructokinase, recognized as the rate-limiting enzyme of glycolysis. The regulating signal might be ascribed to impaired lactate transport out of the muscle, owing to an unfavourable proton gradient across the cell membrane that could decrease the activity of the lactate–H^+ co-transporter.

The process might be one of the mechanisms underlying the phenomenon of the so-called 'lactate paradox'. This term is used to indicate the observed decrease in lactate accumulation in human blood during exercise, in spite of chronic hypoxia. Indeed, such a decrease might be considered a result of the insufficient proton buffer capacity or of the transport of protons out of the cell. In fact, some authors (Bender et al., 1989; Brooks et al., 1992, 1998) have demonstrated that the low blood lactate concentration primarily depends on a reduced net lactate release from muscle fibres. The decrease in lactate accumulation could also be explained by the reduced alkalic reserves at high altitude (Edwards, 1936; Cerretelli, 1967; West, 1986). Although Kayser et al. (1993) did not observe an increase in the peak of blood lactate concentration after ingestion of bicarbonate, it must be considered that bicarbonate is effective in muscle pH regulation only when it really enters the muscle cell and, after its conversion to CO_2, leaves the cell in the form of dissolved CO_2. Such transmembrane exchanges are dependent on co-transporters mediating proton-equivalent efflux and influx, whose important role might be modulated by hypoxia.

Moreover, acid buffering might be more effective in the interstitial space. Nevertheless, the buffer capacity of the interstitial space seems to be rather limited compared with the blood buffer capacity. Therefore, high perifusion rates of skeletal muscle, by reducing proton accumulation in the interstitial space, may play a role in keeping the intramuscular pH balance.

Recently, van Hall et al. (2001) observed that the lactate paradox is a transient phenomenon in the course of acclimatization to severe hypoxia. They suggested that its transient nature is the result of a temporary severe disturbance in muscle acid–base balance. The gradual return to normal maximal lactate concentration observed with the time of acclimatization may be caused by, among other things, upregulation of the lactate–H^+ co-transporter system. In skeletal muscle, this is governed *via* H^+-linked monocarboxylate transporters 1 and 4 (MCT-1 and MCT-4; Juel, 1997). The expression of MCT-4, the transporter specialized in the extrusion of lactate out of the muscle, is induced by anaerobic stress (Bonen, 2001). Alterations in the number of MCT-4 transporters may potentially explain the adaptive change occurring during a long acclimatization period. This might be in accordance with the observation (Itoh et al., 1990) in rat muscles exposed to simulated altitude (4000 m) for a long period (10 weeks), of a significant increase in the percentage of type II fibres, the only ones able to express MCT-4 in mammals (Bonen, 2001; Fishbein et al., 2002).

However, the exact mechanisms explaining the changes in the blood lactate level during hypoxic exercise must still be a matter for further investigation. The results of the present work might contribute to examining the problem from a point of view that, up until now, has not, to our knowledge, been stressed enough.

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