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Muscle bio-energetics in acute glycolytic block: in vivo phosphorus-nuclear magnetic resonance study of iodo-acetate injected rats

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Summary. In vivo phosphorus nuclear magnetic resonance spectroscopy of muscle was performed at rest, during work and during postexercise recovery in rats injected with iodo-acetate (IA) (35–40 mg·kg^{-1}, intra-arterially), in order to follow bio-energetic changes in muscle with acute glycolytic block. Three animals with contracture had very low ratios of phosphocreatine:inorganic phosphate (PCr:Pi) at rest (0.5–0.9). The PCr:Pi were normal at rest (6.9±2.0, ±2 SD) in all other rats. Exercise-induced continuous accumulation of phosphomonoesters (PME), the characteristic finding of glycolytic block, was observed. The end-exercise levels of PME correlated with the degree of block measured in vitro. During steady-state work, induced by nerve stimulation at four frequencies, PCr:Pi values were significantly lower ($p < 0.02$) than the control values at 0.25, 1.0 and 2.0 Hz. The ATP levels fell during exercise to reach 75%±7% of initial values. The recovery of PCr:Pi from exercise and the disappearance of PME were slow. Two animals which survived the IA injection demonstrated much lower PME accumulation 18 h later. It is concluded that in acute muscle glycolytic block: (1) energy metabolism is impaired during exercise and also at rest, (2) accumulating PME can serve as an indicator of the degree of glycolytic block, (3) ATP levels fall during work, and (4) postexercise recovery is slow. The findings are compared with ^{31}P-NMR observations in chronic muscle glycolytic disorders.

Key words: Phosphomonoesters — Muscle adenosine 5′-triphosphate — Phosphocreatine — Muscle exercise — Iodo acetate

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Introduction

Glycolysis is one of the major pathways of fuel supply for muscle energy metabolism during exercise. Thus, it is not surprising that human muscle disorders of blocked glycolysis [phosphofructokinase (PFK), phosphoglycerate mutase (PGAM) and phosphoglycerate kinase (PGK) deficiencies] are associated with exercise-induced pain, excessive fatigue and contractures (rigor, stiff muscles) (DiMauro et al. 1984). The mechanisms associated with these features are, however, not clear (DiMauro et al. 1984). Several patients with these disorders have been investigated by in vivo phosphorus nuclear magnetic resonance spectroscopy (^{31}P-NMR) in order to characterize the bio-energetic changes that occur in muscle deficient of glycolytic activity (Argov et al. 1987a, b; Chance et al. 1982; Duboc et al. 1987; Edwards et al. 1982).

In human PFK, PGAM and PGK deficiencies, exercise-induced accumulation of phosphomonoesters (PME) was recorded by ^{31}P-NMR. This is the most typical ^{31}P-NMR feature of glycolytic blocks at or distal to the PFK reaction (Chance et al. 1982; Edwards et al. 1982), probably reflecting the accumulation of the glycolytic intermediates (sugar phosphates) proximal to the block site. The continually increasing PME during submaximal exercise did not interfere with the ability to maintain a steady-state in the ratio of phosphocreatine:inorganic phosphate (PCr:Pi) in these human inborn errors of metabolism (Argov et al. 1987b; Duboc et al. 1987). Initial experiments suggested that during a short exercise bout Pi remained lower than expected in PFK deficiency (Chance et al. 1982). However, later studies showed that Pi rose continuously with prolonged, graded, steady-state exercise (Argov et al. 1987b).

The PCr:Pi is the ^{31}P-NMR measured value that permits the estimation of the cytosolic phosphorylation potential in vivo (Chance 1984; Chance et al. 1981). This potential is considered to be the best reflection of the bio-energetic state of muscle tissue (Gibbs 1985).

Dogs with inherited muscle PFK deficiency (<1% of normal activity) also demonstrate the typical ^{31}P-NMR findings of blocked glycolysis, an increase in PME during exercise. Unlike the changes in human disorders, the PME increase in the dogs did not start immediately at the beginning of exercise (Giger et al. 1989). The accumulation of PME occurred in spite of the fact that dogs have a very high muscle oxidative capacity and lack fast glycolytic muscle fibers (Maxwell et al. 1977). The PCr:Pi was, however, better maintained in affected dogs than in controls at comparable work loads even during prolonged exercise (Giger et al. 1989). It is not known whether the degree of PME accumulation correlates with the degree of glycolytic block. One patient with partial PGAM deficiency did demonstrate less excessive PME accumulation during muscle work compared with other patients with full blocks (Argov et al. 1987a). In addition, it is not clear to what degree an acute block of glycolysis impairs the bio-energetics of mixed fiber type muscle and whether the changes are similar to those observed in the chronic disorders.

In order to investigate the above questions, we studied a model of acute glycolytic block, induced by intra-arterial injection of iodo-acetate (IA) in an in vivo rat muscle (Brumback 1980). As early as 1931 IA was shown to block muscle glycolysis (for review see Webb 1966). Recently in vivo ^{31}P-NMR in IA injected rat muscles demonstrated moderately increased PME levels but only in association with irreversible contracture (Kuwabara et al. 1986). The IA effects on the kinetics of phosphate-containing compounds were, however, not studied during muscle work. Our investigations of the acute IA model also examined the ^{31}P-NMR changes during work and recovery.

Methods

Adult Sprague-Dawley rats (250–300 g) were anesthetised by chloral hydrate (250–350 mg·kg^{-1}) intraperitoneally. The lower abdominal aorta was exposed through a midabdominal incision. The aorta was briefly clamped while IA (35–40 mg·kg^{-1} dose in 0.15 cc) was injected to 29 animals (Brumback 1980). Pressure was applied to the injection site until bleeding stopped and the abdomen was then closed in layers. The ^{31}P-NMR studies were performed about 1 h after the IA injection. The findings were compared with 4 control rats who had a sham intra-aortic injection (0.15 cc of saline) and 20 rats studied without any surgery.

The protocol for the NMR studies has been recently described (Argov et al. 1987c) and only a brief description will be given here. The right hindleg was studied in all animals, using a Helmholtz coil in a 1.9 tesla magnet interfaced with a spectrometer (Oxford Research Systems TMR-32 Oxford Instruments England). Phosphobronze nonmagnetic electrodes were percutaneously inserted at the sciatic notch for nerve stimulation. Exercise was induced in all muscles below the knee by supramaximal sciatic nerve stimulation. Rectangular pulses of 30 V and 1 msec-duration were transferred through radiofrequency filters to the stimulating electrodes. At the end of the study, the animals were allowed to recover from the anesthesia (1–2 h) and an attempt was made to restudy them 18 h later, if data indicated a full block (see Results). Three animals with a

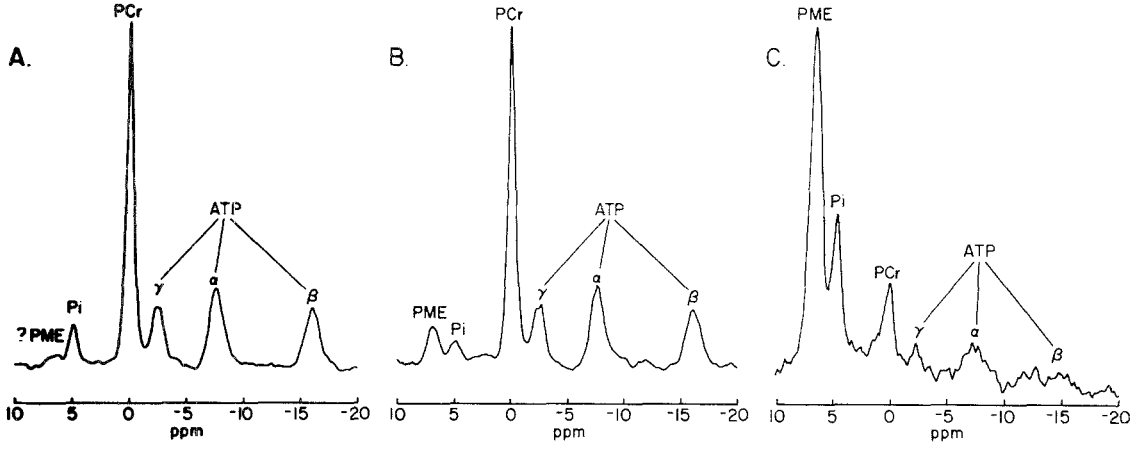

Fig. 1. Phosphorus nuclear magnetic resonance spectra of muscle at rest: (**A**), normal rat, (**B**), iodo-acetate-injected rat of group B, and (**C**), iodo-acetate-injected rat with muscle contracture.
PCr = phosphocreatine, *Pi* = inorganic phosphate, *PME* = phosphomonoester, *ATP* = adenosine 5′-triphosphate, ppm = parts per million
Note that in the control animal little *PME* is present at rest. The *PME* is well defined in the non-contracted muscle of group B and is markedly elevated in the contracted muscle, where reduction of *PCr:Pi* is observed

different type of response to IA (see Results) were immediately sacrificed after the first ^{31}P-NMR experiment and a biopsy was taken from the gastrocnemius muscle.

For ^{31}P-NMR studies, radiofrequency pulses of 20 μs duration (90° pulse) were given every 3 s. This pulsation pattern resulted in a reduction in the various phosphate peaks to 72%, 78% and 83% (for PCr, Pi and adenosine 5′-triphosphate (ATP) respectively) of their fully relaxed amplitudes (saturation factor). Thus, to convert NMR measured ratios to estimated concentrations, correction factors of 1.12 and 1.04 were used for PCr and Pi (Argov et al. 1987c). The in vivo saturation factor of PME is unknown and difficult to determine because PME increases only transiently with exercise. To calculate concentrations of PME in the cell from partially saturated ^{31}P-NMR spectra, we used the PME:ATP ratio without a correction factor for different saturations. This results in lower estimated PME concentrations than actually exist in the cell because the saturation factor of PME is known to be longer than that of ATP. Data were collected in 1-min blocks, thus, each 1-min spectrum contained 20 scans. In order to obtain a better signal:noise ratio during analysis of the exercise data, adjacent pairs (2 min) of data blocks were added at each point. The additions progressed by 1-min steps to maintain the 1-min resolution.

Quantitative data analyses of phosphorus compounds during exercise and recovery were based on the relevant peak areas. The Fourier transformed spectra were apodized with 15 Hz line broadening, phased and plotted. The area was calculated as the peak height × the width at half height (Argov et al. 1987c). The beta peak of ATP was used for the calculation of ATP content. Micropipets filled with a solution of sodium dimethylphosphonate (DMP) were put inside the coil for calibration of changes in ATP content (Argov et al. 1987b). Resting muscle data were measured from a 10-min spectrum (200 scans). Other kinetic measurements were done using 2 min spectra as mentioned above.

Muscle samples from the 3 IA injected rats and 3 control animals were homogenized and tested for lactate production in vitro, using the method described by Layzer et al. (1967), Two substrates were used: 0.15% glycogen and 10 mM fructose 6-phosphate. Each test for each sample was performed in triplicate and the result is the average of the three repetitions expressed in μmol lactate·gm^{-1} muscle·30 min^{-1}.

Differences between data of injected and control rats were evaluated for statistical significance using the non-paired *t*-test and $p < 0.05$. All results are expressed as mean ±2 SD unless stated otherwise.

Results

Twenty-eight animals were injected with IA, but only 21 survived the dose with no generalized side effects (Webb 1966). The 4 animals with sham injections showed no difference in their ^{31}P-NMR features when compared to non-operated animals, thus IA data will be compared with all the 24 control rats.

A normal ^{31}P-NMR spectrum of rat muscle at rest is shown in Fig. 1 A and the peaks of PCr, Pi and ATP are identified. Three animals developed irreversible contracture of the leg muscles shortly after the IA injection, before muscle work was induced. In these animals a very high PME peak was observed at rest, with a ratio of PCr:Pi markedly reduced to 0.5–0.9 (Fig. 1 C). The ATP peak was very small, indicating loss of this compound. These rats were not submitted to nerve stimulation and are referred to as group A.

The ^{31}P-NMR spectra of the other 18 animals showed high PCr:Pi and small PME at rest (Fig. 1 B). These animals were further subdivided according to the behavior of the PME peak during muscle exercise. In 6 rats (group B), there was a continuous, exponential increase in PME during muscle exercise, without achieving steady-state, reaching levels of 2–3 times that of the ATP peak by the end of the stimulation protocol (Fig. 2a). In

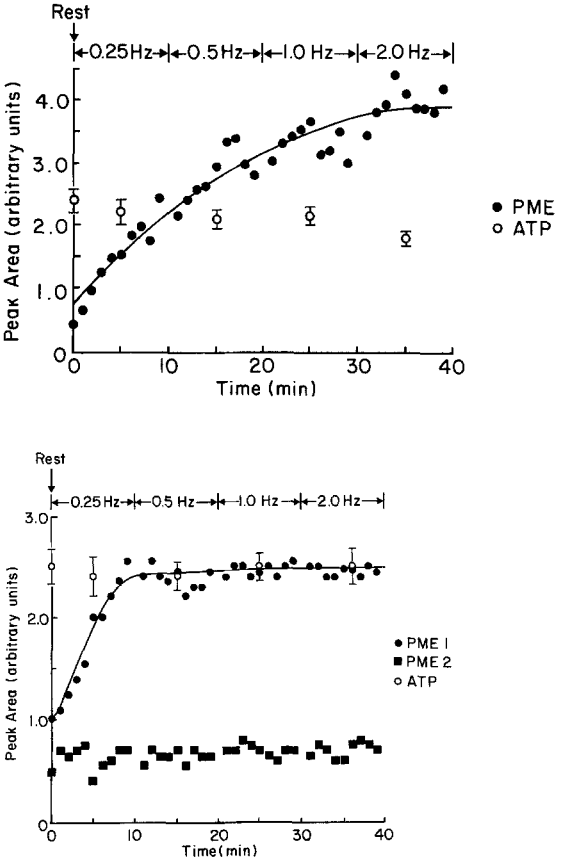

Fig. 2a–b. The accumulation of phosphomonoesters (*PME*) during one experiment. The levels of *PME* were measured from the respective peak areas of 2 min ^{31}P-NMR spectra. *ATP* areas were averaged over each 10 min stimulation frequency period, *bars* = ±1 SD.

a One rat of group B. Note the progressive, continuous increase of *PME* reaching about twice the *ATP* concentration by the end of stimulation protocol.

b Two rats of group C. In one (*PME 1, closed circles*), the increase in *PME* was only at the beginning of exercise and leveled out at a concentration similar to *ATP*. In the second (*PME 2, squares*), no increase above resting values was observed during exercise (Definitions as in Fig. 1)

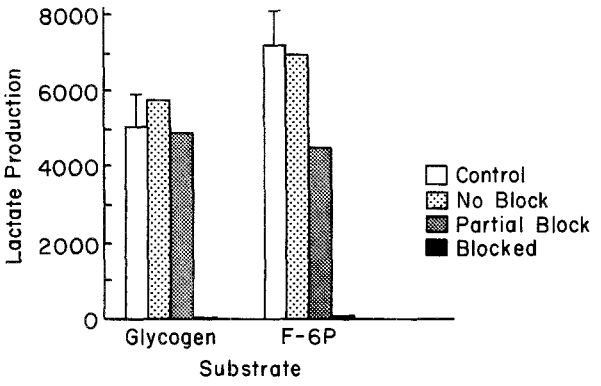

Fig. 3. in vitro lactate production in muscle samples of the rats in Fig. 2.
Lactate production is in $\mu mol \cdot gm^{-1} \cdot muscle \cdot 30\ min^{-1}$ in response to additions of 0.15% glycogen and 10 mM fructose-6-phosphate (*F-6P*) to the medium. Controls were 3 normal rats (*bars* = 1 SD). *Blocked* rat was that of Fig. 2a. The *no block* (no rise in PME) and *partial block* (PME = ATP) are those shown in Fig. 2b

12 other rats (group C), different responses of PME to stimulation were observed (Fig. 2b). In 8 rats the increase in PME amplitude was evident only at the beginning of the muscle work, reached maximal values of PME:ATP = 1 and then remained constant. There was a minimal or no increase in PME above rest values in the remaining 4 rats.

Muscle samples taken from an animal representative of the above groups differed in their glycolytic activity, as measured by lactate production in vitro (Fig. 3). In an animal with a continuous PME increase (group B), lactate production was less than 1.5% of the control values. In an animal with no increase in PME, lactate production was within the normal range. In a rat with some PME elevation (PME:ATP = 1 at the end of exercise), a partial block was probably present since only 55% of control lactate production with fructose 6-phosphate occurred.

Muscles of group B rats had PCr:Pi and PCr:ATP ratios of 6.9 ± 2.0 and 2.4 ± 0.2 at rest respectively, compared with normal ratios of 5.88 ± 1.5 and 2.3 ± 0.34 (differences not significant). There was, however, a smaller than normal ATP peak indicating lower [ATP] concentrations. Normal rat muscle has 8.05 mM [ATP] (Veech et al. 1979). The ^{31}P-NMR ratio of ATP:PCr + Pi + PME) was 0.27 ± 0.05 in this IA injected group, compared with normal values of 0.37 ± 0.06. If one assumes similar overall [PCr] + [Pi] + [PME] at rest, then [ATP] fell to 73% of normal levels or to 5.9 mM 1 h after the IA injection (the assumption of a similar overall sum at rest is reasonable as can be seen in the Table 1). The PME peak at rest had variable heights; [PME] concentration at rest was estimated to be 2.9 ± 1.2 mM. In normal nonoperated rats, PME was usually not above noise level and probably well below 0.3 mM. The latter value is the minimal concentration that can be detected by in vivo ^{31}P-NMR.

During stimulation PCr levels fell and Pi rose in group B, as expected in any exercising skeletal muscle. In order to assess the changes in bio-energetic state (PCr:Pi) in relation to muscle exercise (as estimated from the stimulation rate — Argov et al. 1987b) steady-state conditions need to be maintained. We defined steady-state as a change in the PCr and Pi levels of less than 10% from min to min during continuous stimulation at a constant rate (Argov et al. 1987c; Chance et al. 1981). A steady-state condition occurred for the last 3 min of exercise in normal rats during 10 min of constant nerve stimulation (Argov et al. 1987c). Steady-state conditions were also obtained in group B of IA blocked rats. Figure 4 shows the

Table 1. The estimated concentrations (in mM) of phosphate containing compounds in control ($n = 24$) and group B of iodo-acetate-injected rats ($n = 6$)

	ATP	PCr	Pi	PME	TP*	pH
Control	8.05	20.4 ± 3.0	3.2 ± 1.0	< 0.3	23.6 ± 4.0	7.14 ± 0.07
Group B	5.9 ± 0.3	15.9 ± 1.3	2.1 ± 2.1	2.9 ± 1.2	20.9 ± 3.0	7.0 ± 0.1
End-exercise						
Control	8.2 ± 1.1	11.2 ± 1.2	12.8 ± 2.4	< 0.3	24.0 ± 3.6	7.05 ± 0.05
Group B	4.4 ± 0.4	5.8 ± 1.3	9.5 ± 2.6	11.1 ± 2.3	26.4 ± 5.0	6.9 ± 0.1

* = PCr + Pi + PME
The concentrations were calculated from the ^{31}P-NMR spectral ratios, correcting fo differential saturation of PCr and Pi but not PME. The calculations assumed resting control ATP of 8.05 mM, unchanged rest TP in group B. The ATP changes during exercise were correlated to an external calibration signal (see Methods for the basis of assumptions). pH was calculated as suggested by Taylor et al. (1983). (For definitions see Fig. 1)

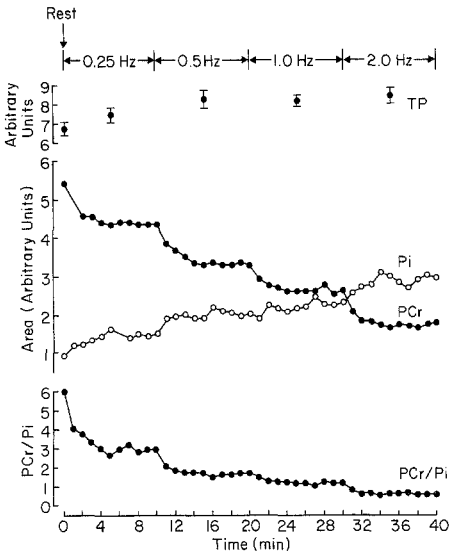

Fig. 4. The changes of *PCr, Pi, PCr:Pi* and *PCr+Pi+PME* (*TP*) during one experiment in a group B rat.
PCr and *Pi* areas were measured from 2-min spectra. *TP* was averaged for each 10-min stimulation frequency, *bars* = ±2SD. Note that during the last 3 min of each frequency period steady-state was obtained in *PCr* and probably also *Pi* (Definitions as in Fig. 1)

PCr, Pi and PCr:Pi changes with time in one experiment. The four levels of stimulation yielded four different PCr:Pi ratios that had become stable by the end of each stimulation period. Figure 5 shows the last 2 min ^{31}P-NMR spectra of each stimulation level from such an experiment. The relationship between exercise (stimulation rate) and PCr:Pi, taken from the last 2 min of each steady-state period in group B rats, is shown in Fig. 6. PCr:Pi ratios were significantly reduced ($p<0.01$) at 0.25, 1.0 and 2.0 Hz, compared with controls.

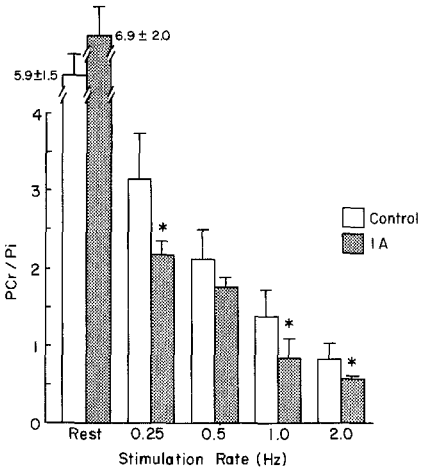

Fig. 6. Average *PCr:Pi* at the end of each stimulation frequency period in control ($n=24$) and group B *IA*-injected rats ($n=6$). *Bars* = ±2SD, * $p<0.02$. Note that the *PCr:Pi* ratios were lower in group B rats reaching significance in 3 of 4 frequencies of stimulation (Definitions as in Fig. 1)

The ATP levels were not maintained during muscle exercise in the IA blocked rats, in contrast to normal controls (Fig. 7). The reduction in area of the ATP peak was observed in all the animals of group B. When compared to the external calibration standard of DMP, ATP had decreased to 75%±7% ($n=5$) of its initial values at the end of exercise (to an estimated [ATP] of 4.4±0.4 mM). At the end of exercise, accumulated PME had reached an estimated concentration of 11.1±2.3 mM (Table 1) in group B muscles. The sum of [PCr]+[Pi]+[PME] increased significantly during muscle exercise, from 20.9±3.0 mM to 26.4±5.0 mM ($p<0.05$). The increase in this sum could not be accounted for by the decrease in [ATP] (Table 1).

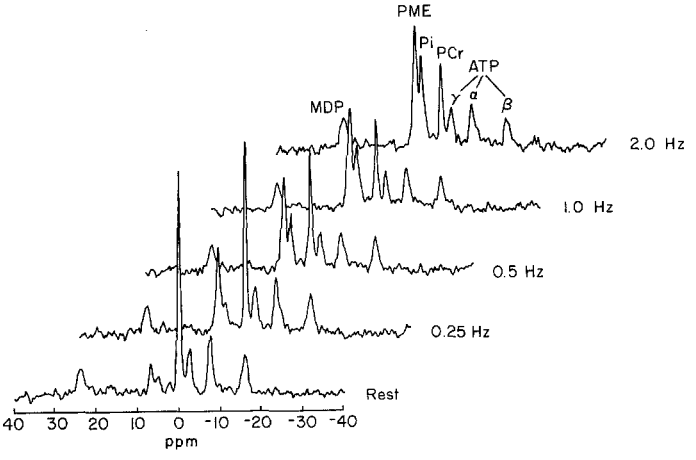

Fig. 5. 2 min ^{31}P-NMR spectra at each stimulation frequency during one experiment in group B rat.
MDP = methylenediphosphonate, an external calibration signal (Definitions as in Fig. 1)

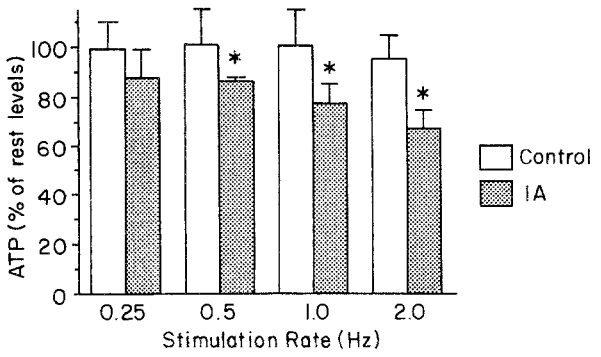

Fig. 7. *ATP* levels in normal ($n=24$) and group B *IA* rats ($n=6$) at each stimulation frequency.
ATP area was averaged over the whole period and is represented as % of value at rest. *Bars* = ±2 SD. * $p<0.05$. Note the continuous fall in *ATP* as muscle exercise continues in the *IA*-injected rats (Definitions as in Fig. 1)

Recovery from exercise was slow and was not complete even after 20 min. The kinetics of the recovery of PCr and Pi showed exponential behavior. The $t_{1/2}$ (time to recover to 50% of the change from rest to end-exercise) was prolonged for both PCr (176±55 s) and Pi (170±3 s) compared with control values (69±5.5 s for both, $p \leq 0.005$). However, the PCr:Pi remained low at 2.9±0.6, as further recovery was minimal. PME did not recover during the 20 min-follow-up.

Most blocked animals of group B died within the first 18 h of injection. A repeat study 18 h later was possible in only 2 animals. There was very little PME accumulation during the second study, which was comparable to the group C findings.

Discussion

The aim of this study was to characterize the bio-energetic changes in muscle during an IA-induced acute glycolytic block. Responses to intra-arterial injection of IA were variable, most probably due to differences in cellular penetration of IA (Webb 1966). Confirmation of a glycolytic block is mandatory if this model is to be used.

We have subdivided our results into three groups: group A those animals with muscle contracture and very low PCr:Pi at rest. It is assumed that the glycolytic block induced by IA was complete in these animals and that it was associated with the inability to maintain phosphorylation potential after the surgery, even at rest. Muscles of this group demonstrated marked accumulation of PME and a decrease in ATP levels. Some ATP was probably metabolized to inosine monophosphate (IMP) (Mineo et al. 1985) and may have contributed to the PME peak (IMP is identified in the PME range of ^{31}P-NMR spectra and at 1.9 T cannot be separated from the glycolytic intermediates — the hexose monophosphates). There was no recovery from the contracture after 1 h therefore, no stimulation was performed. These findings are similar to those of Kuwabara et al. (1986), who tried to evaluate muscle energy metabolism in IA-injected rats but obtained a muscle contracture after minimal stimulation.

Groups B and C were subdivided on the basis of the behavior of the PME peak during muscle exercise. Previous studies on PFK deficiency in humans (Argov et al. 1987b; Chance et al. 1982; Duboc et al. 1987; Edwards et al. 1982) and animals (Giger et al. 1989) and human PGK deficiency (Duboc et al. 1987) have shown a marked and continuous increase of PME during exercise, reaching concentrations of 2–3 times the ATP levels. The PFK and PGK activities have been below 1% of control values in these cases. A case of partially blocked glycolysis due to PGAM deficiency (6% residual enzyme activity) induced a less marked and non-continuous rise in PME (Argov et al. 1987a). To ensure that IA induced a significant block in glycolysis, we selected only rats showing continuous and large increases in PME (PME:ATP>2 or more at the end of exercise). Six animals showed this pattern and were in group B, considered fully blocked. In these animals pH did not change with increased exercise. The pH, however, cannot be used as an indicator of glycolytic block since in our model submaximal frequencies were used and even control animals did not show significant acidosis [Table 1 and see Argov et al. (1987c)]. All animals who had less accumulation of sugar phosphates were termed group C and probably reflected only a partial block in glycolysis.

Glycolytic activity was not measured in all animals, but only in a representative muscle sample of each group. Because we wanted to assess the degree of block after 18 h, we did not take biopsies from the animals at the end of the first ^{31}P-NMR study. Thus, we cannot prove that each rat in group B was completely blocked. However, our results from lactate production in vitro suggest that the kinetics of the PME increase are good indicators of the degree of block in glycolysis in both human and animal muscle.

Group B rats were, therefore, used for examining the influence of acute glycolytic block on muscle bio-energetics. There was no interference with phosphorylation potential at rest, as the ratio of PCr:Pi was within the normal range. This ratio

was also normal at rest in humans with various chronic blocks in glycolysis (Argov et al. 1987b; Duboc et al. 1987). There were, however, two abnormalities in the IA model at rest: ATP levels were low; this was also found in human and dog PFK deficiency (Argov et al. 1987b; Giger et al. 1989). Secondly there was a mild accumulation of PME, which was also observed in human PFK deficiency but not in affected dogs. The contribution of glycolysis to energy metabolism at rest is minor and only a small increase in PME would be expected during this phase.

A steady-state for PCr and Pi could be maintained during muscle exercise in IA blocked animals, despite the gradual decrease in ATP and the lack of substrates normally supplied by glycolysis (pyruvate and nicotinamide adenine dinucleotide, reduced). This suggests that glycolysis was not mandatory to the maintenance of steady-state muscle phosphorylation potential under our experimental conditions. Although IA did not affect PCr:Pi at rest, this ratio was lower during work, compared with normal muscles at similar levels of stimulation. In human and animal disorders of glycolysis, muscle PCr:Pi ratios are maintained at normal values during submaximal exercise, suggesting some metabolic adaptation may have occurred in these chronic diseases.

The accumulation of PME changes the sum of [PCr]+[Pi]+[PME] by at least 26%. It was most probably underestimated as the true [PME] at this stage was higher than we estimated (see Methods). This increase may indicate trapping of intracellular phosphates, although a change in saturation characteristics by IA (change in relaxation time T1) is also possible, since we have not measured the T1 before and after IA injection. However, several bio-energetic calculations are based on the assumption that the sum of phosphate containing compounds remains constant during short exercise (Chance et al. 1981). The assessment of the stoichiometry of the reactions that are monitored by ^{31}P-NMR in IA blocked muscle is difficult because this assumption may not hold.

The recovery of PCr and Pi after exercise was incomplete. The pattern of that recovery was exponential, as seen in normal muscles. This suggests that the creatine kinase system was still operative. The dephosphorylation of PME was extremely slow. Elimination of PME is also slow in human and dog PFK deficiency with an estimated $t_{1/2}$ of 6.5–9 min (Argov et al. 1987b; Giger et al. 1989). The IA may have blocked the phosphatases that are involved in the dephosphorylation of PME, but not the creatine kinase system.

Two animals that survived IA injection showed an acute, almost complete glycolytic block, but did not demonstrate the same pattern 18 h later. There was only a mild increase in PME during the second study, suggesting that some resumption of glycolytic activity had occurred.

In vivo IA blocked glycolysis in the rat mimics some, but not all, features seen in chronic glycolytic disorders of muscle, as measured by ^{31}P-NMR. The results obtained in our group B animals suggest that acute glycolytic block impairs muscle energy metabolism to a greater degree than that observed in the chronic diseases of man and animals.

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