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Fructose 2,6-bisphosphate and glycolytic flux in skeletal muscle of swimming frog

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Glycolytic flux in skeletal muscle is controlled by 6-phosphofructokinase but how this is achieved is controversial. Brief exercise (swimming) in frogs caused a dramatic increase in the phosphofructokinase activator, fructose 2,6-bisphosphate, in working muscle. The kinetics of phosphofructokinase suggest that in resting muscle, the enzyme is inhibited by ATP plus citrate and that the increase in fructose 2,6-bisphosphate is part of the mechanism to activate phosphofructokinase when exercise begins. When exercise was sustained, fructose 2,6-bisphosphate in muscle was decreased as was the rate of lactate accumulation. Glycolytic flux and the content of fructose 2,6-bisphosphate appear to be closely correlated in working frog muscle in vivo.

Skeletal muscle; Glycolysis; Fructose 2,6-bisphosphate; 6-Phosphofructokinase; Exercise; *Rana temporaria*

1. INTRODUCTION

Glycolysis is an important pathway for ATP production in vertebrate muscle. Much has been learned about muscle metabolism using isolated frog muscle, but how glycolysis is regulated in working muscle could not be elucidated from this nor from any other preparation and is still controversial. The main control element of glycolysis is 6-phosphofructokinase (PFK; EC 2.7.1.11) [1,2]. In vitro, PFK activity can be modulated by a variety of ligands such as the inhibitors, ATP and citrate, and the activators (deinhibitors), inorganic phosphate, AMP, fructose 1,6-bisphosphate ($F1,6P_2$) and glucose 1,6-bisphosphate ($G1,6P_2$) [3–7]. However, it has repeatedly been emphasized that the changes in effectors of PFK that have been observed in muscle are not sufficient to bring about the changes in PFK activity that occur in working muscle [1,8–10]. In 1980, $F2,6P_2$, a most potent activator of PFK, was detected in rat liver and subsequently found in all animal tissues studied so far. $F2,6P_2$ is synthesized and degraded by a separate enzyme system and, being neither an intermediate nor a product of glycolysis, qualifies as an external regulator (signal) of glycolysis (for review, see [11–13]). In gastrocnemius muscle of anesthetized rat, $F2,6P_2$ was increased about 2-fold upon electrical stimulation in situ [14]. Nevertheless, $F2,6P_2$ has not been accepted as an important factor in regulating glycolysis in working muscle because: (i) the increase in $F2,6P_2$ was small and seen only if stimulation was at low frequency; (ii) glycolytic flux (lactate accumulation) and the content of $F2,6P_2$ in muscle could not be correlated when rat muscle was stimulated at high frequency [14–16]; (iii) the content of $F1,6P_2$ in muscle is much higher than that of $F2,6P_2$, so that $F1,6P_2$, which competes with $F2,6P_2$ for PFK [17], would displace $F2,6P_2$.

We wanted to study a physiological form of exercise. So we made frogs swim for 1 s–10 min and followed $F2,6P_2$ in gastrocnemius muscle. The content of $F2,6P_2$ and the rate at which lactate was accumulated in working muscle were closely correlated over the 10 min of exercise.

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Abbreviations: $F1,6P_2$, fructose 1,6-bisphosphate; $F2,6P_2$, fructose 2,6-bisphosphate; $G1,6P_2$, glucose 1,6-bisphosphate; PFK, 6-phosphofructokinase (EC 2.7.1.11)

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

Biochemicals and coupling enzymes were obtained from Boehringer Mannheim, fructose 2,6-bisphosphate and the pyrophosphate dependent 6-phosphofructokinase from Sigma (Munich). The other substances were purchased from Merck (Darmstadt), Q-Sepharose and Blue Sepharose were from Pharmacia (Freiburg).

2.2. *Experimental animals and swimming studies*

Common frogs (*Rana temporaria*) were kept separately and rested individually for 24 h before the experiments. The control animals were decapitated with a small guillotine and their legs were immediately freeze-clamped. The experimental animals were placed into a long narrow plastic groove to swim spontaneously against a slow stream of water (at 18°C). After various periods of swimming the animals were decapitated and treated in the same way as the controls. About 6 s elapsed between removing a swimming frog from the water and clamping its muscle.

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2.3. *Determination of metabolites and fructose 2,6-bisphosphate*

The frozen muscle was ground in liquid nitrogen and the tissue compounds were extracted with 5 vols of 1 M $HClO_4$, neutralized and determined by specific enzymatic methods [18]. For measuring $F2,6P_2$, frozen muscle was extracted in 5 vols of 50 mM NaOH by sonication and heated for 6 min at 80°C. $F2,6P_2$ was identified on the basis of its instability under mild acidic conditions (10 min at pH 2, 30°C) and measured by its ability to activate the pyrophosphate-dependent PFK from potato tubers as described [19].

2.4. *Purification and assay of 6-phosphofructokinase*

Leg muscle of frog was homogenized with an Ultra-Turrax disintegrator in a medium comprising 10 mM imidazole buffer, pH 7.6, 2 mM EDTA, 1 mM fructose 6-phosphate and 30 mM 2-mercaptoethanol, and the tissue extract was fractionated with saturated $(NH_4)_2SO_4$. The preparation was further purified by ion-exchange chromatography on Q-Sepharose (elution by a linear gradient of 0–500 mM KCl, 200 ml) and affinity chromatography on Blue Sepharose (elution by 5 mM ADP). The final preparation had a specific activity of about 80 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (at 25°C) and it was free from aldolase and contained less than 0.1% adenylate kinase.

PFK was assayed at pH 7.2 as described [20]. PFK activity was regarded optimum (V_{opt}) at 2 mM F6P, 2 mM $MgATP^{2-}$, 1 mM AMP and 10 mM inorganic phosphate.

3. RESULTS

3.1. *The effects of swimming on the contents of metabolites and fructose 2,6-bisphosphate in muscle*

Common frogs tend to rest motionless for long periods, yet they are ready for vigorous motor activity. This appeared promising for obtaining muscle in a state of physiological rest or physiological activity. Carefully rested frogs were induced to swim for various time spans from 1 s to 10 min. Then they were quickly decapitated, their legs freeze-clamped and their gastrocnemius muscles were analyzed for $F2,6P_2$ and some metabolites as described in section 2. Rested frogs would not move if picked up carefully. Also during the transfer from the water to the guillotine or upon decapitation no contractions of leg muscles were noted.

Swimming for 1–5 s brought about changes in metabolites indicating a rapid activation of both glycogen phosphorylase and PFK (see Table I). No lactate was formed in the muscle during the first 5 s of exercise, but lactate was conspicuously increased after 30 s of swimming and was further accumulated, although at a diminishing rate, for the rest of the exercise (Fig. 1A).

The content of $F2,6P_2$ in gastrocnemius muscle from rested frog was 0.043 ± 0.015 nmol/g, i.e. 4–10-fold lower than had been reported for control muscles of mammals [14–16,21]. One second of swimming, however, triggered a dramatic increase in $F2,6P_2$ which amounted to more than 40-fold its resting level at the time of freeze clamping. After 30 s, $F2,6P_2$ in exercising muscle started to decrease and reached control levels after 5–10 min of swimming when the frogs showed fatigue (Fig. 1B).

Glycolytic flux in the working gastrocnemius can be estimated from the rate of lactate accumulation, because glycolysis proceeds mainly anaerobically and lactate efflux from frog muscle is slow [22,23]. At the initiation of exercise the increase in $F2,6P_2$ preceded the formation of lactate. With the exception of this alactogenic period, the content of $F2,6P_2$ in muscle was closely correlated with the rate of lactate accumulation (Fig. 1).

3.2. *Fructose 2,6-bisphosphate and regulatory properties of 6-phosphofructokinase*

$F2,6P_2$ proved a most potent activator of PFK purified from frog muscle. $F1,6P_2$, a product of the PFK reaction, is also an activator of frog muscle PFK. But, at near-physiological concentrations of substrates and effectors, the activating effect of $F1,6P_2$ (or $G1,6P_2$, results not shown) is almost eliminated by citrate. $F2,6P_2$ was able to overcome the inhibition by citrate (Fig. 2). Hence, the capacity of $F1,6P_2$ (or

Table I

Effect of swimming on the contents of some metabolites in gastrocnemius muscle from frog ($\mu mol \cdot g^{-1}$ muscle)

	Metabolite contents in muscle (μmol/g)				
	Rested control (n = 5)	Duration of swimming			
		1 s (n = 3)	3 s (n = 4)	5 s (n = 4)	30 s (n = 4)
Glucose 6-phosphate	0.26 ± 0.10	0.58 ± 0.14[b]	1.02 ± 0.17[c]	1.54 ± 0.30[c]	2.55 ± 0.30[c]
Fructose 6-phosphate	0.08 ± 0.02	0.14 ± 0.08	0.17 ± 0.06[a]	0.17 ± 0.06[a]	0.27 ± 0.09[a]
$F1,6P_2$	0.15 ± 0.08	0.18 ± 0.07	0.30 ± 0.07[a]	0.44 ± 0.12[b]	0.26 ± 0.11
Glycerol 3-phosphate	<0.05	0.29 ± 0.05[c]	0.38 ± 0.07[c]	0.41 ± 0.17[c]	0.75 ± 0.13[c]
Citrate	0.45 ± 0.10	0.67 ± 0.21	0.62 ± 0.12	0.48 ± 0.04	0.35 ± 0.10

Activation of glycogen phosphorylase in working muscle is indicated by the increase in glucose 6-phosphate, activation of phosphofructokinase by the increase in fructose 1,6-bisphosphate and glycerol 3-phosphate. Results are means ± SD of n separate animals. [a,b,c]Value significantly different from rested control according to Student's t-test at $P \leq 0.05$, 0.01, 0.001, respectively. Glycerol 3-phosphate in the control was below the limit of detection, for statistical calculations the value 0.05 ± 0.05 $\mu mol \cdot g^{-1}$ was used

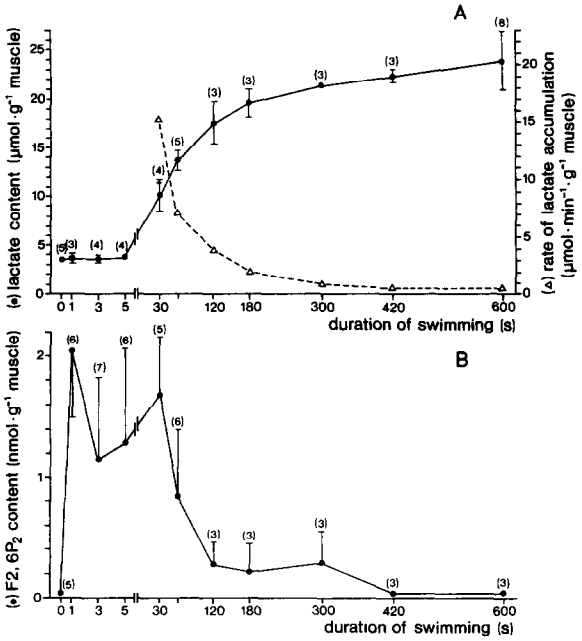

Fig. 1. (A) Effect of swimming on the content of lactate and the rate of lactate accumulation in gastrocnemius muscle from frog. The rate of lactate accumulation at a given point was calculated from the increase in lactate content during the preceding time interval and is given in μmol lactate per min per g muscle. (B) Effect of swimming on the content of fructose 2,6-bisphosphate in gastrocnemius muscle from frog. $F2,6P_2$ increased rapidly upon swimming, and before lactate was increased. After this alactogenic period, the rate of lactate accumulation (see Fig. 1A) was closely correlated with the content of $F2,6P_2$ in muscle. Frogs were treated as described in section 2. Each observation is presented as the mean ± SD and the number of observations on separate animals is given in parentheses.

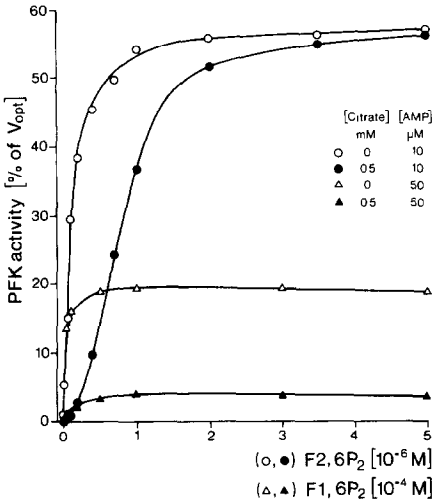

Fig. 2. Citrate differently affects the activation of frog muscle phosphofructokinase by fructose 2,6-bisphosphate and fructose 1,6-bisphosphate. Citrate induced cooperativity with respect to $F2,6P_2$ and increased the concentration of $F2,6P_2$ required for half-maximum effect, but did not affect the maximum activity which, under the given conditions, was about 55% of the optimum activity, V_{opt}. $F1,6P_2$ (up to 500 μM) elicited only some 20% of V_{opt} and this was reduced to about 4% of V_{opt} by 0.5 mM citrate. Assays were performed at 0.1 mM fructose 6-phosphate, 7.5 mM ATP, 2 mM inorganic phosphate, and [AMP] as indicated.

$G1,6P_2$) for modulating the activity of PFK in vivo may be small.

4. DISCUSSION

Fructose 2,6-bisphosphate is a potent activator of 6-phosphofructokinases from all animal tissues studied so far [11–13]. In mammalian liver, the function of fructose 2,6-bisphosphate in controlling the rates of glycolysis and gluconeogenesis is well established [12,13]. In all other tissues its function is unclear [13].

Our findings suggest that $F2,6P_2$ is important in regulating PFK activity in working frog muscle in vivo. This is in contrast to the current opinion that, in mammals, $F2,6P_2$ is of minor if any importance in working muscle [7,12–16]. Although a definite answer cannot yet be given, it seems unlikely that the different results are due solely to species differences. The kinetic properties of PFK from mammalian and frog muscle are very similar, and this includes, as has recently been shown with rat PFK, the capacity of citrate to almost eliminate the activating effects of $F1,6P_2$ [24] and $G1,6P_2$ [25]. Hence the hexose 1,6-bisphosphates may not have the strong activating effect on PFK in mammalian muscle that has been ascribed to them on grounds of kinetics performed in the absence of citrate. The different results could then be due, at least in part, to the different experimental approaches. For instance, the low level of $F2,6P_2$ in control muscle from frog was found only if the frogs were carefully rested. This suggests that experimental stress may obscure the degree to which exercise can affect the $F2,6P_2$ content of muscle. Indeed, catecholamines have been shown to increase the content of $F2,6P_2$ in rat muscle [15,21].

We propose the following working hypothesis as to possible functions of $F2,6P_2$ in skeletal muscle. (i) A rapid increase in the content of $F2,6P_2$ in muscle can be triggered by exercise and this contributes to the activation of PFK in working muscle. The effect of $F2,6P_2$ is probably synergistically reinforced by an increase in inorganic phosphate and particularly AMP. At the initiation of work, the content of inorganic phosphate in muscle is increased due to a breakdown of phosphocreatine while a small fractional decrease in ATP is supposed to bring about, via the adenylate kinase reaction, an increase in the content of AMP (for discussion, see [26,27]). (ii) Upon stress or excitation the increase in $F2,6P_2$ may precede muscular work as part of a mechanism, mediated by catecholamines or related factors, to prepare the animal for 'fight or flight'. (iii) In sustained exercise, $F2,6P_2$ in muscle is decreased and this contributes to decreasing glycolytic flux which, depending on muscle type, may serve protective or integrative functions. In burst work muscle with its high anaerobic potential, glycolytic flux must be checked to prevent tissue damage from excessive lactate production. In aerobic muscles capable of using

substrates other than carbohydrate, a decrease in $F2,6P_2$ could be part of a mechanism to spare carbohydrate. The flight muscle of locust is a case in point because this muscle changes from carbohydrate to fat as the main fuel during sustained flight and this is correlated with a marked decrease in $F2,6P_2$ [28].

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