Biochem. J. (1984) **223**, 73–79
Printed in Great Britain

Fructose 2,6-bisphosphate in rat skeletal muscle during contraction

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(*Received 16 April 1984/Accepted 12 June 1984*)

Fructose 2,6-bisphosphate and several glycolytic intermediates were measured in two rat muscles, extensor digitorum longus and gastrocnemius, which were electrically stimulated *in situ*. Both the duration and the frequency of stimulation were varied to obtain different rates of glycolysis. There was no relationship between fructose 2,6-bisphosphate content and the increase in tissue lactate in contracting muscle. However, in gastrocnemius stimulated at low frequencies ($\leqslant 5$ Hz), there was a 2-fold increase in fructose 2,6-bisphosphate at 10s, followed by a return to basal values, whereas lactate increased only after 1 min of contraction. The concentrations of hexose 6-phosphates, fructose 1,6-bisphosphate and triose phosphates were all increased during the 3 min stimulation. During tetanus (frequencies $\geqslant 10$ Hz) fructose 2,6-bisphosphate was not increased, whereas glycolysis was maximally stimulated and resulted in an accumulation of tissue lactate, mostly from glycogen. The concentrations of hexose 6-phosphate increased continuously during the 1 min tetanus, whereas fructose 1,6-bisphosphate was increased at 10s and then decreased progressively. It therefore appears that fructose 2,6-bisphosphate does not play a role in the stimulation of glycolysis during tetanus; it may, however, be involved in the control of glycolysis when the muscles are stimulated at low frequencies for short periods of time.

Muscle glycogenolysis and glycolysis are largely stimulated during contraction and after adrenaline treatment (Danforth & Helmreich, 1964; Karpatkin *et al.*, 1964; Richter *et al.*, 1982). The degradation of glycogen depends on the activity of glycogen phosphorylase (for a review, see Griffiths, 1981), whereas the rate of glycolysis is generally assumed to depend on the activity of phosphofructokinase, a rate-limiting enzyme in this pathway (Newsholme & Start, 1973).

Fructose 2,6-bisphosphate is a potent stimulator of muscle phosphofructokinase (Van Schaftingen *et al.*, 1981; Uyeda *et al.*, 1981; Foe *et al.*, 1983), and is therefore a possible candidate for the control of glycolysis in muscle. A previous study (Hue *et al.*, 1982) has indicated that, in perfused rat hindlimb muscles, adrenaline treatment and electrical stimulation both stimulated lactate production; however, adrenaline increased, whereas 2 min of electrical stimulation decreased, the concentration of fructose 2,6-bisphosphate. It was therefore concluded that fructose 2,6-bisphosphate might be involved in the stimulation of glycolysis by adrenaline, but not during contraction (Hue *et al.*, 1982). However, after 2 min of violent contraction, the muscles reach a state of relative anoxia which is known to decrease fructose 2,6-bisphosphate, at least in the liver (Hue, 1982). It was therefore decided to repeat these experiments under different conditions. Experiments with frog sartorius muscle have shown that the rate of glycolysis could be increased 10–100-fold over the resting rate by stimulation at increasing frequencies; lactate formation was proportional to the frequency of stimulation (Karpatkin *et al.*, 1964), and phosphorylase activation was proportional to the energy consumed per twitch (Mommaerts *et al.*, 1975). In the present study, the muscles were electrically stimulated *in situ* in anaesthetized rats, and both the duration and the frequency of stimulation were varied to obtain different rates of glycolysis. Results reported below show that there is no relationship between the rate of lactate production and the concentration of fructose 2,6-bisphosphate.

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Methods

Preparations of muscles

Experiments were carried out on fed male Wistar rats (200–300g body wt.) which were anaesthetized by pentobarbital (60mg/kg intraperitoneally). The skin from the right leg was removed; the sciatic nerve and extensor digitorum longus or gastrocnemius muscles were exposed. Caution was taken to keep the vascularization of the muscles intact. The leg was fixed to a platform by a metal clamp, and the distal tendon of the muscle was cut and attached to a fixed steel arm. The muscles were kept moistened by a drip, installed at the top of the muscle, which delivered Krebs–Henseleit (1932) bicarbonate buffer without Ca^{2+}; this buffer was kept at 37°C and in equilibrium with O_2/CO_2 (19:1). The rats were allowed to recover for 20min, and isometric contractions were then induced by electrical stimulation. Muscles were stimulated for different periods of time and at different frequencies to produce contractions ranging from a series of twitches to tetanic contractions. At the appropriate time, the contracting muscles were frozen *in situ* between stainless-steel clamps precooled in liquid N_2 (Wollenberger *et al.*, 1960).

Preparation of extracts and measurement of metabolites

For the measurement of fructose 2,6-bisphosphate, frozen samples of muscle (about 30mg) were homogenized (Branson sonicator, 3 × 10s at 100W) in 9vol. of hot 50mM-NaOH and kept at 80°C for 10min. The extracts were neutralized at 0°C by the addition of 1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH8. Insoluble material was removed by centrifugation at 0°C for 5min in an Eppendorf Microfuge, and fructose 2,6-bisphosphate was measured in the supernatant (Van Schaftingen *et al.*, 1982). For the measurements of other metabolites, frozen samples (about 250mg) were homogenized in 10vol. of 5% (v/v) $HClO_4$ with a Potter–Elvehjem homogenizer kept at about −5°C in an ice bath containing salt. Metabolites were measured in the supernatants after neutralization with 3M-KOH/1M-Hepes. For the extraction of glycogen, frozen samples were dissolved in 1M-KOH and kept for 15min at 100°C. The extracts were then neutralized by the addition of 1M-acetic acid. Glycogen was measured as described previously (Hue *et al.*, 1975).

Glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1963*a*), fructose 1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Bücher & Hohorst, 1963), phosphoenolpyruvate and pyruvate (Czok & Lamprecht, 1974) and lactate (Hohorst, 1963*b*) were measured enzymically. ATP was measured by the luciferin/luciferase bioluminescence assay with a kit purchased from Boehringer, Mannheim, Germany. All biochemicals and enzymes were either from Boehringer or from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results and discussion

Stimulation of extensor digitorum longus

This muscle contains more than 90% of type II b fibres and is considered as a model of fast-twitch white glycolytic muscles (Close, 1972).

The muscles were stimulated for different periods of time (up to 5min); the electrodes were directly applied on the muscles, and isometric contractions were induced by shock (10V, 20ms) at different frequencies (0.5–25Hz). The concentrations of fructose 2,6-bisphosphate, lactate and ATP were measured, and the results are presented in Fig. 1. Although the concentration of fructose 2,6-bisphosphate tended to increase after stimulation, the difference between the control and all the stimulated muscles was not statistically significant. Similar results were obtained when the muscles were stimulated for 10–300s at 1 or 10 shocks/s (results not shown). Fig. 1 also shows that the concentration of ATP was little affected during the 3min period of stimulation at 0.5Hz, whereas it reached 40% of the control value after 30 or 60s of stimulation at higher frequencies. The change in lactate concentration in stimulated muscles was also dependent both on the duration of stimulation and on the frequency. At low frequency, lactate concentration decreased during the first 1min and started to accumulate only after 3min of continuous stimulation, with a rate of 3μmol of lactate/min per g observed between 3 and 5min of stimulation. At higher frequencies (5 and 25Hz) lactate concentration increased immediately after stimulation, with a maximal rate of about 20μmol/min per g observed during the first 1min. Thereafter a high steady state was maintained.

The data clearly show that the concentration of fructose 2,6-bisphosphate is not related to the accumulation of tissue lactate and that fructose 2,6-bisphosphate is probably not a major stimulator of the glycolytic flux during contraction. It is questionable whether the rise in total lactate concentration in muscle does indeed represent the true glycolytic rate. It does in muscles contracting under anaerobic conditions (Karpatkin *et al.*, 1964; Danforth & Helmreich, 1964). In the present study, it is reasonable to assume that tissue lactate gives a good estimate of the glycolytic flux during tetanus, as is the case at high frequencies of stimulation. Under these conditions, muscle con-

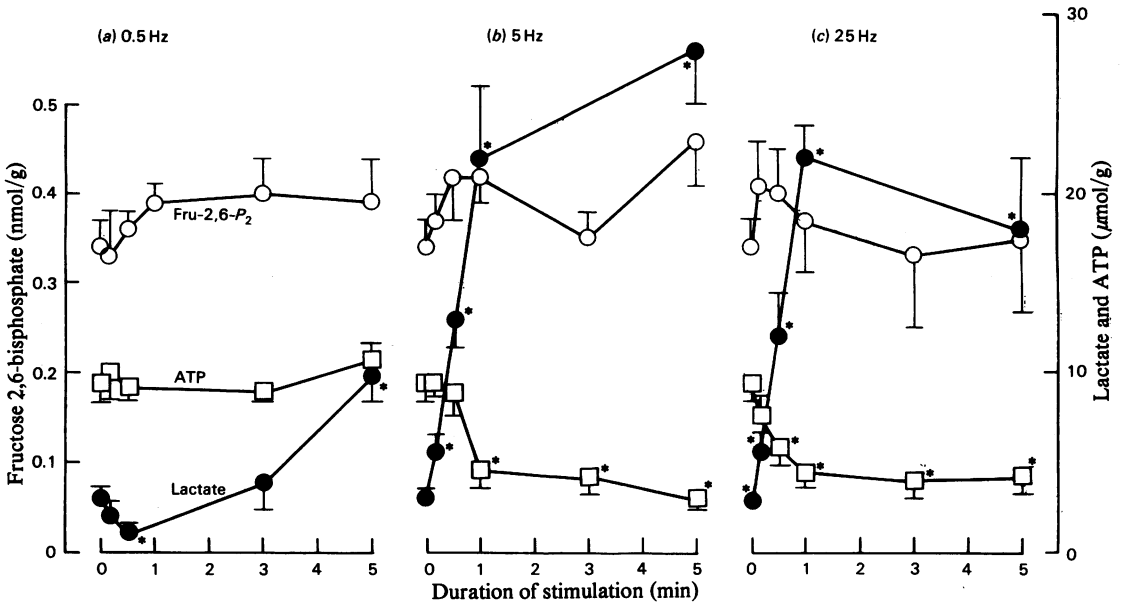

Fig. 1. *Changes in the concentrations of lactate* (●), *ATP* (□) *and fructose 2,6-bisphosphate* (○, *Fru-2,6-P_2*) *in extensor digitorum longus during isometric concentration*

The muscles were stimulated at 0.5 (*a*), 5 (*b*) and 25 (*c*) Hz for different periods of time. Values shown are means for four to nine different muscles. Bars represent S.E.M. Control values are the averages of 19 different samples: * refers to values which are statistically different from control values ($P<0.05$).

traction restrains or even stops the blood flow and lactate cannot be washed out. At lower frequencies this may not be true. Nevertheless, it is well established that stimulation of isolated muscles is characterized by a large increase in the glycolytic flux, which depends on the frequency of stimulation (see, e.g., Karpatkin *et al.*, 1964).

Stimulation of gastrocnemius

A second series of experiments were performed on gastrocnemius muscle. This muscle was chosen because of its relatively large size, which allows more determinations of metabolites to be made on a single sample, and because its composition differs from that of extensor digitorum longus: gastrocnemius consists mainly of fast-twitch red (oxidative, type IIa) fibres and of fast-twitch white (glycolytic, type IIb) fibres (Close, 1972; Hickson *et al.*, 1975). The muscles were stimulated (5 V, 1 ms) via the sciatic nerve.

The time course of the changes in metabolite concentrations was studied in muscles stimulated at 5 Hz (sub-tetanic contractions), and the results are shown in Table 1. When gastrocnemius muscle was stimulated for different periods of time at 5 shocks/s, the glycogen concentration progressively decreased (average rate of 4.5 μmol of glucose equivalent/min per g). This corresponds to the production of 9 μmol of lactate/min per g and can be considered as the minimal glycolytic rate, since it does not include the utilization of blood glucose. During the first 1 min, no lactate accumulated, probably because it was either washed out or oxidized. However, between 1 and 3 min the decrease in glycogen corresponded to an increase in lactate which accounted for 80% of the glycogen lost during this period of time. A biphasic pattern was also observed for the change in fructose 2,6-bisphosphate concentration, which increased transiently at 10 s and returned to basal values between 1 and 3 min despite the elevated concentration of fructose 6-phosphate, the substrate of phosphofructokinase-2. This biphasic pattern can be interpreted as two states of the muscle: the first, presumably aerobic, during which lactate does not accumulate and fructose 2,6-bisphosphate is increased, and a second one characterized by an increase in lactate and a decrease in fructose 2,6-bisphosphate. One may speculate that, during this second period, the blood flow is progressively restrained by contraction and the muscle becomes hypoxic, as evidenced by the decrease in ATP concentration at 3 min.

In contrast, the 3–10-fold increase in the concentration of the glycolytic intermediates from glucose 6-phosphate to triose phosphates remained relatively constant during the 3 min period. This may indicate that a new steady state was reached and that the flux through phosphofructokinase was maintained at a constant and increased rate. The

Table 1. *Concentrations of metabolites in gastrocnemius muscle stimulated for different periods of time at a frequency of 5 shocks/s*

Values shown are means ± S.E.M. for three to five different observations. Results are expressed in μmol/g of tissue (wet wt.). Glycogen is expressed as μmol of glucose equivalent/g and fructose 2,6-bisphosphate in nmol/g. Triose phosphate is the sum of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Values shown without S.E.M. refer to the means of two samples.

Duration of stimulation (s) ...	0	10	60	180
Glycogen	26.6 ± 0.8	24.4 ± 1.3	19.8 ± 3.2	13.2 ± 3.8
Lactate	3.8 ± 0.1	4.1 ± 0.2	6.7 ± 2.0	17.4 ± 1.0
ATP	5.9 ± 0.5	5.7 ± 0.5	6.5 ± 0.4	4.5 ± 0.4
Glucose 6-phosphate	0.022 ± 0.004	0.24 ± 0.05	0.23 ± 0.09	0.21 ± 0.05
Fructose 6-phosphate	0.008 ± 0.002	0.042 ± 0.01	0.042 ± 0.01	0.034 ± 0.01
Fructose 1,6-bisphosphate	0.15 ± 0.02	0.62 ± 0.03	0.62	0.48 ± 0.04
Fructose 2,6-bisphosphate	0.40 ± 0.04	0.71 ± 0.09	0.53	0.47 ± 0.05
Triose phosphate	0.066 ± 0.004	0.15 ± 0.02	0.09 ± 0.02	0.11 ± 0.04
Glycerol 3-phosphate	0.031 ± 0.02	0.17 ± 0.03	0.36	0.35 ± 0.02
Phosphoenolpyruvate	0.012 ± 0.003	0.020 ± 0.002	0.014 ± 0.001	0.018 ± 0.003
Pyruvate	0.030 ± 0.004	0.025 ± 0.004	0.035 ± 0.004	0.041 ± 0.001

Table 2. *Concentrations of metabolites in gastrocnemius muscle stimulated for different periods of time at a frequency of 25 shocks/s*

Values shown are means ± S.E.M. for three to five different observations. Results are expressed in μmol/g of tissue (wet wt.). Glycogen is expressed as μmol of glucose equivalent/g and fructose 2,6-bisphosphate in nmol/g. Triose phosphate is the sum of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.

Duration of stimulation (s) ...	0	10	30	60
Glycogen	26.6 ± 0.8	19.0 ± 0.9	15.2 ± 4.0	12.1 ± 3.5
Lactate	3.8 ± 0.1	12.1 ± 1.8	17.0 ± 1.5	27.0 ± 2.7
ATP	5.9 ± 0.5	6.2 ± 0.7	5.9 ± 1.3	5.7 ± 0.6
Glucose 6-phosphate	0.022 ± 0.004	0.70 ± 0.14	1.81 ± 0.25	3.24 ± 0.38
Fructose 6-phosphate	0.008 ± 0.002	0.091 ± 0.016	0.21 ± 0.04	0.30 ± 0.05
Fructose 1,6-bisphosphate	0.15 ± 0.02	0.93 ± 0.18	0.33 ± 0.21	0.22 ± 0.14
Fructose 2,6-bisphosphate	0.40 ± 0.04	0.37 ± 0.06	0.39 ± 0.04	0.24 ± 0.02
Triose phosphate	0.066 ± 0.004	0.29 ± 0.11	0.29 ± 0.08	0.17 ± 0.05
Phosphoenolpyruvate	0.012 ± 0.003	0.012 ± 0.003	0.020 ± 0.003	0.019 ± 0.002
Pyruvate	0.030 ± 0.004	0.032 ± 0.004	0.040 ± 0.001	0.037 ± 0.004

concentrations of pyruvate and phosphoenolpyruvate were little affected. In conclusion, it appears that the biphasic change in fructose 2,6-bisphosphate is not related to changes in the glycolytic flux.

Table 2 shows the changes in metabolite concentrations when gastrocnemius was stimulated at 25 Hz (tetanic contractions). There was a progressive loss of glycogen, 80% of which was recovered as tissue lactate during the 1 min period. The changes in concentrations were maximal during the first 10 s of stimulation, with a rate of lactate accumulation equal to about 50 μmol/min per g. The sum of the concentration of lactate, hexose phosphates and triose phosphates found at 10 s accounted for almost 80% of the glycogen lost, whereas during the 1 min period it was slightly larger than the loss of glycogen, indicating that glucose was used. The glycolytic flux is at least 300-fold larger than the rate of lactate formation measured in isolated muscle (soleus, epitrochlearis and hemi-diaphragm) incubated under control conditions (Challis *et al.*, 1983) and more than 1000-fold greater than in perfused rat skeletal muscle (Berger *et al.*, 1976). It is also 5-fold higher than in gastrocnemius stimulated at 5 Hz (Table 1) and about 2–3-fold higher than in hamstring muscles of mice stimulated at 25 Hz (Rahim *et al.*, 1980). Despite these large changes in flux, fructose 2,6-bisphosphate did not increase, and its concentation even fell between 30 and 60 s, thus confirming the previous observation (Hue *et al.*, 1982). In contrast, and in agreement with previous reports (Karpatkin *et al.*, 1964; Aragon *et al.*, 1980; Rahim *et al.*, 1980), the concentrations of glucose 6-phosphate and fructose 6-phosphate increased dramatically during contraction to reach values which were respectively 150- and 40-fold higher than the initial rest values. It should be noted that the mass-action ratio of the phosphoglucose isomerase reaction was displaced from equilibrium. This indicates that both the supply of glucose 6-

phosphate from glycogen and the rate of removal of fructose 6-phosphate by phosphofructokinase were greatly increased. The continuous increase in hexose 6-phosphates indicates that the supply of glycolytic substrates exceeded the glycolytic capacity. This accumulation of hexose 6-phosphates did not result in an increase in fructose 2,6-bisphosphate, thus suggesting that phosphofructokinase-2 was inhibited and/or fructose-2,6-bisphosphatase was stimulated. There is no obvious explanation for this observation.

The changes in fructose 1,6-bisphosphate concentrations were biphasic: its concentration reached about 1mM at 10s and then decreased progressively. This biphasic pattern suggests that one reaction in the further metabolism of fructose 1,6-bisphosphate was transiently limiting. Since the concentration of triose phosphates changed in parallel with that of fructose 1,6-bisphosphate, the limiting reaction was probably not catalysed by aldolase. Similarly, since the concentrations of phosphoenolpyruvate and pyruvate were much less affected than that of fructose 1,6-bisphosphate, pyruvate kinase was probably not limiting. The limiting reaction could be catalysed by glyceraldehyde 3-phosphate dehydrogenase, and one may speculate that the enzyme could have been limited by the availability of P_i. Numerous ^{31}P-n.m.r. studies (for reviews, see Taylor *et al.*, 1983; Wilkie, 1983) have shown that the concentration of P_i in resting muscle could be lower than that measured in deproteinized extracts, and the actual concentration may well be lower than 2mM. A state of relative P_i depletion could even be reached when hexose phosphates accumulate during the first seconds of contraction. This state would be only transient, since the hydrolysis of phosphocreatine would readily replenish the P_i pool.

Another set of experiments were carried out to study the effect of different frequencies of stimulation on glycolysis and on the concentration of metabolites. For this study, gastrocnemius muscles were stimulated for 10s at different frequencies and then freeze-clamped. Figs. 2 and 3 show that, in these muscles, the concentrations of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, triose phosphates and lactate increased with the frequency of stimulation. Maximal values were obtained at frequencies equal to or higher than 10Hz, which caused tetanic contractions. Changes in lactate and glycogen concentrations were only detectable at these higher frequencies. In contrast, the pattern of changes in fructose 2,6-bisphosphate concentration was the opposite, and a 2-fold increase was apparent only at low, sub-tetanic, frequencies of stimulation. It therefore seems that fructose 2,6-bisphosphate increases transiently in contracting

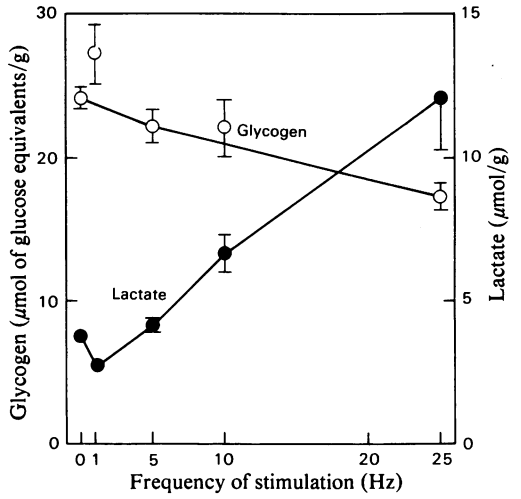

Fig. 2. *Influence of the frequency of stimulation on the concentration of lactate and glycogen in gastrocnemius muscle*

The muscles were stimulated to contract isometrically for 10s. Values shown are means for three to five different muscles. Bars represent S.E.M.

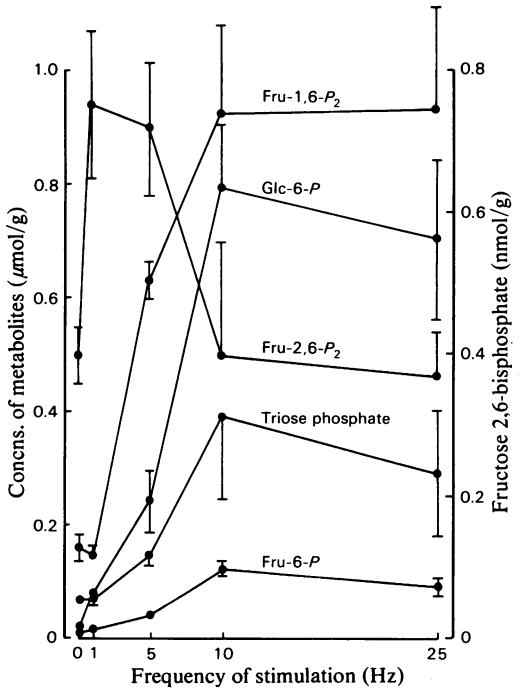

Fig. 3. *Influence of the frequency of stimulation on the concentration of several metabolites in gastrocnemius muscle*

The muscles were stimulated to contract isometrically for 10s. Values shown are means for three to five different muscles. Bars represent S.E.M.

muscles only at low frequencies, when the muscle metabolism is known to be aerobic. At high frequencies (i.e. during anaerobic tetanus) fructose 2,6-bisphosphate is not increased. The first condition is probably more physiological.

General conclusions

Our results clearly show that there is no relationship between the extent of stimulation of glycolysis by contraction and the concentration of fructose 2,6-bisphosphate. However, it appears that when gastrocnemius is stimulated at low frequencies resulting in a small stimulation of glycolysis, fructose 2,6-bisphosphate is increased, and it could then be of some importance in the control of the glycolytic flux. It is noteworthy that in insect muscles the concentration of fructose 2,6-bisphosphate rose 2-fold during exercise (Storey, 1983). It is questionable, however, whether the concentrations of fructose 2,6-bisphosphate in contracting muscle are sufficient to influence the activity of phosphofructokinase. Kinetic and binding studies (Heylen *et al.*, 1982; Foe *et al.*, 1983; Kitajima & Uyeda, 1983) have suggested that the two fructose bisphosphates compete for the same site on muscle phosphofructokinase and that the affinity of the enzyme for fructose 2,6-bisphosphate (half-maximal effect at about 0.1 μM) is about 10-fold greater than for fructose 1,6-bisphosphate. It thus follows that, in the presence of 0.5–1 mM-fructose 1,6-bisphosphate in contracting muscle, the effect of fructose 2,6-bisphosphate (0.4–1 μM) would be negligible. However, when the concentration of fructose 1,6-bisphosphate is low (at frequencies less than 5 Hz) and fructose 2,6-bisphosphate is increased, the latter ester may be of some importance in the stimulation of phosphofructokinase.

What are the mechanisms responsible for the maximal stimulation of glycolysis and phosphofructokinase during contraction? The 10-fold increase in the concentration of the substrate fructose 6-phosphate and the 6-fold increase in the concentration of the stimulator fructose 1,6-bisphosphate do certainly contribute to the overall effect. The importance of fructose 1,6-bisphosphate in the control of muscle glycolysis has been emphasized by Tornheim & Lowenstein (1976). In contrast, adenine nucleotides, which are known to regulate phosphofructokinase, cannot be considered as important regulators during short-term contraction, since their concentrations do not change significantly (the present paper; see also Rahim *et al.*, 1980; Aragon *et al.*, 1980; Gadian *et al.*, 1981; Wilkie, 1983). Are other factors involved, and is there any tight coupling between the contractile system and lactate production, as has been suggested by Wilkie (1983)? It has been shown that phosphofructokinase can be associated with the contractile system (Clarke & Masters, 1975) and that calmodulin binds to phosphofructokinase and, in so doing, affects its state of polymerization (Mayr & Heilmeyer, 1983). Whether these mechanisms play a physiological rôle remains to be demonstrated.

We are greatly indebted to Professor G. Maréchal and Dr. J. Lebacq (Department of Physiology, University of Louvain), who kindly introduced us to the methodology of muscle stimulation *in situ*. We thank Dr. M. H. Rider for his advice and critical reading, and Mrs. Th. Lambert for having typed this manuscript. L. H. is Maître de Recherches du Fonds National de la Recherche Scientifique. This work was supported by the Fonds National de la Recherche Scientifique Médicale (Belgium).

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