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REGULATION OF MUSCLE PHOSPHOFRUCTOKINASE BY PHYSIOLOGICAL CONCENTRATIONS OF BISPHOSPHORYLATED HEXOSES: EFFECT OF ALKALINIZATION

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SUMMARY: To clarify the role of glucose-1,6-P_2, fructose-2,6-P_2 and fructose-1,6-P_2 in the control of the glycolytic flux during muscle contraction, we have determined the activity of muscle phosphofructokinase in the presence of physiological concentrations of these bisphosphorylated hexoses and other allosteric effectors, and at increasing pH values. In the presence of fructose-2,6-P_2, both glucose-1,6-P_2 and fructose-1,6-P_2 can additionally activate the enzyme and partially counteract citrate inhibition. Activation of phosphofructokinase produced by alkalinization increases in the presence of the bisphosphorylated hexoses. It is suggested that the hexose bisphosphates could play a significant role in the initial burst of the glycolytic flux during muscle contraction, when an alkaline pH shift is produced.

It is accepted that activation of phosphofructokinase (PFK, EC 2.7.1.11), the key regulatory enzyme of the glycolytic pathway, is involved in the enhancement of the glycolytic flux that takes place during muscle contraction, but the mechanism of this activation remains not fully understood. Among the allosteric effectors that modulate PFK activity (1,2), two sugar bisphosphates, Fru-2,6-P_2 and Glu-1,6-P_2, have been found in addition to the product Fru-1,6-P_2. Fru-2,6-P_2 could be implicated in the effects produced by insulin and adrenaline on skeletal muscle (3), but its role in the stimulation of glycolysis during mammalian muscle contraction is unclear (3-6). No relationship has been found between muscle Fru-2,6-P_2 content and lactate accumulation during electrical stimulation of rat gastrocnemius muscle (4,6).

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Abbreviations used: Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-P_2; Fru-2,6-P_2, fructose-2,6-P_2; Glu-1,6-P_2, glucose-1,6-P_2, Glu-6-P, glucose-6-phosphate; PFK, phosphofructokinase.

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Muscle Glu-1,6-P_2 concentration changes under several physiological, hormonal and pathological conditions, leading to concomitant changes in PFK activity (7). It has been shown that muscle Glu-1,6-P_2 concentration increases during rat (6) and human (8,9) muscle contraction, suggesting the physiological importance of this bisphosphorylated sugar as activator of muscle PFK in this situation.

In order to clarify the role of Fru-2,6-P_2 and Glu-1,6-P_2 as activators of the glycolytic flux during muscle contraction, we have studied the activity of rat muscle PFK in the presence of physiological concentrations of these sugars and other allosteric effectors of the enzyme at resting and contracting conditions. The effects of the bisphosphorylated hexoses on PFK activity have been also studied at increasing pH values, in an attempt to simulate *in vitro* the alkalinization that takes place during the initial phases of muscle contraction (10-14).

MATERIALS AND METHODS

Auxiliary enzymes were purchased from Boehringer. Ammonium sulphate was removed by gel filtration on a column of Sephadex G-25 Fine. ATP, AMP, NADH, phosphoenolpyruvate, Fru-6-P, Glu-6-P, Glu-1,6-P_2 and Fru-1,6-P_2 were also from Boehringer. Fru-2,6-P_2 was from Sigma and citrate was from Merck. All other chemicals were reagent grade. In order to remove possible Fru-2,6-P_2 contamination, the stock solutions of the sugars were treated as previously described (15).

Preparation of PFK. PFK was partially purified from rat skeletal muscle essentially as described by Tornheim and Lowenstein (16). The yield was 50% and the purification was about 40-fold. The activity was assayed as previously described (15) and protein concentration was determined according to Lowry *et al.* (17). The preparation had a specific activity of 18-20 units/mg protein.

Assay for PFK activity. Two assays were used (A and B). The reaction mixture for assay A contained 25 mM imidazole-HCl buffer (at the indicated pH values), ATP and Fru-6-P as indicated, Glu-6-P at a concentration 3-fold that of Fru-6-P, 8 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 0.2 mM NADH, 0.25 mM phosphoenolpyruvate, 1 mM triethanolamine, 6 mM ortophosphate, 40 μg/ml aldolase, 10 μg/ml glycerolphosphate dehydrogenase, 2 μg/ml triosephosphate isomerase, 20 μg/ml pyruvate kinase, and 5 μg/ml phosphoglucoisomerase. Other additions are indicated in the legends.

In the experiments with added Fru-1,6-P_2, assay B was used. The reaction mixture had the same composition as that for assay A (pH 7.0), except that NADH was 0.1 mM, and the auxiliary enzymes were pyruvate kinase (20 μg/ml), lactate dehydrogenase (20 μg/ml), aldolase (12 μg/ml), and phosphoglucoisomerase (5 μg/ml). Equilibrium concentrations of Fru-1,6-P_2 in the reaction mixture were determined as described by Tornheim and Lowenstein (16).

All assays were performed using a Kontron Uvikon 810 spectrophotometer. PFK was diluted in 50 mM α-glycerolphosphate, 2 mM EDTA, 2 mM ATP, 20% glycerol (w/v), pH 7.2. The reaction mixture (1 ml final vol.) was preincubated at 30 °C for 5 min. The reaction was started by adding PFK (to give a final concentration of 0.02-0.04 units/ml) and the linear decrease in absorbance at 340 nm was measured continously (30 °C). One unit of PFK activity corresponds to the production of 1 μmol of Fru-1,6-P_2/min.

RESULTS

Activation of muscle PFK in the presence of physiological concentrations of bisphosphorylated hexoses and other allosteric effectors.

Table 1 summarizes the effects produced by physiological concentrations of several allosteric effectors on muscle PFK activity at resting and at contracting conditions. These concentrations were calculated from the reported values in rat skeletal muscle (3,4,6,18-20), corrected by: an intracellular water content of 0.67 ml/g (21); a wet weight/dry weight ratio of 4.97 (22); and a 90% binding of Fru-1,6-P_2 to muscle aldolase and PFK (16). It has been considered that 50 μM Fru 6-P, 4 μM Fru-1,6-P_2, 30 μM Glu-1,6-P_2 and 50 μM citrate represent basal (resting) concentrations, and that 2 mM Fru-6-P, 37 μM Fru-1,6-P_2, 80 μM Glu-1,6-P_2 and 250 μM citrate represent contracting conditions. Fru-2,6-P_2 has been kept constant (0.3 μM), since its value did not vary significantly during muscle contraction (4,6).

As shown, at basal Fru-6-P concentrations, both Glu-1,6-P_2 and Fru-1,6-P_2 increasing from resting to contracting levels activated muscle PFK (experiment 1 *vs* experiments 2-6, $P<0.001$). In the presence of resting Fru-1,6-P_2 concentrations, Glu-1,6-P_2 produced an additional activation (experiment 7 *vs* experiment 4, $P<0.001$). However, in the presence of contracting Fru-1,6-P_2, Glu-1,6-P_2 did not have any additional effect (experiment 10 *vs* experiment 5). The increase of Glu-1,6-P_2 and Fru-1,6-P_2 also partially counteracted citrate inhibition (experiments 11 and 12 *vs* experiments 8 and 9, $P<0.001$).

At contracting Fru-6-P concentrations, PFK activity was similar in the absence and in the presence of bisphosphorylated hexoses at the highest levels (experiments 14 and 18 *vs* experiments 15 and 19, $P<0.001$). Citrate inhibition was also strongly reduced (experiments 20 and 21 *vs* experiments 16 and 17).

Activation of muscle PFK by Glu-1,6-P_2 and Fru-2,6-P_2 at increasing pH values.

As shown in Fig. 1, alkalinization produced an important activation of PFK both in the presence and in the absence of bisphosphorylated hexoses.

TABLE 1. Activity of muscle PFK in the presence of physiological concentrations of bisphosphorylated hexoses and other allosteric effectors

Experiment	$F2,6P_2$ (μM)	$G1,6P_2$ (μM)	$F1,6P_2$ (μM)	F6P (mM)	Citrate (mM)	U/mg (X ±SE)	Citrate inhibition (%)
1	0.3	0	0	0.05	0	0.10 ± 0.002	-
2	"	30	0	"	"	0.16 ± 0.002	-
3	"	80	0	"	"	0.21 ± 0.005	-
4	"	0	4	"	"	0.17 ± 0.007	-
5	"	0	37	"	"	0.40 ± 0.016	-
6	"	0	127	"	"	0.36 ± 0.005	-
7	0.3	30	4	0.05	0	0.28 ± 0.009	-
8	"	"	"	"	0.05	0.16 ± 0.007	43
9	"	"	"	"	0.25	0.12 ± 0.004	58
10	0.3	80	37	0.05	0	0.40 ± 0.008	-
11	"	"	"	"	0.05	0.35 ± 0.015	12
12	"	"	"	"	0.25	0.18 ± 0.004	54
13	"	"	"	"	1	0.12 ± 0.001	70
14	0.3	0	0	0.5	0	5.70 ± 0.13	-
15	"	80	37	"	0	5.80 ± 0.11	-
16	"	"	"	"	0.25	3.30 ± 0.08	44
17	"	"	"	"	1	1.10 ± 0.04	81
18	0.3	0	0	2	0	5.80 ± 0.07	-
19	0.3	80	37	2	0	5.80 ±0.09	-
20	"	"	"	"	0.25	5.70 ± 0.05	2
21	"	"	"	"	1	5.00 ± 0.09	14

Note. Assay B was used (pH 7.0), in the presence of 4 mM MgATP, 50 μM AMP and the indicated additions. The number of assays for each experiment was 5-6. Abbreviations are: $F2,6P_2$, fructose-2,6-P_2; $G1,6P_2$, glucose-1,6-P_2; $F1,6P_2$, fructose-1,6-P_2; F6P, fructose-6-P.

However, in the presence of these sugars, the activatory effect was greater and present even at the lowest pH values.

DISCUSSION

The results reported here clearly show that in the presence of physiological concentrations of Fru-2,6-P_2 and at Fru-6-P concentrations corresponding to those of resting muscle, both Glu-1,6-P_2 and Fru-1,6-P_2 activate muscle PFK and partially counteract citrate inhibition. These results support our previous suggestion (6) that the increase in Glu-1,6-P_2

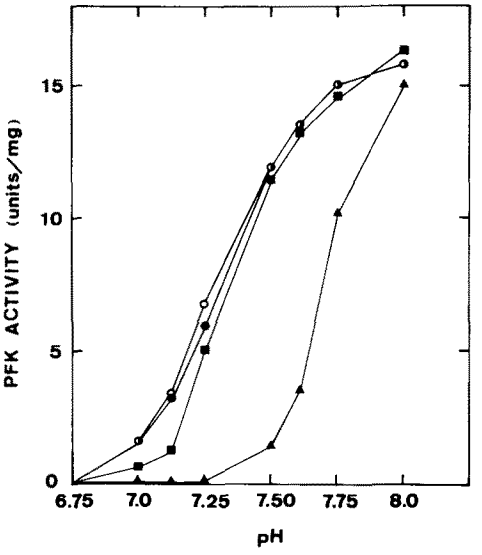

FIGURE 1. Activation of muscle PFK by Glu-1,6-P_2 and Fru-2,6-P_2 at increasing pH values. PFK activity was measured (assay A) at the indicated pH values and in the presence of 2 mM MgATP, 0.3 mM Fru-6-P and the following additions: none (▲); 100 μM Glu-1,6-P_2 (■); 0.3 μM Fru-2,6-P_2 (●); 100 μM Glu-1,6-P_2, 0.3 μM Fru-2,6-P_2 (○).

concentration that occurs at the onset of muscle contraction (6,8,9) could play a role on PFK stimulation and on the activation of the glycolytic flux.

Moreover, our results show that both the inhibition of PFK by citrate and its activation by Fru-1,6-P_2 and Glu-1,6-P_2 decrease as Fru-6-P concentration increases. It has been reported that Glu-6-P concentration increases during muscle contraction (4,6,18). Due to the equilibrium in the phosphoglucomutase reaction, Fru-6-P concentration must concomitantly increase. This suggests that the increase in Fru-6-P levels linked *in vivo* to increased glucose transport or glycogenolysis could be the main factor responsible for the increased glycolytic flux in contracting muscle.

As indicated above, a number of studies have described an initial alkaline pH shift and a subsequent acidification during continous stimulation of skeletal muscle (10-14), and it has been suggested that this initial alkalinization, together with the increase in ADP and AMP levels, could be a major regulatory factor in the early burst of glycolysis during a rest-to-work transition in muscle (23). Our results indicate that Glu-1,6-P_2, Fru-2,6-P_2, and probably Fru-1,6-P_2, could potentiate the activatory effect produced by the initial alkaline pH shift. It has been already suggested by others (24) that Glu-1,6-P_2 and Fru-2,6-P_2, in combination with other positive modulators, can be

the main means by which PFK achieves significant activity *in vivo* despite the intracellular acidification that takes place during prolonged muscle contraction.

From all these results we tentatively conclude that the bisphosphorylated hexoses could play an important role in the activation of PFK in the transition from resting to contracting muscle at two levels: a) by counteracting citrate inhibition, and b) by potentiating the activatory effect produced by the intracellular alkalinization during the initial phases of muscle contraction. Fru- 6-P seems to be the main factor responsible for the maintenance of PFK activity as contraction proceeds.

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