The control of glucose 1,6-bisphosphate by developmental state and hormonal stimulation in cultured muscle tissue

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1. The concentration of glucose 1,6-bisphosphate, a potent regulator of muscle glucose metabolism, was examined in embryonic muscle cells in culture. 2. The concentration in fused myotubes was twice that in unfused myoblasts. 3. The effect of various hormones and agonists on the glucose 1,6-bisphosphate concentration in both pre- and post-fusion muscle cells was examined. In pre-fusion cells no effect of adrenaline or cyclic AMP was observed, but stimulation by vasopressin, adrenaline + propranolol, ionophore A23187 and dibutyryl cyclic GMP significantly decreased glucose 1,6-bisphosphate. In post-fusion cells similar effects were observed, except that stimulation by adrenaline and by dibutyryl cyclic AMP significantly increased metabolite concentration. 4. All effects increased with time (over a 1 h period), except for that of vasopressin, which was transient. 5. The changes in glucose 1,6-bisphosphate concentration were accompanied by changes in the fructose 1,6-bisphosphate/fructose 6-phosphate ratio, implying an effect on phosphofructokinase activity.

Glucose 1,6-bisphosphate (Glc-1,6-P_2) is a potent regulator of several enzymes in carbohydrate metabolism (reviewed by Beitner, 1979). In muscle it has been shown to be one of the strongest activators (de-inhibitors) of phosphofructokinase (Hofer & Pette, 1968), and to be a potent inhibitor of type II hexokinase (Haberman & Beitner, 1975) and of 6-phosphogluconate dehydrogenase (Beitner & Nordenberg, 1979). It has also been shown to exert a de-inhibitory effect on the ATP-inhibited activity of phosphoglucomutase (Livini & Beitner, 1975).

The cellular concentrations of Glc-1,6-P_2 in muscle have been shown to be lowered in conditions such as muscular dystrophy and diabetes and to be increased by adrenaline and cyclic AMP (see Beitner, 1979). In addition, the calcium ionophore A23187 (Beery *et al.*, 1980) and cyclic GMP (Beitner & Cohen, 1980) have both been shown to decrease Glc-1,6-P_2 in the isolated diaphragm muscle. We decided to examine the concentrations of this metabolite in myoblasts and myotubes in culture. The effect of differentiation by fusion, and of various hormonal stimuli, on the cellular concentration were also examined. Some of the data

have been presented in a preliminary form (Wakelam *et al.*, 1981).

Materials and methods

Materials

Culture media were from Seromed G.m.b.H., 8000 München 71, Germany, except for horse serum, which was from GIBCO G.m.b.H., 7500 Karlsruhe 21, Germany. Enzymes and biochemicals were from Boehringer Mannheim G.m.b.H., 6800 Mannheim 31, Germany. All other chemicals were of the highest analytical grade available and were from Merck G.m.b.H., 6100 Darmstadt, Germany.

Methods

Myoblasts were prepared from 12-day chick-embryo breast muscles as previously described (van der Bosch *et al.*, 1972). They were plated (10 cm dishes) in a medium containing 5% (v/v) dialysed chick-embryo extract and 10% (v/v) dialysed horse serum at a Ca^{2+} concentration of approx. 10 μM. Where necesary, fusion was initiated after 50 h by raising the Ca^{2+} concentration to 1.4 mM (van der Bosh *et al.*, 1972). Hormones and effectors were dissolved in 0.9% NaCl (saline) at high concentrations, except for the Ca^{2+} ionophore, A23187,

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which was dissolved in ethanol (which was added to separate plates as a control); they were added to the plates in a volume of $10\,\mu l$. At the end of the incubation period the medium was removed, and the plates were rapidly washed in ice-cold saline and immediately frozen in liquid N_2.

Cell extracts were prepard by sonication (Branson Sonifer, $3\times30\,s$, $50\,W$) in 0.5 ml of 5.5% (v/v) $HClO_4$, the samples centrifuged ($14\,000\,g$ for 15 min) and the precipitates taken for protein determination. The supernatant was neutralized by adding 0.5 mM-KOH containing 0.05 mM-triethanolamine (free base), and the concentration of glucose 1,6-bisphosphate was determined fluorimetrically (Lowry & Passonneau, 1972). Concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate were determined fluorimetrically after amplification by enzymic cycling (Lowry & Passonneau, 1972). Protein was determined by the method of Bradford (1976).

Statistical analysis

Statistical analysis was performed by Student's t test.

Results

Treatment of solutions at pH 3 destroys fructose 2,6-bisphosphate (Van Schaftingen et al., 1980a). Table 1 shows the effect of acid treatment on the measurement of Glc-1,6-P_2 concentrations in a liver extract, with or without added Glc-1,6-P_2. The acid treatment, which would destroy all the fructose 2,6-bisphosphate present, has no effect on the Glc-1,6-P_2 measurements, and we therefore conclude that in all our Glc-1,6-P_2 measurements this is the sole compound being determined by the phosphoglucomutase reaction. This experiment also

Table 1. *Effect of acid treatment to remove fructose 2,6-bisphosphate on the measurement of glucose 1,6-bisphosphate*

A $HClO_4$ extract of rat liver was prepared by the same method used for muscle cells as described in the Materials and methods section. Glc-1,6-P_2 was added to some of these fractions. The total Glc-1,6-P_2 concentration was determined with or without a 30 min preincubation at 20°C at pH 3.0. Values represent means of duplicate determinations.

Concn. of Glc-1,6-P_2 added (pM)	pH 3.0 pretreatment	Concn. of Glc-1,6-P_2 measured (pM)
—	—	0.92
0.5	—	1.38
—	+	0.88
0.5	+	1.36

shows that quantitative recovery of Glc-1,6-P_2 is achieved by the extraction procedure.

The data for all measurements of Glc-1,6-P_2 concentrations are shown in Table 2. An approximate doubling in concentration of the metabolite was observed after fusion. Pre-fusion refers to cells that are still myoblasts and have been cultured for 24 h; post-fusion cells have been cultured for 96 h and exist as multinucleate myotubes.

In pre-fusion cells there is no effect on Glc-1,6-P_2 concentration of the addition of $10\,\mu$M-adrenaline or $10\,\mu$M-dibutyryl cyclic AMP. However, hormonal stimulation by those agents that are thought to act by raising intracellular Ca^{2+} concentrations (adrenaline + propranolol and vasopressin) decreases Glc-1,6-P_2 concentration. The Ca^{2+} ionophore A23187 and dibutyryl cyclic GMP mimic these effects.

The results of experiments where the same hormones and effectors were added to post-fusion

Table 2. *Effect of hormones and effectors on the concentration of glucose 1,6-bisphosphate in pre- and post-fusion muscle cells*

Cells were cultured and the concentrations of glucose 1,6-bisphosphate determined as described in the Materials and methods section. Except where stated incubations were for 60 min. Results are expressed as means ± S.E.M.; $n = 12$ in each case. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Addition	Glucose 1,6-bisphosphate (pmol/mg of protein)	
	Pre-fusion	Post-fusion
None	26.9 ± 2.4	46.3 ± 4.5
Adrenaline ($10\,\mu$M)	24.1 ± 2.5	62.4 ± 4.3***
Adrenaline ($10\,\mu$M) + propranolol ($10\,\mu$M)	17.4 ± 2.0**	29.3 ± 2.6***
Dibutyryl cyclic AMP ($10\,\mu$M)	25.0 ± 1.3	57.8 ± 4.9*
Dibutyryl cyclic GMP ($10\,\mu$M)	17.0 ± 2.3***	28.1 ± 1.7***
Ionophore A23187 ($5\,\mu$g/ml)	18.3 ± 1.5**	24.7 ± 2.3***
Vasopressin (100 munits/ml), 15 min	16.9 ± 1.8***	21.7 ± 3.5***
Vasopressin (100 munits/ml), 30 min	15.9 ± 1.2***	16.3 ± 1.3***
Vasopressin (100 munits/ml), 60 min	15.2 ± 1.0***	40.4 ± 3.5
Ethanol ($1\,\mu$l/ml)	25.9 ± 1.5	47.9 ± 4.9

Table 3. *Effect of hormones and effectors on the fructose 1,6-bisphosphate/fructose 6-phosphate ratio in pre- and post-fusion muscle cells*

Muscle cells were cultured as described in the Materials and methods section. The cells were incubated with the hormone or effector stated for 60min or for the time stated. Fructose 1,6-bisphosphate and fructose 6-phosphate concentrations were determined as described in the Materials and methods section. Results are means ± S.E.M. of eight determinations. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

| | Fructose 1,6-bisphosphate/fructose 6-phosphate | |
Addition	Pre-fusion	Post-fusion
None	0.766 ± 0.023	0.776 ± 0.011
Adrenaline (10 μM)	0.786 ± 0.021	1.280 ± 0.005***
Adrenaline (10 μM) + propranolol (10 μM)	0.694 ± 0.010**	0.697 ± 0.020**
Dibutyryl cyclic AMP (10 μM)	0.733 ± 0.017	1.030 ± 0.010**
Dibutyryl cyclic GMP (10 μM)	0.674 ± 0.043**	0.684 ± 0.037**
Vasopressin (100 munits/ml), 15 min	0.658 ± 0.016***	0.714 ± 0.039**
Vasopressin (100 munits/ml), 30 min	0.568 ± 0.005***	0.699 ± 0.007**
Vasopressin (100 munits/ml), 60 min	0.684 ± 0.006**	0.742 ± 0.007*
Ionophore A23187 (5 μg/ml)	0.698 ± 0.012**	0.701 ± 0.015**
Ethanol (1 μl/ml)	0.770 ± 0.020	0.778 ± 0.022

muscle cells are also shown in Table 2. In post-fusion cells similar effects of adrenaline + propranolol, vasopressin, ionophore A23187 (for which the control had ethanol alone added) and dibutyryl cyclic GMP on Glc-1,6-P_2 concentrations were observed as in pre-fusion cells, i.e. a lowering. Stimulation by adrenaline or by dibutyryl cyclic AMP was also observed; these two agents both raise the Glc-1,6-P_2 concentration.

The effects of these agents on Glc-1,6-P_2 concentration increase with time of incubation up to 1 h (results not shown), except for vasopressin stimulation of post-fusion cells. In these cells vasopressin lowers the Glc-1,6-P_2 concentration to an apparent minimum after 30 min; however, after 60 min the metabolite concentration recovered to approximately normal values (Table 2).

Glc-1,6-P_2 is an activator of phosphofructokinase (Hofer & Pette, 1968; Beitner, 1979). This activation is reflected in the effects of the additions to the culture on the fructose 1,6-bisphosphate/fructose 6-phosphate ratio (Table 3). Addition of adrenaline and cyclic AMP to fused cells results in an increase in the ratio, whereas all other additions cause a decrease. Measurement of the glucose 6-phosphate concentration gave no clear trends (results not shown).

Discussion

Glc-1,6-P_2 has several effects on glucose metabolism (Beitner, 1979). The regulation of this metabolite is therefore important to the cell. Hormones that affect glucose metabolism might thus be expected to regulate its concentration.

In this paper we show that the concentration of Glc-1,6-P_2 increases after fusion (Table 2). This probably reflects the differentiation of embryonic muscle tissue from mononucleate myoblasts to multinucleate myotubes (the precursors of muscle fibres) and the accompanying changes in energy metabolism (see Schudt & Pette, 1978). Depending on the developmental state, there are differences in responsiveness to various hormones and agonists in the regulation of this metabolite.

In pre-fusion cells, no response is observed to stimulation by either adrenaline or dibutyryl cyclic AMP (Table 2). Stimulation by adrenaline + propranolol or by by vasopressin lowers the Glc-1,6-P_2 concentration, and this effect is mimicked both by the calcium ionophore A23187 and by dibutyryl cyclic GMP. In the post-fusion cells the responsiveness to adrenaline + propranolol, vasopressin, ionophore A23187 and dibutyryl cyclic GMP is as in pre-fusion cells. Stimulation by adrenaline and by dibutyryl cyclic AMP results, in post-fusion cells, in a significant increase in Glc-1,6-P_2 concentration (Table 2). The different responsiveness to adrenaline can, in part, be explained by the observation by Parent et al. (1980) that β-adrenergic receptors and catecholamine-responsiveness only appear after fusion in avian muscle cells. However, some part of the mechanism whereby cyclic AMP activates an increase in Glc-1,6-P_2 concentration must also be absent, perhaps a protein kinase. The result that adrenaline in the absence of a β-blocker has no effect on Glc-1,6-P_2 concentration before fusion would, however, suggest that β-receptors are indeed present, but that they are functionally inactive before fusion.

The effect of all the treatments increased with time of incubation, except for incubation with vasopressin in myotubes (Table 2). This appeared to

produce a maximum decrease in Glc-1,6-P_2 concentration after 30 min, but by 60 min the metabolite concentration had recovered to control values. This suggests a transient response of these cells to this hormone; it is notable in this context that Bréant et al. (1981) and Keppens et al. (1981) have observed similar effects in the response of glycogen phosphorylase to vasopressin in isolated rat hepatocytes. However, those authors also noted a transient response to α-agonists, but we have not observed this in the present work. The regulation of Glc-1,6-P_2 concentrations and the fructose 1,6-bisphosphate/fructose 6-phosphate ratio by vasopressin also shows that this hormone has a role in the regulation of muscle glucose metabolism. Such a role was suggested by the studies in vivo by Hems & Whitton (1973) and Hems et al. (1975) (for review, see Hems & Whitton, 1980), and our studies in vitro thus support their hypothesis.

Regulation of Glc-1,6-P_2 concentration could be due to an effect on either synthesis or degradation. Synthesis of this compound appears to be achieved by phosphofructokinase catalysing the transfer of the γ-phosphate of ATP to glucose 1-phosphate (Eyer et al., 1971). Degradation appears to be performed by glucose 1,6-bisphosphate phosphatase (Beitner & Cohen, 1979). Beitner & Cohen (1980) have provided evidence that this enzyme is activated by cyclic GMP and inactivated by cyclic AMP. This interpretation would explain the results in Table 2. However, the data in Table 2 suggest that the intracellular Ca^{2+} concentration has a regulatory role. Hormones such as vasopressin and adrenaline (acting through α-receptors) are thought to produce both a rise in intracellular free Ca^{2+} concentration and a rise in cyclic GMP concentration (Berridge, 1980); both could be affecting either phosphofructokinase activity or glucose 1,6-bisphosphate phosphatase activity. Preliminary results show that the latter appears to be a Ca^{2+}-sensitive enzyme (M. J. O. Wakelam & D. Pette, unpublished work). It is noteworthy, however, that Beitner et al. (1977) have shown an inhibitory effect of cyclic GMP on phosphofructokinase activity.

The cellular concentration of Glc-1,6-P_2, calculated by assuming a protein content of 200 mg/g wet wt., would be 3–10 nmol/g of cells. Beitner et al. (1977) have shown that, when measured under regulatory conditions, this change in Glc-1,6-P_2 concentration causes a 3-fold activation of phosphofructokinase activity. This activation is reflected in the concomitant effects of the treatments on fructose 6-phosphate and fructose 1,6-bisphosphate concentrations (Table 3). These changes can be interpreted as either activation or inhibition of phosphofructokinase activity. Differing effects of α- and β-stimulation on Glc-1,6-P_2 concentration could

also therefore provide an appropriate tool for distinguishing between α- and β-receptor stimulation in muscle.

It has been shown (Claus et al., 1981; Furuya & Uyeda, 1980; Pilkis et al., 1981a,b; Uyeda et al., 1981; Van Schaftingen & Hers, 1981; Van Schaftingen et al., 1980a,b, 1981) that fructose 2,6-bisphosphate is a potent regulator of phosphofructokinase and of fructose 1,6-bisphosphatase activities in liver (Mörikofer-Zwez et al., 1981; Pilkis et al., 1981a,b; Van Schaftingen et al., 1981a). We have shown that we are not measuring the presence of this metabolite in our studies (Table 1). Vasopressin, phenylephrine (Hue et al., 1981a,b), adrenaline and cyclic AMP (Richards et al., 1981) have been shown to regulate fructose 2,6-bisphosphate concentration in liver. Richards et al. (1981) propose that it is regulated by both cyclic AMP-dependent and -independent mechanisms. Although a potent stimulatory effect of fructose 2,6-bisphosphate has also been shown (Pilkis et al., 1981c; Uyeda et al., 1981) on purified muscle phosphofructokinase, the presence of and therefore the physiological relevance of this metabolite in muscle tissue has yet to be demonstrated.

The parallel increases in both the fructose 1,6-bisphoshate/fructose 6-phosphate ratio and the Glc-1,6-P_2 concentration could reflect two phenomena. Firstly, Glc-1,6-P_2 is produced by a side reaction of phosphofructokinase (Eyer et al., 1971). Secondly, its increase causes a further stimulation of the main reaction of phosphofructokinase. Taking into account the effects of Glc-1,6-P_2 on several enzymes (Beitner, 1979), the role of fructose 2,6-bisphosphate may be to provide independent regulation of phosphofructokinase, as opposed to co-ordinate regulation of several enzymes by Glc-1,6-P_2.

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