The control of glucose 1,6-bisphosphatase by Ca^{2+} and calmodulin

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1. The control of glucose 1,6-bisphosphatase activity, the enzyme that degrades glucose 1,6-bisphosphate, a metabolite that regulates hexose phosphate metabolism, has been examined in a rat muscle extract. 2. The enzyme has been found to be activated by physiological Ca^{2+} concentrations. Ca^{2+} can be replaced by Sr^{2+} and Mn^{2+} , but the effects are not so pronounced. 3. The Ca^{2+} effect is inhibited by EGTA. Trifluoperazine also inhibits enzyme activity. This effect can be reversed by adding exogenous calmodulin.

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

We have recently shown that stimulation of muscle cells in culture with adrenaline and with cyclic AMP results in an increase in cellular Glc-1,6- P_2 concentration, whereas stimulation with adrenaline acting through α -receptors, with vasopressin and with the Ca^{2+} -ionophore A23187 results in a reduction in the cellular Glc-1,6- P_2 concentration (Wakelam & Pette, 1982). This raised the possibility of the control by $[\text{Ca}^{2+}]$ of the enzyme degrading this metabolite.

Materials and methods

Glc-1,6- P_2 -ase was measured in an extract prepared from skeletal muscle. A 10% (w/v) homogenate of adult-rat quadriceps muscle was prepared in a 20 mM-triethanolamine/HCl buffer, pH 7.4, containing 150 mM-KCl and 1 mM-EDTA with a Polytron disintegrator (4 × 5 s, with cooling). This was centrifuged for 1 h at 105 000 g in a Sorvall OTD-75B ultracentrifuge. When not used the high-speed supernatant was stored in portions at -80°C .

Glc-1,6- P_2 -ase activity was measured by incu-

bating portions of the high-speed supernatant in a 20 mM-triethanolamine/HCl buffer, pH 7.4, containing 150 mM-KCl, 20 mM-MgCl₂ with Glc-1,6- P_2 and other additions at the concentrations stated in the Results section. At zero time and after 10 min at 37°C , incubations were stopped by the addition of 5.5% (v/v) HClO₄ and the Glc-1,6- P_2 concentration determined fluorimetrically as described previously (Wakelam & Pette, 1982). All solutions were made by using triple-quartz-distilled deionized water that had a $[\text{Ca}^{2+}]$ of approx. 1 nM as determined by flame photometry. The Ca^{2+} concentration was controlled by the use of Ca^{2+} -EGTA buffers. Protein concentration was determined by the method of Peterson (1977), with bovine serum albumin (fraction V) as standard.

The source of most materials is as previously described (Wakelam & Pette, 1982). Trifluoperazine was a gift from Dr. H. Metzger, Hoechst AG, Frankfurt a.M., Germany, EGTA was from Serva G.m.b.H., Heidelberg, Germany, bovine brain calmodulin was prepared by the method of Grand *et al.* (1979) and was a gift from Dr. C. P. Downes and Dr. R. H. Michell, Department of Biochemistry, University of Birmingham, Birmingham, U.K.

Results and discussion

The substrate concentration and the time dependence of the degradation of Glc-1,6- P_2 by the phosphatase enzyme present in the muscle extract was examined. Saturation was observed in the range above $150\text{ }\mu\text{M}$ and the reaction was found to be linear over the examined time (0–20 min). A Lineweaver–Burk plot of these data yielded an apparent K_m of $22\text{ }\mu\text{M}$.

Subsequent measurements were performed by using a glucose 1,6-bisphosphate concentration of

Abbreviations used: Glc-1,6- P_2 , glucose 1,6-bisphosphate; Glc-1,6- P_2 -ase, glucose 1,6-bisphosphatase.

Fig. 1. Effect of metal ion concentration on Glc-1,6- P_2 -ase activity

The Glc-1,6- P_2 -ase activity of a 100 μ l sample of the muscle extract was determined as described in the Materials and methods section. The assay mixture contained 200 μ M-Glc-1,6- P_2 and the stated metal ion concentration. $n = 3$ in each case. ●, Ca^{2+} ; ▲, Mn^{2+} ; ○, Sr^{2+} .

200 μ M and an incubation time of 10 min, since the course of the reaction was linear over this time. The non-stimulated activity of Glc-1,6- P_2 -ase was found to be 0.2 m-units/mg of muscle protein. This activity is adequate to degrade efficiently Glc-1,6- P_2 at physiological concentrations (Wakelam & Pette, 1982).

Fig. 1. shows the Ca^{2+} -dependence of the Glc-1,6- P_2 degradations. As can be seen from the Figure, a 6-fold activation of Glc-1,6- P_2 breakdown is observed when the $[\text{Ca}^{2+}]$ is raised from 0.1 μ M to 10 μ M. This range of Ca^{2+} -activation is in the range of Ca^{2+} concentrations that are thought to be achieved in cell after stimulation by hormones such as vasopressin and adrenaline acting through α -receptors.

Fig. 1 also shows that the stimulation can be mimicked by Sr^{2+} and by Mn^{2+} . The activation of the enzyme in both cases is lower than with Ca^{2+} : about 70% by Mn^{2+} and about 55% for Sr^{2+} . Sr^{2+} is frequently able to replace Ca^{2+} in biological systems, having a similar atomic structure. The acti-

Table 1. The effects of EGTA, calmodulin, trifluoperazine and phenylmethanesulphonyl fluoride (PMSF) on Glc-1,6- P_2 -ase activity

The Glc-1,6- P_2 -ase activity of a 100 μ l sample of the muscle extract was determined as described in the Materials and methods section. The Glc-1,6- P_2 -ase concentration was 200 μ M. In experiments involving trifluoperazine the inhibitor was incubated with the muscle extract for 10 min at 37°C before determination of enzyme activity. $n = 3$ in each case. One milli-unit of Glc-1,6- P_2 -ase activity is the amount of enzyme that catalyses the degradation of 1 nmol of Glc-1,6- P_2 per min at 30°C.

Additions to the assay	Glc-1,6- P_2 -ase activity (m-units/ml)
None	0
1 μ M- Ca^{2+}	0.06 $\pm$ 0.01
1 mM- Ca^{2+}	0.18 $\pm$ 0.01
1 μ M- Ca^{2+} + 2 μ M-EGTA	0
1 mM- Ca^{2+} + 2 μ M-EGTA	0
1 μ M- Ca^{2+} + 1 μ M-trifluoperazine	0
1 mM- Ca^{2+} + 1 μ M-trifluoperazine	0
1 μ M- Ca^{2+} + 0.1 μ M-calmodulin	0.18 $\pm$ 0.01
1 mM- Ca^{2+} + 0.1 μ M-calmodulin	0
1 μ M- Ca^{2+} + 1 μ M-trifluoperazine, 0.1 μ M-calmodulin	0.19 $\pm$ 0.02
1 mM- Ca^{2+} + 1 μ M-trifluoperazine, then 1 μ M-calmodulin	0.08 $\pm$ 0
1 μ M- Ca^{2+} + 0.1 μ M-calmodulin + 0.5 mM-PMSF	0.14 $\pm$ 0.04
1 mM- Ca^{2+} + 0.1 μ M-calmodulin + 0.5 mM-PMSF	0.23 $\pm$ 0
1 μ M- Ca^{2+} + 0.5 mM-PMSF	0

vation by Mn^{2+} suggests an involvement of calmodulin in the process, since Mn^{2+} can replace Ca^{2+} in the activation of calmodulin-dependent enzymes (Stewart *et al.*, 1982).

Table 1 shows that this effect of Ca^{2+} is abolished by incubation with excess EGTA. Table 1 also shows the results of studies of the effects of trifluoperazine and purified calmodulin on the activity of the enzyme. Trifluoperazine (1 μ M), an inhibitor of calmodulin function (Livini & Weiss, 1977), abolishes enzyme activity at both 1 μ M- and 1 mM- Ca^{2+} . Exogenous calmodulin activates (approx. 2-fold) the enzyme at 1 μ M- Ca^{2+} but inhibits at 1 mM. This unexpected result could possibly be explained by a Ca^{2+} -calmodulin activation of neutral proteinases, which could be degrading the enzyme during the incubation. This conclusion is supported by an experiment (also shown in Table 1) where incubation of the muscle extract containing 1 mM- Ca^{2+} and 0.1 μ M-calmodulin with phenylmethanesulphonyl fluoride (a proteinase inhibitor) results in activation of the enzyme by calmodulin at the higher Ca^{2+} concentration. Phenylmethanesulphonyl fluoride in the absence of calmodulin inhibits

the enzyme, but this is probably due to a general non-specific effect of this inhibitor.

The addition of 0.1 μM -calmodulin to an extract that had been pre-incubated for 10 min with 1 μM -trifluoperazine restores Glc-1,6- P_2 -ase activity (Table 1).

Despite the fact that these experiments have been performed with an unpurified enzyme, Glc-1,6- P_2 -ase thus appears to be a Ca^{2+} - and calmodulin-controlled enzyme. We have previously shown that in cultured muscle tissue stimulation by vasopressin and by adrenaline acting through α -receptors lowers the cellular concentration of Glc-1,6- P_2 (Wakelam & Pette, 1982). In view of the present findings the mechanism of control appears therefore to be through activation of the phosphatase by Ca^{2+} and calmodulin. Variations in $[\text{Ca}^{2+}]$ could therefore influence the activity of Glc-1,6- P_2 -ase and thus exert an important control on muscle carbohydrate metabolism. In addition, Nordenberg *et al.* (1981) have shown that local anaesthetics that produce an increase in intracellular free $[\text{Ca}^{2+}]$ cause a fall in muscle Glc-1,6- P_2 concentration. This is probably due to Ca^{2+} -activation of the Glc-1,6- P_2 -ase.

We have recently discussed the differing roles of Glc-1,6- P_2 and fructose 2,6-bisphosphate in regulating carbohydrate metabolism (Wakelam & Pette, 1982). In this context, it is interesting that van Schaftingen *et al.* (1982) have observed no Ca^{2+} -activation of fructose 2,6-bisphosphatase; the con-

trol of the two regulators of glycolysis, which act in different ways, is thus achieved in differing fashions.

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References

- Beinter, R. (1979) *Trends Biochem. Sci.* **4**, 228–230
Beitner, R. & Nordenberg, J. (1979) *Biochim. Biophys. Acta* **583**, 266–269
Grand, R. J. A., Perry, S. V. & Weeks, R. A. (1979) *Biochem. J.* **177**, 521–529
Haberman, S. & Beitner, R. (1975) *Isr. J. Med. Sci.* **11**, 1180
Hofer, H. W. & Pette, D. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1378–1392
Livini, L. & Beitner, R. (1975) *Isr. J. Med. Sci.* **11**, 1181
Livini, R. M. & Weiss, B. (1977) *Mol. Pharmacol.* **13**, 690–697
Nordenberg, J., Klein, S., Beery, E., Kaplansky, M. & Beitner, R. (1981) *Int. J. Biochem.* **13**, 1005–1009
Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B. & Cohen, P. (1982) *FEBS Lett.* **137**, 80–84
van Schaftingen, E., Davies, D. R. & Hers, H.-G. (1982) *Eur. J. Biochem.* **124**, 143–149
Wakelam, M. J. O. & Pette, D. (1982) *Biochem. J.* **204**, 765–769