EFFECT OF EPINEPHRINE AND INSULIN ON THE PHOSPHORYLATION OF PHOSPHORYLASE PHOSPHATASE INHIBITOR 1 IN PERFUSED RAT SKELETAL MUSCLE

Balwant S. KHATRA, Jean-Louis CHIASSON, Hisataka SHIKAMA, John H. EXTON and Thomas R. SODERLING
Howard Hughes Medical Institute Laboratories, Department of Physiology, Vanderbilt University, Nashville, TN 37232, USA

Received 12 April 1980

1. Introduction

The two heat-stable trypsin-labile proteins which inhibit the low molecular weight phosphorylase phosphatase [1–4] have been isolated from rabbit skeletal muscle. The activity of one of the inhibitors, termed inhibitor 1 (I_1), is controlled by phosphorylation catalyzed by cAMP-dependent protein kinase and dephosphorylation catalyzed by a Mn^{2+}-dependent phosphatase [2,5,6]. The phosphorylated form of I_1 is inhibitory whereas the dephosphorylated form is not active [2,6].

In view of high concentration of I_1 in skeletal muscle, its high potency as an inhibitor, and control of its activity by phosphorylation, it has been suggested that I_1 may play an important role in the regulation of phosphoprotein phosphatase [2,6,7]. Normal rabbits injected with epinephrine have increased levels of phosphorylated form of I_1 [7]. However, insulin increases the rate of activation of glycogen synthase in tissues of control and diabetic animals [8–10] and may act by inhibiting the cAMP-dependent protein kinase [11] or by activating a phosphoprotein phosphatase. Both of these changes may result in a decrease in the phosphorylation state of I_1.

Here we have studied the effects of epinephrine and insulin on the phosphorylation state of I_1. The hindlimb perfusion technique was used to investigate the effects of these hormones in a controlled situation in intact rat muscle.

2. Materials and methods

2.1. *Preparation of enzymes and ^{32}P substrates*

High molecular weight phosphoprotein phosphatase was prepared as in [12]. Low molecular weight phosphoprotein phosphatase was prepared from the supernatant of amylase-treated glycogen–protein complex of rabbit muscle [13]. The supernatant was concentrated by ammonium sulfate, treated with 70% ethanol and the phosphatase purified by DEAE-cellulose chromatography as in [14]. Skeletal muscle phosphorylase kinase, phosphorylase *b* and the catalytic subunit of cAMP-dependent protein kinase were prepared by the methods in [15], [16] and [17], respectively. [γ-^{32}P]ATP and ^{32}P phosphorylase *a* were prepared as in [18] and [19], respectively.

2.2. *Hindlimb perfusion*

Hindlimbs were perfused following [20] as modified [21]. Epinephrine (10^{-9}, 10^{-8}, 10^{-7} M) with or without insulin (1 mU/ml), with or without propranolol (10^{-6} M) was infused for 30 min in the presence of 10 mM glucose. At the end of the perfusion period, muscle tissue was quick frozen in liquid nitrogen for determination later of phosphorylation state of I_1.

2.3. *Preparation of samples and assays*

Phosphorylase phosphatase I_1 was extracted from skeletal muscle as in [7]. The activity of I_1 was determined by its ability to inhibit the purified low molecular weight phosphatase. Phosphorylase phosphatase assay conditions and definition of unit activity were as in [22] except that 3.75 mM EDTA was included in the assay. One unit of inhibitor activity is defined as the amount of inhibitor which inhibits 7.5 mU phosphatase activity by 50%. All enzyme or inhibitor dilutions were made in 50 mM Tris–HCl (pH 7.5) containing 10% sucrose, 1 mM EDTA, 1 mg/ml BSA and 30 mM 2-mercaptoethanol.

2.4. *Phosphorylation and dephosphorylation of inhibitor I_1*

Phosphorylation of the I_1 was carried out in a mixture containing 50 mM Tris buffer (pH 7.5), 30 mM 2-mercaptoethanol, 10 mM magnesium acetate, 0.5 mM ATP, 20 μl inhibitor sample and 2500 units of catalytic subunit of cAMP-dependent protein kinase in 0.1 ml total vol. The dephosphorylation of inhibitor was carried out in 100 μl mixture containing 50 mM Tris buffer (pH 7.5), 30 mM 2-mercaptoethanol, 1 mM $MnCl_2$, 50 μl of sample and 2.5 units of purified high molecular weight phosphoprotein phosphatase. Both phosphorylation and dephosphorylation were at 30°C for 90 min and terminated by boiling. Appropriate dilutions were then made to assay the activity of the inhibitor. Preliminary experiments showed that the amount of protein kinase or phosphatase utilized were far in excess of that required for maximal phosphorylation or dephosphorylation, respectively, of the I_1.

3. Results and discussion

The basic strategy for determining the degree of phosphorylation of I_1 in perfused rat muscle was that in [7] for rabbit skeletal muscle. The homogenization of frozen muscle powder in 2% trichloroacetic acid-solubilized I_1, but little or none of inhibitor 2 (I_2). The degree of phosphorylation of I_1 in extracts was determined by assaying the extracts for I_1 activity prior to and after phosphorylation and dephosphorylation of extracts (section 2). Dephosphorylation of the extracts by high molecular weight phosphatase led to complete loss in inhibitor activity thus establishing the absence of any I_2. This loss of activity could however be reversed by phosphorylation by cAMP-dependent protein kinase. Units of I_1 activity prior to phosphorylation divided by units of activity obtained after maximal phosphorylation gave the ratio of I_1 in the phosphorylated state. When purified I_1 was added to the phosphatase reaction mixture in increasing concentration, the inhibition was linear up to 50–60% loss of phosphatase activity. Therefore the amount of inhibitor sample added to the phosphatase assay mixture was such that inhibition was ⩽20–40%.

Fig.1 shows the effect of perfusing rat skeletal muscle with epinephrine on the phosphorylation state of I_1. In the absence of epinephrine, only 5–10% of the inhibitor was phosphorylated suggesting little inhibition of phosphatase by I_1 in the absence of hormones. The phosphorylation state of I_1 increased 2–3-fold over this basal level at the lowest dose of epinephrine tested (10^{-9} M) and reached 50–60% at 10^{-7} M epinephrine. This implies that the phosphorylation and hence the inhibitory activity of I_1 is very sensitive to epinephrine. As shown in fig.1 propranolol markedly inhibited the phosphorylation of I_1 by 10^{-7} M epinephrine, thus suggesting that the stimulation of phosphorylation of I_1 by epinephrine in skeletal muscle is through the classical β-adrenergic mechanism. This is also in agreement with [23] which shows that in skeletal muscle activation of phosphorylase by epinephrine occurs through the β-adrenergic mechanism only. Perfusion with 1 mU insulin/ml in the presence or absence of epinephrine did not affect the phosphorylation state of I_1.

Although the physiological function of phosphatase inhibitors is not well understood, several properties of I_1 suggest that it may be involved in the regulation of

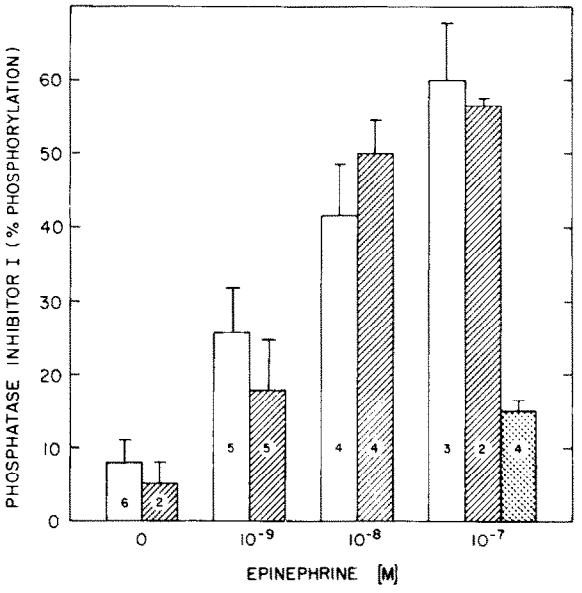

Fig.1. The effect of saline or epinephrine (open bars), epinephrine plus insulin (hatched bars) and epinephrine plus propranolol (crossed bars) on the phosphorylation state of phosphorylase phosphatase I_1 in rat skeletal muscle. The hindlimbs were perfused for 30 min with the hormones in the presence of 10 mM D-glucose and muscle tissue was freeze clamped at the end of the study. Values are expressed as mean ± SEM and the no. perfusions are listed within each bar.

glycogen metabolism. The concentration of I_1 in skeletal muscle (1.4 μM, [6]) is comparable to the concentration of a general phosphoprotein phosphatase which has been shown to dephosphorylate glycogen synthase, phosphorylase *a* and phosphorylase kinase [3,4]. The I_1 has a relatively low K_i of ~2 nM [4] suggesting that it could inhibit a large proportion of phosphatase in muscle. The control of I_1 activity by covalent modification catalyzed by cAMP-dependent protein kinase [2,5,6] suggests that its activity could be turned on and off rapidly by β-agonists such as epinephrine. In spite of these considerations which suggest a role of I_1 in glycogen metabolism in skeletal muscle, there is evidence [24] that phosphorylase phosphatase activity in freshly prepared skeletal muscle extracts is not inhibited by I_1. No data were given, however, on the possible effect of endogenous phosphatases and proteases on the added I_1. In contrast, I_1 inhibits phosphorylase phosphatase activity by 50–70% in tissue extracts of brain, liver, heart, mammary gland and skeletal muscle [25].

On the basis of the above results, it is proposed (fig.2) that cAMP-dependent protein kinase regulates the phosphorylation and hence the inhibitory activity of I_1. An increase in cAMP levels in response to increased epinephrine levels would increase phosphorylase kinase activity and lower phosphoprotein phosphatase activity thus allowing activation of phosphorylase.

Acknowledgements

This work was supported in part by the National Institutes of Health grant AM 17808. We wish to express our appreciation to Ms Martha Bass for her excellent technical assistance.

References

[1] Huang, F. L. and Glinsmann, W. (1976) FEBS Lett. 62, 326–329.
[2] Huang, F. L. and Glinsmann, W. (1976) Eur. J. Biochem. 70, 419–426.
[3] Cohen, P., Nimmo, G. A. and Antonio, J. F. (1977) Biochem. J. 162, 435–444.

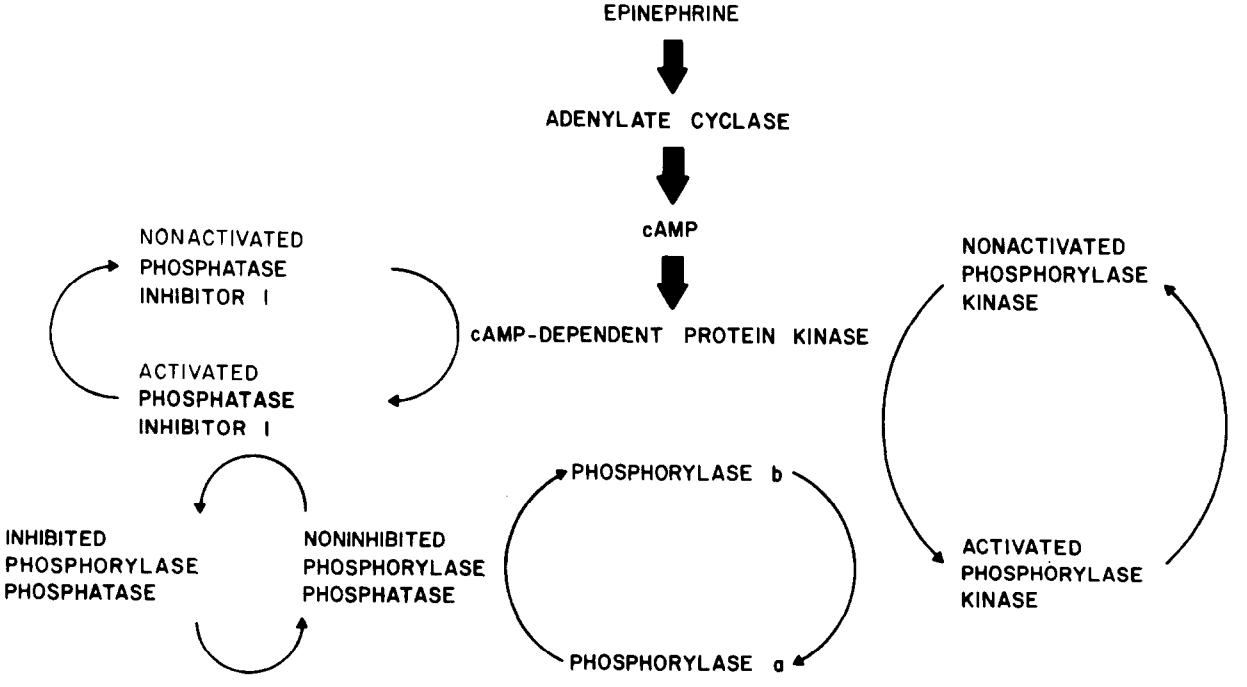

Fig.2. Proposed scheme for the mechanism of inhibition of phosphorylase phosphatase activity by cAMP in rat skeletal muscle.

[4] Nimmo, G. A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[5] Huang, F. L. and Glinsmann, W. (1975) Proc. Natl. Acad. Sci. USA 72, 3004–3008.
[6] Nimmo, G. A. and Cohen, P. (1978) Eur. J. Biochem. 87, 353–365.
[7] Foulkes, J. G. and Cohen, P. (1979) Eur. J. Biochem. 97, 251–256.
[8] Nuttall, F. Q., Gammon, M. C., Corbett, V. A. and Wheeler, M. P. (1976) J. Biol. Chem. 251, 6724–6729.
[9] Bishop, J. S. (1970) Biochim. Biophys. Acta 20, 208–218.
[10] Miller, T. B., Rannels, S. R. and Jefferson, L. S. (1977) Diabetes 26, 365.
[11] Walkenbach, R. J., Hagen, R. and Larner, J. (1978) Mol. Cell. Biochem. 19, 31–41.
[12] Khatra, B. S. and Soderling, T. R. (1978) J. Biol. Chem. 253, 5247–5250.
[13] Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkler, F. L., Walsh, D. A. and Krebs, E. G. (1970) J. Biol. Chem. 245, 6317–6328.
[14] Brandt, H., Capulong, Z. L. and Lee, E. Y. C. (1975) J. Biol. Chem. 250, 8058–8064.
[15] Brostrom, C. O., Hunkeler, F. L. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1961–1967.
[16] Fischer, E. H. and Krebs, E. G. (1958) J. Biol. Chem. 321, 65–71.
[17] Reimann, E. M. and Corbin, J. D. (1976) Fed. Proc. FASEB 35, 1384.
[18] Walseth, T. F. and Johnson, R. A. (1979) Biochim. Biophys. Acta 562, 11–31.
[19] Krebs, E. G., Kent, A. B. and Fischer, E. H. (1958) J. Biol. Chem. 231, 73–83.
[20] Ruderman, N. B., Houghton, C. R. S. and Hems, R. (1971) Biochem. J. 124, 639–651.
[21] Caldwell, M. D., Lacy, W. W. and Exton, J. H. (1978) J. Biol. Chem. 253, 6837–6844.
[22] Khatra, B. S. and Soderling, T. R. (1978) Biochem. Biophys. Res. Commun. 85, 647–654.
[23] Dietz, M. R., Chiasson, J. L., Soderling, T. R. and Exton, J. H. (1980) J. Biol. Chem. in press.
[24] Laloux, M. and Hers, H. G. (1979) FEBS Lett. 105, 239–243.
[25] Burchell, A., Foulkes, J. D., Cohen, P. T. W., Condon, G. D. and Cohen, P. (1978) FEBS Lett. 92, 68–72.