Control of Rat Skeletal-Muscle Phosphorylase Phosphatase Activity by Adrenaline

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(Received 20 July 1978)

Administration of adrenaline to an isolated rat hindlimb preparation rapidly decreased muscle phosphorylase phosphatase (EC 3.1.3.17) activity and increased heat-stable and trypsin-labile phosphatase inhibitor activity. This was associated with increased tissue cyclic AMP concentrations, phosphorylase (EC 2.4.1.1) activation and glycogen synthase (EC 2.4.1.11) inactivation.

Stimulation of muscle glycogenolysis by adrenaline involves multiple-protein-phosphorylation reactions initiated by activation of the cyclic AMP-dependent protein kinase (Krebs *et al.*, 1973; Soderling & Park, 1974; Cohen, 1974, 1976). In addition to previously described cyclic AMP-controlled reactions, the possibility exists that a similar reaction inactivates phosphorylase phosphatase (Chelala & Torres, 1970; Merlevede & Riley, 1966; Merlevede *et al.*, 1976). Our studies on rabbit skeletal muscle have shown that the effect of cyclic AMP is mediated by the cyclic AMP-dependent protein kinase and phosphorylation of a relatively heat-stable protein inhibitor of the phosphatase (Huang & Glinsmann, 1975). These findings prompted us to investigate: (1) whether adrenaline would cause a decrease in phosphorylase phosphatase activity, and (2) whether the inactivation of phosphorylase phosphatase would be associated with an increase of the heat-stable and trypsin-labile phosphatase inhibitor activity. We used an isolated rat hindlimb perfusion system to avoid complications of secondary responses that occur when adrenaline is given to intact animals.

Materials and Methods

Hindlimb perfusions and sample handling

A Mortimore-type perfusion apparatus (Glinsmann *et al.*, 1969) was used with minor modifications. Male rats (200–300 g) of the Osborne–Mendel strain were used. The perfusion was started between 10 and 20 s after interrupting aortic flow and inserting a polyethylene cannula just above the aortic bifurcation. The eviscerated lower portion of the animal was washed out with 20 ml of perfusate before

recirculating the perfusate to an oxygenating reservoir. The perfusion medium (Glinsmann *et al.*, 1969) containing 15 mM-glucose was pumped at a flow rate of 10 ml/min. At intervals indicated, a portion of thigh muscle was removed from one leg, a drug or an equivalent amount of 0.9% NaCl was injected, and a second sample of muscle was obtained from the second leg. All muscle was frozen immediately between liquid-N₂-chilled Wollenberger tongs and was stored in liquid N₂ until homogenization.

The perfusate was assayed for glucose (Beckman glucose analyser). The frozen muscle was powdered in liquid N₂ and was immediately homogenized with a Sorvall Omni-Mixer in 3 or 5 vol. (w/v) of 40 mM-imidazole/HCl buffer (pH 7.4) containing 10 mg of Norit A/ml, 10 mM-EDTA, 0.5 mM-EGTA, 0.4 mM-dithiothreitol and 0.2 mM-phenylmethanesulphonyl fluoride in the presence and absence of 50 mM-NaF. The homogenate was centrifuged at 8000g for 1 min in a micro-centrifuge (Brinkman Instruments); the supernatant fraction was frozen immediately in liquid N₂ and stored at –100°C. Fractions containing NaF were used for assays of phosphorylase and glycogen synthase; fractions without NaF were for assays of phosphatase and phosphatase inhibitor. Fractions for inhibitor assay were heated to between 90 and 100°C for 5 min, centrifuged, and the supernatant fraction was dialysed against 5 mM-Tris buffer for 20 h and assayed.

Assays

Phosphorylase activity was measured in the direction of glycogen synthesis by the method of Cori *et al.* (1943) in the absence and presence of 1 mM-5'-AMP by using [¹⁴C]glucose 1-phosphate and product separation as described by Huang & Cabib (1973). A unit of phosphorylase is defined as the amount of enzyme that incorporates 1 μmol of

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[^{14}C]glucose 1-phosphate into glycogen per min at 30°C. Glycogen synthase was determined (Thomas *et al.*, 1968; Huang & Cabib, 1973) in the presence of either 10mM- SO_4^{2-} (I form) or 10mM-glucose 6-phosphate (D form). Enzyme activity was expressed as μmol of UDP-[^{14}C]glucose incorporated into glycogen per min at 30°C.

Phosphorylase phosphatase activity was measured by measuring the decrease of phosphorylase *a* activity in the absence of 5'-AMP. Muscle phosphorylase *a* (Sigma Chemical Co.) was recrystallized in glycerol phosphate buffer after treatment with neutral AG-1 resin (Bio-Rad) to remove AMP (Gilboe & Nuttall, 1972). The phosphatase reaction mixture (60 μl) contained 20mM-imidazole/HCl, pH 7.4, 3.9mM-EDTA, 0.2mM-EGTA, 0.2mM-dithiothreitol, 20mM-glucose, 3.5mg of phosphorylase *a*/ml, and phosphatase. All reagents were warmed to 30°C for 5min before assay to convert endogenous tissue phosphorylase *a* into the *b* form and to minimize temperature-dependent aggregation of phosphorylase. The reaction at 30°C was started by the addition of phosphorylase *a*, and was stopped by dilution (1:50) with ice-cold 10mM-sodium phosphate buffer, pH 7, containing 200mM-NaF and 7.5mM-EDTA. In all experiments the linearity of the reaction with time was verified. One unit of phosphorylase phosphatase activity is defined as the amount of enzyme that lowers phosphorylase *a* activity by 1 unit per min.

Phosphorylase phosphatase inhibitor activity was expressed as units of phosphorylase phosphatase activity inactivated per min. In this case phosphorylase phosphatase activity was measured by the release of [^{32}P]P_i from [^{32}P]P_i-labelled phosphorylase *a* in a 50 μl assay system to which 6 units of partially purified rabbit skeletal-muscle phosphorylase phosphatase was added (Huang & Glinsmann, 1975). Inhibition was proportional to the inhibitor concentration if the inhibitor was diluted to an amount that produced less than 40% inactivation of the phosphatase. [^{32}P]P_i-labelled phosphorylase *a* was prepared by enzymic phosphorylation of phosphorylase *b* as described by Torres & Chelala (1970). Cyclic AMP was determined by radioimmunoassay (Schwartz/Mann). Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Reagents

L-Adrenaline bistartrate, L-isoproterenol D-bitartrate, and phosphorylases *a* and *b* were obtained from Sigma Chemical Co. Phosphorylase was recrystallized and treated with AG-1 resin (Bio-Rad). Insulin was a gift from the Eli Lilly Research Laboratory [γ - ^{32}P]ATP, uridine diphosphate [^{14}C]glucose and [^{14}C]glucose 1-phosphate were pur-

chased from New England Nuclear Corp. Tests of significance are based on Student's *t* distribution of paired differences.

Results

Evaluation of the perfused system

Glucose consumption was measured in the presence and absence of insulin. We observed a control glucose-uptake rate of 1.7–2.3 μmol of glucose/g of tissue per h and a 5-fold increase in this rate in the presence of 20nM-insulin. This is in good agreement with responses in comparable systems (Jefferson *et al.*, 1972; Berger *et al.*, 1975). Total phosphorylase and glycogen synthase activities, and the percentage of these enzymes in the active form, remained stable during control perfusion for up to 2h, and tissue cyclic AMP concentrations were in the range found in normal resting muscle. Fifteen minutes after the addition of insulin, a small but consistent increase of glycogen synthase in the I form was observed, whereas total synthase activity remained unchanged. Phosphorylase activity was used as an indicator to find the minimum time required for adrenaline to produce a maximum effect in the present system; a maximum was approached 2min after adrenaline administration. Since the mechanism of the effect of adrenaline on phosphorylase activation in normal muscle is through a β -receptor-cyclic AMP response, we verified that this was also the case in our system by using the β -adrenergic agonist, isoproterenol, and by demonstrating increased concentrations of tissue cyclic AMP. The inactivation of glycogen synthase, an additional consequence of increased tissue concentrations of cyclic AMP, also was noted (Table 1). Thus we had a system that appeared suitable and that behaved in response to adrenaline via a mechanism involving the β -adrenergic receptor, increased cyclic AMP concentrations and phosphorylase activation.

Regulation of phosphorylase phosphatase and phosphatase inhibitor activities

After the administration of adrenaline at the 2min time point, phosphorylase phosphatase activity was decreased and phosphorylase phosphatase inhibitor activity was increased (Table 2). There was a direct correlation of the percentage of activity changes between these two events by regression analysis ($r = 0.8755$). The β -agonist isoproterenol also caused an increase in phosphatase inhibitor activity and a decrease in phosphorylase phosphatase activity (results not shown). Phosphatase inhibitor activity was stable to prolonged dialysis and was totally destroyed by treatment with trypsin.

Table 1. *Effects of adrenaline on cyclic AMP concentrations and phosphorylase and glycogen synthase activities in the isolated perfused hindlimb*

After a 30 min control perfusion period, muscle samples from isolated perfused hindlimbs were removed before and 2 min after adrenaline administration. Phosphorylase and glycogen synthase activities are expressed as the percentage of enzymes in the active forms, i.e. $a\%$ = (phosphorylase activity in the absence of AMP)/(phosphorylase activity in the presence of AMP) $\times 100$, and 1% = (synthase activity in the absence of glucose 6-phosphate)/(synthase activity in the presence of glucose 6-phosphate) $\times 100$. Total enzyme activities did not change with treatment (phosphorylase = 1.4 units/mg of protein, glycogen synthase = 45.8 munits/mg of protein). Values are mean $\pm$ S.E.M. *Significantly different from control at $P < 0.001$.

Treatment	No. of rats	Cyclic AMP concn.		Phosphorylase (a%)	Glycogen synthase (1%)
		pmol/mg of protein	pmol/mg of muscle		
None	8	0.8 $\pm$ 0.11	0.1 $\pm$ 0.01	32.5 $\pm$ 1.10	14.2 $\pm$ 1.18
Adrenaline (100 μ g/kg body wt.)	8	9.3 $\pm$ 2.72*	1.5 $\pm$ 0.44*	55.0 $\pm$ 2.30	6.5 $\pm$ 0.62*

Table 2. *Changes in phosphorylase phosphatase and phosphorylase phosphatase inhibitor activities by adrenaline in isolated perfused hindlimb*

Experimental conditions and expression of results are as indicated in Table 1 and the Materials and Methods section. Significantly different from control at: *, $P < 0.05$; †, $P < 0.02$.

Treatment	No. of rats	Phosphorylase phosphatase (unit/mg of protein)	Phosphorylase phosphatase inhibitor (munits/mg of protein)
None	8	0.65 $\pm$ 0.07	3.0 $\pm$ 0.50
Adrenaline (100 μ g/kg body wt.)	8	0.41 $\pm$ 0.04*	4.3 $\pm$ 0.26†

Discussion

The idea that phosphorylase phosphatase may be an important site of control of glycogenolysis has been raised repeatedly (Fischer *et al.*, 1971; Brandt *et al.*, 1974; Huang & Glinsmann, 1975; Brandt *et al.*, 1975; Lee *et al.*, 1976; Glinsmann *et al.*, 1977); however, to our knowledge no evidence existed that demonstrated such a response in intact tissue. In previous experiments we found with partially purified proteins from rabbit skeletal muscle that phosphorylase phosphatase can be inactivated by an inhibitor that is active only after phosphorylation by the cyclic AMP-dependent protein kinase (Huang & Glinsmann, 1975). Thus we considered that this mechanism may exist *in vivo* and that further investigation of the control of phosphorylase phosphatase on the basis of our understanding of this mechanism may allow us to demonstrate hormonal control of this reaction. We chose to examine the effects of adrenaline in intact skeletal muscle, since the mechanisms by which adrenaline stimulates cyclic AMP synthesis and activates the cyclic AMP-dependent kinase are well defined in this tissue (Krebs *et al.*, 1973; Soderling & Park, 1974; Cohen, 1976),

and since this approach has been used for establishing the regulation *in vivo* of other glycogen-metabolizing enzymes (Danforth, 1965; Posner *et al.*, 1965; Lyon & Mayer, 1969; Yeaman & Cohen, 1975).

The results of the present investigation are in all respects consistent with the possibility that skeletal-muscle phosphorylase phosphatase activity is controlled in the intact cell by adrenaline via a cyclic AMP-mediated reaction that involves changes in heat-stable protein phosphatase inhibitor activity. These findings support the idea that control of glycogenolysis by the cyclic AMP-dependent protein kinase effectively controls in concert all of the reactions involved in the synthesis and degradation of glycogen. It also foregrounds the importance of further studies with phosphorylase phosphatase-inhibitor proteins. The phosphorylated inhibitor was shown to be phosphorylated at a threonine residue by Cohen *et al.* (1977). It is a distinct entity from the heat-stable protein inhibitor of the cyclic AMP-dependent protein kinase (Huang & Glinsmann, 1976b; Nakai & Glinsmann, 1977). The potential role of heat-stable phosphatase-inhibitor proteins as regulatory subunits of phosphoprotein phosphatases has been raised (Huang & Glinsmann, 1975; Brandt *et al.*, 1975), but definitive evidence for such a role remains to be demonstrated. These proteins have been shown to exist in skeletal muscle and liver (Huang & Glinsmann, 1975, 1976a,b; Brandt *et al.*, 1975; Goris *et al.*, 1976; Nakai & Glinsmann, 1977). Thus these inhibitors may be general in their tissue distribution and may contribute to substrate specificity in the control of phosphoprotein phosphatase activity.

S.-H. T. is a recipient of a National Institutes of Health post-doctoral fellowship HD-05062.

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