Epinephrine Regulation of Skeletal Muscle Glycogen Metabolism

STUDIES UTILIZING THE PERFUSED RAT HINDLIMB PREPARATION

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Studies of rat skeletal muscle glycogen metabolism carried out in a perfused hindlimb system indicated that epinephrine activates phosphorylase via the cascade of phosphorylation reactions classically linked to the \(\beta\)-adrenergic receptor/adenylate cyclase system. The \(\beta\) blocker propranolol completely blocked the effects of epinephrine on cAMP, cAMP-dependent protein kinase, phosphorylase, and glucose-6-P, whereas the \(\alpha\) blocker phentolamine was totally ineffective. Omission of glucose from the perfusion medium did not modify the effects of epinephrine.

Glycogen synthase activity in control perfused and nonperfused muscle was largely glucose-6-P-dependent (\(-\text{glucose-6-P}+/\text{glucose-6-P}\) activity ratios of 0.1 and 0.2, respectively). Epinephrine perfusion caused a small decrease in the enzyme's activity ratio (0.1 to 0.05) and a large increase in its \(K_v\) for glucose-6-P (0.3 to 1.5 mM). This increase in glucose-6-P dependency correlated in time with protein kinase activation and was totally blocked by propranolol and unaffected by phentolamine.

Comparison of the kinetics of glycogen synthase in extracts of control and epinephrine-perfused muscle with the kinetics of purified rat skeletal muscle glycogen synthase \(\alpha\) phosphorylated to various degrees by cAMP-dependent protein kinase indicated that the enzyme was already substantially phosphorylated in control muscle and that epinephrine treatment caused further phosphorylation of synthase, presumably via cAMP-dependent protein kinase. These data provide a basis for speculation about in vivo regulation of the enzyme.

It is well established that epinephrine causes glycogenolysis in skeletal muscle via a cascade of phosphorylation reactions triggered by interaction of the catecholamine with the \(\beta\)-adrenergic receptor. The same mechanism is thought to decrease glycogen synthesis in skeletal muscle, because phosphorylation of glycogen synthase by the cyclic adenosine 3':5'-monophosphate-dependent protein kinase results in inactivation of the enzyme.

Several groups (1-3) working with purified preparations of rabbit skeletal muscle glycogen synthase, have recently shown that measurement of the enzyme's \(K_v\) for glucose-6-P is more sensitive than the \(-\text{glucose-6-P}+/\text{glucose-6-P}\) activity ratio as an index of its phosphorylation state (1, 3). It has also been shown that the kinase used to phosphorylate the enzyme plays a major role in regulating its \(K_v\) for glucose-6-P and activity ratio (3, 4).

Most of the studies heretofore carried out to examine catecholamine effects on muscle glycogen metabolism have utilized isolated diaphragms (5-9), in vivo injection of hormone (10-13), and perfused hearts (14-17). In the present study, we have employed the perfused rat hindlimb preparation. Until recently (18), this has not been used to examine the regulation of glycogen metabolism. We have focused our studies on the effects of epinephrine on muscle glycogen synthase. Comparison of perfusion data with observations from phosphorylation studies on purified rat skeletal muscle glycogen synthase and other data (1-4, 19) have allowed us to speculate on the in vivo regulation of glycogen synthase by catecholamine.

**EXPERIMENTAL PROCEDURES**

**Hindlimb Perfusion**—The method used for hindlimb perfusion was a modification of Ruderman's technique (20) developed by Caldwell (21). During the experimental period, saline (0.9% NaCl) or hormone was infused into the aortic line at a constant rate using a Harvard model 940 infusion pump. When adrenergic blockers were used, they were added to the perfusate as a single bolus and allowed to equilibrate throughout the system 10 min prior to hormone infusion. Hormone and blocker solutions were made up as previously described (22). Experiments were performed without recirculation of perfusion medium, to prevent accumulation of the catecholamine in the perfusate.

**Enzyme Assays**—Phosphorylase was assayed by a modification (23) of the procedure of Gilboe et al. (24). For glycogen synthase assay, an aliquot of the phosphorylase homogenate was diluted with buffer containing 50 mM Tris, 5 mM Na,EDTA, 50 mM NaF, 50 mM \(\beta\)-mercaptoethanol, and 1 mg of albumin/ml at pH 7.8 (synthase dilution buffer). The assay was essentially that of Thomas et al. (25). In some experiments, supernatants were filtered through Sephadex G-25 columns (1 x 20 to 25 cm) to remove glucose-6-P and other small molecules prior to assay. These filtered supernatants were used initially to assess the effects of glucose-6-P concentration on glycogen synthase activity and to estimate a \(K_v\) for the enzyme by utilizing assays varying the glucose-6-P concentration from 0 to 10 mM. When initial experiments showed that Sephadex G-25 filtration did not lower the base-line activity ratio, gel filtration of supernatants prior to assay was no longer carried out.

Approximate \(K_v\) values for glucose-6-P of glycogen synthase were obtained by plotting the ratio \(v/v_{\text{max}} = c/\text{counts per min at zero glucose-6-P} - v\) against glucose-6-P concentration. The concentration giving a ratio of 1 was taken as the \(K_v\). Values used were: \(v_0 = \text{counts per min at zero glucose-6-P} = \text{counts per min at 10 mM glucose-6-P}\).V

\(v = \text{counts per min at a given}\)

\(K_v\) refers to that concentration of allosteric modifier which gives half-maximal activation of the enzyme.
glucose-6-P concentration.
cAMP-dependent protein kinase was assayed according to the method of Keely and Corbin (23).

Analytical Methods—cAMP was purified (26) and assayed by the protein-binding method of Gilman (27). Glucose-6-P and lactate were measured according to the methods of Hohorst (28, 29).

Perfusate oxygen tension and flow rate were measured as described previously (21). For perfusion pressure, a transducer (model 467A, Sanborn Co., Waltham) was inserted into the aortic line just proximal to the aortic cannula, and attached to a Sanborn model 60 Twin-Viso Recorder with a strain-gage amplifier model 64-500-C50.

Partial Purification and Phosphorylation of Rat Skeletal Muscle Glycogen Synthase—Glycogen synthase a' was purified from rat hindlimb skeletal muscle essentially by the procedure described for the rabbit muscle enzyme (4). The final purification step entailed sulfate polyacrylamide gel electrophoresis showed a pattern similar to that obtained for the rabbit muscle enzyme (30). Preparations contained no detectable cAMP-dependent synthase kinase activity and only trace activities of cAMP-independent synthase kinase and synthase phosphatase.

Phosphorylation of synthase a was performed as described previously (4) with the addition of 20 mM NaP to the reaction mixture. Phosphorylation was stopped by the addition of excess EDTA to aliquots from which 32P incorporation, activity ratio, K₅, for glucose-6-P, and trypsin effects were assessed. The assay of 32P-labeled glycogen synthase for synthase activity was carried out as described by Hutson et al. (31); trypsin effects were assessed as described previously (32).

Sources of Chemicals—(-)-Epinephrine bitartrate, (+)-propranolol HCI, type II-A histone from calf thymus, a-d-glucose-6-P (the disodium salt), UDP-glucoze, and oyster glycogen were from Sigma. a-d-Glucose-1-P (the disodium salt) was from Grand Island Biological Co. Phenolamine mesylate was a gift from Ciba-Geigy. Bovine serum albumin (Pentex, Fraction V) was from Miles. UDP-[U-¹⁴C]glucose was from Amersham/Searle or New England Nuclear. a-d-[U-¹⁴C]-glucose-1-P was from New England Nuclear. Catalytic subunit of cAMP-dependent protein kinase (purified according to the method of Miller et al. (35)).

RESULTS

Examination of Epinephrine Effects on Perfusion Pressure, Flow Rate, and Oxygen Consumption—Since epinephrine can have both vasoconstrictive and vasodilatory effects in skeletal muscle, preliminary experiments were done to determine the changes in perfusion pressure and flow rate in hindlimbs perfused with epinephrine concentrations up to 2 × 10⁻⁶ M. The data (not shown) indicated that epinephrine, at concentrations greater than 5 × 10⁻⁸ M, increased perfusion pressure, but neither reduced flow rate nor changed arterial and venous oxygen tensions. Thus, it appeared that the tissues would be adequately perfused in our studies.

Time Course of Effects of Epinephrine on cAMP-dependent Protein Kinase, Glycogen Synthase, Levels of Tissue Glycolytic Intermediates, and Perfusate Lactate Levels—Epinephrine at 10⁻⁷ M, a submaximally effective dose (see below), increased the cAMP-dependent protein kinase and phosphorylase activity ratios significantly by 1 min (Fig. 1, upper and lower panels). The protein kinase activity ratio was maximally elevated, about 2-fold, by 5 min and remained so throughout 20 min. The maximal activity ratio observed for phosphorylase, about a 4-fold increase, was seen at 10 min and remained so throughout 20 min. This dose of epinephrine also caused a small, but significant, decrease in the activity ratio of glycogen synthase by 5 min (Fig. 1, middle panel). Significant activation of protein kinase and phosphorylase was observed with epinephrine concentrations as low as 2 × 10⁻⁶ M and maximal activation of protein kinase occurred with 2 × 10⁻⁷ M catecholamine (data not shown).

Glucose-6-P in tissue perfused with 10⁻⁷ M epinephrine (Fig. 2) increased significantly above control levels at 1 min and continued to rise with time. At 15 or 20 min there was about a 5-fold increase in the level of this metabolite. Tissue lactate levels (not shown) were significantly increased (about 1.5-fold)
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FIG. 2. Time course of effects of 10^{-7} M epinephrine (Epi) on tissue glucose-6-P. Experimental procedure was as in Fig. 1. The metabolite was assayed as described under "Experimental Procedures." For each point, n = 3. * p < 0.05 versus appropriate saline control; ** p < 0.025 versus appropriate saline control.

by epinephrine at 15 min. Changes at earlier times were not significant.

The arterial lactate concentration remained constant over the perfusion period both in the control and in the epinephrine-treated preparations, but the venous lactate concentration was significantly increased above the arterial concentration in the epinephrine-treated animals after 5 min (data not shown).

Effects of α- and β-Adrenergic Blockade on Epinephrine Actions—In order to characterize the adrenergic response of perfused rat skeletal muscle, the α blocker phentolamine and the β-blocker propranolol were used. A short time period, 2 min, was utilized, in order to facilitate the comparison of data before and after epinephrine treatment in the same animal.

The 6-fold rise in CAMP produced by 10^{-7} M epinephrine at 2 min was completely blocked by propranolol, and was unchanged by phentolamine, in agreement with the classic theory of β receptor linkage to adenylate cyclase (Fig. 3, lower panel). Similarly, the doubling of the protein kinase activity ratio caused by epinephrine was completely blocked by propranolol, but unaffected by phentolamine (Fig. 3, middle panel). The phosphorylase activity ratio changes (Fig. 3, upper panel) showed a corresponding pattern. Glycogen synthase changes (not shown), were not significant, due to the short (2 min) exposure to epinephrine (cf. Fig. 1).

Epinephrine treatment doubled the glucose-6-P level, as expected from the phosphorylase changes. Phentolamine did not affect this increase, whereas propranolol completely prevented it (data not shown). Consistent with previous observations, tissue and perfusate lactate were not significantly affected by epinephrine treatment at this early time point (data not shown).

Perfusion without glucose in the medium did not alter epinephrine's effects on any of the above parameters at 2 min and did not change the effects of the blockers (data not shown).

Effect of Glucose-6-P Concentration on the Activity of Glycogen Synthase in Extracts from Control and Epinephrine-treated Muscle—Although the previous data showed that in control muscle phosphorylase was mainly in the dephosphorylated form, this did not appear to be the case for glycogen synthase. The activity ratio of this enzyme from control tissue was already very low (about 0.1) resembling that of the phosphorylated b form of the enzyme rather than that of the dephosphorylated α form (about 0.7 or higher).

Since the various forms of glycogen synthase can be distinguished by their sensitivity to activation by glucose-6-P as well as by their (-glucose-6-P/+glucose-6-P) activity ratio, we studied the effects of different concentrations of glucose-6-P on the enzymes from saline- and epinephrine-treated tissues.

Fig. 4 shows the activity ratio of glycogen synthase from control and epinephrine-treated tissues assayed at increasing glucose-6-P concentrations. The activity ratios of enzymes
Fig. 4. Effect of glucose-6-P concentration on the activity of glycogen synthase from saline- and epinephrine-treated muscle. Muscle samples were from rats perfused for up to 20 min with epinephrine (Epi) or saline (Control) (experimental procedure as in Fig. 1). These samples were assayed for glycogen synthase, as described under "Experimental Procedures," at the indicated glucose-6-P concentration ([Glucose-6-P]). Some samples were assayed after passage over a Sephadex G-25 column. The activity ratios shown from epinephrine-treated tissues assayed with no added glucose-6-P were decreased at infusion times of 15 and 20 min (Panel F); data for the last time point are not shown because they were not different from those for 15 min. At 5 min and longer (Panels D through F), the activity ratios for enzymes from epinephrine-treated tissues were lower at all glucose-6-P concentrations between 0.02 mM and 5 mM. Even at 2 min (Panel C), the activity ratios for enzymes from epinephrine-treated tissues were lower at glucose-6-P concentrations of 1 and 2 mM than were those from control tissues.

The conclusion from the above data that the sensitivity of synthase to activation by glucose-6-P was lowered in epinephrine-treated tissues was reinforced by the calculation of $K_s$ values for experimental and control tissue enzymes (see "Experimental Procedures") (Table I). The $K_s$ for glucose-6-P of enzyme from epinephrine-treated muscle was increased over the control value at 2 min and was progressively increased by longer epinephrine treatment. The maximum increase in $K_s$ observed was about 4 times the control value at 5 min or longer of epinephrine exposure.

<table>
<thead>
<tr>
<th>Time of infusion</th>
<th>Approximate $K_s$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>0 min</td>
<td>0.28 ± 0.03$^a$</td>
</tr>
<tr>
<td>1 min</td>
<td>0.35</td>
</tr>
<tr>
<td>2 min</td>
<td>0.49 ± 0.07</td>
</tr>
<tr>
<td>5 min</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td>10 min</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>15 &amp; 20 min</td>
<td>0.32 ± 0.07</td>
</tr>
</tbody>
</table>

$^a$ Standard error of the mean.

$^b$ $p > 0.05$ versus control.

$^c$ $p < 0.05$ versus control.

$^d$ $p < 0.0005$ versus control.
To determine whether the changes in the sensitivity of glycogen synthase to glucose-6-P induced by epinephrine were mediated by the β receptor-cAMP-linked phosphorylation system, the effects of adrenergic blockers were examined. Fig. 5 shows that propranolol prevented the shift in the glucose-6-P sensitivity curve caused by epinephrine at 10 min, whereas phentolamine had no effect. As may be deduced from the figure, propranolol completely prevented the increase in $K_m$ for glucose-6-P caused by epinephrine at 10 min, whereas phentolamine was without effect.

**Kinetics of Purified Rat Skeletal Muscle Glycogen Synthase and Effects of Phosphorylation by cAMP-dependent Protein Kinase**—In order to relate the changes in the kinetics of glycogen synthase produced by epinephrine in the perfusion experiments with alterations in the kinetics of the enzyme produced by cAMP-dependent protein kinase, glycogen synthase $a$ was purified from rat hindlimb skeletal muscle as described under “Experimental Procedures,” and phosphorylation studies were done. Table II presents kinetic parameters for the $a$ form of the rat enzyme. The data resemble those previously found for the rabbit skeletal muscle enzyme, namely an activity ratio of 0.80 and a $K_m$ for glucose-6-P of 0.015 mM. The alkali-labile phosphate content of the preparations was also determined and found to be 0.3 mol/90,000 g of enzyme.

Phosphorylation of synthase $a$ by incubation with [γ-32P]-ATP and purified catalytic subunit of cAMP-dependent protein kinase (see “Experimental Procedures”) resulted in progressive incorporation of 32P into the enzyme as a function of time. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the phosphorylated enzyme showed incorporation of 32P into a protein with a molecular weight of approximately 90,000 (data not shown). Increasing phosphorylation caused concurrent increases in the $K_m$ for glucose-6-P and decreases in the activity ratio for the enzyme (Table II). Incorporation of 32P into synthase $a$ to the extent of 2.1 mol/90,000 g of enzyme produced a $K_m$ of 0.36 mM and an activity ratio of 0.07. As expected from the studies with rabbit muscle enzyme (4), about 70% of the 32P incorporated into synthase $a$ was released by trypsin treatment (20 μg/ml incubated with 0.3 mg/ml of synthase for 15 min at 30°C) (data not shown).

3 J. L. Chiasson, unpublished observations.

### Table II

<table>
<thead>
<tr>
<th>Form of synthase</th>
<th>32P incorporation</th>
<th>$K_m$ for glucose-6-P</th>
<th>Activity ratio glucose-6-P/glucose-6-P</th>
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<tbody>
<tr>
<td>a</td>
<td>0.015</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.14</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.17</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.21</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.36</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

*Alkali-labile phosphate was about 0.3 mol/90,000 g of synthase.*

**DISCUSSION**

The data presented in this study support the generally accepted mechanism for epinephrine activation of phosphorylase in skeletal muscle and provide evidence that the perfused rat hindlimb preparation is a valid experimental system for *in vitro* study of this tissue. Values for cAMP, cAMP-dependent protein kinase, phosphorylase, glycogen synthase, and glucose-6-P measured in the perfused tissues agree well with values for muscle *in vivo* (17, 38–38).

**β-Adrenergic Mediation of Epinephrine Effects**—The data presented clearly support the classical β-adrenergic cascade mechanism for phosphorylase activation and indicate absence of the α-adrenergic activation of phosphorylase recently identified in liver, heart, and adipose tissue (39–41).

It has been reported that glucose can reduce the level of phosphorylase $a$ in the incubated diaphragm (42) and decrease phosphorylase $a$ and increase glycogen synthase $a$ in liver (43). No evidence was found in the present experiments, however, for a role of plasma glucose in regulation of the enzymes of skeletal muscle glycogen metabolism.

**Hormone Effects on Glycogen Synthase**—Extracts prepared from perfused muscle exhibited lower basal glycogen synthase activity ratios (0.09 ± 0.01, $n = 4$) than did extracts from unperfused muscle (0.19 ± 0.01, $n = 7$). Neither ratio, however, corresponded to that obtained for purified synthase $a$ (about 0.8). The low ratio in perfused muscle was not due to the presence of allosteric modifiers in the extracts since passage over Sephadex G-25 columns caused no increase. Apparently, then, glycogen synthase ordinarily exists in a comparably glucose-6-P-dependent form in the resting muscle cell.

As illustrated in Fig. 4, glycogen synthase in extracts from muscle perfused with epinephrine had properties different from those of enzyme extracted from saline-perfused muscles. Epinephrine treatment resulted in greatly reduced sensitivity to activation by glucose-6-P (Fig. 4 and Table I), but only slightly decreased activity ratios (Fig. 1). Our studies of the kinetics of perfused rat skeletal muscle glycogen synthase and of the effects of phosphorylation by cAMP-dependent protein kinase (Fig. 4 and Table II) thus support the previous findings of Roach and colleagues (1, 2), Brown *et al.* (3), and Soderling (5) that the enzyme probably exists in vivo as a mixture of variously phosphorylated forms and that the data given in Table I and Fig. 4 probably reflect average values for all the forms.
et al. (4) obtained with the rabbit skeletal muscle enzyme. Roach and colleagues (1, 2) and Brown et al. (3) concluded that the $K_i$ for glucose-6-P was a more sensitive index of the phosphorylation state of the enzyme than was the activity ratio, and Soderling et al. (4) have pointed out that the site at which phosphate is incorporated into the enzyme (which is determined by the kinase used to carry out the phosphorylation) is more important in defining the enzyme’s activity ratio than is the number of phosphate molecules incorporated per enzyme subunit.

Using data from Tables I and II and Fig. 4 as a basis, one can speculate about the phosphorylation state of the enzymes in control and epinephrine-perfused muscle. It appears that a phosphorylation state of 1 to 2 phosphates/subunit (taken from Table II) corresponds with the activity ratio and $K_i$ for glucose-6-P of the enzyme assayed in control perfused muscle (Table I and Fig. 4). Comparison of the changes in synthase induced by epinephrine infusion (Table I and Fig. 4) with those caused by phosphorylation of synthase a by CAMP-dependent protein kinase (Table II) provides evidence that, as expected, the changes in synthase induced by the hormone are consistent with increased activity of CAMP-dependent protein kinase. This observation receives strong support from the fact that the increase in $K_i$ for glucose-6-P induced by epinephrine was totally blocked by propranolol which abolished activation of protein kinase (Fig. 3).

The present findings as well as the conclusions of Brown et al. (3) and Roach and Larner (2) indicate that glycogen synthase in the resting muscle cell is probably partially phosphorylated. Although it is impossible to conclude from the present data which site(s) is (are) phosphorylated, one can speculate that the basal phosphorylation state of synthase might be due to the activity of a CAMP-independent synthase. Alternatively, the CAMP-dependent protein kinase might maintain the basal phosphorylation state of glycogen synthase since the basal activity ratio of this kinase (Fig. 3) indicates that it is fairly active in the cell.

In summary, it is proposed that glycogen synthase is present in the cell in a phosphorylated state. Epinephrine treatment increases the phosphorylation of the enzyme, probably via the CAMP-dependent protein kinase, and results in a form with a greatly increased $K_i$ for glucose-6-P, but an apparently little changed activity ratio.

It is probable that the balance between glycogen synthesis and breakdown in the cell is partly dependent on the level of glucose-6-P. Since epinephrine treatment produces a large increase in the intracellular glucose-6-P concentration, this could result paradoxically in increased glycogen synthesis if glycogen synthase were not simultaneously made less sensitive to this effector. Other factors, however, probably regulate glycogen synthase in the cell. The enzyme is sensitive to many other allosteric effectors (2), and the phosphorylated enzyme is more readily inhibited by phosphate, adenosine nucleotides, and uridine diphosphate than is the dephosphorylated enzyme (2). This means that, although the intracellular glucose-6-P level appears to have risen high enough to apparently completely activate the enzyme in our perfused samples (cf. Figs. 2 and 4), the enzyme may have remained inactive in the intact tissue because of inhibition by other allosteric modifiers.

Another factor which would be important in vivo is the interplay between insulin and epinephrine and perhaps other hormones in the regulation of the enzyme. Further work is being directed toward defining the phosphorylation state of the enzyme in resting muscle and toward correlating hormone effects on the enzyme with possible alterations in its phosphorylation state.

REFERENCES


6 This possibility is diminished, however, by recent measurements by T. R. Soderling of the CAMP-dependent protein kinase activity ratio using purified rabbit skeletal muscle glycogen synthase a rather than histone as a substrate which have given values between 0.1 and 0.2.
Epinephrine regulation of skeletal muscle glycogen metabolism. Studies utilizing the perfused rat hindlimb preparation.
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