Hormonal Regulation of Adipocyte Enzymes

THE EFFECTS OF EPINEPHRINE AND INSULIN ON THE CONTROL OF LIPASE, PHOSPHORYLASE KINASE, PHOSPHORYLASE, AND GLYCOGEN SYNTHASE*

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SUMMARY

The effects of epinephrine and insulin on three enzyme systems in adipocytes were investigated with regard to the role of adenosine 3',5'-monophosphate (cyclic AMP) in their control and the possibility that differential regulatory mechanisms might operate beyond cyclic AMP.

When rat adipocytes were incubated with increasing concentrations of epinephrine (0.01 to 100 μM) elevation of cyclic AMP and glycerol production, activation of phosphorylase and deactivation of glycogen synthase appeared to be closely correlated. Activatability of hormone-sensitive lipase in cell extracts by cyclic AMP-dependent protein kinase decreased with increasing concentrations of epinephrine indicating conversion of the nonactivated to the activated form during incubation of the cells. Activation of lipase in cell-free extracts by cyclic AMP and MgATP was promptly arrested by addition of protein kinase inhibitor. This finding, together with results of previous studies, rules against the involvement of a "lipase kinase" analogous to that of phosphorylase kinase in phosphorylase activation. Phosphorylase kinase activities measured between pH 6.0 and 9.2 were unaffected at any concentration of epinephrine. Moreover, activation of phosphorylase in cell-free extracts was not inhibited by addition of protein kinase inhibitor. When adipocytes were incubated in Ca2+-free Krebs-Ringer bicarbonate medium, activation of phosphorylase in response to epinephrine was not impaired. Phosphorylase kinase assay in cell-free extracts did not require Ca2+ for the activation of endogenous phosphorylase. However, some Ca2+ dependence was shown when exogenous skeletal muscle phosphorylase b was used as substrate. Thus, Ca2+ does not appear to regulate the catalytic activity of adipocyte phosphorylase kinase, nor could any change in the activation state of this enzyme in response to epinephrine be observed. These results distinguish adipocyte phosphorylase kinase from that in muscle and in heart which requires Ca2+ and is clearly convertible to an activated form by epinephrine.

Incubation of adipocytes with glucose (10 mM) significantly reduced the phosphorylase activity ratio (activity in the absence of 5'-AMP divided by that in its presence). Epinephrine stimulated increases in lipolysis and phosphorylase activity in the presence or absence of glucose, but its effect on glycogen synthase was apparent only in the presence of glucose. The insulin-induced increase in glycogen synthase activity ratio (activity in the absence of glucose 6-phosphate divided by that in its presence) was also dependent on glucose.

Insulin (0.4 nM, 50 microunits per ml), in the presence of glucose, almost completely antagonized the epinephrine-induced increases in lipolysis, activation of phosphorylase and deactivation of glycogen synthase. The antilipolytic action of insulin in cells previously exposed to epinephrine was associated with reversion of the activated lipase to its nonactivated form. However, insulin antagonized the elevation of cyclic AMP induced by epinephrine only marginally. This dissociation between the antagonism of insulin and epinephrine on the activities of the three enzymes on the one hand and the concentration of cyclic AMP on the other hand makes it seem unlikely that the action of insulin is mediated by a reduction in cyclic AMP concentration.

Phosphorylase kinase, glycogen synthase, and hormone-sensitive lipase are examples of enzymes regulated by covalent modification, specifically via phosphorylation catalyzed by adenosine 3',5'-monophosphate-dependent protein kinase (1-6). All three are found in adipose tissue and the latter two have been shown directly to respond to a variety of hormonal stimuli (7, 8). Changes in phosphorylase kinase activity have not been directly demonstrated although the responsiveness of phosphorylase to hormones implies that it is regulated similarly (9, 10). Protein kinase-dependent activation of purified lipase has been directly demonstrated (4-6). Phosphorylase kinase and glycogen synthase have not been purified from adipose tissue and a role for protein kinase in their regulation rests on correlations with changes in cyclic AMP levels and on analogy with their regulation in other tissues (11-13). The present studies were

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The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.
undertaken to characterize further the regulation of these enzymes in a single tissue with a view to determine whether their regulation is in all cases concerted or whether there may be sites of regulation beyond cyclic AMP formation. One such site may be the regulation of phosphorylase kinase activity by Ca²⁺ (14-16), whereas reactions catalyzed by cyclic AMP-dependent protein kinases (lipase activation and glycogen synthase deactivation) do not require Ca²⁺ (2, 5). A preliminary report on part of these studies has appeared (17).

**EXPERIMENTAL PROCEDURE**

**Materials**

Rabbit skeletal muscle was the source of the following: (a) protein kinase, partially purified by the procedure of Walsh et al. through the first DEAE-cellulose chromatography step (18); (b) protein kinase inhibitor, purified to the maximum extent as described by Walsh et al. (19); (c) phosphorylase b, purified by a modification (11) of the method of Fischer and Krebs (20); (d) phosphorylase kinase, purified by the method of Krebs et al. (21). Shellfish glycogen was obtained from Sigma Chemical Company and further purified by the procedure of Somogyi (22). ³²P-labeled phosphorylase a was prepared by a slight modification of the method of Krebs et al. (23). After crystallization, the enzyme was dialyzed in a cellulose hollowfiber apparatus (Bio-Rad Laboratories, Los Angeles), until the ³²P content was less than 1% that of the protein-bound ³²P. [γ-³²P]ATP was prepared by modification (24) of the method of Glynn and Chappell (25). L-Epinephrine (adrenaline HCl) was obtained from Parke-Davis and Co.; insulin from Calbiochem, [¹⁴C]triolein (170 mCi per mmole) from DOHM products Ltd., Hollywood, UDP-glucose and collagenase (Lot C-6855) from Sigma Chemical Co., St. Louis, UDP-[¹³C]glucose (248 mCi per mmole) from Amersham-Searle Corp., Chicago.

**Methods**

Adipocytes were prepared from epididymal fat pads of Sprague-Dawley rats (150 to 180 grams) according to Rodbell (20). The cells were washed twice with 5 volumes of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 3% bovine serum albumin and 10 mM glucose. The washed cells were resuspended to about 2 mg of cell protein per ml. Aliquots of cell suspension (5 ml) were pipetted into siliconized flasks using a polystyrene pipette. The cell suspensions were incubated with or without hormones for exactly 5 min under a gas phase of 95% O₂-5% CO₂ at 37°C. At the end of incubation, 1 ml of each cell suspension was pipetted into a tube containing 0.1 ml of 100% tri chloroacetic acid for cyclic AMP and glyceral determinations.

Cyclic AMP was determined by the method of Wastila et al. (27), which is based on measurement of cyclic AMP-dependent protein kinase activity. Glyceral was measured fluorometrically by a modification of Wieland's procedure (28). Total cyclic AMP and glyceral (cells plus medium) were determined, and concentrations are expressed per mg of cell protein.

For assays of phosphorylase, phosphorylase kinase, and glycogen synthase, an aliquot from each cell suspension was removed and centrifuged at 1000 × g for 1 min. The infranatant incubation medium was drawn off, and the packed cells were homogenized in an equal volume of a solution containing 50 mM β-glycerophosphate, 15 mM mercaptoethanol, and 1 mM EDTA, pH 6.8. The homogenate was then centrifuged at 1000 × g for 10 min. The infranatant fluid was aspirated and the enzymes were assayed within 2 hours. For hormone-sensitive lipase assay, the packed cells were homogenized in 2 volumes of 10 mM Tris buffer (pH 7.4) containing 0.25 mM sucrose and 1 mM EDTA. This homogenate was centrifuged at 1000 × g for 10 min and the infranatant fraction was aspirated for lipase assay.

Phosphorylase activity was assayed in the direction of glucose-1-P formation in the absence and in the presence of 5'-AMP (29). The ratio of former to the latter is termed phosphorylase activity ratio. Increases in this ratio are presumed to reflect relative increases in the percentage of phosphorylase in the activated form. Activity assayed in the presence of 5’-AMP is designated total phosphorylase activity. The incubation mixture consisted of 10 mg per ml of glycogen, 50 mM potassium phosphate buffer, pH 6.7, 1 mg per ml of bovine serum albumin, and 5 µl of enzyme in a total volume of 55 µl with or without addition of 2 mM 5'-AMP. The reaction was carried out at 30°C for 30 min.

Phosphorylase kinase activity was measured by a modification (30) of the method of Krebs et al. (21) based on the conversion of skeletal muscle phosphorylase b to a. The reaction mixture contained 0.042 mM-Tris-0.042 mM β-glycerophosphate at pH 6.0, 6.8, or 8.2; 100 units per ml of purified skeletal muscle phosphorylase b; 10 mM Mg²⁺; 3 mM ATP; and 25 µl of enzyme in a total volume of 150 µl and was incubated for 10 min at 30°C.

Glycogen synthase activity was assayed in terms of the rate of incorporation of UDP-[¹³C]glucose radioactivity into glycogen using the method of Thomas et al. (31). Activity was measured in the absence and in the presence of glucose-6-P. The ratio of the former to the latter is designated synthase activity ratio. A decrease in this ratio is assumed to reflect a relative increase in the fraction of the enzyme in the D form (glucose-6-P dependent). The activity assayed in the presence of glucose-6-P is assumed to represent total synthase activity. The 125 µl of reaction mixture contained 25 mM Tris, 1 mM EDTA, 0.4% glycogen, 4.5 mM UDP-glucose, 54,800 cpm of UDP[¹³C]glucose, and 25 µl of enzyme and was incubated at pH 7.8 for 15 min at 30°C with or without 7.5 mM glucose-6-P.

Phosphorylase phosphatase was assayed in terms of the release of ³²P; from [³²P]labeled phosphorylase a by the method of Hurd et al. (32). The 100 µl of incubation mixture contained 4 mg per ml of [³²P]-labeled phosphorylase a, 10 µl of enzyme, 10 mM β-glycerophosphate, and 15 mM mercaptoethanol, pH 6.8. This reaction mixture was incubated at 30°C for 15 min.

Hormone-sensitive lipase was assayed as described previously by Khoo et al. (33) using [¹⁴C]triolein as substrate and isolating free fatty acids released by the method of Kelley (34). Activation of lipase by cyclic AMP-dependent protein kinase was carried out for 10 min at 30°C in an incubation mixture containing 10 mM Tris, 7.4, 1 mM dibutylriboitol, 0.5 mM EGTA, 0.25 mg/ml bovine serum albumin, 2.5 mM phosphopyruvate, 20 units per ml of pyruvate kinase, 1 mM theophylline, 5 mM MgCl₂, 0.5 mM ATP, 1 µM cyclic AMP, 8 µg per ml of skeletal muscle protein kinase, and 100 µl of lipase in a final volume of 200 µl. In control incubations, protein kinase and cyclic AMP were omitted. The lipase assay was initiated by adding 2 ml of a sonicated emulsion containing 12.5 mg per ml of gum arabic, 0.84 mM [¹⁴C]triolein, 40 mM sodium phosphate, pH 6.8, 70 mg per ml of bovine serum albumin, and 30 mM EDTA. A decrease in the activity of the enzyme observed in this system (comparing extracts from epinephrine-treated and control cells) is interpreted to reflect an increase in the ratio of activated to nonactivated forms of the lipase.

Glycogen was determined using the anthrone reaction (35) and protein by the method of Lowry et al. (36).
Effects of Epinephrine on Intact Adipocytes—When rat adipocytes were incubated with graded concentrations of epinephrine from 0.01 to 100 $\mu M$ for 5 min, the increase in cyclic AMP production closely paralleled the increase in glycerol production (Fig. 1). The epinephrine-stimulated conversion of the nonactivated form of lipase to its activated form was reflected by the progressive decrease in the activatability of the enzyme produced by protein kinase in cell-free extracts. In the absence of epinephrine, lipase was activated 60% but after exposure to a high concentration of epinephrine (100 $\mu M$) it was activated only 16%.

The effects of epinephrine on glycogen phosphorylase and glycogen synthase are shown in Fig. 2a; the changes in cyclic AMP and glycerol production, determined under the same conditions, are shown in Fig. 2b. The phosphorylase activity ratio ($-5'$-AMP: +5'-AMP) increased as a function of epinephrine concentration in a manner parallel to the dose-response curve for glycerol production. Total phosphorylase activity also increased although not to the same extent.

The glycogen synthase activity ratio decreased with increasing concentrations of epinephrine. There was no measurable change in total glycogen synthase activity.

Effects of Protein Kinase Inhibitor on Activation of Hormone-sensitive Lipase and Phosphorylase—Previous studies have established that activation of lipase in crude 100,000 $\times g$ supernatant fractions is dependent on protein kinase. Because even the most highly purified kinase directly phosphorylates and activates the lipase itself or an intermediate "lipase kinase" (37), it is not clear whether the protein kinase may be activating an intermediate "lipase kinase" or whether, in analogy with the phosphorylase system in muscle, activation of lipase in crude 100,000 $\times g$ supernatant fractions has properties compatible with those of a multienzyme complex (37). If an analogous lipase kinase were involved here, then once the activation process was initiated by addition of cyclic AMP, protein kinase, and MgATP, it might be expected to continue after protein kinase activity was blocked by protein kinase inhibitor. The inhibitor was added to 100,000 $\times g$ supernatant extracts of fat cells at time zero and at intervals after the onset of activation by cyclic AMP and MgATP (Fig. 3). When protein kinase inhibitor was added at zero time, little or no activation occurred. When the inhibitor was added at any time during the course of activation, the process was arrested immediately. In two additional experiments of this kind (not shown), addition of the inhibitor at 0 or 10 min (approximately 50% of maximum activation) again blocked activation at once. However, in these experiments lipase activity either plateaued or dropped to a lesser extent than in the case of the experiment shown in Fig. 3. Soderling et al. (24) have shown that when protein kinase inhibitor was added during the course of the conversion of purified muscle glycogen synthase I to D catalyzed by cyclic AMP-dependent protein kinase, the reaction was immediately blocked. Glycogen synthase I is a substrate, directly, of protein kinase. The effects of protein kinase inhibitor on the activation of hormone-sensitive lipase from adipocytes and the deactivation of glycogen synthase from skeletal muscle appear to be similar and the postulation of a lipase kinase seems unnecessary.

Protein kinase inhibitor was without effect on the activation of adipocyte phosphorylase, affecting neither changes in phosphorylase activity ratio nor total activity (Fig. 4, a and b). When only MgATP and cyclic AMP were added, the phosphorylase activity ratio increased from 0.049 to 0.429 in 20 min.
and total enzyme activity from 20 to 100 milliunits per mg of protein. Addition of protein kinase inhibitor was without effect on activation. Parallel studies of histone phosphorylation and of lipase activation showed that the protein kinase inhibitor added was sufficient to block the activity of endogenous protein kinase. Addition of muscle protein kinase did not increase the rate of activation of phosphorylase. However, the phosphorylase activity ratio and total activity increased more rapidly when purified skeletal muscle phosphorylase kinase (1 μg per ml) was added to the incubation mixture. The amount of exogenous phosphorylase kinase activity added was estimated to be about four times that present in the homogenate.

Properties of Phosphorylase Kinase—Although there was a good correlation between increases in cyclic AMP production and activation of phosphorylase in response to increasing concentrations of epinephrine, there was no detectable change in phosphorylase kinase activity measured at pH 6.0, 6.8, or 8.2 (data not shown). The basal pH 6.8:8.2 ratio was high (0.34) compared to the ratios found in resting muscle (0.02) (38) and heart (0.00) (13). This ratio remained constant (0.33 to 0.94) despite incubation of adipocytes for 5 min in the presence of epinephrine at concentrations ranging from 0.01 to 100 μM. No changes in activity were observed at intermediate pH values nor at higher pH values up to 9.2. The maximal phosphorylase kinase activity in adipocytes (1.3 to 1.8 units per mg of protein) was obtained at pH 8.2, an optimum similar to that in muscle and heart.

Muscle phosphorylase kinase can be activated by autocatalysis (11) and proteolytic attack (16) as well as by phosphorylation catalyzed by protein kinase (18). Since the crude collagenase used to prepare the adipocytes contained proteolytic enzymes, we had to consider the possibility that phosphorylase kinase was artifically activated. However, homogenates prepared from fat pads, without collagenase treatment, also showed a high pH 6.8:8.2 ratio and prior incubation of the fat pads with epinephrine did not increase the ratio. Treatment of the rats with a β-adrenergic blocking agent (propranolol) and anesthesia with chloroform before removal of fat pads, freezing of the fat pads in situ and homogenization of fat pads in a buffer containing protein kinase inhibitor did not lower the pH 6.8:8.2 ratio.

**Effect of Ca**²⁺ on Phosphorylase and Lipase Activation—At the normal concentration of Ca**²⁺** used in Krebs-Ringer bicarbonate buffer (1.3 mM), epinephrine stimulated a 67% increase in total phosphorylase activity and a 5-fold increase in cyclic AMP (Table I). Lipase in extracts from control cells was activated 63% by the protein kinase system while lipase from epinephrine-treated cells was activated by only 34%. Omission of Ca**²⁺** and the addition of EGTA (1 mM) did not significantly affect the epinephrine activation of phosphorylase or lipase (as indicated by the comparable values for percentage of activation obtained after exposure to hormone).

Adipocytes incubated in a medium containing a high concentration of Ca**²⁺** (4.8 mM) showed a decrease in basal concentration of cyclic AMP. The high Ca**²⁺** medium also reduced the ability of epinephrine to stimulate the production of cyclic AMP and activation of phosphorylase in response to increasing concentrations of epinephrine, there was no detectable change in phosphorylase kinase activity measured at pH 6.0, 6.8, or 8.2 (data not shown). The basal pH 6.8:8.2 ratio was high (0.34) compared to the ratios found in resting muscle (0.02) (38) and heart (0.00) (13). This ratio remained constant (0.33 to 0.94) despite incubation of adipocytes for 5 min in the presence of epinephrine at concentrations ranging from 0.01 to 100 μM. No changes in activity were observed at intermediate pH values nor at higher pH values up to 9.2. The maximal phosphorylase kinase activity in adipocytes (1.3 to 1.8 units per mg of protein) was obtained at pH 8.2, an optimum similar to that in muscle and heart.

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**Table I**

**Effects of Ca**²⁺** on concentration on cyclic AMP, total phosphorylase activity, and activatability of hormone-sensitive lipase by cyclic AMP-dependent protein kinase in adipocytes**

Fat cell suspensions were prepared in Ca**²⁺**-free Krebs-Ringer bicarbonate buffer. At the end of 5 min incubation aliquots of each cell suspension were processed for cyclic AMP, lipase activity, and phosphorylase assays as described under "Methods."

<table>
<thead>
<tr>
<th>Addition</th>
<th>Epinephrine</th>
<th>Cyclic AMP</th>
<th>Total phosphorylase activity</th>
<th>Lipase activatability by protein kinase in cell extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca<strong>²⁺</strong>, 1.3 mM</td>
<td>8.6</td>
<td>11.9</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>45.2</td>
<td>12.4</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>EGTA, 1 mM</td>
<td>57.2</td>
<td>11.4</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 3.** Effect of protein kinase inhibitor on the activation of hormone-sensitive lipase. The source of lipase was the 100,000 × g supernatant fraction. Lipase activation was carried out as described under "Methods" except that no exogenous protein kinase was added and the concentration of cyclic AMP was 1 μM. At various time intervals during the activation process, aliquots of the incubation mixture were removed and added to protein kinase inhibitor (PKI) at a final concentration of 8 μg per ml. The percentage activation represents the increase in lipase activity over that assayed in the absence of cyclic AMP.

**Fig. 4.** Effect of protein kinase inhibitor on phosphorylase activity measured as the activity ratio (−AMP: +AMP) (left) and total activity (in the presence of 2 mM AMP) (right). The 100,000 × g supernatant fraction was preincubated with the following cofactors for 0, 5, 10, and 20 min at 30° and subsequently assayed for phosphorylase activity as described under "Methods." The tubes contained: no additions (----); 3 mM Mg**²⁺**, 0.5 mM ATP, and 0.8 μg per ml of protein kinase inhibitor (PKI) (□ □); 3 mM Mg**²⁺**, 0.5 mM ATP, 1 μM cyclic AMP, and 1 μg per ml of skeletal muscle protein kinase (■ ■); 3 mM Mg**²⁺**, 0.5 mM ATP, and 1 μg per ml of purified activated skeletal muscle phosphorylase kinase (△ △). The phosphorylase kinase (1 μg per ml) was first converted to its activated form by incubation with MgATP, cyclic AMP, and protein kinase for 15 min at 30°. The enzyme was diluted 1,000-fold for use.
and blocked the increase in total phosphorylase activities (results not shown).

The effect of Ca\(^{2+}\) on the activity of adipocyte phosphorylase kinase was further evaluated in cell-free extracts. The effects of EGTA on the activation by MgATP of adipocyte phosphorylase, as well as skeletal muscle phosphorylase added to the adipocyte extracts, are described in Table II. Although no \(\text{Ca}^{2+}\) was added to the activation mixture, the ion was present in the final incubation mixtures at concentrations from 70 to 240 nM. The phosphorylase activity ratio increased from 0.12 to 0.67 in 15 min when 3 mM \(\text{Mg}^{2+}\) and 1 mM ATP were added, and total phosphorylase activity increased from 57 to 82 milliunits per mg of protein. Addition of EGTA (0.01 mM to 1 mM) had little or no effect on the activation of adipocyte phosphorylase expressed either in terms of activity ratio or total activity.

In contrast, when a 25-fold excess of purified skeletal muscle phosphorylase b was incubated for 10 min with the adipocyte extract in the presence of MgATP and various concentrations of EGTA, it was found that 1 mM EGTA inhibited the rise in phosphorylase activity ratio by 60%. Slight inhibition was noted at 0.01 mM EGTA (Table II). There was no change in total phosphorylase activity before and after activation nor in the presence of increasing concentrations of EGTA. Thus, \(\text{Ca}^{2+}\) did not seem to be required for the activation of adipocyte phosphorylase but enhanced the conversion of skeletal muscle phosphorylase b to a, presumably catalyzed by adipocyte phosphorylase kinase.

### Table II

**Effect of varying Ca\(^{2+}\) concentration by addition of EGTA on activation of adipocyte phosphorylase and skeletal muscle phosphorylase in adipocyte extracts**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Substrate</th>
<th>Adipocyte phosphorylase</th>
<th>Skeletal muscle phosphorylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in mM</td>
<td>AMP: + AMP</td>
<td>AMP: + AMP</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+}) (3 mM)</td>
<td>37</td>
<td>0.12 ± 0.02</td>
<td>2.7 ± 0.03</td>
</tr>
<tr>
<td>MgATP + 0.01 mM EGTA</td>
<td>85</td>
<td>0.88 ± 0.02</td>
<td>2.7 ± 0.12</td>
</tr>
<tr>
<td>MgATP + 0.1 mM EGTA</td>
<td>92</td>
<td>0.64 ± 0.02</td>
<td>3.3 ± 0.13</td>
</tr>
<tr>
<td>MgATP + 1 mM EGTA</td>
<td>75</td>
<td>0.67 ± 0.02</td>
<td>3.1 ± 0.10</td>
</tr>
</tbody>
</table>

*Inubation medium contained 70 to 240 \(\mu\)M \(\text{Ca}^{2+}\), determined by atomic absorption spectrophotometry.*

### Table III

**Effect of insulin and epinephrine on adipocyte phosphorylase, glycogen synthase, glycerol, and cyclic AMP concentration in absence and presence of 10 mM glucose**

Adipocyte suspensions were incubated with and without hormones for 5 min at 37\(^\circ\). Other conditions were as described for Figs. 1 and 2. Each value represents the mean (±SE) of four experiments.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phosphorylase activity ratio (AMP: + AMP)</th>
<th>Glycogen synthase activity ratio (glucose-6-P: + glucose-6-P)</th>
<th>Glycerol</th>
<th>Cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No glucose</td>
<td>10 mM glucose</td>
<td>No glucose</td>
<td>10 mM glucose</td>
</tr>
<tr>
<td>None</td>
<td>0.38 ± 0.03</td>
<td>0.10 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Epinephrine (0.5 (\mu)M)</td>
<td>0.57 ± 0.06</td>
<td>0.30 ± 0.06</td>
<td>0.15 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Epinephrine (0.5 (\mu)M) + insulin (0.4 (\mu)M)</td>
<td>0.46 ± 0.04</td>
<td>0.24 ± 0.04</td>
<td>0.17 ± 0.02</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Insulin (0.4 (\mu)M)</td>
<td>0.37 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>0.35 ± 0.01</td>
</tr>
</tbody>
</table>

*\(p < 0.05\)*  
*\(p < 0.01\)*  
*\(p < 0.001\)*  
*Significance of differences from results in absence of any additions ("None").*  
*Group means are not significantly different (\(p > 0.2\)). However when the data from individual experiments (using a single preparation of adipocytes) are subjected to paired comparisons, \(p < 0.001\).*
buffer containing epinephrine, hormone-sensitive lipase was converted to its activated form. However, when insulin was added to cells that had been first exposed to epinephrine for 5 min, the activated lipase rapidly reverted to its nonactivated form (Table IV). The activated form of phosphorylase, induced by epinephrine treatment, also reverted rapidly to its nonactivated state on exposure to insulin.

Comparison of Concentrations of Cyclic AMP, Phosphorylase Kinase, Phosphorylase, Phosphorylase Phosphatase, and Glycogen Synthase from Skeletal Muscle and Adipocytes—When compared on a total cell protein basis, skeletal muscle and adipocytes contain about the same concentration of glycogen but cyclic AMP was greater in adipocytes (Table V). The activities of phosphorylase kinase and phosphorylase per mg of protein are about 1% of those found in muscle. It is notable that the activity of phosphorylase phosphatase from adipocytes assayed with a high concentration of muscle phosphorylase (4 mg per ml) was very high in relation to the phosphorylase kinase activity and lOO-phosphorylase phosphatase from adipocytes assayed with a high concentration of glycogen but cyclic AMP was greater in adipocytes (Table V).

Table IV

Effect of insulin on epinephrine-induced activation of hormone-sensitive lipase and phosphorylase in intact adipocytes

The cells were incubated at 37°C for the times specified. When both hormones were used the cells were first exposed to epinephrine for 5 min, then insulin was added and the cells were then allowed to incubate for an additional 5 min. Adipocytes were processed and assayed for hormone-sensitive lipase and phosphorylase as described under "Methods."

<table>
<thead>
<tr>
<th>Addition</th>
<th>Time</th>
<th>Hormone-sensitive Lipase activation by protein kinase in cell extract (milliunits/mg)</th>
<th>Phosphorylase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>%</td>
<td>Total activity</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>mg-AMP: +AMP</td>
</tr>
<tr>
<td>Epinephrine (0.5 μM)</td>
<td>5</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>Epinephrine (0.5 μM)</td>
<td>10</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>Epinephrine (0.5 μM) + insulin (4 nm)</td>
<td>5 + 5</td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>Insulin (4 nm)</td>
<td>50</td>
<td>10</td>
<td>17</td>
</tr>
</tbody>
</table>

Table V

Comparison of skeletal muscle and adipocytes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rabbit muscle</th>
<th>Rat adipocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic AMP (pmole/mg)</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Phosphorylase kinase (units/mg)</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>Phosphorylase (units/mg)</td>
<td>1</td>
<td>0.013</td>
</tr>
<tr>
<td>Phosphorylase phosphatase (units/mg)</td>
<td>1</td>
<td>1-2</td>
</tr>
<tr>
<td>Glycogen synthase (units/mg)</td>
<td>0.01</td>
<td>0.032</td>
</tr>
<tr>
<td>Glycogen (μg/mg)</td>
<td>30-50</td>
<td>25-45</td>
</tr>
</tbody>
</table>

a In fat-free infranatant fraction expressed per mg of protein.
b Unit = converts 1 unit of phosphorylase b to a per min (61).
c Unit = forms 1 μmol of glucose-1-P per min (61).
d Unit = release 1 nmole of 32P from [32P]phosphorylase a per min (61).
e Unit = incorporates 1 μmol of glucose into glycogen from UDP-glucose per min (61).

The close correspondence between increases in cyclic AMP and increases in lipolysis, activation of phosphorylase, and deactivation of glycogen synthase (Figs. 1 and 2) can be interpreted as evidence for the dependence of these multiple events on cyclic AMP concentration in the cell. This is in harmony with the present view that cyclic AMP is the mediator of epinephrine action on these and other metabolic processes. Incubation in the presence of epinephrine was limited to 5 min, at which time cyclic AMP accumulation was maximal (40). This should have prevented interference with assessment of the hormone effect by the inhibitor of cyclic AMP formation in fat cells described by Ho and Sutherland (41) and by Manganiello et al. (40).

Adipocyte phosphorylase appears to be similar to the skeletal muscle enzyme in that it was stimulated by 5'-AMP and there was an increase in the activity ratio (-5'-AMP: +5'-AMP) subsequent to exposure to epinephrine. However, the total phosphorylase activity (measured in the presence of 5'-AMP) increased significantly in adipocytes (Fig. 2) while it increased only slightly in skeletal muscle. In the latter respect, adipocyte phosphorylase is more nearly similar to liver phosphorylase (42). On the other hand, the activated liver enzyme was only slightly stimulated by 5'-AMP while the activated adipocyte enzyme showed a greater response. Thus, the phosphorylase from adipocytes appears to be of a type intermediate between that in muscle and that in liver and is probably similar to the enzyme from spleen (43) and kidney (44).

The stimulation of lipolysis in the intact cells by increasing concentrations of epinephrine was correlated with a decrease in the activatability of hormone-sensitive lipase by protein kinase in cell extracts (Fig. 1), indicating the conversion of the nonactivated form of lipase to its activated form. Further study of the mechanism of lipase activation in cell-free extracts showed that protein kinase inhibitor not only prevented lipase activation but also blocked the activation process immediately when added at various time intervals during the course of activation. Previous studies have shown that activation can be effected equally well in crude extracts and in purified preparations of hormone-sensitive lipase (6, 45). It seems unlikely that a second enzyme required for activation is present in all of these preparations. Thus the lipase activation system is similar to the deactivation system for glycogen synthase in that protein kinase appears to work directly on the operative enzyme.

In contrast, protein kinase inhibitor had no effect on the phosphorylase activation system (Fig. 4, a and b). This implies that phosphorylase in adipose tissue, as in other tissues (11-13), is activated through an intermediate enzyme, phosphorylase kinase, and that this enzyme, once activated by cyclic AMP and protein kinase, is not blocked by protein kinase inhibitor.

The nonactivated form of skeletal muscle phosphorylase kinase has relatively little activity at or below pH 6.8 but its activity approaches that of the activated form at pH 8.2. The activated form has 25 to 30 times more activity than the nonactivated form at pH 6.8 and its activity is increased 2-fold at pH 8.2 (16). Efforts to demonstrate activation of adipocyte phosphorylase kinase measured over a range of pH values, either after incubation of cells with epinephrine or after incubation of extracts with cyclic AMP, MgATP, and protein kinase were unsuccessful. This failure to distinguish between the activated and nonacti-
vated forms could be because skeletal muscle phosphorylase \( b \) was not a suitable substrate for adipocyte phosphorylase kinase. However, as shown in Fig. 4, a and b, even when endogenous phosphorylase was used as substrate, there was no apparent protein kinase-dependent activation. To test whether an increase in phosphorylase kinase activity would indeed have been detected under the conditions used, exogenous phosphorylase kinase (activated form) was added. This increased the rate of phosphorylase activation, indicating that the test system was adequate to show appreciable phosphorylase kinase activation had it occurred.

One might postulate that adipocyte phosphorylase kinase is always in its activated form. However, it is difficult to explain the observation that phosphorylase remains nonactivated in intact fat cells in the absence of epinephrine. A second possibility is that the enzyme was artifically activated in the course of preparation of cell extracts. However, none of our attempts to modify this procedure changed the pH 6.8:8.2 activity ratio or the levels of enzyme activity assayed at either pH. Another possibility is that the criterion used to differentiate the activated form of phosphorylase kinase from the nonactivated form in muscle and heart cannot be applied to adipocyte phosphorylase kinase. Krebs (1) reported that it had not been possible to detect a form of liver phosphorylase kinase having a low ratio of activity at pH 6.8 to activity at pH 8.2. Activation of liver phosphorylase kinase by MgATP and cyclic AMP has not been described.

It is now well established that the catalytic activity of phosphorylase kinase in muscle and heart requires Ca\(^{2+}\) (14–16). Ozawa et al. (15) found that skeletal muscle phosphorylase kinase had very high affinity for free Ca\(^{2+}\) (more than half of the maximum activation could be demonstrated at 0.1 \( \mu \)M) and that the effect of Ca\(^{2+}\) was reversible. Nammi et al. (14) found that removal of Ca\(^{2+}\) from the medium perfusing isolated rat hearts blocked the conversion of phosphorylase b to a while the formation of cyclic AMP and activation of phosphorylase kinase were not attenuated. Adipocyte phosphorylase kinase appears to be quite different from the enzyme in these contractile tissues, in that no Ca\(^{2+}\) requirement could be demonstrated for activation of endogenous phosphorylase. However, the rate of activation of skeletal muscle phosphorylase b by adipocyte phosphorylase kinase was reduced 60% by addition of 1 mM EGTA. Krebs et al. (16) reported that EGTA (0.10 mM) inhibited skeletal muscle phosphorylase kinase by 75% and that the inhibition could be reversed by adding back Ca\(^{2+}\). Thus, while some dependence is demonstrable with exogenous substrate it seems clear that phosphorylase kinase in adipocytes does not require Ca\(^{2+}\). In this way fat cells are similar to liver. Exton et al. (16) found that omission of Ca\(^{2+}\) from perfusion medium did not alter the control of cyclic AMP-stimulated glycerogenolysis and gluconeogenesis in rat liver.

It has been widely held that the antilipolytic action of insulin is secondary to a decrease in intracellular cyclic AMP concentration. It was first demonstrated by Butcher et al. (47, 48) that in the presence of caffeine insulin lowered cyclic AMP concentration in epinephrine-treated cells. However, the effects of insulin on lipolysis were not correlated with its effects on cyclic AMP concentration. Jaret et al. (49) recently showed that insulin could inhibit epinephrine- or adrenocorticotropin hormone-induced lipolysis without effect on the elevated cyclic AMP concentrations. Illiano and Cuatrecasas (50) reported that insulin inhibited the epinephrine-induced activation of adenylate cyclase of liver and fat cell membrane only at low concentrations (0.04 to 0.8 nM) but not at unphysiologically high (>1 nM) concentrations. Contrary to these findings, several other investigators (51–53) have been unable to demonstrate an inhibitory effect of insulin on adenylate cyclase. Kone (54) reported that insulin at a very high concentration actually potentiated the stimulation of lipolysis induced by epinephrine. The concentration of insulin used in the present studies was usually limited to 0.4 nM (50 microunits per ml) to maximize the antilipolytic effect and the effects of adenylate cyclase.

Insulin (0.4 nM) was strongly antilipolytic both in the presence and in the absence of glucose. Cyclic AMP levels in the presence of epinephrine were significantly decreased (\( p < 0.001 \) based on paired comparisons) but not at all in proportion to the reduction in lipolysis (Table III). Thus, although concerted increases in cyclic AMP concentration and glycerol production in response to epinephrine were demonstrated (Fig. 1), the correlation between cyclic AMP and glycerol was poor after insulin treatment.

It has been shown that insulin arrests glycerol production within 1 to 2 min after an epinephrine-stimulated rate of glycerol release has been established (40). This antilipolytic action of insulin could be due to interference with the activity of protein kinase in the presence of a given level of cyclic AMP or to the activation or unmasking of a lipase phosphatase. In either case, the lipase might be expected to revert to its nonactivated form. Another possibility is that insulin leads to the production of an inhibitor acting on hormone-sensitive lipase directly. In this latter case insulin need not cause reversion of the enzyme to its nonactivated form. Our studies on the state of activation of lipase in cells treated with epinephrine and then briefly with insulin appear to rule against the latter possibility (Table IV). Insulin also caused rapid deactivation of phosphorylase in adipocytes, in agreement with the findings of Jungs with epididymal fat pads (8).

Insulin increased glycogen synthase I activity in adipocytes without significantly lowering the basal cyclic AMP levels. Similar results were obtained in muscle by Craig et al. (55) and Goldberg et al. (56). Insulin increased glycogen synthase I activity of rat diaphragm in the presence or absence of epinephrine without lowering the basal or epinephrine-elevated level of cyclic AMP.

The presence of glucose alone in the medium increased glycogen synthase I activity and decreased phosphorylase activity ratios. The finding of Hers et al. (57) may be relevant in that glucose caused activation of synthase phosphatase in liver. Glucose also has been reported to activate phosphorylase phosphatase in isolated rat diaphragm as described by Holmes and Mansour (58). However, the recent findings of Barry et al. (59) and Bailey et al. (60) suggest that the glucose effect on phosphorylase phosphatase activity may be mediated through conformational changes in the substrate, phosphorylase. Glucose favors the dissociation of the tetrameric form of phosphorylase a to the dimeric form (60) which is a better substrate for the phosphatase.

The physiological role of glycogenolysis in relation to lipolysis remains to be explored, particularly as to whether the parallel increases in lipolysis and glycogenolysis are obligatorily linked.

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Hormonal Regulation of Adipocyte Enzymes: THE EFFECTS OF EPINEPHRINE AND INSULIN ON THE CONTROL OF LIPASE, PHOSPHORYLASE KINASE, PHOSPHORYLASE, AND GLYCOGEN SYNT HASE

John C. Khoo, Daniel Steinberg, Barbara Thompson and Steven E. Mayer


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