Insulin-dependent and Insulin-independent Low $K_m$ Cyclic AMP Phosphodiesterase from Rat Adipose Tissue*

(Received for publication, December 21, 1981)
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Chromatographic analysis of a soluble extract of rat adipose tissue on DEAE-Sephacel resolves four distinct peaks of 3':5'-cyclic-nucleotide phosphodiesterase (EC 3.1.4.17) activity. Kinetic investigation indicates that two of these fractions have a high affinity for cyclic AMP and show negative cooperative kinetic behavior at high substrate concentration. They differ in the degree of inhibition by cyclic GMP and in their response to insulin. If rat epididymal fat pads are incubated with insulin prior to homogenization, only one of the low $K_m$ cyclic AMP phosphodiesterase forms is stimulated.

In 1965, Butcher, Sneyd, Park, and Sutherland (1) first suggested that some of the actions of insulin might be mediated by a decrease in intracellular cAMP levels. Many investigators have, since then, examined the effects of insulin on cyclic nucleotide phosphodiesterases, the only enzymes that break down cyclic nucleotides in mammalian cells. Loten and Sneyd (2) demonstrated that homogenates of insulin-tREATED adipocytes have increased cAMP phosphodiesterase activity. Although many investigators have observed increased high affinity or "low $K_m$" cAMP phosphodiesterase activity after the treatment of intact fat cells with insulin (3-7), the specific form of the enzyme which responds to the hormone has not been resolved chromatographically from other phosphodiesterase activities.

We observe that after treatment of rat epididymal fat pads with insulin in vitro, there is an increase in cAMP phosphodiesterase activity, measured at low cAMP substrate concentration (0.1 µM), in the soluble extract. Ion exchange chromatography resolves four distinct cyclic nucleotide phosphodiesterase activities from this preparation. Although two of these activities are very similar high affinity cAMP phosphodiesterases, we are able to distinguish between them because they are differentially inhibited by the presence of a 10-fold excess of cGMP in the assay for cAMP hydrolysis. Furthermore, only one of these phosphodiesterase activities is responsive to insulin treatment. After in vitro insulin treatment of the rat epididymal fat pads, we are able to chromatographically resolve in the soluble extract a low $K_m$ cAMP phosphodiesterase which is stimulated and a low $K_m$ cAMP phosphodiesterase which is unaffected by the insulin treatment.

*This work was supported by National Institutes of Health Grants AM 20978 and AM 16367 and by the American Diabetes Association, Southern California Affiliate, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS

Epididymal adipose tissue was removed from 200-300-g Sprague Dawley rats. One pad from each rat was used as a control, while the other was used as the insulin-treated experimental tissue. The minced tissue was first incubated at 37 °C under an O$_2$ atmosphere in Krebs-Ringer-Hepes buffer (pH 7.4) which contained 1 mM glucose, 1 mg/ml of bovine serum albumin (Fraction V, Sigma) and 50 mM benzamidine. The incubation was then continued for 10 min more with or without 1 millimolar/ml of insulin (glucagon-low bovine insulin lot #615-70N-80 was kindly supplied by Eli Lilly). Experiments also were performed on isolated adipocytes prepared by the collagenase method of Rodbell (8), washed, and then resuspended in Krebs-Ringer-Hepes buffer as above but containing 10 mg/ml of albumin instead of 1 mg/ml. The subsequent incubation procedures were the same as those used for epididymal fat pads.

The tissue or cells were then homogenized in the incubation buffer with a Brinkman polytron homogenizer using two 10-s bursts at full speed. The homogenate was chilled and centrifuged at 3,000 g for 10 min. The infranatant was removed from under the fat cake and centrifuged for 30 min at 105,000 × g. The supernatant fraction contained more than 80% of the cAMP phosphodiesterase activity measured at 0.1 µM substrate concentration and more than 95% of the cGMP phosphodiesterase activity measured at 1.0 µM substrate concentration. The resulting fat pad extract was diluted with 0.05 M sodium acetate buffer, pH 6.4, (containing 5 mM MgCl$_2$ and 5 mM β-mercaptoethanol) and applied to a DEAE-Sephacel chromatography column (1 × 15 cm) which had been equilibrated with the sodium acetate buffer. After washing with 0.05 M sodium acetate buffer, a 100-ml gradient of 0.15 to 1.2 M sodium acetate was initiated.

Two-ml fractions were collected and assayed for cAMP and cGMP phosphodiesterase activity according to the two-step assay procedure of Thompson and Appleman (9). As the first step of the assay, [3H]cAMP is converted by phosphodiesterase to [3H]AMP which is subsequently converted to [3H]adenosine by the added nucleotidase in the second step. In the first step, the 0.4-ml assay contained 50 mM Tris (pH 7.4), 5 mM MgCl$_2$, 0.1 µM cAMP or 1.0 µM cGMP, 120,000 cpm of [3H]cAMP or [3H]GMP, respectively, and 0.05 ml of the fractions to be assayed. After incubation at 30 °C for 20 min, the reaction was terminated by boiling for 2 min. The second step was completed by the addition of 0.1 ml of snake venom solution (1 mg/ml of Crotaulus atrox, Sigma), incubation for 10 min at 30 °C, and removal of residual substrate with 1 ml of 50% slurry of anion exchange resin, BioRad AGX2 (chloride form in 50% ethanol). Aliquots of the supernatant were counted by liquid scintillation. Product recoveries were calculated using [3H]adenosine. Phosphodiesterase activity units are in picomoles hydrolyzed/min; the relatively high cyclic GMP phosphodiesterase activities reflect the 16-fold higher substrate concentration than is used in the assay for cyclic AMP phosphodiesterase.

RESULTS

DEAE-Sepacel column chromatography of a rat epididymal fat pad extract at pH 6.4 resolves four distinct peaks of cyclic nucleotide phosphodiesterase activity (Fig. 1). These phosphodiesterase activities elute from the ion exchange column at 0.17 M, 0.35 M, 0.6 M, and 0.9 M sodium acetate concentrations. The first peak is minor and only exhibits cGMP hydrolytic activity. The second form of phosphodiesterase is of low specificity and low affinity, hydrolyzing both cGMP and cAMP. The cGMP phosphodiesterase activities of both peaks are Ca$^{2+}$-calmodulin sensitive. The third form is a high affinity cAMP phosphodiesterase which exhibits negatively cooperative kinetic behavior and is also a high affinity cAMP phosphodiesterase (app $K_m = 0.2$ µM and app $K_m = 7.3$ µM).

In the presence of a 10-fold excess of cGMP, the hydrolysis
of cAMP by the separated phosphodiesterases is differentially affected (Fig. 2); while cAMP phosphodiesterase activity of the second peak is stimulated 2-fold, that of the third peak is reduced by half and that of the fourth peak is totally inhibited. The two low Km cAMP phosphodiesterase activities (the third and fourth peaks), while very similar in substrate affinity, are inherently different in their ability to be inhibited by cGMP. The possibility of an artifact due to partial contamination of the third peak by the cyclic nucleotide phosphodiesterase (second peak) is eliminated since cGMP inhibits at a constant percentage across the entire third peak. There are previous reports of this differential effect of cGMP on the cAMP hydrolytic ability of phosphodiesterases of rat liver (10, 11).

Table I summarizes the kinetic and regulatory properties displayed by the various peaks of cyclic nucleotide phosphodiesterase activity.

In an effort to delineate a specific enzyme target of insulin action, epididymal fat pads which had been incubated for 10 min with 1 miliunit/ml of insulin were compared with paired control pads which had been incubated in the Krebs-Ringer-Hepes buffer alone. The preparations were homogenized and centrifuged as described under "Materials and Methods." The resulting supernatant and high speed pellet fractions were assayed for cAMP phosphodiesterase activity with 0.1 μM cAMP as substrate. Table II gives the results of two such experiments. The supernatants from the pooled fat pads of the insulin-treated animals showed greater cAMP phosphodiesterase activity than those of control animals. Similar results were seen when isolated adipocytes were incubated with insulin.

Table II

Effects of insulin treatment of epididymal fat pads on cyclic AMP phosphodiesterase activity

<table>
<thead>
<tr>
<th></th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>units/g of tissue*</td>
<td></td>
<td></td>
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<tr>
<td>1st Experiment (10 rats, 13.2 g tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>259</td>
<td>36</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>319</td>
<td>41</td>
</tr>
<tr>
<td>2nd Experiment (5 rats, 8.4 g tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>236.5</td>
<td>25.8</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>327.0</td>
<td>29.7</td>
</tr>
</tbody>
</table>

*An accurate determination of the endogenous protein is not possible because bovine serum albumin is present throughout the experiment.
Insulin-stimulated Cyclic AMP Phosphodiesterase

**DISCUSSION**

The homogenization methods utilized in this investigation produce an adipose tissue extract containing greater than 85% of the cyclic AMP phosphodiesterase activity (measured at 0.1 μM substrate) in a soluble form. The addition of the protease inhibitor, benzamidine, to the Krebs-Ringer homogenization buffer appears not only to stabilize all soluble phosphodiesterase activities but also to stabilize the observed insulin stimulation of the phosphodiesterase activity through subsequent analysis. Loten et al. (12) found that insulin-stimulated cAMP phosphodiesterase activity could not be released from a liver particulate fraction by hypotonic buffer if benzamidine was present. Further analysis will be necessary to determine if the apparent activation of a single fraction of phosphodiesterase by both Ca²⁺-calmodulin and cGMP represents cochromatography of two enzyme forms or a single enzyme responding to bifunctional regulation.

Ion exchange chromatography clearly shows that only one form of the cAMP phosphodiesterase is increased by treatment of rat epididymal tissue with insulin. In the chromatographic profiles of the supernatants from a number of untreated and treated tissues, we have found the amount of phosphodiesterase activity in the second peak to be constant. If the activity of this second peak is normalized to 1, the ratios of the activities of the three major cAMP phosphodiesterases may be expressed as 1/2.5/2.6; for insulin-treated tissues the ratios are 1/2.6/6.0. This indicates that the phosphodiesterase activity of the second and third peaks is unaffected by insulin treatment, while the activity of the fourth peak is increased more than 100%.

Recently, Marchmont and Houslay (13) reported that insulin treatment of liver plasma membranes causes an increase in low Kᵦ, cAMP phosphodiesterase through protein phosphorylation. In the liver system, elevated cAMP levels are required in order to observe insulin action. The intact adipose cell seems to be more sensitive since insulin action can be observed without prior elevation of the cAMP levels. Although the elution of the insulin-stimulated cyclic AMP phosphodiesterase at high salt concentrations from the anion exchange chromatographic system would be consistent with it being a phosphorylated form of the enzyme, we have no evidence for this mechanism of regulation in adipose tissue.

Our results indicate that three different forms of enzyme can contribute to the cAMP phosphodiesterase activity even when the soluble adipose tissue extract is assayed at low substrate concentration. Comparison of the chromatographic activity profiles derived from the soluble fraction of untreated and insulin-treated epididymal fat pads reveals two forms of low Kᵦ, cAMP phosphodiesterase activity, an insulin-independent and an insulin-stimulated enzyme. It is possible, therefore, to discriminate between the two low Kᵦ, cAMP phosphodiesterases on the basis of 1) salt concentration needed for elution from the ion exchange column, 2) effect of cGMP on their cAMP hydrolytic ability, and 3) response of their activity to prior in vitro insulin treatment of the epididymal fat pads.

**REFERENCES**


Fig. 3. The effect of insulin treatment of rat epididymal tissue on phosphodiesterase activity. Extraction preparation, DEAE-Sephacel chromatography, and enzyme assays as in Fig. 1. A, control tissue. B, tissue treated with 1 milliunit/ml of insulin for 10 min prior to homogenization. Recovery of activity from the control and experimental columns were equal. † — o, cAMP hydrolysis (--1 M); o — o, cGMP hydrolysis (-1 M).
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