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Putative mediators of insulin action: Regulation of pyruvate dehydrogenase and adenylate cyclase activities

(liver plasma membranes/adipocytes/biphasic dose–response/hormone receptors/protein phosphorylation)

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ABSTRACT **Recent evidence suggests that certain actions of insulin may be mediated by the selective generation of chemically undefined intracellular substances. Incubation of rat liver particulate fraction with low concentrations of insulin enhances the release into the supernatant of a substance that stimulates mitochondrial pyruvate dehydrogenase. Higher concentrations of insulin release less stimulating activity. It is possible to resolve activities that stimulate and inhibit pyruvate dehydrogenase by differential ethanol extraction of the supernatant solutions. The elaboration of both factors is dependent upon the presence of insulin in a dose-dependent manner. Moreover, fractions that contain the pyruvate dehydrogenase-inhibiting activity also inhibit adipocyte basal and hormonally stimulated adenylate cyclase. The production of this adenylate cyclase inhibitory activity is also stimulated by insulin. Cyclase inhibition is virtually abolished when the nonhydrolyzable ATP analog, 5′-adenylyl imidodiphosphate, is included in the assay. These results indicate that the bimodal effects of insulin on certain functions may be ascribed to the generation of at least two distinct chemical substances that show opposing activities, which may operate by regulating phosphorylation reactions.**

Our understanding of the molecular events involved in the physiological actions of insulin remains incomplete. The binding of insulin to its receptor on the target cell surface, and the modulation by insulin of a number of intracellular enzymes, have been studied extensively (1, 2). Several of these enzymes, such as glycogen synthetase (3), pyruvate dehydrogenase complex (PDHase; EC 1.2.2.2 and 1.2.4.1) (4), and pyruvate kinase (5), appear to be regulated by phosphorylation-dephosphorylation reactions. Similar processes have been postulated for Ca^{2+}, Mg^{2+}-ATPase (6), cyclic AMP phosphodiesterase (7), and adenylate cyclase (EC 4.6.1.1) (8, 9). It recently has been shown that exposure of plasma membranes from rat adipocytes (10, 11), skeletal muscle (3, 12), and liver (13) to insulin results in the release of one or more low-molecular-weight substances, which in turn can affect certain insulin-sensitive enzymes in broken cell preparations. It has been proposed that these chemical mediators act on PDHase (14) and glycogen synthetase (3) by controlling their state of phosphorylation.

Because insulin-sensitive target tissues are known to be under the regulatory influence of cyclic nucleotides, the effects of insulin on the levels of cyclic AMP have been examined extensively. Early reports (15, 16) demonstrated that insulin inhibited the catecholamine- or glucagon-stimulated rise in cellular cyclic AMP. It was later shown in intact fat and liver cells that insulin could activate the high-affinity form of cyclic AMP phosphodiesterase (17). Furthermore, other evidence indicated that insulin could directly inhibit the adenylate cyclase activity of isolated membranes from adipocytes (18), liver cells (19, 20), and fibroblasts (21). Although the changes in cyclic nucleotide levels probably cannot account for all of the numerous actions of insulin (22), it was of interest to determine whether the mediators regulating PDHase also might modulate adenylate cyclase activity. Here we report the separation of two chemical mediators of insulin action. One of these stimulates PDHase while the other inhibits both PDHase and adenylate cyclase activities. Both factors are released from isolated liver plasma membranes by physiological concentrations of insulin. Preliminary studies suggest that a mechanism of phosphorylation-dephosphorylation may be involved in the inhibition of adenylate cyclase.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (100–125 g) were used. Most reagents were from Sigma, except for collagenase and coenzyme A (P-L Biochemicals), [1-^{14}C]pyruvic acid (Amersham), porcine insulin (Eli Lilly), pyruvate kinase (Boehringer Mannheim), and [α-^{32}P]ATP and cyclic [2,8-^{3}H]AMP (New England Nuclear).

Generation of Insulin Mediators. These were prepared by a modification of the method as described (13). Rat livers were washed in 10 mM potassium phosphate buffer (pH 7.4) and homogenized by using a Teflon/glass homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 30,000 × *g* for 25 min. The pellet was rehomogenized and sedimented at 1,100 × *g* for 10 min. The supernatant was centrifuged again at 30,000 × *g* for 25 min. The pellet was resuspended in the same buffer (2–4 mg of protein per ml, referred to as "particulate fraction") and incubated with insulin for designated times at 37°C. An equal volume of 0.2 M acetate buffer, pH 3.75/0.1 mM dithiothreitol/0.1 mM EDTA was then added. After centrifugation at 30,000 × *g* for 15 min, the aspirated supernatant was treated with activated charcoal for 10 min at 4°C. Charcoal was removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant was lyophilized. The stimulatory and inhibitory factors were separated by extracting the lyophilized powder twice with 100% ethanol. The powder was suspended in ethanol, sonicated for 5 min, and centrifuged at 500 × *g* for 20 min. The ethanol supernatants from two such procedures were pooled and centrifuged again before drying under N_2 and suspending in 1 mM formic acid. The nonextractable residue, which contained the stimulatory activity, was suspended in 1 mM formic acid and centrifuged at 10,000 × *g* for 10 min. Neither the stimulatory or inhibitory activities were destroyed when the extracted supernatants were boiled for 5 min.

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Abbreviations: PDHase, pyruvate dehydrogenase; EC_{50}, half-maximal effective concentration; p[NH]ppA, 5′-adenylyl imidodiphosphate.

Assay of PDHase. This activity was assayed by the conversion of [1-^{14}C]pyruvic acid to $^{14}CO_2$ (13) by rat liver mitochondria that had been stripped of their outer membrane (23). Rat livers were washed extensively in 20 mM potassium phosphate buffer (pH 7.4), trimmed of fat, chopped into small pieces, homogenized in 10 vol of buffer A (0.21 M mannitol/0.075 M sucrose/1 mM Tris/0.1 mM EDTA, pH 7.2) and centrifuged at 500 × *g* for 10 min. The supernatant was centrifuged at 9,000 × *g* for 10 min. The pellet was homogenized in an equal volume of buffer A, and the two centrifugation steps were repeated. The fluffy layer above the pellet was discarded, and the pellet was homogenized in buffer A and centrifuged at 9,000 × *g* for 10 min. The pellet consisted of whole mitochondria whose PDHase activity was sensitive to the extracted mediators. To enhance enzyme sensitivity, the outer mitochondrial membranes were detached by swelling in 10 vol of 20 mM potassium phosphate/0.02% bovine serum albumin, pH 7.2 (buffer B). Buffer B was added gradually over 45 min at 4°C, and the pellets were gently agitated at 5-min intervals. The suspension was centrifuged (40,000 × *g* for 20 min) to shear the outer membranes, and the pellets were suspended in buffer B. This mixture of inner and outer mitochondrial membranes was separated by centrifugation at 1,900 × *g* for 15 min. The resulting pellet, containing inner mitochondrial ghosts (mitoplasts) highly enriched in PDHase activity, could be frozen for up to 2 wk with no significant loss of enzyme activity.

For measurements of the PDHase-stimulating activity, mitoplasts were preincubated with 0.2 mM ATP for 10 min in the presence of 50 μM $CaCl_2$/50 μM $MgCl_2$ to convert the enzyme to its inactive, phosphorylated form (14). After this incubation, the solutions were cooled to 4°C and washed free of unbound nucleotide by centrifugation and resuspension in 10 mM potassium phosphate (pH 8.0). The inhibitor was assayed with mitoplasts not preincubated with ATP; thus, the "basal" state was that of the active, dephosphorylated form. The inhibitory activity was detected best when 0.2 mM ATP and 0.2 mM $MgCl_2$ were included in the assay.

Subfractionation of Adipocytes. Highly purified plasma membranes were prepared from adipocytes (24) by a modification of the method of Torres *et al.* (18). Packed adipocytes were diluted 1:10 in 1 mM sodium bicarbonate (pH 7.5) and homogenized with 10 strokes in a Teflon/glass homogenizer. After centrifugation at 36,000 × *g* for 10 min, the pellet was rehomogenized and purified on discontinuous sucrose gradients containing 1.2 M, 0.86 M, 0.52 M, and 0.26 M sucrose. An aliquot (0.8 ml) of each solution was layered in polyallomer tubes (11 × 60 mm) and allowed to equilibrate for 1 hr. Aliquots (0.6 ml) of the membrane suspension were centrifuged at 58,000 rpm for 15 min in a SW 60 rotor. The material at the interface of the 1.2 and 0.86 M sucrose fractions was diluted 1:10 in 50 mM Tris·HCl/1 mM dithiothreitol, pH 7.6. The suspension, after centrifugation (36,000 × *g* for 10 min) and resuspension, contained highly enriched adenylate cyclase activity that was sensitive to inhibition by insulin if prepared in the absence of dithiothreitol (18).

Adenylate Cyclase Activity. This was assayed at 30°C as described (18). Unless specified otherwise, reactions contained 5 mM phospho*enol*pyruvate, 5 units of pyruvate kinase per ml, 0.2 mM 3-isobutyl-1-methylxanthine, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.10 mM ATP, 1–3 × 10^6 cpm [α-^{32}P]ATP, and 1–2 × 10^5 cpm of cyclic [2,8-^{3}H]AMP (50 nM). Reactions were started by the addition of membranes (15–20 μg of protein) to prewarmed reaction mixtures and were terminated after 5 min by immersion in a dry ice/acetone bath. Cyclic AMP was separated as described by Salomon *et al.* (25). Results are expressed as the means of triplicate determinations. In general, variability was less than 5%.

RESULTS

Separation of the Stimulatory and Inhibitory Activities of PDHase. We have demonstrated (13) that both rat liver and adipocyte mitochondrial PDHase activities are sensitive to material released from liver particulate fractions incubated with insulin. Addition of the released material to mitochondria resulted in a biphasic dose–response curve for PDHase; the activity increased in proportion to the concentration of the crude extract but then declined at high concentrations, suggesting the existence of two opposing mediating activities. To separate these two putative antagonistic activities, the lyophilized supernatant of material released from liver particulate fractions was extracted with 100% ethanol (Fig. 1). As described earlier for adipocyte mitochondria, the crude extracts produced a biphasic dose–response curve in liver mitoplasts (Fig. 1*A*). However, the ethanol-extracted residue caused only a dose-dependent stimulatory effect on the PDHase activity of mitoplasts deactivated by pretreatment with 0.2 mM ATP; no inhibitory phase was detected (Fig. 1*B*). In contrast, the ethanol-extractable material caused a dose-dependent decrease in PDHase activity (Fig. 1*C*). This inhibition was most pronounced when ATP was present in the assay medium. Thus, the crude material released from liver membranes contains both inhibitory and

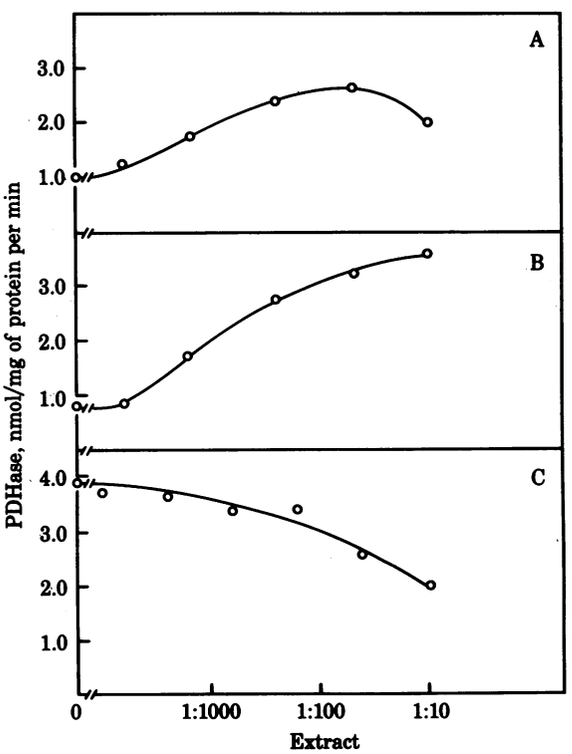

FIG. 1. Separation of a stimulator and inhibitor of PDHase. Liver particulate fractions were incubated with 2 nM insulin for 15 min at 37°C. After centrifugation the supernatant was acidified with acetate buffer, absorbed to charcoal, and lyophilized. (*A*) The crude extract, suspended in 1 mM formic acid, was added to preparations of liver mitoplasts; prior to assay, mitoplasts were incubated with 0.2 mM ATP. (*B*) The crude extract was further extracted with 100% ethanol; the ethanol residue was suspended in 1 mM formic acid and added to mitoplasts pretreated with ATP as in *A*. (*C*) The ethanol extract was dried under N_2, dissolved in 1 mM formic acid, and added to untreated mitoplasts. In *A* and *B*, PDHase was assayed in the absence of ATP; in *C*, ATP was added into the reaction mixture. Results are the means of triplicate determinations.

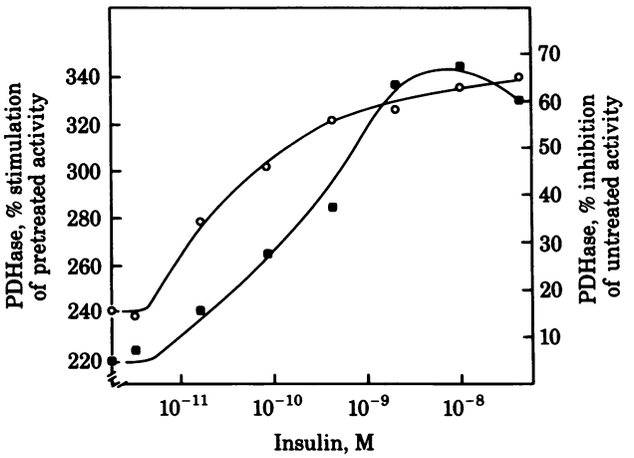

FIG. 2. Effect of varying the concentrations of insulin on the generation of stimulatory and inhibitory activities of PDHase. Liver particulate fractions were incubated with the indicated concentration of insulin for 5 min at 37°C. The supernatants were extracted, and the inhibitory and stimulatory fractions were separated by ethanol extraction. The ethanol residue (○) was suspended in 1 mM formic acid and added at a final dilution of 1:10 to mitoplasts pretreated with ATP. The ethanol extract (■) was dried under N_2, dissolved in 1 mM formic acid, and added at a final dilution of 1:10 to untreated mitoplasts. Results are expressed as percent stimulation (○) or inhibition (■) of control activity.

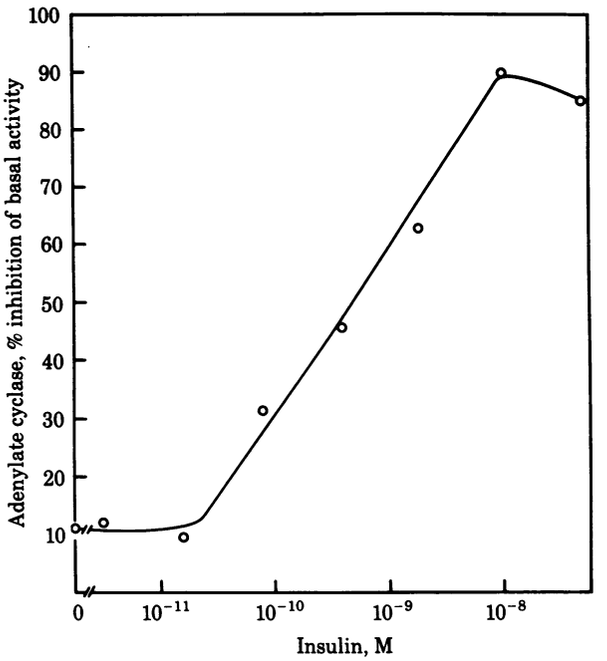

FIG. 3. Effect of varying the concentration of insulin on the generation of an inhibitor of adenylate cyclase. Liver particulates were incubated with varying concentrations of insulin, as described in Fig. 2. The ethanol-extracted material was added at a final dilution of 1:10 to purified plasma membranes from adipocytes, and the adenylate cyclase activity was assayed for 5 min at 30°C. Results are the means of triplicate determinations, expressed as percentage inhibition of control activity.

stimulatory components that are easily separable by alcohol extraction.

Incubation of rat liver particulate fractions with increasing concentrations of insulin resulted in a dose-dependent generation of both the stimulatory and inhibitory factors (Fig. 2). The concentration of insulin required for the half-maximal production of stimulatory activity (EC_{50}) was about 0.03 nM, whereas that for the inhibitory activity was about 0.18 nM. The apparent biphasic nature of the insulin dose–response curves of crude extracts may be due in part to this differential sensitivity to insulin (13).

Inhibition of Adenylate Cyclase Activity. The material not extractable by ethanol had no effect on the adenylate cyclase activity of purified adipocyte membranes (data not shown); it is difficult to exclude the possibility that a stimulatory activity in this fraction was masked by other inhibitory agents. On the other hand, the ethanol-extracted material markedly inhibited the enzyme (Table 1). The basal and isoproterenol-stimulated activities are decreased in the presence of this extract by 60–70%. However, fluoride-stimulated activity, was inhibited by only 30%. As described (26), GTP inhibited adipocyte adenylate cyclase with a maximal effect at 1–2 μM. In the presence of 0.5 μM or 10 μM GTP, the ethanol-extractable material further inhibited the activity (Table 1). In the presence of isoproterenol, the GTP dose–response curve for adipocyte cyclase is biphasic (27). The ethanol extract can depress the activity of adenylate cyclase in the presence of either stimulatory (0.5 μM) or inhibitory (10 μM) concentrations of GTP (Table 1).

Effect of Insulin on the Generation of an Inhibitor of Adenylate Cyclase. Insulin produced a dose-dependent increase in the ethanol-extractable inhibitory activity released from liver particulate fractions (Fig. 3). Half-maximal stimulation of the production of adenylate cyclase inhibitory activity occurred with 0.4 nM insulin. The material extracted from liver particulate material in the absence of insulin produced some (<15%) inhibition of cyclase activity, but no activity was detected in parallel extractions with equal volumes of buffer in the absence of liver particulates. Under the conditions used, insulin did not inhibit cyclase activity when added directly to the assay of the adipocyte membranes (which had been prepared in the presence of 1 mM dithiothreitol).

ATP Requirement for the Action of the Adenylate Cyclase Inhibitory Activity. It has been suggested that adenylate cyclase may be inhibited by phosphorylation of the enzyme (8, 9). To determine whether phosphorylation might be involved in the

Table 1. Effect of liver-derived ethanol extract on adipocyte adenylate cyclase activity

	Adenylate cyclase activity						
Preparation	No additions	10 mM NaF	10 μM iPT	0.5 μM GTP	10 μM GTP	0.5 μM GTP + 10 μM iPT	10 μM GTP + 10 μM iPT
Control	344.2 ± 12.7	660.4 ± 36.3	410.6 ± 8.2	134.5 ± 1.8	29.8 ± 2.9	480.8 ± 33.6	236.1 ± 9.4
Ethanol extract	112.9 ± 1.8	444.8 ± 9.9	153.9 ± 15.1	51.3 ± 7.9	14.7 ± 1.3	133.4 ± 13.3	91.0 ± 6.5

Liver particulate fractions were treated with 2 nM insulin for 15 min at 37°C, and the resulting supernatant was lyophilized, extracted with ethanol, dried under N_2, and dissolved in 1 mM formic acid. Adenylate cyclase activity was assayed in 0.1 ml for 5 min at 30°C in the presence of the designated additions. To each assay was added 10 μl of 1 mM formic acid (control) or 10 μl of the ethanol-extracted material. Results are the means of triplicate determinations, expressed as pmol of cyclic AMP per mg of protein per min ± SD. iPT, *l*-Isoproterenol.

inhibitory activity studied here, the effects of ATP and the nonhydrolyzable substrate analog 5'-adenylyl imidodiphosphate (p[NH]ppA) were studied (Fig. 4). The ethanol-extracted material produced a dose-dependent decrease in adenylate cyclase activity in the presence of ATP. However, virtually no inhibition occurred when p[NH]ppA was substituted for ATP.

Studies using various substrate concentrations demonstrated that adenylate cyclase activity reaches its maximal level with 0.2 mM ATP, with an apparent K_m for ATP of about 5×10^{-5} (Fig. 5). The ethanol-extractable inhibitory activity decreased the V_{max} for the enzyme, with no apparent effect on the K_m.

Further Characterization of Insulin Mediators. We have shown (13) that the crude extract from liver membranes that modulates PDHase can be chromatographed on an HPLC molecular sieve column (I-60, Waters Associates). Comparison of its elution profile with parallel chromatography on Sephadex G-25 suggested that the molecular weight of the mediator was ≈1,000–2,000. The two resolved modulators of PDHase described in the present studies were coeluted on an HPLC I-60 column with the same retention time as that previously observed for the crude extract. Moreover, the adenylate cyclase inhibitor was eluted similarly, suggesting that the inhibitor(s) and stimulator may be of similar molecular weight.

DISCUSSION

Recent attempts to explain the biochemical actions of insulin on cellular metabolic processes have suggested the existence of a chemical mediator that is released from the plasma membranes of target cells upon incubation with the hormone (3, 10–14, 28–30). The known effects of insulin on glycogen synthetase from skeletal muscle (3) and on PDHase from adipocytes (10–14) and liver (13) can be reproduced qualitatively by this

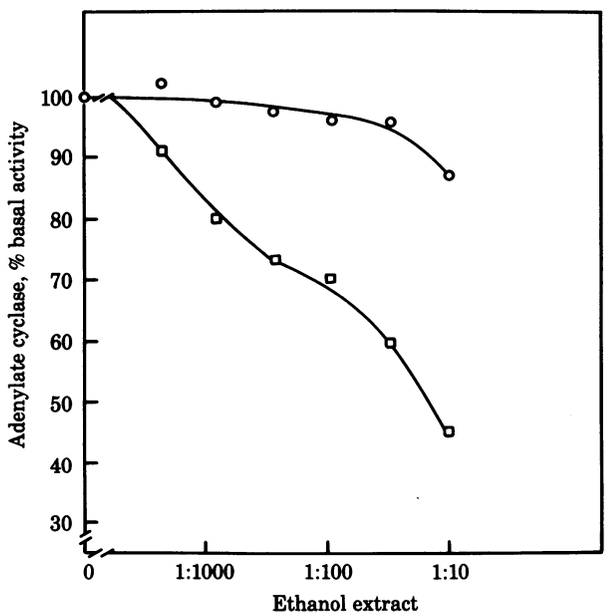

FIG. 4. Requirement for ATP in inhibition of adenylate cyclase by the liver-derived inhibitor. Liver particulates were incubated with 2 nM insulin for 15 min at 30°C, and the supernatant was extracted. The ethanol extract, dissolved in 1 mM formic acid, was added to adipocyte plasma membranes at the designated concentrations. Adenylate cyclase activity was assayed in the presence of unlabeled 0.1 mM ATP (□) or unlabeled 0.1 mM p[NH]ppA (○); [α-^{32}P]ATP was used as tracer in all assays. Results are means of triplicate determinations, expressed as percentage of control activity. Basal activity with ATP was 25% higher than with p[NH]ppA.

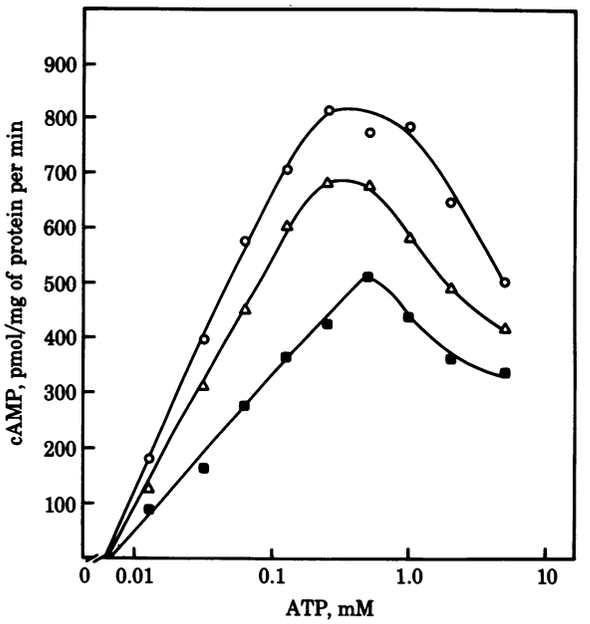

FIG. 5. Effect of ATP concentration on adenylate cyclase activity in the presence of liver-derived inhibitor. Liver particulates were incubated with 2 nM insulin for 15 min at 30°C and extracted as described in Fig. 4. Adenylate cyclase activity was assayed in the presence of increasing concentrations of ATP with no additions (○), with a 1:50 final dilution of ethanol extract (△), or with a 1:10 final dilution of ethanol extract (●). In all assays, $MgCl_2$ was 5 mM.

substance. In nearly all cases, the effects of insulin on the generation of this substance appear to be biphasic with respect to the concentration of hormone (10, 11, 13, 14, 30). Low concentrations of insulin elaborate a stimulator of PDHase, whereas the degree of stimulation is decreased significantly at higher concentrations. Insulin at 7–10 nM produces no detectable PDHase "stimulator" from adipocyte membranes, although there is a 2- to 3-fold increase in this activity at low concentrations (30). It has been suggested (13, 28–30) that these paradoxical effects of insulin might be attributable to the release of two antagonistic substances. Cheng *et al.* (29) described the separation of stimulatory and inhibitory activities of glycogen synthetase phosphatase by high-voltage paper electrophoresis. Seals and Czech (30) speculated that both an inhibitor and an activator of PDHase were released from adipocyte plasma membranes and that these could be distinguished by the kinetics of their generation by insulin.

Data presented here confirm these suggestions by achieving a separation of these two antagonistic activities. Extraction of the lyophilized supernatant of liver membrane incubations with 100% ethanol removes the inhibitory from the stimulatory activity, resulting in monophasic dose–response curves (Fig. 1). The production of both components is regulated by physiological concentrations of insulin, although production of the stimulator appears to be more sensitive to the hormone after 5 min of incubation. Although the relationship between the two factors is not yet clear, these results imply the existence of an intrinsic homeostatic mechanism for the regulation of PDHase by insulin and might perhaps be related to the paradoxical effects of insulin on lipolysis in adipocytes (31, 32).

Insulin can inhibit the rise in cyclic AMP levels produced by lipolytic hormones in fat cells (15). Although this perhaps can be explained in part by activation (*in vivo*) of the high-affinity form of cyclic AMP phosphodiesterase (17), insulin can still reduce the catecholamine-induced rise in cyclic AMP in the presence of phosphodiesterase inhibitors (16). Also, insulin added

directly to adipocyte (18) or liver (19, 20) plasma membranes can decrease adenylate cyclase activity.

The liver-derived ethanol extract described here inhibited all states of adenylate cyclase activity in adipocyte membranes, even in the presence of maximally effective inhibitory concentrations (10 μM) of GTP (Table 1). We also have observed inhibitory effects of this extract on adenylate cyclase of rat erythrocytes and hepatocytes (unpublished). Interestingly, the inhibition of adipocyte adenylate cyclase occurs at doses of extract equivalent to those which inhibit PDHase. The adenylate cyclase inhibitor and the PDHase inhibitor also have been coextracted from isolated fat cell plasma membranes and intact rat hepatocytes upon treatment with insulin (unpublished data). The quantity of adenylate cyclase inhibitory activity generated from adipocyte and liver membranes occurs with concentrations of insulin approximately equal to those that produce the PDHase inhibitor. These findings, together with the apparent coelution of the PDHase and adenylate cyclase inhibitors on HPLC, suggest that perhaps a single insulin-dependent substance may be responsible for both of these activities.

It has been proposed that adenylate cyclase may be regulated by phosphorylation-dephosphorylation reactions (8, 9). Notably, the action of the insulin-stimulated inhibitor of adenylate cyclase does not proceed if p[NH]ppA, a nonhydrolyzable analog of ATP, is used in the adenylate cyclase assay (Fig. 4). Thus, the inhibitor may well exert its action through the control of a protein phosphorylation step, in analogy to that postulated for the PDHase regulator (14).

Although the chemical nature of these two antagonistic substances remains elusive, some things are known regarding their production and properties. The material that stimulates PDHase is not absorbed to charcoal, is insensitive to boiling, and has a molecular weight of 1,000–3,000 (3, 10–14). Seals and Czech (30) have further suggested that it may be peptidic in nature and that it exhibits a net negative charge at neutral pH. Although the relationship of this substance to the PDHase inhibitor is not known, it has a similar molecular weight, is not absorbed to charcoal, and is also boiling-insensitive. Because this substance can be extracted differentially with ethanol, it is probably somewhat more hydrophobic. Moreover, the inhibitor of PDHase appears to copurify with the inhibitor of adenylate cyclase, and it exhibits very similar chemical properties and responsiveness to insulin. Endogenous adenylate cyclase inhibitors have been identified earlier (33–35). Whether the substance described here is related to other, previously described inhibitors will become evident with further purification and characterization.

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