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Activation of pyruvate dehydrogenase by direct addition of insulin to an isolated plasma membrane/mitochondria mixture: Evidence for generation of insulin's second messenger in a subcellular system

[phosphorylation/concanavalin A/antibody to insulin receptor/pyruvate dehydrogenase (lipoamide)]

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ABSTRACT **The addition of insulin to a mixture of plasma membrane and mitochondrial fractions from rat adipocytes results in a decrease in the phosphorylation of a mitochondrial protein identified as the α subunit of pyruvate dehydrogenase [pyruvate:lipoamide oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1] (Seals, J. R., McDonald, J. M. & Jarett, L. (1979) *J. Biol. Chem.* 254, 6991–6996). This study confirms the prediction that a corresponding increase in pyruvate dehydrogenase activity can be effected by insulin treatment of this preparation. Incubation of the plasma membrane/mitochondria mixture with ATP inhibited pyruvate dehydrogenase activity as measured in a subsequent enzyme assay. The presence of insulin during this incubation with ATP resulted in a 24.5% stimulation of enzyme activity compared to incubation without insulin ($n = 9$, $P < 0.001$). The effect was specific for biologically active insulin and was insulin dose-dependent in the physiological range of insulin. Supermaximal doses of insulin produced reduced effects. An insulin effect of similar magnitude could also be observed when the plasma membrane/mitochondria mixture was incubated without ATP. Two insulin mimickers, concanavalin A and antibody to insulin receptor, stimulated pyruvate dehydrogenase by 30.4% ($n = 6$, $P < 0.001$) and 28.1% ($n = 8$, $P < 0.001$), respectively. Both of these agents also produced reduced effects at supermaximal concentrations. The effects of all three agents required plasma membranes and could not be produced by treatment of mitochondria alone. The results suggest that a mechanism common to all three agents is responsible for transmitting the stimulation from the plasma membrane to the mitochondrial components of the mixture.**

Analysis of the mechanism of insulin action on its target cells has been hindered by the complex interactions among metabolic and regulatory factors in the intact cell. However, insulin action has recently been demonstrated in a simplified subcellular system consisting of a mixture of purified plasma membrane and mitochondrial fractions from the rat adipocyte (1, 2). Addition of insulin to this subcellular system caused a plasma membrane-mediated decrease in the phosphorylation of a mitochondrial protein, identified as the α subunit of pyruvate dehydrogenase [pyruvate:lipoamide oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1]. This effect was consistent with the proposed model of insulin action on pyruvate dehydrogenase in the intact cell (3, 4). From these data, it was predicted that the activity of pyruvate dehydrogenase should be stimulated by the addition of insulin to the plasma membrane/mitochondria mixture. This study investigates the activity of pyruvate dehydrogenase in the plasma membrane/mitochondria mixture and the effect of insulin and other agents on this enzyme.

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EXPERIMENTAL PROCEDURES

Materials. Male Sprague–Dawley rats (120 g) were obtained from Eldridge Laboratory Animals (Barnhardt, MO). Collagenase, bovine serum albumin (fraction V), ATP (disodium salt), pyruvic acid (sodium salt), dithiothreitol, coenzyme A, cocarboxylase (thiamin pyrophosphate chloride), β-NAD, and concanavalin A were purchased from Sigma. The lots of collagenase and albumin used were chosen as described (2). Porcine insulin was a gift from R. Chance, Eli Lilly. Serum containing antibody to insulin receptor (5) was the gift of Pedro Cuatrecasas, Burroughs Wellcome Research, (Triangle Park, NC). The serum was diluted for use without further purification. Omnifluor and [1-^{14}C]pyruvic acid were purchased from New England Nuclear. Hydroxide of Hyamine (10-X) was from Packard Instrument (Downers Grove, IL). Other reagents and chemicals were from standard sources.

Preparation of Adipocyte Subcellular Fractions. Adipocytes were prepared from rat epididymal fat pads digested with collagenase according to a modification of previous methods (6, 7). Fat pads from all rats were minced and placed in a vial containing modified Krebs–Ringer phosphate buffer (1.5 ml per pair of pads) containing 11 mM D-glucose, 3% albumin, 1 mg of collagenase per ml, and half the normal amount of Ca^{2+}. The pads were digested with shaking (120 cycles per min) at 37°C until only small pieces of tissue remained (40–50 min). The isolated adipocytes were washed as described (7). Plasma membranes and mitochondria were prepared as described by Jarett and McKeel (7, 8) with minor modifications, including omission of EDTA from the fractionation medium (2, 9) and isolation of fractions from the nuclei-free particulate fraction on a discontinuous Ficoll/sucrose gradient (7). The purity of these fractions is documented elsewhere; there was less than 10% cross-contamination between plasma membranes and mitochondria (8, 10). Protein content of the fractions was determined by the method of Lowry *et al.* (11) with albumin as a standard. Pyruvate dehydrogenase activity was expressed per mg of mitochondrial protein in the sample except when the plasma membrane fraction alone was tested. Fractions were routinely frozen in a dry ice/ethanol bath, stored at −70°C, and used within 1 week after preparation.

Assay of Pyruvate Dehydrogenase Activity. Pyruvate dehydrogenase activity was assayed as the release of $^{14}CO_2$ from [1-^{14}C]pyruvic acid. The assay included two incubation steps. First, the fractions to be assayed were incubated with ATP and any agents whose effects were being tested. Incubation was carried out in polypropylene tubes (17 × 100 mm) in a final volume of 200 μl. The standard medium was 50 mM Tris·HCl (pH 7.4 at 37°C)/50 μM $MgCl_2$/50 μM $CaCl_2$/250 μM ATP.

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Other agents or their respective controls were included as noted. The reaction was begun by the addition of the fraction to be assayed and was allowed to continue at 37°C for 2–10 min. In the second incubation step, constituents for the assay of pyruvate dehydrogenase were added to the tubes and the incubation was continued. The assay was begun by the addition of 25 μl of an assay buffer to give final concentrations of 50 mM potassium phosphate buffer at pH 8.0, 1 mM dithiothreitol, 0.1 mM coenzyme A, 0.1 mM cocarboxylase, 0.25 mM [1-^{14}C]-pyruvate [1 mCi/mmol (1 Ci = 3.7×10^{10} becquerels)], and 0.5 mM β-NAD. The tube was immediately sealed with a rubber cap that suspended a plastic well containing a 25 × 75 mm piece of filter paper (Whatman no. 1) inside the tube. Subsequent additions were made by syringe through the rubber cap. The assay incubations were at 37°C for 1–5 min; then reactions were stopped by adding 200 μl of 3 M H_2SO_4 to the tube and placing the tubes on ice. Hydroxide of Hyamine (250 μl) was added to the well and filter paper of each sample and allowed to absorb $^{14}CO_2$ at room temperature with gentle shaking for 1 hr. The filters were then removed and radioactivity was measured in Omnifluor/toluene (4 g/liter). The specific activity of [1-^{14}C]pyruvate in the assay was determined by measuring the radioactivity of an aliquot of the assay buffer. Background values were determined from samples that were incubated exactly as described above but without the addition of tissue; they ranged from 5 to 10% of the values for samples with tissue. The background values also showed a slight drift during the assay, increasing by ¼ to ½ during the experiment. This drift was minimized by keeping the assay buffer on ice until addition to the samples. Corrections for this drift were made by determining background values at the beginning and end of the assay, interpolating values throughout the experiment, and subtracting the appropriate background value from each experimental sample. This small correction improved the consistency of results throughout the experiment; the variation between identical samples taken at different times during the assay was less than 10%. The mean coefficient of variation for triplicate samples in this study was less than 5%.

RESULTS

Pyruvate Dehydrogenase Activity in the Plasma Membrane/Mitochondria Mixture. The activity of pyruvate dehydrogenase in the plasma membrane/mitochondria mixture used in this study was inhibited by incubation with ATP. In the absence of ATP, enzyme activity was constant through 5 min of incubation, averaging 3.8 nmol/mg of mitochondrial protein per min (Fig. 1). The bulk of this activity was accounted for by the mitochondrial fraction; the plasma membrane fraction alone possessed less than 3% of the activity of the mitochondrial fraction alone. Incubation of the plasma membrane/mitochondria mixture with 250 μM ATP for 5 min inhibited pyruvate dehydrogenase activity by more than 70% compared to samples incubated without ATP (Fig. 1). After incubation with ATP, enzyme activity was linear through 2 min of further incubation, averaging 1.1 nmol/mg of mitochondrial protein per min. Inhibition of enzyme activity by ATP was dependent on the time of incubation (Fig. 2). Maximal inhibition was reached within 5 min of incubation. The standard procedure in subsequent experiments employed an incubation of 5 min and then an assay incubation of 2 min.

Stimulation of Pyruvate Dehydrogenase in the Plasma Membrane/Mitochondria Mixture by Insulin, Concanavalin A, and Antibody to Insulin Receptor. The presence of insulin during incubation of the plasma membrane/mitochondria mixture with ATP stimulated the activity of pyruvate dehydrogenase measured during a subsequent 2-min assay compared

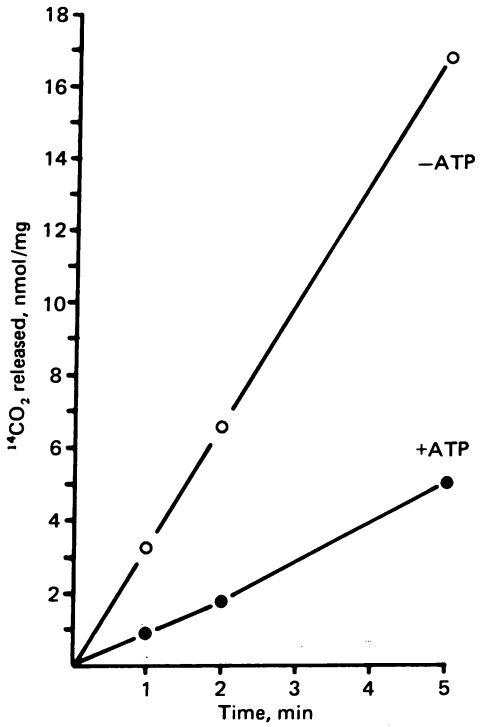

FIG. 1. Inhibition of pyruvate dehydrogenase activity by ATP. The plasma membrane (200 μg/ml)/mitochondria (500 μg/ml) mixture was incubated in the presence (●) or absence (○) of ATP (250 μM) for 5 min, then assayed for pyruvate dehydrogenase activity as described in the text.

to samples incubated without insulin (Fig. 3). The effect of insulin was dose-dependent and biphasic in the range from 10 to 500 microunits/ml. Pyruvate dehydrogenase activity increased with increasing insulin concentrations to a maximal level, then decreased at higher insulin doses. The concentration of insulin that produced maximal enzyme stimulation varied among different preparations of plasma membranes and mitochondria from 50 to 500 microunits/ml, most commonly occurring at 100 microunits/ml. The average maximal stimulation of enzyme activity was 24.5%, and this effect was highly

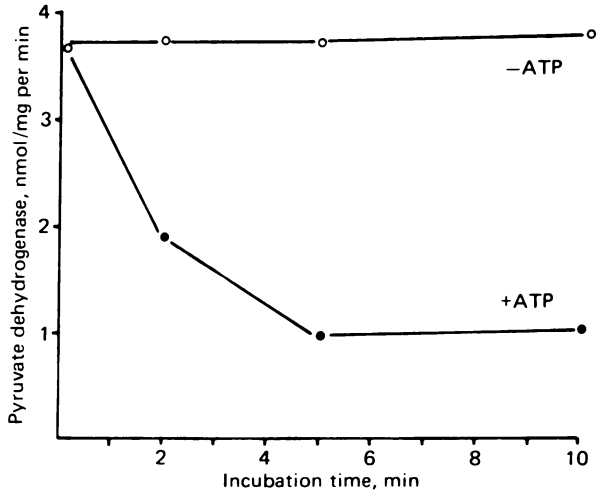

FIG. 2. Time course of pyruvate dehydrogenase inhibition by ATP. The plasma membrane (200 μg/ml)/mitochondria (500 μg/ml) mixture was incubated in the presence (●) or absence (○) of ATP (250 μM) for the time indicated, then assayed for pyruvate dehydrogenase activity for 2 min as described in the text.

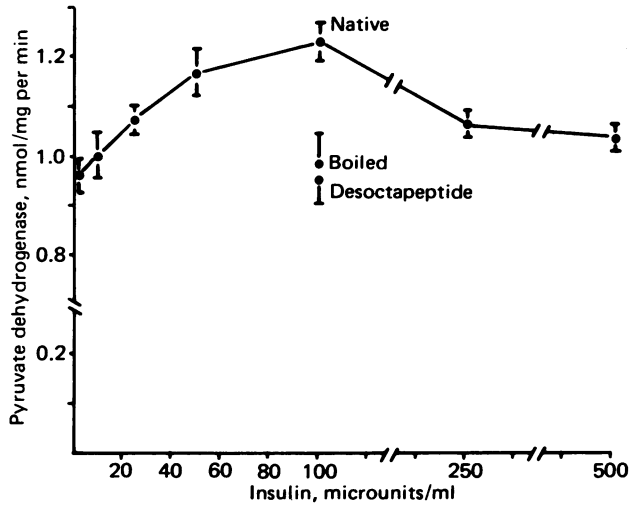

FIG. 3. Stimulation of pyruvate dehydrogenase activity by insulin. The plasma membrane (200 μg/ml)/mitochondria (500 μg/ml) mixture was incubated for 5 min with ATP (250 μM) and the indicated concentration of insulin, boiled insulin, or desoctapeptide-insulin, then assayed for pyruvate dehydrogenase activity for 2 min. All samples, including control, contained 0.0005% albumin. The figure shows the results of a single representative experiment. Error bars indicate SEM.

statistically significant (Table 1). In contrast, the insulin derivative desoctapeptide-insulin, which possesses only 1% of the activity of nature insulin, had no effect on pyruvate dehydrogenase activity at a concentration equivalent to 100 microunits/ml (Fig. 3). In addition, insulin that had been inactivated by boiling had no effect on pyruvate dehydrogenase activity (Fig. 3).

The conditions under which the insulin effect on pyruvate dehydrogenase activity was observed were chosen on the basis of previously reported effects on the phosphorylation of the enzyme (1, 2). These experiments required the presence of [γ-^{32}P]ATP as a means of measuring phosphorylation. As shown in Figs. 1 and 2, the activity of the enzyme was greater in the absence of ATP. This activity does not represent the maximal activity of the enzyme, but only that which is in the active, nonphosphorylated form when the mitochondria are isolated from the cell. Incubation in the presence of high levels of Mg^{2+} and Ca^{2+} converts all of the enzyme to the active, nonphosphorylated form and produces a 4- to 5-fold increase in enzyme activity (data not shown). This indicates that even before incubation with ATP, 80% of the enzyme is already in the inactive, phosphorylated form. The effect of insulin also was tested in the absence of ATP at low Mg^{2+} and Ca^{2+} concentrations to determine if the process of phosphorylation by added ATP was necessary for insulin to activate pyruvate dehydrogenase. It was found that insulin (100 microunits/ml) produced effects of similar or greater absolute magnitude in the absence of ATP compared to the presence of ATP in each of five experiments (data not shown), and the insulin effect was statistically significant ($P < 0.01$).

Table 1. Stimulation of pyruvate dehydrogenase by insulin, concanavalin A, and antibody to insulin receptor

Agent	n	Maximal stimulation, %	P
Insulin	9	24.5 ± 3.8	<0.001
Concanavalin A	6	30.4 ± 6.4	<0.001
Antibody to insulin receptor	8	28.1 ± 7.9	<0.001

The plasma membrane (200 μg/ml)/mitochondria (500 μg/ml) mixture was incubated for 5 min with ATP (250 μM) and a range of concentrations of insulin (10–500 microunits/ml), concanavalin A (10–250 μg/ml), or antibody to insulin receptor (1:2000–1:100 dilution), then assayed for pyruvate dehydrogenase activity for 2 min. In each experiment, the maximal stimulation (mean ± SEM) by each agent compared to control over the range tested was determined and the statistical significance of the difference between these values was determined by the paired t test for the number of experiments indicated (n).

Two agents that have been reported to mimic insulin action in other systems, concanavalin A (12, 13) and antibody to insulin receptor (5), also stimulated pyruvate dehydrogenase activity in a manner similar to that of insulin (Fig. 4). Stimulation by both agents was dose-dependent, with supermaximal doses eliciting reduced effects. The concentration of each agent required for maximal stimulation varied among tissue preparations from 50 to 250 μg/ml for concanavalin A and from 1:500 to 1:100 dilution for the receptor antibody. The average maximal stimulations for concanavalin A and for the antibody were 30.4% and 28.1%, respectively, and both effects were statistically significant (Table 1).

Requirement of Plasma Membranes for Stimulation of Pyruvate Dehydrogenase. The effects of insulin, concanavalin A, and antibody to insulin receptor on pyruvate dehydrogenase activity of the plasma membrane/mitochondria mixture were

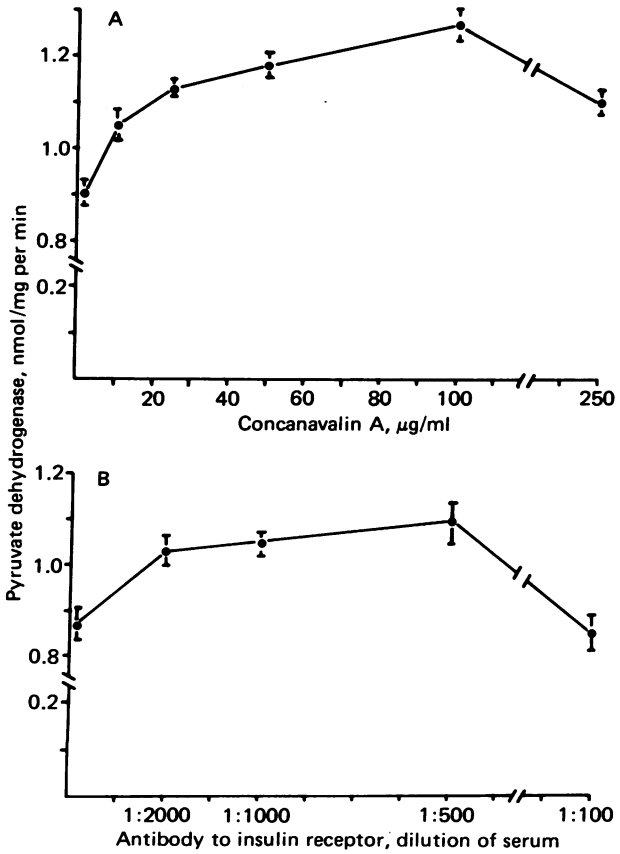

FIG. 4. Stimulation of pyruvate dehydrogenase activity by concanavalin A and antibody to insulin receptor. The plasma membrane (200 μg/ml)/mitochondria (500 μg/ml) mixture was incubated for 5 min with ATP (250 μM) and the indicated concentration of concanavalin A (A) or antibody to insulin receptor (B) then assayed for pyruvate dehydrogenase activity for 2 min. Control samples for the antibody contained a 1:100 dilution of normal rabbit serum, which alone had no effect on enzyme activity at any dilution. The figure shows the results of a single representative experiment for each agent. Error bars indicate SEM.

Table 2. Requirement of plasma membranes for stimulation of pyruvate dehydrogenase

Sample	Pyruvate dehydrogenase, nmol/mg per min	Stimulation, %		
		Insulin (100 microunits/ml)	Concanavalin A (100 μg/ml)	Antibody to insulin receptor (1:500)
Plasma membranes	0.04 ± 0.02	5.5 ± 29.6	—	—
Mitochondria	0.93 ± 0.05	−6.6 ± 6.7	−1.6 ± 2.3	2.2 ± 3.5
Plasma membranes + mitochondria	1.19 ± 0.09	28.2 ± 6.9	25.6 ± 1.7	23.6 ± 2.0

Samples consisting of plasma membranes (200 μg/ml), mitochondria (500 μg/ml), or of a mixture of these fractions were incubated with ATP (250 μM) in the presence or absence of the indicated agents for 5 min then assayed for pyruvate dehydrogenase activity for 2 min. The stimulation by each agent relative to its respective control was determined for each sample. Results represent the mean ± SEM of three experiments.

dependent on the presence of plasma membranes and could not be produced by treatment of the isolated mitochondrial fraction alone (Table 2). The plasma membrane fraction alone possessed little pyruvate dehydrogenase activity and the effects of insulin were difficult to quantitate accurately under these conditions. The mitochondrial fraction alone possessed substantial enzyme activity, but the addition of insulin, concanavalin A, or antibody to receptor at concentrations that produced significant effects on the plasma membrane/mitochondria mixture had no effect on enzyme activity.

DISCUSSION

Previous investigations demonstrated that a combination of mitochondrial and plasma membrane subcellular fractions could be used as a model system for studying an action of insulin similar to one observed in the intact adipocyte (1, 2). Addition of insulin to a mixture including both organelles, ATP, and ions resulted in decreased phosphorylation of two proteins, one of which was identified as a mitochondrial component, the α subunit of pyruvate dehydrogenase. Phosphorylation of this protein has been shown to inhibit the activity of the enzyme (14, 15). Further, incubation of the intact adipocyte with insulin stimulates pyruvate dehydrogenase activity by decreasing the proportion of the enzyme in the phosphorylated, inactive form (16, 17). The similarity between the insulin effect in the intact cell and in the subcellular system suggested that the same insulin-sensitive mechanisms were acting in both cases. This interpretation was supported by the observation that insulin action on the plasma membrane/mitochondria mixture required plasma membranes and could not be affected by treatment of mitochondria alone. From these data, it was predicted that the activity of pyruvate dehydrogenase should be stimulated by the addition of insulin to the plasma membrane/mitochondria mixture. The present study examines the activity of pyruvate dehydrogenase in this system and determines its insulin sensitivity, confirming the hypothesis proposed on the basis of the phosphorylation data.

The pyruvate dehydrogenase assay used in this study was modified from previous methods in order to facilitate measurement of the enzyme during relatively short incubation periods that conform to the conditions under which insulin decreased the phosphorylation of the α subunit. As predicted, the activity of pyruvate dehydrogenase was inhibited by incubation with ATP under these conditions (Figs. 1 and 2). Inhibition of the enzyme reached its greatest extent within 5 min and was constant through at least 10 min of incubation. Enzyme activity was routinely assayed after a 5 min incubation by using a 2-min assay incubation, during which the enzyme reaction was linear.

Direct addition of insulin to the plasma membrane/mitochondria mixture resulted in an increase in pyruvate dehydrogenase activity under the assay conditions described. The specificity of the insulin effect was indicated by several observations. First, the insulin concentration range for increasing pyruvate dehydrogenase activity (Fig. 3) was the same range over which insulin causes increasing effects on fat cell metabolism (18, 19). Second, the low-activity insulin derivatives, desoctapeptide-insulin and boiled insulin, had no effect on enzyme activity (Fig. 3). And finally, two agents that are known to mimic insulin action on various processes in the intact cell, concanavalin A (12, 13) and antibody to insulin receptor (5), produce the same effect as insulin on pyruvate dehydrogenase in this system (Fig. 4).

The insulin-induced increase in pyruvate dehydrogenase activity may be accounted for by an inhibition of the protein kinase that phosphorylates the enzyme (20, 21) or by stimulation of the phosphatase that dephosphorylates the enzyme (22, 23). Investigation of insulin action on pyruvate dehydrogenase in the intact adipocyte has demonstrated no alteration of these enzymes that is sufficiently stable to survive isolation of the mitochondria (24, 25). The present data suggest that insulin activates the phosphatase, because the insulin effect on pyruvate dehydrogenase was observed in the absence of added ATP, indicating that insulin does not affect the process of enzyme phosphorylation but increases the rate of dephosphorylation of the already phosphorylated component. This conclusion is compatible with the observation of McDonald *et al.* (26) that insulin treatment of the intact adipocyte caused a shift in mitochondrial Ca^{2+} from a stable to a free or more labile form. This increase in intramitochondrial free Ca^{2+} could result in stimulation of the Ca^{2+}-sensitive phosphatase. However, the stimulation would not be expected to persist during fractionation as free Ca^{2+} is washed out of the organelle (26).

The decreased insulin effect observed at supermaximal concentrations of insulin is a phenomenon that has been observed in other insulin-sensitive systems and suggests associations between the behavior of insulin in the subcellular system used here and other related systems. In the intact adipocyte, high concentrations of insulin have been reported not to inhibit lipolysis as do low concentrations (27, 28). In isolated plasma membranes, high concentrations of insulin enhance rather than inhibit activation of adenylate cyclase by catecholamines (29). The mechanism of these biphasic effects is unknown, but each suggests that reversal of the action of insulin at the plasma membrane accounts for the biphasic effect. In addition, a partially purified insulin-dependent cytoplasmic factor that stimulates Ca^{2+} uptake into liver mitochondria also produces a biphasic dose response when added to isolated mitochondria (30). This suggests that paradoxical effects of insulin may be accounted for by the behavior of messengers subsequent to the hormone rather than by the hormone itself.

The biological significance of the insulin effect in the plasma membrane/mitochondria mixture is suggested by the observation that two agents that mimic insulin action in adipocytes,

concanavalin A (12, 13) and antibody to insulin receptor (5), also stimulate pyruvate dehydrogenase activity (Fig. 4). The magnitude of the effects of these agents is similar to that of insulin (Table 1), and both agents exhibit decreased effects at supermaximal concentrations (Fig. 4). The similar characteristics of the effects of insulin, concanavalin A, and antibody to insulin receptor suggest that all three agents act by the same mechanism in this system. This conclusion implies that insulin action does not involve mandatory processing of the insulin molecule itself, by internalization of the hormone (31), cleavage of a piece of the molecule to act as an intracellular messenger (32), or by degradation and uptake (33). This last conclusion has also been confirmed in intact adipocytes by the observation that inhibition by chloroquine of insulin degradation and processing by lysosomes did not block insulin action on glucose transport, protein synthesis, or antilipolysis (34).

The requirement of plasma membranes for observation of the insulin effect on pyruvate dehydrogenase activity in the plasma membrane/mitochondria mixture suggests that the generation or modulation of a second messenger may be responsible for mediating the hormone's action. Further, the similarity of insulin action in the model system and in the intact cell raises the possibility that the mechanism acting in the subcellular system is representative of that in the intact cell. Although a second messenger for insulin has often been proposed (35–37), its identity remains unknown. A cytoplasmic factor that inhibits glycogen synthase kinase (38, 39) and an insulin-dependent cytoplasmic component that stimulates Ca^{2+} uptake by mitochondria (30) are two potential second messengers that have been partially purified but not thoroughly evaluated. The simplified subcellular system described here may provide a unique means of identifying and isolating the second messenger for insulin.

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