Journal of Cellular Biochemistry 45:374–380 (1991)

The Specific Protein Phosphatase Inhibitor Okadaic Acid Differentially Modulates Insulin Action

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Abstract The pleiotropic nature of insulin action suggests diverse mechanisms of signal transduction for the hormone. The specific protein phosphatase inhibitor, okadaic acid, is utilized to differentiate metabolic pathways that may be regulated by phosphorylation or dephosphorylation of key enzymes. In H-35 hepatoma cells, okadaic acid inhibits insulin-stimulated glycogen synthesis with an IC_{50} of 400 nM. In contrast, activation of lipogenesis by insulin is inhibited with an IC_{50} of 50 nM okadaic acid. The toxin also inhibits stimulation of lipogenesis in these cells by the insulin-sensitive inositol glycan enzyme modulator. In isolated rat adipocytes, insulin-stimulated lipogenesis is also inhibited by okadaic acid with an IC_{50} of approximately 1,700 nM. The antilipolytic effect of insulin in these cells is more sensitive to okadaic acid, exhibiting an IC_{50} of 150 nM. Maximal activation of lipogenesis by insulin is dramatically reduced by okadaic acid with no effect on the concentration required for half-maximal activation, whereas the sensitivity of insulin-induced antilipolysis is attenuated by okadaic acid, with no apparent reduction in the maximal effect of the hormone. Taken together, these data suggest that specific phosphatases may be differentially involved in some of the metabolic pathways regulated by insulin.

Key words: lipogenesis, lipolysis, protein phosphorylation, second messenger, glycogen synthesis, adipocytes

The molecular mechanisms by which insulin regulates metabolic processes remain uncertain. Numerous investigations have indicated that the acute metabolic effects of the hormone may be mediated through phosphorylation and dephosphorylation of proteins. Insulin stimulates the net dephosphorylation on serine/threonine residues of several key rate-limiting enzymes, including glycogen synthase [1,2], acetyl-CoA carboxylase [3–7], pyruvate kinase [8], pyruvate dehydrogenase [9,10], hydroxylmethylglutaryl CoA reductase [11], and hormone-sensitive lipase [12], thereby modulating the activity of each of these enzymes. Dephosphorylation of enzymes by insulin could result from activation of specific phosphatases or inhibition of kinases [13,14]. In addition, insulin also stimulates the phosphorylation of several proteins on serine residues, including ribosomal S6 [15], adenosine triphosphate (ATP) citrate lyase [16], acetyl-CoA carboxylase [17,18], and others [13,14]. While protein dephosphorylation is clearly linked to regulation of the catalytic activities of key rate-limiting enzymes, the functional roles of increases in protein phosphorylation remain largely unknown.

Okadaic acid, a polyether fatty acid isolated from the black sea sponge *Halichondria okadaii*, is a potent tumor promoter and diarrheal agent that is an inhibitor of serine/threonine type 1 and type 2A phosphatases [19]. This toxin promotes the phosphorylation of a number of proteins, such as acetyl-CoA carboxylase [20] and tyrosine hydroxylase [21], promotes smooth muscle contraction [22], and increases the activation of maturation promoting factor [23] and MAP kinase [24].

To clarify the role of protein phosphorylation and dephosphorylation in insulin action, we have exploited the specificity of okadaic acid as a serine/threonine phosphatase inhibitor. In this report, we confirm and extend previous observations [20] demonstrating that okadaic acid differentially antagonizes some but not all of the effects of insulin, suggesting the involvement of

Abbreviations used: CoA, coenzyme A; ATP, adenosine triphosphate; IPG, inositol phosphate glycan; PL-PLC, phosphatidylinositol-specific phospholipase C.

Received September 28, 1990; accepted December 7, 1990.

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specific protein phosphatases in mediating some of the actions of this hormone.

METHODS

Materials

Crystalline porcine insulin was obtained from Eli Lilly and Co. (Indianapolis, IN). Okadaic acid was purchased from Moana Bioproducts, Inc. (Honolulu, HI). Bovine serum albumin (BSA, fraction V) was from Calbiochem Corp. (La Jolla, CA). Collagenase, (−)-isoproterenol hydrochloride, β-nicotinamide adenine dinucleotide, adenosine triphosphate (disodium salt), glycine, HEPES, hydrazine hydrate, and the enzymes glycerokinase (from *Candida mycoderma*), and α-glycerophosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). D-[U-^{14}C] glucose and [^{14}C] acetate were obtained from New England Nuclear (Boston, MA). All other reagents were analytical grade.

Cell Culture

H-35 hepatoma cells were grown to confluence on Corning 35 mm 6-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin in a 5% CO_2 humidified atmosphere.

Preparation of Isolated Adipocytes

Isolated adipocytes were prepared from epididymal adipose tissue of male Sprague-Dawley rats (150–200 gm) according to the collagenase method of Rodbell [25] with some modifications. Cells were digested with collagenase (1 mg/ml) in Krebs-Ringer HEPES buffer (pH 7.4) containing 0.5% (w/v) bovine serum albumin (Fraction V) for 15–20 min at 37°C. Cells were filtered through nylon mesh and washed four times by centrifugation, and subsequently resuspended in Krebs-Ringer HEPES buffer (pH 7.4) containing 0.5% bovine serum albumin. This buffer was used in subsequent studies involving isolated adipocytes. When measuring the rate of lipolysis, the cells were preincubated for 30 min at 37°C in the above buffer, prior to a final wash before use. In the case of lipogenesis, adipocytes were preincubated for 5 min at 37°C, and then washed a final time before use.

Assay of Glycogen Synthesis

Glycogen synthesis was determined by a modification of the method of Lawrence et al. [26]. Hepatoma cells were serum-deprived overnight and washed twice with Krebs-Ringer buffer (pH 7.4). Thereafter, the cells were incubated for 2 h at 37°C in the above buffer containing 11 mM D-[U-^{14}C]glucose (1 μCi/ml) in the presence of 10 nM insulin at varying concentrations of okadaic acid. The reaction was stopped by the addition of 0.7 ml of 30% KOH, and cells were transferred into test tubes (capped), and heated in a dry bath for 20 min at 100°C. After digestion was completed, 2.2 ml of 95% ethanol was added to the tubes, boiled, and then immediately removed and placed in an ice bath for 20 min to allow precipitation of glycogen. The samples were then centrifuged at 2,200 rpm for 10 min at 0°C, and the precipitate was dissolved in distilled water. The total sample was counted by measurement of the rate of incorporation of D-[U-^{14}C]glucose into glycogen. The protein concentration was determined by the method of Bradford [27]. Glycogen synthesis is expressed as percent of maximal stimulation of insulin in the absence of okadaic acid.

Assay of Lipogenesis in Hepatoma Cells

The incorporation of D-[U-^{14}C]glucose into lipids was determined by the procedure of Moody et al. [28]. Hepatoma cells were serum-deprived overnight, and then washed twice with Krebs-Ringer buffer (pH 7.4). The cells were preincubated for 15 min at 37°C in the above buffer containing either 2 nM insulin or inositol glycan and okadaic acid, followed by incubation for 30 min with reaction mixture containing 5 mM D-[U-^{14}C]glucose. The reaction was terminated by the addition of trypsin-EDTA, followed by toluene-based scintillation cocktail. The results are expressed as percent of maximal stimulation of insulin in the absence of okadaic acid.

Assay of Lipogenesis in Isolated Adipocytes

A fat cell suspension (approximately 4×10^6 cells/ml) was incubated at 37°C for 60 min in a reaction mixture containing 2 mM D-[U-^{14}C]glucose in Krebs-Ringer HEPES buffer (pH 7.4) containing 0.5% BSA in the presence of 0.2 nM insulin at varying concentration of okadaic acid. The reaction was stopped by the addition of toluene-based scintillation cocktail.

Assay of Lipolysis

Adipocytes (approximately 10^6/ml) were preincubated in Krebs-Ringer HEPES buffer (pH 7.4) containing 0.5% BSA, with okadaic acid and

insulin for 10 min at 37°C, followed by the addition of isoproterenol for an additional 30 min. The assay was terminated by the addition of 50 μl of 4.6 M perchloric acid, followed by 50 μl of chloroform. The mixture was then vortexed and centrifuged, and an aliquot from the aqueous portion was neutralized with 5 N KOH. The rate of lipolysis was determined by measurement of the release of glycerol into the incubation medium [41].

Production of the Insulin-Sensitive Inositol Phosphate Glycan

This was prepared as previously described [29]. Briefly, a particulate fraction from ten rat livers [30] was suspended in 10 mM ammonium bicarbonate, pH 7.4, and lyophilized. The lyophilized powder was extracted by stirring for 15 min in 400 ml of chloroform/methanol/1 N HCL (100:200:1). Following filtration of particulate material, the extract was evaporated and resuspended in 200 ml of 10 mM ammonium bicarbonate buffer, pH 7.4, and treated with 1.0 μg/ml Phosphatidylinositol-Phospholipase C (PI-PLC) for 6 h at 37°C. The suspension was then extracted with 200 ml chloroform/methanol/1N HCL (200:100:1), and the upper aqueous phase was aspirated, evaporated to remove methanol, and purified by sequential chromatography on DEAE-Cellulose, C-18 reversed phase resin, QAE-Sephadex, SAX HPLC, and P-2 gel filtration [29]. The enzyme-modulating activity was monitored by the stimulation of the membrane-bound rat adipocyte cAMP phosphodiesterase [31]. The generation of the inositol glycan was dependent upon PI-PLC and was identical to that produced by insulin in membranes or intact cell [30,32].

Statistics

Data were analyzed by Students paired t test where indicated.

RESULTS

Previous studies have shown that insulin may regulate certain metabolic pathways of intermediary metabolism through dephosphorylation of key rate-limiting enzymes [14]. To determine whether phosphatases play a role in modulating insulin-stimulated glycogen synthesis, the effect of insulin on H-35 hepatoma cells was assayed in the presence of increasing concentrations of okadaic acid. Okadaic acid produced a concentration-dependent decrease in insulin-stimulated synthesis of glycogen from [^{14}C]glucose (Fig. 1). An IC_{50} for this inhibition was calculated to be 400 nM okadaic acid, with maximal inhibition occurring at approximately 1 μM. Basal glycogen synthesis was unaffected by the addition of okadaic acid to the cells (data not shown).

The effect of okadaic acid on insulin-stimulated lipogenesis was also evaluated in H-35 hepatoma cells. The stimulation of lipogenesis by insulin in these cells was also inhibited by okadaic acid in a dose-dependent manner (Fig. 1). Half-maximal inhibition was observed at 50 nM okadaic acid, and the effect was maximal between 100 and 500 nM. The addition of okadaic acid alone to hepatoma cells had no effect on lipid synthesis (data not shown). These results are consistent with the increased phosphorylation and subsequent inactivation of acetyl CoA carboxylase by okadaic acid [20].

A comparison of the effect of okadaic acid on insulin-stimulated synthesis of glycogen and lip-

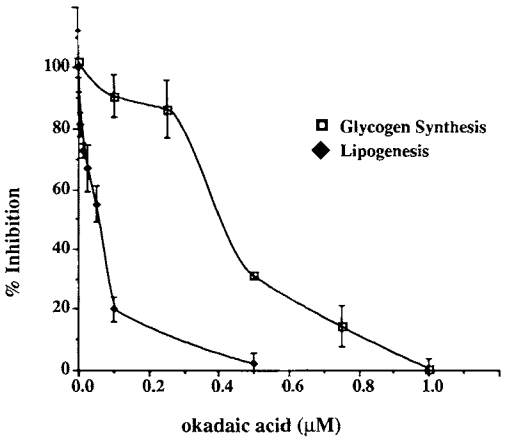

Fig. 1. The stimulation of lipogenesis and glycogen synthesis by insulin in H-35 hepatoma cells is differentially sensitive to okadaic acid. Serum-deprived cells were preincubated for 10 min with 10 nM insulin in the presence and absence of varying concentrations of okadaic acid, followed by incubation for 2 h in reaction mixture containing 11 mM [^{14}C]glucose. Glycogen synthesis was assayed by the incorporation of [^{14}C]glucose into glycogen. (Basal activity = 1.4 nmol glycogen/mg-min; insulin-stimulated activity = 2.43 nmol glycogen/mg-min). For lipogenesis experiments, serum-deprived cells were preincubated with 10 nM insulin in the presence or absence of varying concentrations of okadaic acid, followed by incubation with 5 mM [^{14}C]glucose for 30 min at 37°C. Lipogenesis was assayed by measuring the incorporation of [^{14}C]glucose into lipids. (Basal activity = 25 ± 7 dpm, insulin stimulated = 1,460 ± 52 dpm). Results were repeated in three (glycogen synthesis) or five (lipogenesis) separate experiments, and are expressed as the percent of maximal stimulation by insulin in the absence of okadaic acid, presented as the mean ± SD of triplicate determinations.

ids from [^{14}C]glucose in hepatoma cells revealed significant differences (Fig. 1). The apparent sensitivity of lipogenesis to okadaic acid was greater than that for glycogen synthesis, perhaps indicating the involvement of different protein phosphatases.

Numerous studies have indicated a role for an inositol phosphate glycan (IPG), in mediating some of the metabolic actions of insulin through changes in protein phosphorylation [33]. This modulator was shown to modify the activities and phosphorylation states of a number of insulin-sensitive enzymes, assayed in cell-free systems [30,34–36], as well as mimic several metabolic actions of insulin in intact cells, including lipogenesis [29] and antilipolysis [37]. To determine whether the stimulation of lipogenesis by the IPG is also inhibited by okadaic acid, hepatoma cells were treated with insulin or IPG, in the presence and absence of okadaic acid, and the incorporation of [^{14}C]acetate into lipids was measured (Table I). Okadaic acid (100 nM) significantly attenuated insulin-stimulated lipogenesis (141 ± 54 dpm, $P < 0.0005$) compared to the control in the absence of okadaic acid (474 ± 85 dpm). The toxin similarly reduced IPG-stimulated lipogenesis to basal levels (238 ± 34 dpm vs. 93 ± 7 dpm, $P < 0.0005$), which were unaffected by the addition of okadaic acid to cells.

The effect of okadaic acid on insulin-regulated lipogenesis and lipolysis was also examined in isolated rat adipocytes. The addition of okadaic acid to fat cells attenuated insulin-stimulated lipogenesis (Fig. 2). This inhibition exhibited an apparent IC_{50} of 1.7 μM, and was maximal at 2.2 μM. Okadaic acid alone had no effect on basal lipogenesis (data not shown). This is consistent with a previous report [20], in which the inactivation of acetyl-CoA carboxylase by okadaic acid paralleled that of the toxin on insulin-stimulated lipogenesis. The requirement for higher concentrations of okadaic acid to attain inhibition of insulin-stimulated lipogenesis in adipocytes probably reflects cell-specific differences in solubility. It is likely that the high neutral lipid content of fat cells may result in decreased potency of the lipid soluble compound, due to sequestration of the okadaic acid in cellular lipids.

TABLE I. Effect of Okadaic Acid on Insulin and IPG-Stimulated Lipogenesis in H-35 Hepatoma Cells

	Conversion of [^{14}C]acetate into lipids (dpm)	
Addition	Control	Okadaic acid
None	148 ± 16	139 ± 36
Insulin	474 ± 85	141 ± 54*
IPG	238 ± 34	93 ± 7*

Cells were preincubated for 15 min at 37°C with 2 nM insulin or IPG in the presence and absence of 100 nM okadaic acid, followed by incubation for 30 min with reaction mixture containing 5 mM [^{14}C]acetate. Lipogenesis was assayed by measuring incorporation of [^{14}C]acetate into lipids. Results are expressed as the means ($n = 6$) ± standard deviation.

*$P < 0.0005$, significantly different from control values (based on paired t-test).

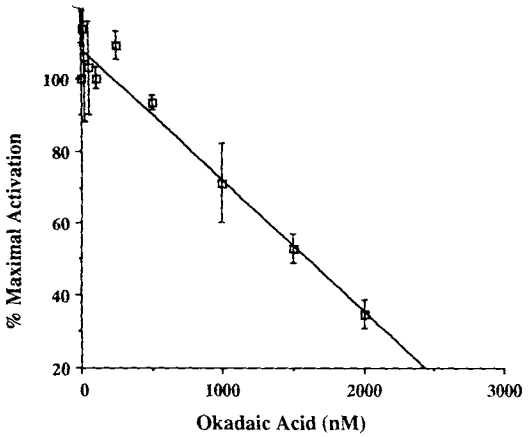

Fig. 2. Okadaic acid inhibits insulin-stimulated lipogenesis in rat adipocytes. Cells were incubated for 60 min at 37°C in a reaction mixture containing 0.2 nM insulin and 2 mM [^{14}C]glucose in the presence of the indicated concentrations of okadaic acid. Lipogenesis was assayed by measuring the incorporation of [^{14}C]glucose into lipids. Basal activity = 974 dpm; insulin-stimulated activity = 8,095 dpm. The results are expressed as percent of maximal activation by insulin in the absence of okadaic acid and are presented as the mean ± SD of triplicate determinations. Results were repeated in more than five separate experiments.

In order to explore the mechanism of inhibition of insulin-stimulated lipogenesis by the toxin in greater detail, the effect of okadaic acid on the insulin concentration response was examined in rat adipocytes. In the absence of okadaic acid, the incorporation of [^{14}C]glucose into lipids was stimulated by increasing concentrations of insulin. The half-maximal effect was observed at approximately 0.02 nM and was maximal at approximately 5 nM (Fig. 3). Addition to cells of 1.25 μM okadaic acid caused a reduction in the maximal response to insulin, without any significant effect on the EC_{50}.

Because hormone-sensitive lipase undergoes dephosphorylation with concomitant inactiva-

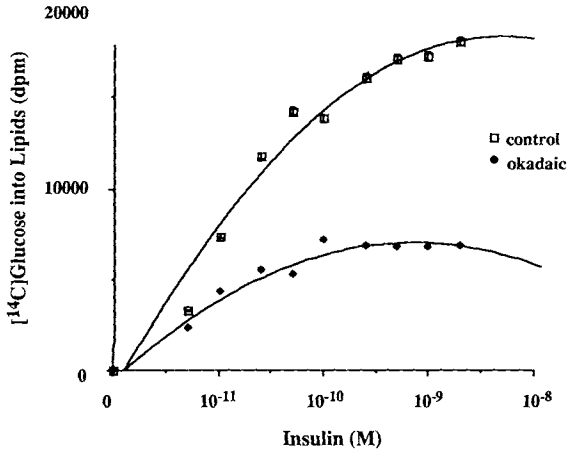

Fig. 3. Okadaic acid reduces the maximal lipogenic response to insulin in rat adipocytes. Cells were incubated for 60 min at 37°C with 500 μM [^{14}C]glucose, at the indicated concentrations of insulin, in the presence (◆) and absence (□) of 1.25 μM okadaic acid. Lipogenesis was assayed as described in Figure 4. Results are expressed as dpm [^{14}C]glucose incorporated into lipids and are presented as the mean ± SD of triplicate determinations. Results were repeated in three separate experiments.

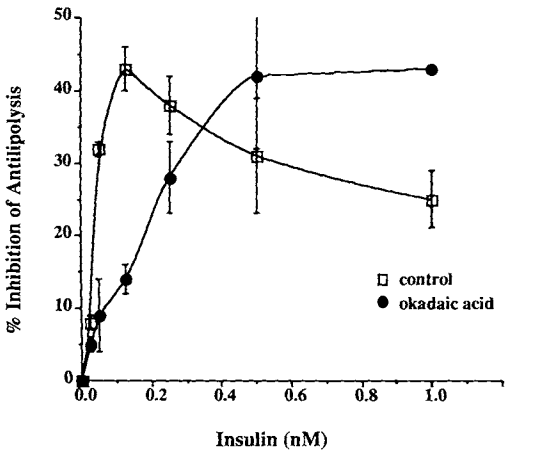

Fig. 5. Okadaic acid reduces the sensitivity of the antilipolytic response to insulin in rat adipocytes. Cells were exposed to the indicated concentrations of insulin in the presence (●) and absence (□) of 150 nM okadaic acid for 10 min at 37°C, followed by the addition of isoproterenol (50 nM). After 30 min, glycerol release was assayed. Results are expressed as percent inhibition of antilipolysis in the absence of okadaic acid and are presented as the mean of triplicate determinations in which error was less than 5%. Results were repeated in three separate experiments.

tion in response to insulin [12], the effect of okadaic acid on isoproterenol-activated lipolysis was evaluated in the presence and absence of insulin in rat adipocytes. Okadaic acid blocked the antilipolytic effect of insulin in isolated rat adipocytes (Fig. 4). Half-maximal inhibition of the antilipolytic effect by insulin was attained at approximately 150 nM okadaic acid, with complete inhibition at 500 nM. In the absence of insulin, up to 1 μM okadaic acid had no detectable effect on isoproterenol-stimulated lipolysis.

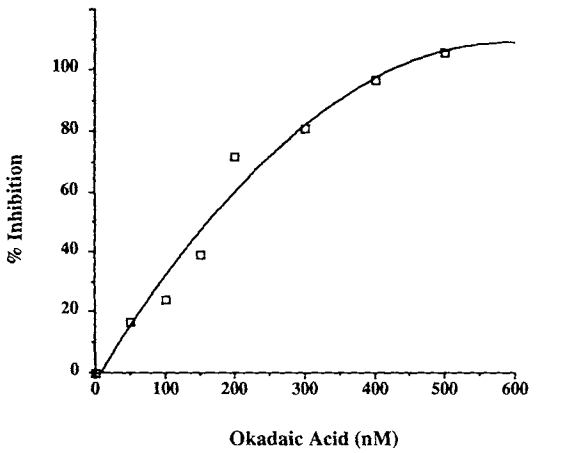

Fig. 4. Okadaic acid attenuates insulin-stimulated antilipolysis in rat adipocytes. Cells were preincubated at 37°C for 10 min with 0.2 nM insulin in the presence of the indicated concentrations of okadaic acid, followed by the addition of isoproterenol (50 nM). Following a 30 min incubation, the rate of lipolysis was evaluated by assay of glycerol release into the incubation medium. The results are expressed as percent inhibition of insulin-stimulated antilipolysis in the absence of okadaic acid and are presented as the mean of triplicate determinations in which error was less than 5%. Results were repeated in four separate experiments.

To further evaluate the mechanism by which okadaic acid attenuates the antilipolytic effect of insulin, the effect of the toxin on the insulin dose response was examined (Fig. 5). Insulin exerted a biphasic effect on lipolysis. The maximal inhibition of isoproterenol-stimulated lipolysis (45%) was observed at 0.15 nM insulin and declined at higher concentrations, consistent with previous reports [38–41]. Interestingly, addition to cells of 0.15 μM okadaic acid caused a rightward shift in the dose response curve, increasing the concentration of insulin required for maximal inhibition to approximately 1 nM. In contrast to the effect of okadaic acid on insulin-stimulated lipogenesis, the maximal inhibition of lipolysis induced by insulin (45%) was unchanged by addition of okadaic acid. However, the biphasic nature of the dose response for the antilipolytic effect of insulin makes it difficult to draw meaningful conclusions regarding these kinetic differences. Moreover, another difference was noted by a comparison of the dose response for the inhibitory effect of okadaic acid-treated rat adipocytes on insulin-stimulated lipo-

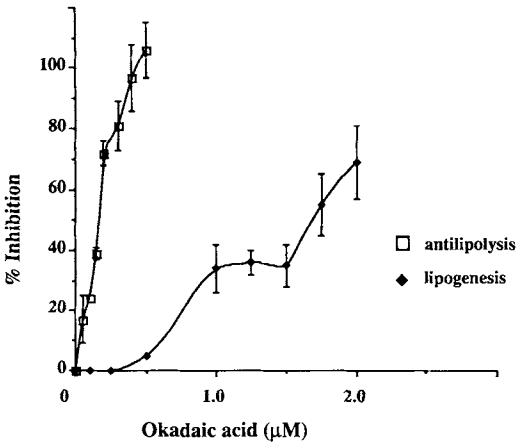

Fig. 6. Insulin-stimulated lipogenesis (□) and antilipolysis (◆) are differentially inhibited by okadaic acid in rat adipocytes. Both of the results here are expressed as percent inhibition of insulin-stimulated lipogenesis or antilipolysis assayed in the absence of okadaic acid.

genesis and antilipolysis (Fig. 6). The antilipolytic response to insulin was considerably more sensitive to inhibition by okadaic acid (IC_{50} = 0.150 μM) than was insulin-stimulated lipogenesis (IC_{50} = 1.7 μM), although the different sensitivities to insulin of these two activities makes meaningful comparisons of the effects of okadaic acid difficult.

DISCUSSION

Although the precise molecular mechanisms of signal transduction in insulin action remain to be elucidated, there is general agreement regarding an important role for changes in protein phosphorylation in at least some of the actions of the hormone. While the tyrosine kinase activity of the insulin receptor itself may be essential for the full expression of insulin's actions [42–44], it is likely that the regulation of key enzyme activities downstream from the receptor are regulated by changes in the phosphorylation of serine or threonine residues [14]. One of the first clues towards understanding the pleiotropic nature of insulin action arose from the apparent paradoxical effect of the hormone on protein serine/threonine phosphorylation. Early reports [13,14] noted that insulin simultaneously produces both the dephosphorylation of some proteins, and the phosphorylation of others. These opposite effects on protein phosphorylation occur over a similar time and concentration range but have not been linked mechanistically. Although the enhanced dephosphorylation of proteins such as glycogen synthase [1,2], hormone-sensitive lipase [12], pyruvate dehydrogenase [9,10], and others [14] appears to play a central role in the acute regulation of carbohydrate and lipid metabolism, by modulating the activity of these key rate-limiting enzymes, the functional consequence of enhanced protein phosphorylation of proteins such as ribosomal S6 [15], ATP-citrate lyase [16], acetyl-CoA carboxylase [17,18], and others [13,14] by insulin is unclear.

We have utilized okadaic acid as a probe to distinguish metabolic actions of insulin that are primarily regulated by phosphatases or kinases. This toxin is a powerful inhibitor of type 1 and 2A, and to a lesser extent type 2B phosphatases [19], but has no effect on type 2C. The compound is ineffective in inhibiting pyruvate dehydrogenase phosphatase [20], protein tyrosine phosphatase [19], acid and alkaline phosphatases [19], and inositol triphosphatase [19] and does not regulate protein ser/thr kinases [19,20,45]. The toxin promotes myosin light chain phosphorylation [22], increases smooth muscle contraction [22], increases the formation of maturation-promoting factor and germinal vesicle breakdown in *Xenopus laevis* oocytes [23], and activates tyrosine hydroxylase [21] and MAP kinase in PC-12 cells [24]. Recent studies have shown that the toxin enhances phosphorylation of many proteins in adipocytes and hepatocytes and prevents many of the acute anabolic effects of insulin [20]. Our data on inhibition of insulin-stimulated lipogenesis and glycogen synthesis in hepatoma cells, and insulin-stimulated lipogenesis and antilipolysis in fat cells, confirm the latter studies, which indicate the involvement of specific phosphatases in mediating insulin action. Moreover, as described previously [20], we have observed that okadaic acid potentiates to some extent the stimulation of glucose transport by insulin (unpublished observation). Additionally, the stimulation of amino acid uptake by insulin in BC_3H1 cells was unaffected by okadaic acid (unpublished observation). The differential sensitivities of these insulin-stimulated metabolic processes to okadaic acid is a further indication of the complexity of insulin signaling, possibly suggesting that while some effects of the hormone, such as glucose or amino acid uptake may not involve protein phosphatases, other processes (i.e., glycogen and lipid synthesis) may be regulated by distinct subtypes of phosphatases. Alternatively, the same subtype of phosphatase may modulate key enzymes in-

volved in these pathways, yet the functional significance of a specific phosphatase may vary in each pathway.

The selective inhibition of insulin action by okadaic acid suggests that the pleiotropic nature of insulin action may reflect, to some extent, differences in the regulation of protein phosphorylation states. These and other observations have led to the proposal that two basic pathways, one to activate specific protein serine/threonine phosphatases and a second to activate protein serine/threonine kinases, may mediate many of insulin's actions. Although it has been difficult to demonstrate substrate specificity for the phosphatases and kinases that are presumably regulated by insulin, it is likely that compartmentalization mechanisms exist in cells to allow for the induction by insulin of a complex pattern of protein phosphorylations and dephosphorylations.

ACKNOWLEDGMENTS

We thank Philip Fishman and Melissa Caccam for excellent technical assistance and Dr. David Misek for valuable advice. This study was supported by a fellowship to S.L.H. (D5-005) from the New York State Health Research Council, DHS grant DK33804 from the NIH, and a grant from the New York Heart Association.

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