Hormone-sensitive Cyclic GMP-inhibited Cyclic AMP Phosphodiesterase in Rat Adipocytes

REGULATION OF INSULIN- AND cAMP-DEPENDENT ACTIVATION BY PHOSPHORYLATION*

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In $^{32}PO_4$-labeled adipocytes, isoproterenol (ISO) or physiologically relevant concentrations of insulin rapidly increased phosphorylation of a particulate 135-kDa protein which has been identified as a cGMP-inhibited "low K_m" cAMP phosphodiesterase (CGI-PDE) by several criteria, including selective immunoprecipitation with anti-CGI-PDE IgG (Degerman, E., Smith, C. J., Tornqvist, H., Vasta, V., Belfrage, P., and Manganiello, V. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 533–537). The time courses and concentration dependences for phosphorylation of CGI-PDE by ISO and insulin correlated with CGI-PDE activation in the presence of these agents; effects of ISO were somewhat more rapid than those of insulin. Adenosine deaminase, which metabolizes the adenylate cyclase inhibitor adenosine, also rapidly induced phosphorylation and activation of CGI-PDE. Phenylisopropyladenosine (an adenosine deaminase-resistant adenosine analog) prevented or reversed both adenosine deaminase-stimulated phosphorylation and activation of CGI-PDE ($IC_{50} \approx 0.2$ nM). Incubation of adipocytes with 0.1 nM insulin in the presence of ISO rapidly produced 30–200% greater activation and phosphorylation of CGI-PDE than the expected added effects of insulin and ISO individually; both effects preceded the insulin-induced decreases in protein kinase A activity and inhibition of lipolysis. These and other results indicate that CGI-PDE can be phosphorylated at distinct sites and activated by cAMP-dependent and insulin-dependent serine kinase(s), that the activation state of CGI-PDE reflects its relative phosphorylation state, and that synergistic phosphorylation/activation of CGI-PDE may be important in the antilipolytic action of insulin.

In rat adipocytes, lipolysis is promoted by agents that increase intracellular cyclic AMP (cAMP) and antagonized by agents that decrease synthesis and/or increase degradation of cAMP (1). Insulin is a physiologically important and potent inhibitor of lipolysis (1). Although description of the molecular mechanisms involved in the antilipolytic action of insulin is incomplete, several studies suggest that insulin activation of the adipocyte hormone-sensitive, cilostamide- and cGMP-inhibited particulate, "low K_m" cAMP phosphodiesterase (CGI-PDE)[1] (2) is an important component (3–6). Insulin and ISO produce a transient and synergistic activation of rat adipocyte CGI-PDE, which temporally correlates with insulin-induced reduction in ISO-activated protein kinase A and inhibition of lipolysis (3). Recent studies indicate that in intact adipocytes incubated with ISO or insulin, CGI-PDE is phosphorylated on serine site(s), presumably via ISO activation of protein kinase A and insulin activation of an unidentified intracellular serine protein kinase, respectively (7).

The studies reported herein demonstrate time- and concentration-dependent relationships between hormone-induced, reversible phosphorylation and activation of the CGI-PDE in rat adipocytes. Our results indicate that ISO (or adenosine deaminase) increases CGI-PDE activity via activation of protein kinase A and that the activation state of CGI-PDE is regulated by phosphorylation and dephosphorylation. Insulin-dependent phosphorylation and activation do not require but may be enhanced by elevated cAMP, consistent with the view that synergistic phosphorylation and activation of CGI-PDE by insulin and ISO are important in insulin-mediated inhibition of lipolysis. To our knowledge, this report is the first direct demonstration of interactive hormonal regulation of a PDE via distinct phosphorylations in intact cells.

EXPERIMENTAL PROCEDURES

Rat Adipocyte Preparation—Adipocytes from overnight-fasted male Sprague-Dawley rats were prepared by the method of Honnor *et al.* (8) in buffers containing 200 nM adenosine and 2 mM glucose. Washed adipocytes (2–4% packed cells/medium (v/v)) were incubated with hormones and other agents for the indicated times in 3 ml of Krebs-Ringer-HEPES (KRH) buffer containing 2 mM glucose, 200 nM adenosine, 4% BSA as described (3). Since there can be consid-

[1] The abbreviations used are: CGI-PDE, cGMP-inhibited low K_m cAMP phosphodiesterase; anti-CGI-PDE, immunoglobulin G fraction of a polyclonal antibody raised in rabbits against the cGMP-inhibited low K_m cAMP phosphodiesterase from bovine adipose tissue; BSA, bovine serum albumin; $C_{13}E_{12}$, heterogeneous, nonionic alkyl polyoxyethylene glycol detergent; EGTA, [ethylenebis(oxyethylenenitrile)] tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ISO, isoproterenol; $K_{0.5}$, concentration for half-maximal activation or phosphorylation; KRH, Krebs-Ringer-HEPES buffer; NLS, *N*-lauroylsarcosine; PBS, phosphate-buffered saline; PIA, N^6-phenylisopropyladenosine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid.

erable variation in adenosine production and/or accumulation in different adipocyte preparations, in some experiments adipocytes were incubated with adenosine deaminase (0.5–1 units/ml), with or without the adenosine deaminase-resistant adenosine analog PIA. Incubations were terminated by homogenization; CGI-PDE was assayed (with 0.5 μM [^{3}H]cAMP as substrate) in particulate fractions (100,000 × g, 60 min), and protein kinase A or glycerol was assayed in supernatant fractions (14,000 × g, 30 min) as previously described (3, 8). Except where noted, results are presented as the mean ± S.E. of values from three to eight different cell preparations (range for n = 2).

^{32}P-Labeling of Adipocytes—Adipocytes were washed twice (500 × g, 30 s) in five to seven volumes of KRH buffer containing 1 mM KH_2PO_4 and 1% BSA, and twice in KRH buffer containing 100 μM KH_2PO_4 and 1% BSA. Adipocytes ($\approx$15% cells/total cell suspension (v/v)) in KRH buffer with 100 μM KH_2PO_4 and 1% BSA were incubated with ^{32}PO$_4$ (10–40 μCi/ml) for 2 h (37 °C, 60-cycle shaking speed) and allowed to float for 2 min, before aspiration of the infranatant. Cells were resuspended in KRH buffer with 100 μM KH_2PO_4 and 4% BSA containing ^{32}PO$_4$ (same specific activity as before washing), pipetted into 25-ml flasks (final volume, 3 ml), and incubated (15 min, 140-cycle shaking speed) before addition of hormones. Incubations were terminated by homogenization of flask contents in 3 ml of TES/sucrose buffer (3) containing 50 mM NaF, 10 mM sodium pyrophosphate, 3 mM benzamidine, 10 μg/ml each pepstatin A and leupeptin, 1 mM EDTA, 0.1 mM EGTA. Particulate fractions (100,000 × g, 60 min) from duplicate incubations (total of $\approx$0.25 ml of packed cells) were combined and solubilized in 0.6 ml of solubilization buffer (50 mM Tris HCl (pH 7.4 at 4 °C), 5 mM MgCl$_2$, 1 mM EDTA, 0.1 mM EGTA, 3 mM benzamidine, 10 μg/ml each leupeptin and pepstatin A, 100 mM NaBr, 50 mM NaF, 10 mM sodium pyrophosphate, 1% (v/v) $C_{13}E_{12}$ detergent). Greater than 85% of the total CGI-PDE activity assayed in the presence of solubilization buffer was recovered as described (7).

Since the anti-CGI-PDE IgG inhibits PDE activity (9), CGI-PDE activity or protein was assayed in solubilized membranes from ^{32}P-labeled cells that were subsequently processed for immunoprecipitation. Protein content of the solubilized membrane fraction did not precisely reflect adipocyte protein, since the amount of BSA in the membrane fraction was high relative to adipocyte membrane protein. Since basal activity of CGI-PDE assayed in the detergent- and salt-containing solubilization buffer was almost twice that in membrane fractions suspended in TES/sucrose/2% BSA/KRH, but stimulated activity was similar, the relative effects of hormones on CGI-PDE activity in solubilized preparations were somewhat smaller than those previously observed (2, 3).

Preparation of Staphylococcus aureus Protein A Reagent—Formalin-fixed S. aureus cells (2.5 g of Sigma P-9151) were manually suspended with a Potter-Elvehjem Teflon pestle in 25 ml of SDS-wash buffer (100 mM Tris-HCl, pH 7.0, 2% SDS, 20 mM dithiothreitol), boiled for 10 min and centrifuged (10 min, 4 °C, SS-34 rotor, 10,000 rpm). The supernatant was discarded, and the wash procedure was repeated eight times. The pellets were washed at 25 °C (three times in 3% Triton X-100, phosphate-buffered saline (PBS), 0.1% N-lauroylsarcosine (NLS) and five times in PBS, 0.1% NLS), suspended in 80 ml of PBS/0.1% NLS ($\approx$10% slurry), and stored at -20 °C (28). Just before use, the slurry was centrifuged (5 min, 14,000 × g) and the PBS/NLS buffer was removed from the protein A pellet.

Immunoprecipitation of Particulate CGI-PDE—To minimize nonspecific precipitation of proteins, solubilized particulate fractions were treated twice (15 min, 4 °C) with protein A ($\approx$300 μl of 10% protein A slurry per 600 μl of solubilized membrane) and centrifuged (5 min, 14,000 × g). Supernatants (plus 1 mM dithiothreitol) were incubated overnight with sufficient anti-CGI-PDE to precipitate >80% of CGI-PDE activity (7), transferred to a fresh protein A pellet, mixed vigorously, and incubated (4 °C, 15–30 min) to form an insoluble immune complex. The immunoprecipitates were collected by centrifugation (15 min, 4 °C, 14,000 × g) and washed five times in 1 ml of PBS/NLS before solubilization for SDS-PAGE (8% gels). Autoradiography was carried out with dried gels on Kodak X-Omat XAR film with an intensifying screen at -70 °C for 2–8 days. As discussed previously (7), and in the experiments carried out during the course of this work, ^{32}P-labeled $\sim$44- and $\sim$116-kDa bands were sometimes immunoprecipitated along with the 135-kDa CGI-PDE. The appearance of these $\sim$44- and $\sim$116-kDa bands was unpredictable and variable (sometimes barely detectable as in Fig. 1). Film exposures of varying intensity for each experiment were analyzed by scanning densitometry (7).

Estimated Stoichiometry of Hormone-induced Phosphorylation of CGI-PDE—Adipocytes (prelabeled in KRH containing 300 μM KH_2PO_4 with 0.4–1.4 mCi/ml ^{32}PO$_4$) were incubated with maximally effective concentrations of ISO (300 nM for 3 min) or insulin (0.3 nM for 12 min). Cells were homogenized, particulate fractions were isolated and solubilized, and CGI-PDE (0.20–0.25 pmol per condition) was immunoprecipitated and subjected to SDS-PAGE as described above. The ^{32}P-labeled 135-kDa band was cut from the gel and quantitatively (80–95% recovery of counts/min) electroeluted in an ISCO concentrating apparatus (3 h/1 watt in 10 mM NH$_4$HCO$_3$ electrode buffer; the cup buffer was supplemented with 0.1% SDS, 1 mM dithiothreitol). The specific activity of cellular ATP was estimated to be $\sim$50% of the extracellular medium ^{32}PO$_4$ based on the results of Denton and Hopkirk (10); enzyme mass was estimated from the enzymatic activity of the adipocyte particulate fraction relative to the specific activity of purified CGI-PDE (2). This estimate of mass could be affected by a number of factors, i.e. if activity in crude particulate fractions were inhibited and/or the pure enzyme were inhibited or activated by proteolysis (19).

Materials—Prestained high molecular weight standards for SDS-PAGE were purchased from Bethesda Research Laboratories; prestained β-galactosidase from Diversified Biotech (Neston Centre, MA); collagenase A (1088–785) from Boehringer Mannheim; DDA from Pharmacia LKB Biotechnology Inc. Sources of other materials are published (3, 7).

RESULTS

Effects of Insulin or Isoproterenol on Phosphorylation and Activation of CGI-PDE—In previous studies with ^{32}P-labeled rat adipocytes, ISO or insulin induced phosphorylation of a 135-kDa protein, which was selectively immunoprecipitated from solubilized particulate fractions by anti-CGI-PDE and identified as native CGI-PDE (7). The concentration dependences for hormone-induced phosphorylation of the immunoprecipitated CGI-PDE are shown in Fig. 1. The $K_{0.5}$ values for ISO- and insulin-induced phosphorylation of CGI-PDE were estimated at 2.5 ± 0.7 nM (n = 2; Fig. 1B) and 7 ± 6 pM (n = 5; Fig. 1C) respectively, which are similar to (insulin) or somewhat lower (ISO) than the previously reported values for hormonal activation of CGI-PDE in intact adipocytes (3). During incubation of [^{32}P-labeled adipocytes for 12 min, maximally effective concentrations of ISO or insulin increased phosphorylation of CGI-PDE to 6.4 ± 1.4 (n = 6) or 8.4 ± 1.2 (n = 8) times basal, respectively. In the same ^{32}P-labeled adipocyte preparations, ISO increased CGI-PDE activity to 1.84 ± 0.07 (n = 3) and insulin increased activity to 1.54 ± 0.06 (n = 4; cf. Ref. 3) times basal values (=1.0). In two other experiments, the stoichiometry of phosphorylation induced by ISO was estimated at 0.54 and 1.3 mol of ^{32}PO$_4$/mol of CGI-PDE and that by insulin was 1.1 and 1.4.

As shown in Fig. 2, a maximally effective concentration of ISO (100 nM) produced a rapid increase in phosphorylation of CGI-PDE. In some preparations, ISO-induced phosphorylation of CGI-PDE was half-maximal within 1–1.5 min and was relatively constant (varying between 80–100% maximal effect) between 2 and 12 min (Fig. 2, lower panel). In the same experiments, ISO-induced activation of CGI-PDE was maximal within 1–1.5 min, after which activity was essentially constant at least until 12 min (Fig. 2, upper panel). In other experiments (with five different time points), however, CGI-PDE activity declined slightly after 2 min and then was relatively stable between 4 and 12 min.

Adenosine deaminase activates adenylate cyclase in rat adipocytes by removal of the inhibitory ligand adenosine (8). Exposure of adipocytes to maximally effective concentrations of adenosine deaminase (1 unit/ml) was associated with a rapid (<2 min) activation of protein kinase A (Ref. 8, data not shown) and, as shown in Fig. 3, rapid phosphorylation and activation of CGI-PDE (4.2 ± 1.0 times basal labeling in five preparations; Ref. 11). The nonhydrolyzable adenosine
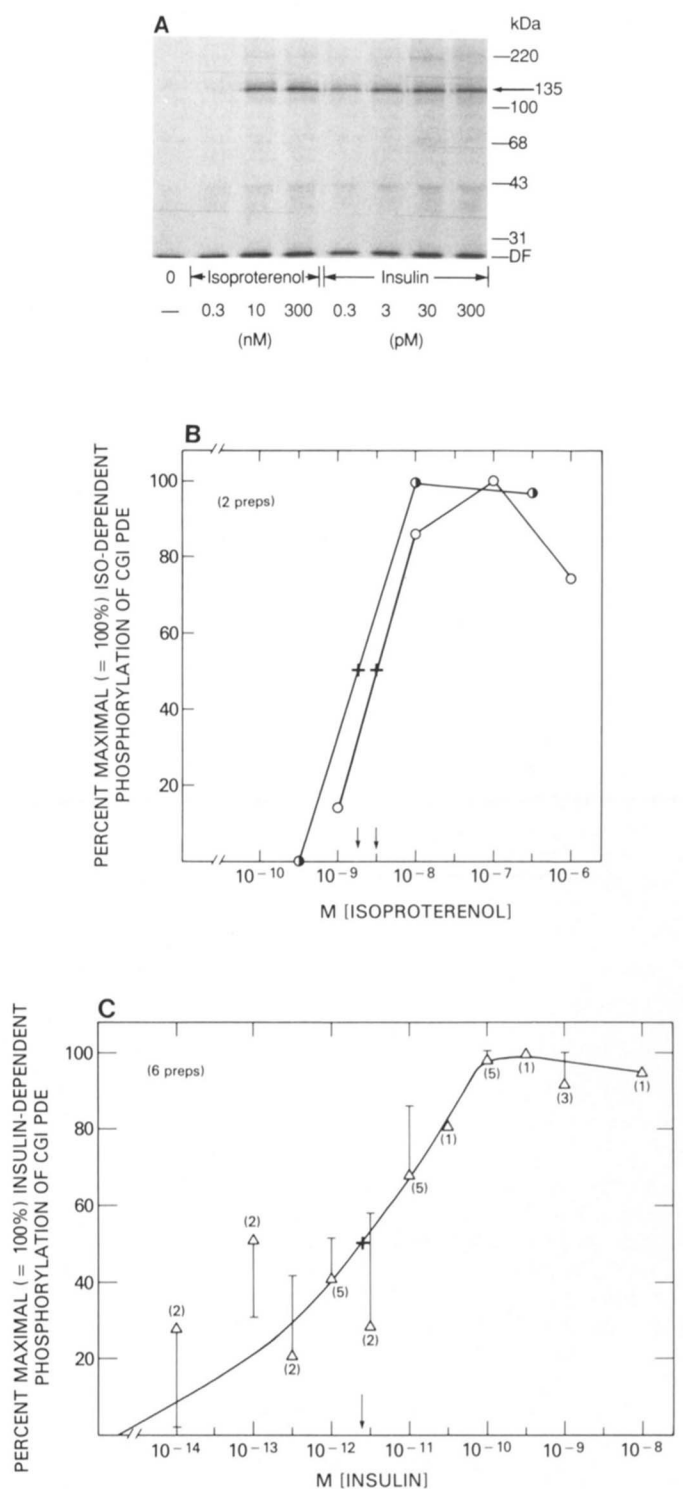
FIG. 1. **Concentration dependence for insulin or β-agonist-induced phosphorylation of the 135-kDa CGI-PDE.** ^{32}P-labeled adipocytes were washed and incubated in duplicate for 12 min as described under "Experimental Procedures," with or without the indicated concentrations of ISO or insulin before homogenization, immunoprecipitation of the 135-kDa CGI-PDE protein, and SDS-PAGE/autoradiography. *A*, positions of standard proteins (kDa) are indicated; *DF*, dye front. Maximal labeling (~8 times basal in this experiment) of the 135-kDa ^{32}P-labeled CGI-PDE was produced by 10 nM ISO or 0.3 nM insulin; effects of 0.3, 3, 30 pM insulin were 42, 58, and 86% of the maximal effect, respectively. Autoradiogram is from a representative experiment repeated several times (*B, C*). *B*, ISO-induced phosphorylation of the 135-kDa ^{32}P-labeled CGI-PDE (two experiments). The maximal increase in agonist-dependent labeling (normalized to the percent maximal effect, after subtraction of basal labeling) was 15-fold in the *open circle* experiment. *C*, insulin-
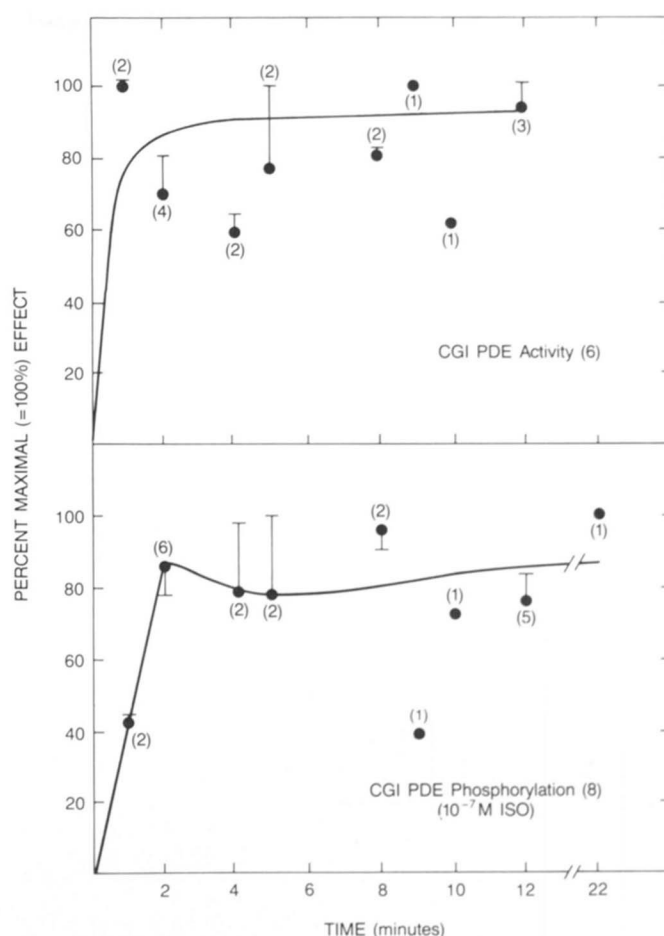
FIG. 2. **Time course of ISO-induced phosphorylation/activation of CGI-PDE.** ^{32}P-labeled adipocytes were incubated as described under "Experimental Procedures" for the indicated times with 100 nM ISO before homogenization of cells. CGI-PDE activity was assayed in the solubilized particulates (*upper panel*) in six out of the eight preparations that were further processed for SDS-PAGE/autoradiography of immunoprecipitated particulate proteins. Basal conditions were defined by either 200 nM adenosine or 3 nM PIA + 1 unit/ml adenosine deaminase; basal values were subtracted before normalizing ISO effects to the maximal effect (=100%) for each experiment. In all preparations, the effects at 2 or 5 min were compared to one point in the 9–12-min range, as well as 1, 4, and 8 min in two individual experiments. Data at each time point are the means ± S.E. (or ± range of two) experiments, of the total of eight different cell preparations.

analog PIA (Fig. 3) and other adenylate cyclase inhibitors (prostaglandin E_1, nicotinic acid or 2',5'-dideoxyadenosine), as well as the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine HCl, blocked the stimulatory effect of adenosine deaminase on CGI-PDE activity and lipolysis (8).[2] However, during incubations of adipocytes for 15–25 min, adenosine deaminase-induced phosphorylation/activation of CGI-PDE (Fig. 3) and activation of protein kinase A (not shown; Ref. 8) declined somewhat more rapidly (as much as 40%) than they did in the presence of ISO (Fig. 2; Refs. 3, 11, 12)). Within 2 min, ISO and adenosine deaminase, alone or in combination, produced maximal increases in protein kinase A activity (3)[2] and, at this early time point, increased

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[2] C. J. Smith and V. C. Manganiello, unpublished data.
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induced phosphorylation of CGI-PDE. Results are from six different preparations in which four to six concentrations of insulin were incubated with adipocytes for 12 min. In *parentheses* are numbers of values represented by mean ± S.E. (or range of *n* = 2).
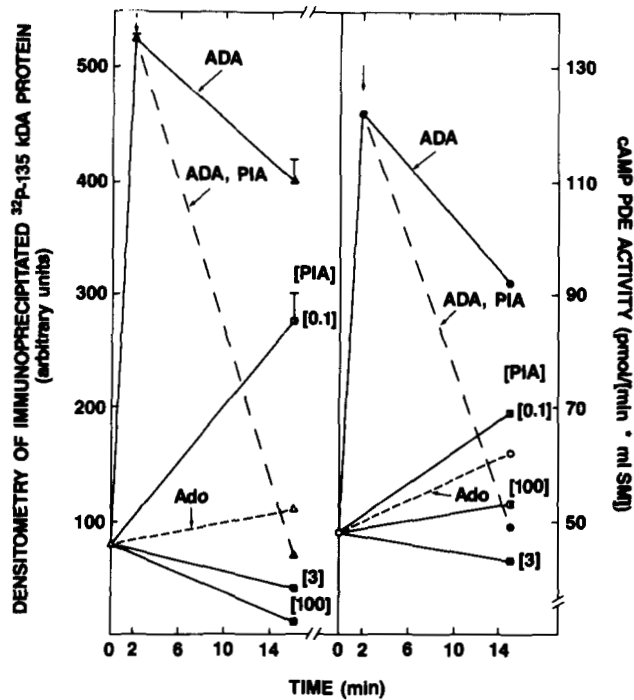
FIG. 3. **Effects of adenosine deaminase and PIA on phosphorylation and activation of CGI-PDE.** ^{32}P-labeled adipocytes were washed and incubated (with ^{32}PO$_4$/100 μM KH$_2$PO$_4$) in duplicate for the indicated times in the presence of 200 nM adenosine (*Ado*). Adenosine deaminase (*ADA*, 1 unit/ml) was added to some incubations as indicated by the *filled* or *half-filled symbols*; PIA was present with adenosine deaminase at 0.1, 3, or 100 nM (PIA concentrations indicated in *brackets* next to the *half-filled squares* at 14 min). To some samples, 100 nM PIA was added 2 min after adenosine deaminase (*dashed line* after *arrow* leading to *half-filled symbol*). Cells were homogenized after 2 or 14 min before preparation of particulate fractions for assay of CGI-PDE and ^{32}P content of the immunoprecipitated 135-kDa CGI-PDE. *Left,* scanning densitometry (arbitrary units after exposure for 3 days) of the immunoprecipitated CGI-PDE; *Right,* CGI-PDE activity of solubilized particulate fractions assayed (in duplicate) before immunoprecipitation. Results are from a representative experiment repeated several times with similar results.

phosphorylation and activation of CGI-PDE to the same extent (data not shown). These latter data suggest that these two effectors may induce phosphorylation of the same site(s) via activation of protein kinase A.

Reversibility of cAMP-dependent Phosphorylation of CGI-PDE—As also seen in Fig. 3, adenosine deaminase stimulation of phosphorylation and activation of CGI-PDE were both prevented or reversed in a dose-dependent manner by PIA (IC$_{50}$ ~ 0.1–0.3 nM, range of two experiments). In ^{32}P-labeled adipocytes (continuously incubated with ^{32}PO$_4$), addition of 100 nM PIA after a 2-min exposure to adenosine deaminase decreased both stimulated CGI-PDE activity and ^{32}P content (Fig. 3), consistent with dephosphorylation of the CGI-PDE. In two experiments with ^{32}P-labeled adipocytes (incubated for 20 min with various agents), the β-blocker propranolol (3 or 10 μM) completely prevented (added 5 min before ISO) or reversed (added 5 min after ISO) the phosphorylation of CGI-PDE induced by 30 nM ISO (data not shown).

Effects of Insulin on the Time Course of Phosphorylation and Activation of CGI-PDE—In the presence of insulin (0.1 nM), the time courses of maximal effects on phosphorylation and activation of CGI-PDE (Fig. 4) were somewhat slower than those with ISO (Fig. 2) or adenosine deaminase (Fig. 3). When CGI-PDE phosphorylations induced by ISO (100 nM) or insulin (0.1 nM) were compared in the same cell prepara-
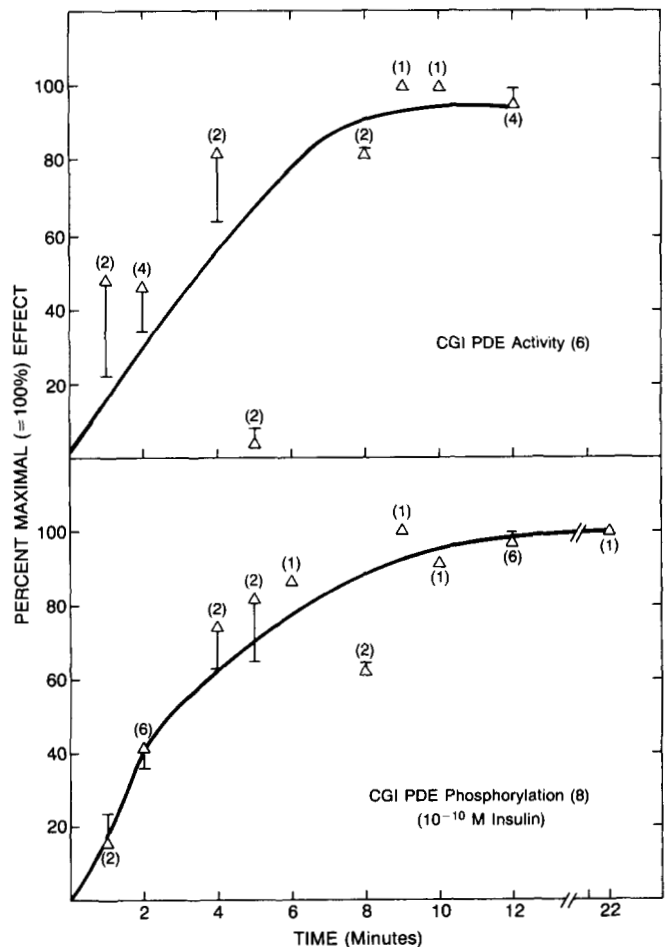

FIG. 4. **Time course of insulin-induced phosphorylation/activation of CGI-PDE.** ^{32}P-labeled adipocytes were incubated as described under "Experimental Procedures" for the indicated times with 0.1 nM insulin before homogenization of cells; data are presented as described for Fig. 2 where six out of the eight preparations destined for CGI-PDE immunoprecipitation (*lower panel*) were also assayed for enzyme activation (*upper panel*).

tions, the effects of insulin were less than those of ISO at 2–5 min (71 ± 9% of ISO; $n = 7$), but as reported (7), were similar to those of ISO by 9–12 min (98 ± 23% of ISO; $n = 6$; see Ref. 7).

Effects of Insulin on Phosphorylation and Activation in the Presence of ISO—Incubation of rat adipocytes with ISO and insulin was associated with a rapid and synergistic activation of CGI-PDE, which temporally correlated with insulin-induced reduction in ISO-stimulated protein kinase A (3). The combination of insulin and ISO not only evoked increases in CGI-PDE activity that were greater than the added effects of each alone, but also produced synergistic phosphorylation of CGI-PDE (Fig. 5, Table I). After 2–5 min with ISO plus insulin, the increase in particulate CGI-PDE activity was almost twice and phosphorylation was two to three times the added effects of the agents (Fig. 5, Table I). Incubation of adipocytes for 2–9 min with ISO plus insulin resulted in ~50–100% larger PDE activation and 30–200% greater phosphorylation than the added effects of each agent alone (Table I), consistent with an earlier report (3). The synergism is more pronounced before 12 min (Table I); effects of ISO and insulin on phosphorylation were approximately additive after 12–16 min (not shown).

The synergistic interaction between insulin and a lipolytic agent was not apparently dependent upon a β-agonist-induced

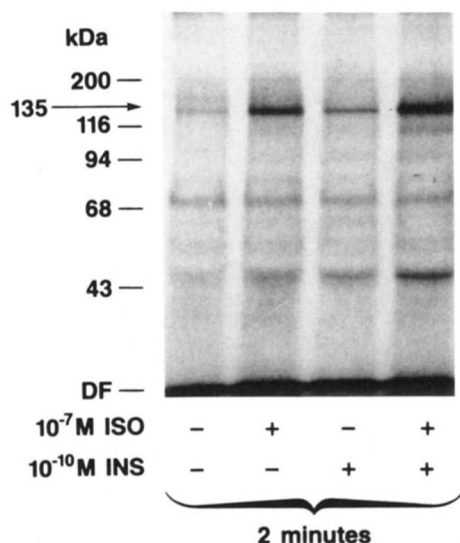net CGI PDE (0) 36 11 67
net ^{32}P label (0) 316 110 556

FIG. 5. **Phosphorylation/activation of CGI-PDE in the presence of ISO and insulin.** ^{32}P-labeled adipocytes were washed and incubated with 3 nM PIA + 1 unit/ml adenosine deaminase, with or without 0.1 nM insulin (*INS*), 100 nM ISO or ISO + insulin for 2 min as indicated before preparation of particulate fractions for assay of particulate CGI-PDE, and ^{32}P content of the 135-kDa CGI-PDE. *Net CGI-PDE* is the difference in activity from the basal (*BAS*) value at 2 min, which was 56 ± 2 pmol of cAMP hydrolyzed/(min ml solubilized membrane); mean ± range of duplicate assays. ^{32}P-labeling of CGI-PDE quantified by scanning densitometry is reported relative to that of basal samples (=100%). Results are representative of those from several preparations (Table I). In additional experiments in which adipocytes were incubated for 5 min with ISO + insulin, the absolute levels of CGI-PDE phosphorylation and activation were 17 ± 8 ($n = 3$) and 2.5 ± 0.2 ($n = 3$) times basal values, respectively.

TABLE I

Synergistic effects of ISO and insulin on phosphorylation and activation of CGI-PDE

^{32}P-labeled adipocytes prepared as described in Fig. 5 were incubated with or without 100 nM ISO, 0.1 nM insulin, or both for indicated times. All incubations contained 3 nM PIA plus 1 unit/ml adenosine deaminase. CGI-PDE activities were assayed in solubilized membranes before immunoprecipitation of ^{32}P-labeled 135-kDa CGI-PDE. In each of five individual experiments illustrated, basal activity (or ^{32}P-labeling) was subtracted from other values and the sum of the net effect of ISO and insulin alone was considered 100%. Synergistic activation/phosphorylation of CGI-PDE in adipocytes incubated simultaneously with ISO and insulin was observed when the actual effects produced by agonists in combination exceeded the expected additive (100%) value.

Expt.	Time	CGI-PDE activity	^{32}P-labeled 135-kDa
	min	%	
I	2	222	222
II	5	209	320
III	2	315	231
	9	98	131
IV	2	143	130
	12	92	165
V	5	248	155
	12	103	103

increase in cAMP, since similar but more rapid (<90 s) effects on CGI-PDE were observed with adenosine deaminase + insulin. Like the ISO experiments, the adenosine deaminase + insulin-induced effects on CGI-PDE phosphorylation/ac-

tivation preceded insulin-induced reductions in protein kinase A and lipolysis (not shown).

DISCUSSION

In order to further our understanding of the molecular mechanisms involved in activation of CGI-PDE by insulin and lipolytic effectors that increase cAMP, we purified the enzyme from rat (2) and bovine adipose tissues (9). Using antibody raised against the CGI-PDE purified from bovine adipose tissue (9), a phosphorylated 135-kDa protein which copurified with CGI-PDE (7) was immunoprecipitated from insulin- or ISO-treated ^{32}P-labeled rat adipocytes. These initial studies identified the 135-kDa protein as native CGI-PDE and suggested that serine phosphorylation of CGI-PDE was a regulatory mechanism shared by both insulin and ISO. In the present studies, the close relationship between phosphorylation and activation of CGI-PDE with regard to pharmacologic specificity, time course, hormone concentration dependence, and synergistic interactions indicates that activation most likely results from increased phosphorylation induced by insulin or lipolytic (cAMP-elevating) agents.

It is generally believed that insulin, by interacting with and activating its membrane receptor, triggers a cascade of events resulting in activation of an unidentified intracellular serine kinase(s) (13), which, as we currently propose, catalyzes phosphorylation and activation of CGI-PDE. Insulin induces phosphorylation and activation of CGI-PDE without changes in protein kinase A activity (3). ISO, which activates adenylate cyclase, and adenosine deaminase, which metabolizes the adenylate cyclase inhibitor adenosine, both increase cAMP as well as stimulate phosphorylation and activation of particulate CGI-PDE via protein kinase A (Figs. 2 and 3; Refs. 3, 8, 11, 12). Insulin and ISO (or adenosine deaminase) synergistically accelerate and enhance phosphorylation and activation of CGI-PDE. These effects precede the insulin-induced reduction in ISO-stimulated protein kinase A activity and inhibition of lipolysis. The synergism between insulin and ISO, which may be important in the antilipolytic action of insulin (3), is consistent with multiple phosphorylations of the same CGI-PDE molecule.

ISO and adenosine deaminase, by increasing cAMP and rapidly activating protein kinase A, may cause phosphorylation of the same site(s) on CGI-PDE. In addition, the adenosine deaminase- and ISO-stimulated phosphorylation of CGI-PDE in intact cells is reversible (Fig. 3) in parallel with changes in CGI-PDE activity. The CGI-PDE is apparently in close functional proximity to both protein kinase A (14) and protein phosphatase(s), since termination of protein kinase A activation (8, 12) by inhibition of adenylate cyclase (3, 15) leads to a rapid dephosphorylation and deactivation of CGI-PDE (Fig. 3). These results, together with recent reports of a stimulatory effect of protein kinase A on CGI-PDE activity in adipocyte microsomal membranes (16) and on phosphorylation and activation of an immunoprecipitated 110-kDa platelet cytosolic CGI-PDE (17, 18), demonstrate that CGI-PDE itself is the target of protein kinase(s) and phosphatase(s) and that cAMP-dependent phosphorylation of CGI-PDE is closely related to its activation.

The concentration dependence for insulin- or ISO-induced phosphorylation is somewhat more sensitive than that for activation of CGI-PDE and may reflect the relative ease in measuring phosphorylation due to the much larger phosphorylation signal (up to 40-fold) as compared with the activation signal (<3-fold) of the adipocyte particulate CGI-PDE. Similar results with respect to cAMP-induced phosphorylation and activation of the CGI-PDE in platelets were reported

by Macphee *et al.* (17). Kono and colleagues (19, 20) have found that variations in homogenization of adipocytes (pH, temperature, EDTA, redox state) or incubation of adipocyte microsomal membranes (detergent solubilization, salts, dithiothreitol, cGMP) can dramatically mimic *or* reduce the apparent relative magnitude of hormonal activation of the CGI-PDE. Perturbations of adipocyte membranes during preparation may account, in part, for the apparently small percentage of activation of the CGI-PDE by hormones in intact adipocytes and by protein kinase A *in vitro* (16).

Whereas phosphorylation of CGI-PDE induced by insulin and/or cAMP in adipocytes, or by cAMP in platelets (17, 18), does coincide with the increase in CGI-PDE activity, phosphorylation of the adipocyte CGI-PDE in the presence of insulin *plus* cAMP is more persistent than the transient synergistic effects on CGI-PDE activity (Table I; Ref. 3). Maximally effective concentrations of insulin or ISO each increased phosphorylation of CGI-PDE to the same extent, but activation was consistently greater with ISO ($\approx$100%) than insulin ($\approx$ 50%) as also reported earlier (3, 7). In addition, ISO-induced activation of CGI-PDE was apparently maximal (in ~1 min) somewhat before maximal CGI-PDE phosphorylation (Fig. 2). Thus, although these results suggest that ISO, adenosine deaminase, and insulin activation of CGI-PDE are associated with phosphorylation of CGI-PDE, a precise quantitative or stoichiometric relationship between the two effects has not been established. This will require isolation and characterization of CGI-PDE phosphopeptides (*i.e.* identification of sites and quantification of phosphorylation regulated by cAMP and insulin alone and in combination). Like the hormonal regulation of glycogen synthase (21–23), ATP citrate lyase (24), microtubule-associated protein kinase (25), or the glycogen-binding phosphatase subunit (26), the sequence and extent of phosphorylation of different sites in the CGI-PDE may be complex, and be different for different effectors or combinations thereof. It is also possible that some phosphorylation sites are not directly involved in regulation of activity (*i.e.* are "silent" phosphorylations), or that certain phosphorylations (perhaps catalyzed by unidentified kinases) directly or indirectly limit or reduce the activation of CGI-PDE. It will be important to compare hormone-dependent phosphorylations in intact adipocytes with *in vitro* labeling (and activation) of CGI-PDE by various protein kinases; these latter studies will be especially valuable for identifying the insulin-dependent serine kinase(s) (7, 13) presumably responsible for insulin-mediated activation (27) of particulate CGI-PDE in intact cells.

In summary, our results suggest that distinct serine kinases activated by low concentrations of insulin or ISO promote rapid phosphorylation and activation of the same CGI-PDE in rat adipocytes. In the presence of insulin and β-agonist, which may be present together *in vivo*, there is a rapid, synergistic phosphorylation and activation of CGI-PDE which may be important in the antilipolytic action of insulin. Whether and how other kinases and phosphatases may influence CGI-PDE remains to be established. The adipocyte CGI-PDE may represent a model system in which to study certain acute insulin-signaling mechanism(s) which modulate cAMP-dependent intracellular effector systems, *i.e.* cAMP-mediated regulation of hormone-sensitive lipase in adipose tissue.

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