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 32PO4-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 Km" 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 adenylyl 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 (IC50 ≈ 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

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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 Km" cAMP phosphodiesterase (CGI-PDE) (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 sites(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 Km cAMP phosphodiesterase; anti-CGI-PDE, immunoglobulin G fraction of a polyclonal antibody raised in rabbits against the cGMP-inhibited low Km cAMP phosphodiesterase from bovine adipose tissue; BSA, bovine serum albumin; C8E10, heterogenous, nonionic alkyl polyoxyethylene glycol detergent; EGTA, [ethylenbis-(oxy-

ethyl)enitrile]] tetraacetic acid; HEPES, N-2-hydroxyethylpipera-

zine-N'-2-ethanesulfonic acid; ISO, isoproterenol; Km, concentration for half-maximal activation or phosphorylation; KRH, Krebs-Ringer-

HEPES buffer; NLS, N-lauroylsarcosine; PBS, phosphate-buffered saline; PIA, N6-phenylisopropyladenosine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, N-

tris(hydroxymethyl) methyl-2-aminothanesulfonic acid.

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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 mM [3H]cAMP as substrate) in particulate fractions (100,000 \( \times g \), 60 min), and protein kinase A or glycerol was assayed in supernatant fractions (14,000 \( \times g \), 30 min) as previously described (3, 8). Except where noted, results are presented as the mean \pm S.E. of values from three to eight different cell preparations (range for \( n = 3 \) to 8).

\[ \text{3P-labeling of Adipocytes—Adipocytes were washed twice (500 \( \times g \), 30 s) in five to seven volumes of KRH buffer containing 1 mM KH2PO4 and 1% BSA, and twice in KRH buffer containing 100 \( \mu \)M KH2PO4 and 1% BSA. Adipocytes (\( \pm 15\% \) cells/total cell suspension \( /v/v\) ) in KRH buffer with 100 \( \mu \)M KH2PO4 and 1% BSA were incubated with \( \text{32P-PO}_4 \) (0.1–10 cpm/ml) for 2 h (37 \( ^\circ \)C, 60-h cycle shaking speed) and allowed to float for 2 min, before aspiration of the infranatant. Cells were resuspended in KRH buffer with 100 \( \mu \)M KH2PO4 and 4% BSA containing \( \text{3}^{32} \text{P-PO}_4 \) (same specific activity as washing), pipetted into 25-ml flasks (final volume, 3 ml), and incubated (15 min, 140-cycling shaking speed) before addition of hormones. Incubations were terminated by homogenization of cell contents in 3 ml of TES/sucrose buffer (5) containing 50 mM NaF, 10 mM sodium pyrophosphate, 3 mM benzamidine, 10 \( \mu \)g/ml each peptatin A and leupeptin, 1 mM EDTA, 0.1 mM EGTA. Particulate fractions (100,000 \( \times g \), 60 min) from duplicate incubations (total of \( \pm 20\% \) of cell suspension) were solubilized in 0.6 ml of solubilization buffer (50 mM Tris HCl, pH 7.4 at 4 \( ^\circ \)C, 5 mM MgCl2, 1 mM EDTA, 0.1 mM EGTA, 3 mM benzamidine, 10 \( \mu \)g/ml each leupeptin and peptatin A, 100 mM NaBr, 50 mM NaF, 10 mM sodium pyrophosphate, 1% (\( /v/v\) ) C6H12O6 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 \( \text{3}^{32} \text{P-labelled} \) cells that were subsequently processed for immunoprecipitation. Protein content of the solubilized membrane fraction did not preclude protein precipitation, 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—For

malin-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/water/2% SDS, 20 mM dithiothreitol, boiled for 10 min and centrifuged (10 min, 4 \( ^\circ \)C, 30,000 rpm). The supernatant was discarded, and the wash procedure was repeated eight times. The pellets were washed at 25 \( ^\circ \)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 50 ml of PBS/0.1% NLS (\( \approx 10^9 \) slurry), and stored at -20 \( ^\circ \)C (28).

Just before use, the slurry was centrifuged (5 min, 14,000 \( \times g \)) and the PBS/NLS buffer was removed from the protein A pellet.

Immunoprecipitation of Particulate CGI-PDE—To minimize non-
specific precipitation of proteins, solubilized particulate fractions were centrifuged twice (15 min, 4 \( ^\circ \)C) with 2% SDS, 20 \( \mu \)l dithiothreitol, and 1 ml of 100 mM Tris HCl, pH 7.4, 1% (\( /v/v\) ) C6H12O6 detergent. Protein A slurry per 600 \( \mu \)l of solubilized membrane) and centrifuged (5 min, 14,000 \( \times g \)). Supernatants (plus 1 ml 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 \( ^\circ \), 20–30 min) to form an insoluble immune complex. The immunoprecipitates were collected by centrifugation (15 min, 4 \( ^\circ \), 14,000 \( \times g \)) 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 -80 \( ^\circ \)C. The discussion was published (7), and in the experiments carried out during the course of this work, \( \text{3}^{32} \text{P-labeled} \) and -116-kDa bands were sometimes immunoprecipitated along with the 135-kDa CGI-PDE. The appearance of these -44- and -116-kDa bands was unpredictable and variable (sometimes barely detectable as in Fig. 1). Film exposure of varying intensities for each experiment were analyzed by scanning densitometry (7).
Regulation of Adipocyte Hormone-sensitive CGI Phosphodiesterase

A

Regulation of Adipocyte Hormone-sensitive CGI Phosphodiesterase

B

Fig. 1. Concentration dependence for insulin or β-agonist-induced phosphorylation of the 135-kDa CGI-PDE. 32P-labeled adipoctyes 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 32P-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 32P-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-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. 2. Time course of ISO-induced phosphorylation/activation of CGI-PDE. 32P-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 E1, 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). 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) and, at this early time point, increased

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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 (100 nM; range of two experiments). In 32P-labeled adipocytes (continuously incubated with 32PO4−), addition of 100 nM PIA after a 2-min exposure to adenosine deaminase decreased both stimulated CGI-PDE activity and 32P content (Fig. 3), consistent with dephosphorylation of the CGI-PDE. In two experiments with 32P-labeled adipocytes (incubated with 32PO4−) 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 preparations, 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 9–12 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...
Fig. 5. Phosphorylation/activation of CGI-PDE in the presence of ISO and insulin. 

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**TABLE I**

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

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 (~100%) than insulin (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 mechanisms (7) which modulate cAMP-dependent intracellular effector systems, i.e., cAMP-mediated regulation of hormone-sensitive lipase in adipose tissue.

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REFERENCES