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Evidence that insulin and isoprenaline activate the cGMP-inhibited low-K_m cAMP phosphodiesterase in rat fat cells by phosphorylation

(lipolysis/cilostamide/immunoprecipitation/cAMP-dependent protein kinase)

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ABSTRACT **Incubation of intact rat fat cells with maximally effective concentrations of insulin (1 nM, 12 min) or isoprenaline (300 nM, 3 min) increased particulate cGMP- and cilostamide-inhibited, low-K_m cAMP phosphodiesterase (cAMP-PDE) activity by about 50% and 100%, respectively. In ^{32}P-labeled cells, these agents induced serine ^{32}P-phosphorylation of a 135-kDa particulate protein and, to a variable and lesser extent, a 44-kDa protein, which were selectively immunoprecipitated by anti-cAMP-PDE, as analyzed by SDS/PAGE and autoradiography. In the absence of hormonal stimulation, little phosphorylation was detected (<10% of that with the hormones). The two phosphoproteins were identified as cAMP-PDE or a closely related molecule (in the case of the 44-kDa species, perhaps a proteolytic fragment) since (*i*) amounts of ^{32}P in the immunoprecipitated 135-kDa protein paralleled enzyme inactivation, (*ii*) prior incubation of the anti-cAMP-PDE with the pure rat or bovine enzyme selectively blocked the immunoprecipitation of the phosphoproteins, (*iii*) 135- and 44-kDa proteins reacted with the anti-cAMP-PDE on Western immunoblots, and (*iv*) the two phosphoproteins copurified with cAMP-PDE activity through DEAE-Sephacel chromatography and were isolated by highly selective affinity chromatography on cilostamide-agarose. Thus, in fat cells, catecholamine- and insulin-induced activation of the cAMP-PDE may be mediated via phosphorylation by cAMP-dependent protein kinase and an insulin-activated serine protein kinase, respectively.**

Although a number of cyclic nucleotide phosphodiesterases (PDEs) have been purified and extensively characterized, little is known concerning the detailed molecular mechanisms for regulation of this complex group of enzymes in intact cells (1). One type, the membrane-associated, cGMP- and cilostamide-inhibited, low-K_m cAMP phosphodiesterase (hereafter referred to as cAMP-PDE) is activated by insulin and, paradoxically, catecholamines and other agents that increase cellular cAMP in adipose tissue and liver (2–6). The latter action most likely represents a feedback mechanism whereby cAMP modulates its own turnover (4, 7). In isoprenaline-stimulated rat fat cells, insulin-induced activation of cAMP-PDE seems to correlate with termination of the cAMP signal and reduction in cAMP-dependent protein kinase and thus may be important in the antilipolytic action of insulin (8).

In both cases, hormone-induced activation of the cAMP-PDE seems to involve covalent modification of the enzyme, since the activation persists through several steps of enzyme purification (9–11). In rat adipose tissue and human platelets, the catecholamine/cAMP-mediated activation of cAMP-PDE apparently may involve phosphorylation of the enzyme by cAMP-dependent protein kinase (12–16). In this report, we demonstrate that, in isolated fat cells, both isoprenaline and insulin induce serine phosphorylation of the cAMP-PDE under conditions that activate the enzyme. From these results it may be inferred that insulin-induced activation of an unidentified serine protein kinase leads to phosphorylation/activation of cAMP-PDE.

MATERIALS AND METHODS

Materials. Specific polyclonal rabbit antibodies were raised against cAMP-PDE purified from bovine adipose tissue (17). IgG fractions purified by chromatography on *Staphylococcus aureus* protein A-Sepharose Cl-4B (Pharmacia) are referred to as anti-cAMP-PDE. Anti-phosphotyrosine monoclonal antibodies immobilized on Sepharose 4B were a generous gift from R. Frackelton (Brown University, Providence, RI). Formalin-fixed *Staph. aureus* (Cowan strain, which produces protein A) was from Sigma; $^{32}P_i$ (HCl-free, carrier-free) and [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), from the Radiochemical Centre, Amersham. Sources of other materials are in refs. 11 and 17.

cAMP-PDE Assay. cAMP-PDE activity was assayed for 12 min at 30°C in a total volume of 0.3 ml of 50 mM Hepes, pH 7.5/0.1 mM EDTA/8.3 mM $MgCl_2$/0.5 μM [^{3}H]cAMP (≈15,000 cpm) (18) without or with the cilostamide derivative OPC 3911 at 300 nM (17). PDE activity is reported as amount of cAMP hydrolyzed per min.

Incubations of ^{32}P-Labeled Fat Cells. Fat cells were prepared by collagenase digestion (8, 19) of epididymal fat pads from Sprague–Dawley rats (150–170 g) fasted overnight. Ten milliliters of a 5–8% (vol/vol) fat cell suspension in Krebs–Ringer Hepes, pH 7.40 (125 mM NaCl/5.0 mM KCl/2.5 mM $CaCl_2$/300 μM KH_2PO_4/2.5 mM $MgCl_2$/25 mM Hepes) containing 200 nM adenosine/2 mM glucose/1% (wt/vol) bovine serum albumin was incubated with $^{32}P_i$ (0.6–1.4 mCi/ml of cell suspension) for 105 min at 37°C. The incubation medium was replaced with one devoid of $^{32}P_i$ but containing 4% bovine serum albumin, followed by addition of insulin or isoprenaline for the indicated times. Incubations were terminated by addition of 10 ml of ice-cold 50 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate (Tes) buffer, pH 7.0/250 mM sucrose/1 mM EDTA/0.1 mM EGTA/3 mM benzamidine/10 mM sodium pyrophosphate/50 mM NaF/7.5 μg of pepstatin per ml/5 μg of leupeptin per ml, followed immediately by homogenization. Particulate

Abbreviations: cAMP-PDE, cGMP-inhibited, low-K_m cAMP phosphodiesterase; anti-cAMP-PDE, specific polyclonal rabbit antibodies, IgG fraction; $C_{13}E_{12}$, heterogeneous, nonionic alkyl polyoxyethylene glycol detergent; CIT-agarose, agarose-immobilized derivative of cilostamide, a specific inhibitor of cAMP-PDE.

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fractions were prepared by centrifugation of the homogenate (50,000 × *g*, 90 min, 4°C). In experiments measuring cAMP-PDE activities $^{32}P_i$ was omitted.

Immunoprecipitation of Solubilized High-Speed Pellet [^{32}P]Phosphoproteins with Anti-cAMP-PDE and Subsequent SDS/PAGE. Particulate fractions, which contained >75% of total homogenate cAMP-PDE (measured as the OPC 3911-inhibitable activity at 0.5 μM [^{3}H]cAMP), were homogenized in 0.7 ml of solubilization buffer (50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/1 mM EDTA/0.1 mM EGTA/100 mM NaBr/50 mM NaF/1% $C_{13}E_{12}$/3 mM benzamidine/10 μg of leupeptin per ml/5 μg of antipain per ml/1 μg of pepstatin per ml) ($C_{13}E_{12}$ is a heterogeneous nonionic alkyl polyoxyethylene glycol detergent). After 1 hr (4°C), supernatants (10,000 × *g*, 15 min, 4°C) from control and hormone-treated preparations, containing >90% of the particulate cAMP-PDE activity, were obtained for immunoprecipitation of phosphoproteins.

To reduce background ^{32}P the supernatants were incubated (15 min, 4°C) with fixed *Staph. aureus* cells (twice with 70 μl of a 10% slurry per 0.7 ml of supernatant) and centrifuged (10,000 × *g*, 5 min, 4°C) before incubation (12 hr, 4°C) with anti-cAMP-PDE (0.2 μg of IgG per pmol/min of cAMP-PDE activity) or the corresponding amount of preimmune IgG. The immunocomplexes were precipitated with *Staph. aureus* (2 μl of a 10% slurry per μg of anti-cAMP-PDE, 15 min, 4°C) and collected by centrifugation. This immunoisolation procedure was developed with purified rat and bovine adipose tissue cAMP-PDE (11, 17) phosphorylated by the catalytic unit of cAMP-dependent protein kinase in the presence of 5 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP. Proportional amounts of cAMP-PDE and IgG were chosen to ensure immunoprecipitation of >90% of [^{32}P]cAMP-PDE from solubilized fat cell membranes. The efficiency of the immunoprecipitation procedure was also monitored by measuring cAMP-PDE activity remaining in the immunosupernatants from the cell samples; in all cases <15% of initial activity remained. Prior incubation of anti-cAMP-PDE with pure antigen (≈1 ng of rat or bovine cAMP-PDE per μg of anti-cAMP-PDE) for 6 hr at 4°C prevented the antibody from immunocomplexing with the ^{32}P-labeled cAMP-PDE.

Immunoprecipitated proteins were washed five times with 1 ml of 0.1 M sodium phosphate buffer, pH 7.5/1% *N*-lauroyl sarcosine, solubilized by boiling in 200 μl of 50 mM Tris·HCl, pH 6.8/2% SDS/2% (vol/vol) 2-mercaptoethanol/5% (vol/vol) glycerol, separated from the *Staph. aureus* by centrifugation, and subjected to SDS/PAGE in 8% gels (17). The gels were dried and autoradiographed with an intensifying screen at −70°C on Kodak X-Omat XAR film, which after development was scanned with an LKB Ultroscan.

RESULTS

Insulin- and Isoprenaline-Induced Activation and [^{32}P]Phosphorylation of cAMP-PDE in Fat Cells. In fat cells incubated with and without maximally effective concentrations of isoprenaline or insulin under the conditions used for activation of the cAMP-PDE (cf. ref. 8), $^{32}P_i$ was incorporated into a number of particulate phosphoproteins (Fig. 1*A*). Anti-cAMP-PDE selectively immunoprecipitated one phosphoprotein with a molecular mass of ≈135 kDa and a minor phosphoprotein with a mass of ≈44 kDa from cells that had been incubated with the hormones (Fig. 1*B*). Preimmune IgG immunoprecipitated negligible amounts of these ^{32}P-labeled phosphoproteins (<5% of that with the anti-cAMP-PDE) from isoprenaline- or insulin-treated cells (data not illustrated). In the absence of hormonal stimulation, little or no ^{32}P phosphorylation of these two proteins occurred; in 15 experiments the hormones induced a >10-fold increase in their phosphorylation. In six experiments, in which fat cells were incubated with maximally effective concentrations of insulin or isoprenaline under identical conditions, the two hormones increased the phosphorylation of the 135-kDa phosphoprotein to the same extent (relation 1.01 ± 0.20, mean ± SEM). The amount of the 44-kDa ^{32}P-labeled phosphoprotein varied considerably between experiments and it was sometimes absent (0–35% of the total immunoprecipitated phosphoprotein ^{32}P in eight experiments).

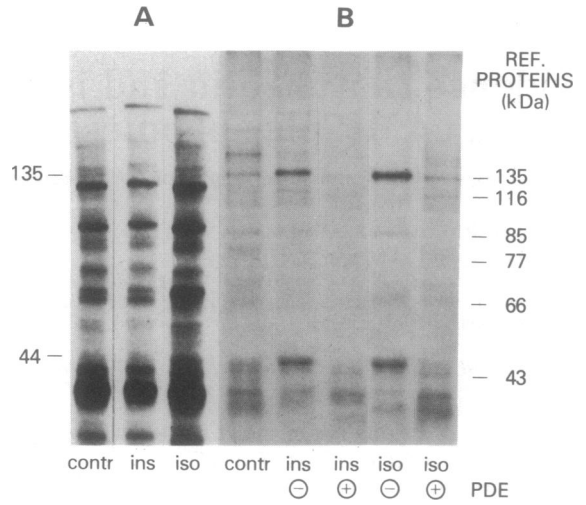

FIG. 1. Insulin- and isoprenaline-induced ^{32}P incorporation into fat cell high-speed pellet proteins immunoprecipitated by anti-cAMP-PDE. Rat fat cells, labeled with $^{32}P_i$, were incubated at 37°C with vehicle (contr), insulin (ins; 1 nM, 12 min), or isoprenaline (iso; 300 nM, 3 min). In four experiments, cAMP-PDE activity was increased 90–100% by isoprenaline and 40–50% by insulin (data not shown; see ref. 8). Particulate fractions were solubilized, and incubated at 100°C for 2 min in 2% SDS/1% 2-mercaptoethanol (*A*), or subjected to immunoprecipitation with anti-cAMP-PDE ⊖ or with antibody which had been blocked by preincubation with pure rat cAMP-PDE ⊕. The solubilized pellets and immunoprecipitates were subjected to SDS/PAGE followed by autoradiography of the dried slab gels. The data from the insulin- and isoprenaline-treated cells are not directly comparable, as ≈40% less protein from insulin-treated cells was applied to the SDS/PAGE gel. Positions of 135- and 44-kDa proteins (left) and of reference proteins (right) are indicated.

Immunoprecipitation of the 135-kDa [^{32}P]phosphoprotein species with increasing amounts of anti-cAMP-PDE closely paralleled the disappearance of cAMP-PDE activity from the pellet extract (data not shown). Furthermore, the immunoprecipitation of both phosphoprotein species was prevented by incubation of the anti-cAMP-PDE with pure rat (Fig. 1*B*) or bovine (data not shown) cAMP-PDE before addition of the ^{32}P-labeled particulate extracts. Blocking of the anti-cAMP-PDE with antigen did not prevent the occasional nonspecific coprecipitation of other ^{32}P-labeled phosphoproteins (mainly a 116-kDa phosphoprotein, which may represent ATP-citrate lyase, and an unidentified ≈42-kDa species).

In detergent extracts of particulate fractions from small-scale adipocyte preparations, 135-kDa material exhibited weak reactivity on Western immunoblots, presumably because the cAMP-PDE is of low abundance (<0.001% total adipocyte protein) as calculated from the specific activity and yield of the purified enzyme (11, 17). To enrich the 135-kDa protein (and cAMP-PDE), solubilized particulate fractions were partially purified by chromatography on DEAE-Sephacel. Whereas 96% of the total protein passed through the column, most (87%) of the cAMP-PDE activity was adsorbed (Fig. 2 *Left*). Over 60% was eluted with 225 mM NaBr, with much less activity recovered in the 100 mM NaBr (14%) and 450 mM NaBr (13%) eluates. In the Western immunoblot of these fractions, an immunoreactive 135-kDa band was observed in the solubilized particulate fraction, was adsorbed by DEAE-Sephacel and not visible in the pass-through, and

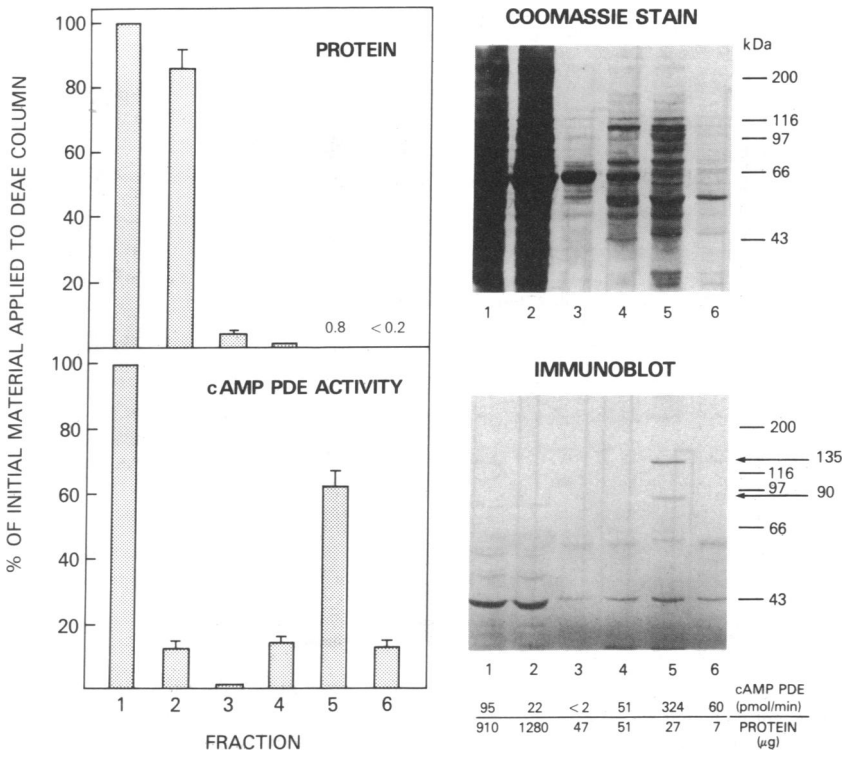

FIG. 2. Western immunoblot of partially purified cAMP-PDE. Solubilized particulate fractions were diluted with a modified solubilization buffer containing phosphatase inhibitors [50 mM Tris·HCl, pH 7.5/5.0 mM $MgCl_2$/1 mM EDTA/0.1 mM EGTA/10 μg of pepstatin per ml/10 μg of leupeptin per ml/3 mM benzamidine/20% (vol/vol) glycerol/0.16% $C_{13}E_{12}$/25 mM NaF/5 mM sodium pyrophosphate/50 mM NaBr] and then applied to a 0.4-ml DEAE-Sephacel column, equilibrated in the same buffer, containing 0.03% $C_{13}E_{12}$ and eluted successively with equilibration buffer containing 50 mM NaBr (4 ml), 100 mM NaBr (4 ml), 225 mM NaBr (2 ml), or 450 mM NaBr (2 ml). Portions of each fraction were assayed for protein content (Bio-Rad) and cAMP-PDE activity (*Left*) or precipitated with trichloroacetic acid for Western immunoblot (*Right*). (*Left*) Solubilized membranes (2.9 ± 0.4 mg of total protein; 397 ± 50 pmol/min cAMP-PDE activity) were applied to and eluted from DEAE-Sephacel. Results are normalized (original = 100%) and expressed as mean ± SEM (three experiments). Fractions: 1, solubilized membranes; 2, pass-through; 3, 50 mM NaBr wash; 4, 100 mM NaBr eluate; 5, 225 mM NaBr eluate; and 6, 450 mM NaBr eluate. (*Right*) The indicated fractions, as described above, from three DEAE columns were combined, precipitated with trichloroacetic acid (7.2%), solubilized with 300 μl of 2% SDS/125 mM Tris·HCl, pH 6.5/50 mM dithiothreitol/10% (vol/vol) glycerol/0.001% bromphenol blue, and heated (2 min, 100°C). Each sample was divided between two large (1.5 × 70 × 140 mm) 8% polyacrylamide gels for electrophoresis in SDS (176 V, 3 hr). After electrophoresis, gels were cut to minigel size (1.5 × 50 × 90 mm) for electrophoretic transfer (3 hr, 50 V) in 10 mM 3-(cyclohexylamino)propanesulfonic acid, pH 10/10% (vol/vol) methanol/1 mM dithiothreitol onto polyvinylidene difluoride membranes (Millipore) by using a Bio-Rad Mini-Protean II apparatus as described (20). One membrane (representing one-fourth of the trichloroacetic acid precipitates) was stained for protein with Coomassie blue R250. The second membrane (representing three-fourths of the trichloroacetic acid precipitates) was utilized for immunostaining with anti-cAMP-PDE as described previously (17). Listed are the amounts of cAMP-PDE activity and protein in the fractions used for immunoblotting.

copurified with cAMP-PDE activity (Fig. 2 *Right*). In the 225 mM NaBr eluate less immunoreactive bands of ≈90 kDa and ≈44 kDa also appeared. Several other immunoreactive bands, especially at ≈42 kDa, were observed in the solubilized particulate and pass-through (nonadsorbed) fractions. In these latter two fractions, however, as much as 0.9–1.2 mg of protein was precipitated with trichloroacetic acid and utilized for Western immunoblots, whereas the 225 mM NaBr fraction contained <30 μg of protein.

Detergent-solubilized particulate fractions from ^{32}P-labeled adipocytes incubated with insulin or isoprenaline were also purified by chromatography on DEAE-Sephacel and cilostamide (CIT)-agarose (11, 17). Both the 135-kDa and the 44-kDa (when present) ^{32}P-labeled phosphoprotein species copurified with cAMP-PDE activity (Fig. 3*A*) on DEAE-Sephacel. In these experiments ^{32}P-labeled 116-kDa protein was also immunoprecipitated with the 135-kDa phosphoprotein; in other experiments the 116-kDa phosphoprotein was only barely visible in immunoprecipitates of the DEAE eluate and was not observed after affinity chromatography on CIT-agarose (see below). No immunoreactive 116-kDa protein was observed in Western blots of partially purified cAMP-PDE (Fig. 2).

All cAMP-PDE activity (as measured by its absence in the pass-through) and both phosphoproteins were adsorbed to the CIT-agarose, and they were apparently bound more tightly than the nonphosphorylated, proteolytically nicked purified PDE (11), since they could not be eluted with 50 mM cAMP. Pure rat and bovine adipose tissue cAMP-PDE phosphorylated with the catalytic subunit of cAMP-dependent protein kinase were also adsorbed tightly to CIT-agarose and could not be eluted with 50 mM cAMP (E.D., unpublished data). A combination of cAMP and anti-cAMP-PDE released the adsorbed 135- and 44-kDa phosphoproteins, which were identified by SDS/PAGE (Fig. 3*B*) after precipitation of the eluted immunocomplexes with *Staph. aureus*. Since immunocomplexing inactivated the cAMP-PDE, the enzyme activity of the ^{32}P-labeled phosphoproteins after elution from CIT-agarose could not be assessed.

[^{32}P]Phosphoamino Acid Analysis. In particulate fractions from cells activated by insulin or isoprenaline, partial acid hydrolysis of the 135-kDa ^{32}P-labeled phosphoprotein followed by two-dimensional thin-layer electrophoresis revealed the presence of [^{32}P]phosphoserine, and no detectable [^{32}P]phosphotyrosine or [^{32}P]phosphothreonine (Fig. 4), whereas phosphorylated insulin receptor yielded [^{32}P]phos-

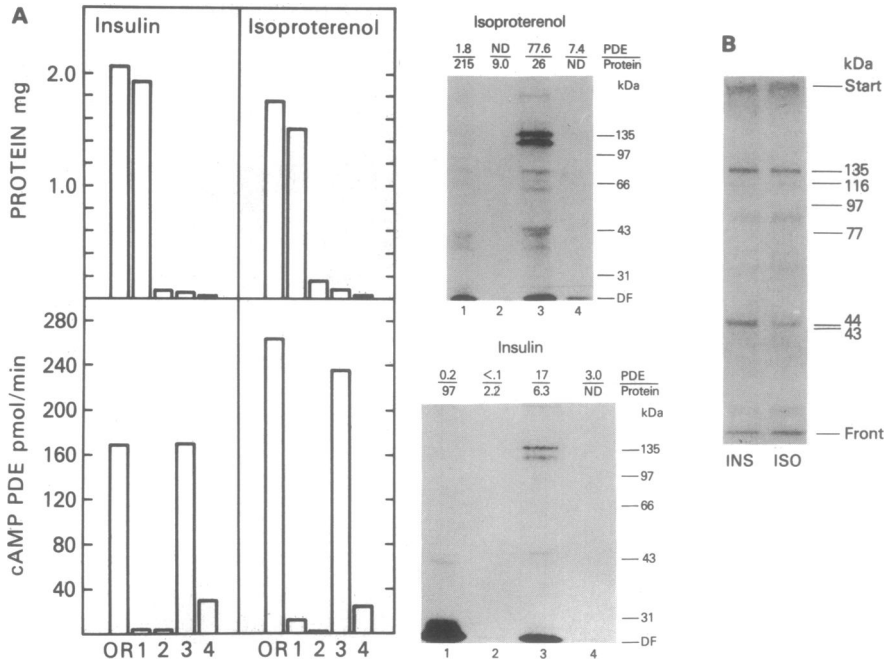

FIG. 3. Copurification of cAMP-PDE activity and 135/44-kDa phosphoproteins during DEAE-Sephacel chromatography, and isolation of the phosphoproteins by subsequent CIT-agarose affinity chromatography. Detergent-solubilized particulate fractions from ^{32}P-labeled insulin- or isoprenaline-treated fat cells, obtained as in Fig. 1, were applied to DEAE-Sephacel equilibrated in the modified solubilization buffer with reduced salt and phosphatase inhibitors, and eluted (cf. Fig. 2). (*A*) Portions were taken for assay of protein and cAMP-PDE activity (*Left*) and for immunoprecipitation, SDS/PAGE, and autoradiography (*Right*). Listed are the amount of protein and cAMP-PDE activity in the fractions: OR, original (solubilized membranes); 1, pass-through; 2, 50 mM NaBr; 3, 225 mM NaBr; 4, 450 mM NaBr. (*B*) In another experiment, 225 mM NaBr eluates from DEAE chromatography of samples from isoprenaline (ISO)- and insulin (INS)-treated ^{32}P-labeled adipocytes were diluted 40-fold with 50 mM Tris·HCl, pH 7.5/1 mM EDTA/5 mM $MgCl_2$/10 μg of antipain per ml/10 μg of leupeptin per ml/10% glycerol/0.03% $C_{13}E_{12}$ and applied to a 0.3-ml CIT-agarose column (cf. refs. 11 and 17). After the pass-through, the column was washed with dilution buffer containing 2 M NaBr (6 ml) and then 100 mM NaBr (3 ml). cAMP-PDE was eluted by incubating CIT-agarose overnight with 0.5 ml of dilution buffer containing 100 mM NaBr/50 mM cAMP/60 μg anti-cAMP-PDE. Portions of fractions were assayed for protein and cAMP-PDE activity and subjected to immunoprecipitation, SDS/PAGE, and autoradiography. Pass-through and 2 M NaBr eluates from CIT-agarose contained neither cAMP-PDE activity nor ^{32}P immunoprecipitated with anti-cAMP-PDE (data not shown). In cAMP and anti-cAMP-PDE eluates, cAMP-PDE was inactivated by anti-cAMP-PDE and the quantity of protein was insufficient to be measured.

photyrosine exclusively (data not illustrated). The cAMP-PDE 135- and 44-kDa phosphoproteins were not adsorbed to a monoclonal anti-phosphotyrosine antibody coupled to agarose (data not shown).

DISCUSSION

This study provides strong evidence that isoprenaline and insulin induce serine phosphorylation of cAMP-PDE in fat cells under conditions in which these hormones cause activation of the enzyme, consistent with a causal relationship between phosphorylation and activation. The ^{32}P-phosphorylated 135- and 44-kDa protein species were identified as, or closely related to, cAMP-PDE, since they were selectively immunoprecipitated by anti-cAMP-PDE (Fig. 1) and copurified with cAMP-PDE activity (Fig. 3). In addition, proteins of the same sizes were detected by the anti-cAMP-PDE in Western immunoblots (Fig. 2) and found to copurify with the enzyme activity.

Although the anti-cAMP-PDE selectively immunoprecipitated a predominant 135-kDa (and perhaps 44-kDa) phosphoprotein, after extensive purification from much larger amounts of rat fat pads (11) or bovine omental fat (17) the pure cAMP-PDE exhibits a molecular mass of ≈100 kDa by gel chromatography and 60- to 80-kDa protein species by SDS/PAGE. Comparable results have been reported with other analogous cGMP-inhibited cAMP-PDEs from human and bovine platelets and bovine cardiac tissue (14, 16, 22, 23). These cGMP-inhibited cAMP-PDEs are apparently exquisitely sensitive to proteolysis during isolation, since native species in the range of 110–130 kDa can be rapidly isolated by immunoadsorption techniques, but time-consuming purification procedures, even with extensive protection from proteases, yield predominantly proteolytically nicked forms (11, 14, 16, 17, 22, 23). Given the sensitivity of the cAMP-PDE to proteolysis, it is possible that proteolysis of the 135-kDa phosphoprotein produces the 44-kDa phosphorylated fragment and a 90-kDa nonphosphorylated, immunoreactive fragment observed in Western immunoblots of the DEAE-Sephacel fraction enriched in cAMP-PDE activity (Fig. 2). The size difference compared to the ≈60-kDa species obtained in preparations from whole rat adipose tissue (11, 17) presumably reflects differences in proteases between fat cells and other cell types in adipose tissue.

The phosphorylation of the 135-kDa cAMP-PDE protein in response to isoprenaline is presumably mediated by cAMP-dependent protein kinase, since the catalytic subunit of this kinase readily catalyzes the phosphorylation of the pure rat and bovine adipose tissue cAMP-PDE on a single serine residue (E.D., unpublished data). However, for technical reasons, it has not been possible to determine if the same site is phosphorylated in intact fat cells. Similar cGMP-inhibited phosphodiesterases from bovine cardiac muscle and bovine and human platelets can apparently be phosphorylated, and activated, by cAMP-dependent protein kinase in broken cell preparations (15, 16, 24) and in intact cells in response to agents which increase cAMP (14, 15, 24). These and other (4, 7, 12, 13) data, and the present results, support the notion that cAMP modulates its own turnover by a feedback mechanism which involves activation of the cAMP-PDE through phosphorylation catalyzed by cAMP-dependent protein kinase.

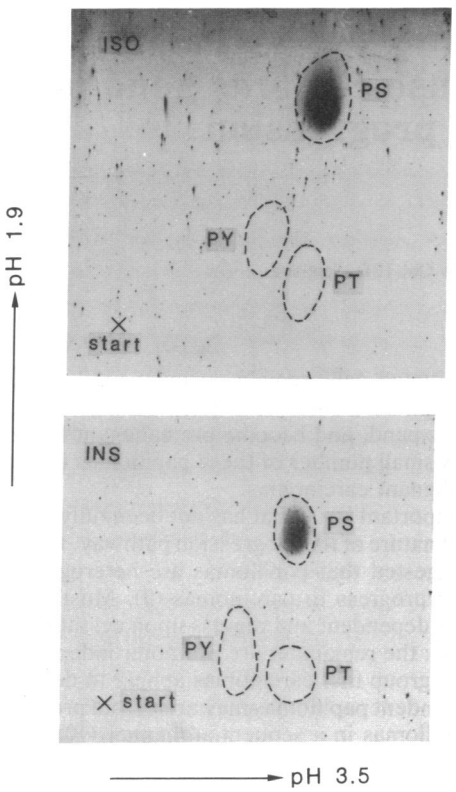

FIG. 4. Phosphoamino acid analysis of the fat cell 135-kDa phosphoprotein identified with cAMP-PDE. Solubilized particulate fractions from fat cells labeled with $^{32}P_i$ and incubated with isoprenaline (ISO) or insulin (INS) were immunoprecipitated and subjected to SDS/PAGE as described in the legend of Fig. 1 and in *Materials and Methods*. The gel segment with the 135-kDa ^{32}P-labeled phosphoprotein was cut out, the protein was electroeluted into 10 mM NH_4HCO_3/1 mM dithioerythritol/0.1% SDS (3 hr, 10°C), dialyzed against 50 mM NH_4HCO_3, lyophilized, and subjected to partial acid hydrolysis with 6 M HCl for 2 hr at 110°C. The solution was evaporated to dryness and ^{32}P-phosphorylated amino acids were analyzed by two-dimensional thin-layer high-voltage electrophoresis in the presence of unlabeled phosphoamino acids (21). The ^{32}P-phosphorylated amino acids were detected by autoradiography. PY, phosphotyrosine; PT, phosphothreonine; PS, phosphoserine.

Our results are consistent with the hypothesis that in rat fat cells insulin, by interaction with its specific cell-surface receptor and activation of receptor tyrosine kinase, increases cAMP-PDE activity by activation of a serine protein kinase responsible for phosphorylation of the particulate 135-kDa cAMP-PDE exclusively on serine residues. Several candidate intracellular serine protein kinases that are activated by insulin have recently been described (25–28). Although isoprenaline and insulin increased incorporation of $^{32}P_i$ into the cAMP-PDE to the same extent, isoprenaline activated the enzyme twice as much as insulin. This is consistent with phosphorylation of separate sites on the cAMP-PDE by the two hormones, as are data demonstrating that in intact adipocytes insulin and isoprenaline increase cAMP-PDE activity with different time-courses of activation, and that the two hormones, when present together, increase cAMP-PDE activity in a synergistic fashion (8).

The number of insulin receptors in a rat fat cell that need to be activated to elicit a full metabolic response (at 1 nM insulin) can be estimated at 500–1000 (29). The number of cAMP-PDE copies can be calculated to be only about 10-fold higher, assuming a native molecular mass of 135 kDa. This suggests a close proximity between the insulin receptor and the cAMP-PDE in an amplifying signal chain—e.g., by coupling them through the postulated serine protein kinase, itself perhaps a substrate for the insulin receptor tyrosine kinase. Clearly, the present findings and the availability of pure cAMP-PDE and its antibody provide new possibilities to examine this hypothesis experimentally.

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