cAMP Counter-regulates Insulin-mediated Protein Phosphatase-2A Inactivation in Rat Skeletal Muscle Cells*

(Received for publication, June 19, 1996, and in revised form, September 11, 1996)

Najma Begum‡§ and Louis Ragolia

From the Diabetes Research Laboratory, Winthrop University Hospital, Mineola, New York 11501 and the §School of Medicine, State University of New York, Stony Brook, New York 11794

In this study, we examined the mechanism of recently reported inactivation of protein phosphatase-2A (PP-2A) by insulin (Srinivasan, M., and Begum, N. (1994) J. Biol. Chem. 269, 12514–12520) and its counter-regulation by cAMP agonists. Exposure of L6 myotubes to insulin resulted in a rapid inhibition of PP-2A that was accompanied by a 3-fold increase in the phosphotyrosine content of the immunoprecipitated PP-2A catalytic subunit. Pretreatment with (S₆)ₐ-cAMP, a cAMP agonist, completely blocked insulin-mediated inhibition of PP-2A activity and decreased the tyrosine phosphorylation of PP-2A catalytic subunit to control levels. To understand the mechanism of counter-regulation of PP-2A by (S₆)ₐ-cAMP, cells were pretreated with sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases. Vanadate prevented the effect of (S₆)ₐ-cAMP on PP-2A activity and increased the phosphorylation status of PP-2A catalytic subunit to the level observed with insulin. Wortmannin, a phosphatidylinositol 3-kinase inhibitor, and rapamycin, an inhibitor of 70-kDa S6 kinase activation, prevented insulin-mediated inactivation of PP-2A, suggesting that these pathways may participate in insulin-mediated phosphorylation and inactivation of PP-2A. These results show that insulin signaling results in a rapid inactivation of PP-2A by increased tyrosine phosphorylation and cAMP agonists counter-regulate insulin's effect on PP-2A by decreasing phosphorylation, presumably via an activated phosphatase.

Protein phosphatase-2A (PP-2A) is one of four major cytoplasmic serine/threonine phosphatases that are known to play an important role in the regulation of diverse cellular proteins, including metabolic enzymes, ion channels, hormone receptors, kinase cascade, and cell growth (1–3). Two forms of PP-2A, PP-2A₁ and PP-2A₂, have been found in the cytosol of all tissues examined (4, 5). The holoenzyme is a heterotrimer consisting of a catalytic subunit “C” with an apparent mass = 36 kDa, a 60-kDa regulatory subunit “A,” and one of several “B” subunits of ~54, 55, 72, and 130 kDa (6). The ABC, AC, and C forms of PP-2A are known to have different substrate specificities based upon in vitro assays with artificial substrates (7, 8). The physiologic importance of these forms and their regulation by hormones and extracellular stimuli has not been established. In vitro studies indicate that the catalytic subunit of PP-2A is regulated by a variety of post-translational modifications, including phosphorylation on tyrosine (9–11) or threonine residues (12, 13), and methylation on the carboxyl-terminal leucine (14). Phosphorylation of PP-2A on tyrosine or threonine residues inactivates the phosphatase (9, 12, 13), while methylation has been reported to activate the enzyme (14).

Recent studies from this laboratory have shown that insulin acutely activates protein phosphatase-1 and concomitantly inhibits PP-2A activity in the rat skeletal muscle cell line, L6, in a differentiation-dependent manner (15). These results, in conjunction with our recent observation, that TNF-α-induced insulin resistance is accompanied by an elevation in PP-2A activity (16), suggested that rapid activation and inactivation of PP-1 and PP-2A, respectively, may be an integral part of the insulin-stimulated signal transduction pathway. Therefore, in this study, we have attempted to examine the mechanism of PP-2A inactivation by insulin and its counter-regulation by cAMP agonists. Furthermore, in order to gain insight into the upstream signaling components that may participate in insulin inactivation of PP-2A, the contributions of PI3-kinase and 70-kDa S6 kinase signaling pathways were evaluated with the use of specific inhibitors of these pathways. The results of this study suggest that insulin rapidly inactivates PP-2A in rat skeletal muscle cells by increasing tyrosine phosphorylation of the catalytic subunit. cAMP agonists counter-regulate the insulin effect by decreasing phosphorylation of PP-2A, thereby resulting in enhanced PP-2A activity. Wortmannin and rapamycin prevent insulin's effect on PP-2A inactivation, suggesting that PI3-kinase and/or 70-kDa S6 kinase generated signals may participate in insulin inactivation of PP-2A.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents, fetal bovine serum, phosphorylase kinase, and phosphorylase b were purchased from Life Technologies, Inc. [γ-³²P]ATP (specific activity > 3000 Ci/mmol), [³²P]orthophosphoric acid, and 125I-protein A were purchased from DuPont NEN, [S₆]ₐ-cAMP, wortmannin, and rapamycin were purchased from Biomol Research (Plymouth Meeting, PA). Electrophoresis and protein assay reagents were from Bio-Rad. Okadaic acid was from Moana Bioproducts (Honolulu, Hawaii). Protease inhibitors, sodium orthovanadate, and all other reagents were from Sigma. Porcine insulin was a kind gift from Eli Lilly Co. Antibody to the catalytic subunit of PP-2A was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture—The spontaneously fusing rat skeletal muscle cell line, L6, was a kind gift from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Canada). Cells were grown and maintained in α-minimal essential medium containing 2% fetal bovine serum and 1% antibiotic/antimycotic mixture in an atmosphere of 5% CO₂ at 37 °C as described previously (15). Completely differentiated myotubes were used for all the experiments after 16–18 h of starvation in serum-free Dulbecco's modified Eagle's medium.

(S₆)ₐ-cAMP and Insulin Treatment and Extraction of PP-2A—Serum-
starved myotubes were fed with serum-free medium containing 5 mM glucose and incubated at 37 °C for 1 h before treatment with insulin or (S₃₂P)AMP. Identical dishes in triplicate were treated with and without (S₃₂P)AMP (10⁻⁴ M) for 0–60 min, followed by 10 mM insulin treatment for 5–10 min. In some experiments, cells were pretreated with freshly prepared orthovanadate (1 mM) for 20 min prior to (S₃₂P)AMP or insulin exposure. At the end of the incubation period, the medium was removed and the cells were rinsed three times with ice-cold phosphate-buffered saline followed by the addition of PP-2A extraction buffer.

**Assay of Cellular PP-2A Activities—Control-, insulin-, and (S₃₂P)AMP-treated cells were scraped off the dishes with 0.3 ml of phosphate extraction buffer containing 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with 10 µg/ml each of aprotinin, leupeptin, antipain, soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM PhMoSO₄.F₄. The cells were sonicated for 10 s and centrifuged at 2000 × g for 5 min, and the supernatants were used for the assay of phosphatase activities. The assay was performed in the presence and absence of 2 mM okadaic acid to inhibit PP-2A activity. The conditions of the assay allow measurement of PP-1 and PP-2A activities and not PP-2B and PP-2C enzymes which require divalent ions. Previous studies from this laboratory have shown that okadaic acid at 2 mM concentration inhibits only PP-2A and the PP-1 activity remaining in the assay was comparable with the activity inhibited by inhibitor 2 (15). PP-2A activity was calculated by subtracting the activity measured in the presence of okadaic acid from the activity measured in the absence of okadaic acid. Purified (S₃₂P)phosphorylase a was used as a substrate (15, 17). (S₃₂P)Labeled phosphorylase a was prepared by reacting [γ-³²P]ATP with purified phosphorylase kinase and phosphorylase b (18).

**Measurement of Immunoprecipitated PP-2A Activity—Control- and agonist-treated cells were lysed in lysis buffer deprived of phosphatase inhibitors. Cell extracts containing equivalent amounts of protein (50–100 µg) were then incubated 2–4 h at 4 °C in the presence of 2 µg of anti-PP-2A C subunit antibody adsorbed overnight at 4 °C to protein A-Sepharose (19). The pellets were washed four times in lysis buffer. After an additional wash in kinase buffer, pellets were resuspended in 40 µl of reaction buffer. The phosphatase assay was initiated by the addition of 20 µl of (S₃₂P)-labeled phosphorylase a. After 10 min at 30 °C, the reaction was stopped with 40 µl of 3 × Laemml sample buffer. In parallel, spontaneous dephosphorylation of (S₃₂P)-labeled phosphorylase a was evaluated by incubating it in the absence of cell extract. Samples were separated by electrophoresis on 7.5% SDS-polyacrylamide gels and then subjected to autoradiography. The specificity of the immunoprecipitation was tested by the addition of 2 mM okadaic acid, a concentration that specifically inhibits PP-2A activity. In all cases, the okadaic acid-resistant phosphatase activity did not exceed 10% of the activity measured in the absence of okadaic acid. After autoradiography, (S₃₂P)phosphorylase dephosphorylation was measured by quantitation of the substrate bands by image analysis. Phosphatase activity was determined as the difference between the initial (S₃₂P)phosphorylase level and the level remaining after the reaction.

**In Vivo Phosphorylation and Immunoprecipitation of PP-2A—L6 cells were serum-starved overnight. Next day, the medium was removed and replaced by 1 ml of phosphate-free Dulbecco’s modified Eagle’s medium and incubation was continued for 1 h. [³²P]Orthophosphate was added (0.5 mM/ml), and the cells were incubated for 4 h followed by sodium orthovanadate (1 mM) or (S₃₂P)AMP (10⁻⁴ M) for 20 min. Insulin (10 mM) was added, and the incubation was continued for an additional 10 min. The cells were rinsed four times with 1 ml of ice-cold phosphate-buffered saline containing phosphatase and protease inhibitors and harvested in 0.5 ml of lysis buffer containing 20 mM triethanolamine, pH 7.2, 0.5 mM EGTA, 1 mM EDTA, 2 mM sodium vanadate, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 40 mM β-glycerophosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin, aprotinin, antipain, soybean trypsin inhibitor, and peptatin A, 100 mM NaCl, 1 mM microcystin, and 1% Triton X-100. The cell lysates were centrifuged at 16,000 × g in a microcentrifuge for 10 min to remove cell debris. 400 µg of cell lysate protein was diluted to 1 ml with lysis buffer and precleared by incubation with rat IgG (5 µg/ml, coupled to protein A-Sepharose) at 4 °C for 1 h. The supernatants were immunoprecipitated with PP-2A catalytic subunit antibody (4 µg/sample) for 8 h at 4 °C, followed by treatment with 50 µl of protein A-Sepharose CL4B (50% slurry) for 1 h. In some experiments, the antibody was preincubated with the competing peptide before adding to cell lysates. The pellets were washed four times with 1 ml of lysis buffer and resuspended in 40 µl of 2 × SDS sample buffer. The samples were boiled for 5 min, followed by centrifugation (10,000 × g for 30 s) to pellet down the Sepharose beads. Electrophoresis of the immunoprecipitates was performed in 10% SDS-polyacrylamide gels, followed by autoradiography (20). The protein and phosphotyrosine contents of PP-2A catalytic subunits were determined by immunoprecipitating unlabeled cell lysates with anti-PP-2A C subunit antibody, followed by separation of immunoprecipitated proteins on SDS-PAGE. After transferring proteins to PVDF membranes, the membranes were probed with 237P-protein A (0.2 µCi/ml) and autoradiography. The intensity of the signal was quantitated by densitometric analysis of the autoradiograms as well as by the “cut and count” technique.

**Protein Assay—The protein contents of the cell extracts were determined by the Bradford assay (21) or by using bicinchoninic acid (22).

**Statistics—Student’s t test and analysis of variance were used to evaluate the significance of the effects of vanadate, (S₃₂P)AMP, and insulin.

**RESULTS**

**Effect of Insulin on PP-2A Activity—**As indicated previously (15), acute exposure of L6 myotubes to physiologic concentrations of insulin for 5–10 min resulted in a rapid activation of PP-1 and an inhibition of PP-2A. In this study, we examined the mechanism of inactivation of PP-2A by insulin and the effect of cAMP analogs on insulin-mediated inhibition of PP-2A activity.

The kinetics of PP-2A inhibition by insulin is shown in Fig. 1. Treatment of L6 cells with 10 mM insulin resulted in a 35% reduction in cytosolic PP-2A activity within 10 min when compared with untreated control cells. A maximal reduction of 43% was observed after 20 min of incubation, and the effect was sustained for the entire 60-min period studied. Insulin doseresponse studies demonstrated an inhibition of PP-2A activity at all concentrations of insulin tested (Fig. 2). Maximal reduction in enzyme activity was observed at 20 mM insulin, and the effect was sustained up to 100 mM insulin tested.

cAMP Agonist, (S₃₂P)-cAMP, Blocks Insulin-induced Decrease in PP-2A Activity—To further investigate whether PP-2A activity could be differentially regulated by insulin and the other counter-regulatory hormones, L6 cells were exposed to (S₃₂P)-cAMP, a cAMP agonist, for 20 min prior to or after treatment with insulin. The presence of (S₃₂P)-cAMP completely abolished the effect of insulin on PP-2A inhibition (Fig. 3). (S₃₂P)-cAMP
alone had very little effect on the basal PP-2A activity. Similar results were obtained with the other cAMP analogues, 8-bromo-cAMP and dibutyryl-cAMP (data not shown).

**Vanadate Prevents (Sp)-cAMP Effect on PP-2A**

To examine whether (Sp)-cAMP blocked insulin inactivation of PP-2A by activating a phosphatase, thereby decreasing phosphorylation of the catalytic subunit, cells were preincubated for 20 min with 1 mM sodium orthovanadate (an inhibitor of tyrosine and dual specific phosphatases) before exposure to (Sp)-cAMP and insulin. As shown in Fig. 4, pretreatment of cells with sodium orthovanadate prevented the effect of (Sp)-cAMP on PP-2A activity. Direct addition of vanadate to cell extracts did not inhibit PP-2A activity. In contrast, treatment of intact cells with sodium vanadate alone decreased PP-2A when compared with control cells.

To directly assess changes in PP-2A activity in response to insulin and (Sp)-cAMP, immunoprecipitation of PP-2A was performed with a peptide antibody directed against the C subunit of human PP-2A (19). The immunoprecipitated PP-2A activity was measured with [32P]phosphorylase a as a substrate. As indicated in Fig. 5, insulin caused 60% decrease in PP-2A activity when compared with control. The presence of (Sp)-cAMP during insulin exposure blocked insulin effect on PP-2A activity. Pretreatment with vanadate prevented (Sp)-cAMP effect on PP-2A. As indicated in Fig. 4, cells incubated with vanadate alone also showed an inhibition of PP-2A activity when compared with control cells. Addition of vanadate to immunoprecipitated PP-2A did not affect the enzyme activity.

Insulin and (Sp)-cAMP treatment did not change the abundance of PP-2A catalytic subunit (Fig. 6).

**Effect of Insulin and (Sp)-cAMP on Tyrosine Phosphorylation of PP-2A Catalytic Subunit**

The results of Figs. 3–5 suggested that insulin and (Sp)-cAMP effects on PP-2A inactivation/reactivation may be mediated by phosphorylation/dephosphorylation mechanism. To examine this, we immunoprecipitated cell extracts with a peptide antibody raised against the catalytic subunit of PP-2A. The immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane, and probed with an anti-phosphotyrosine antibody (Fig. 7A). Insulin treatment resulted in a 3-fold increase in tyrosine phosphorylation of PP-2A catalytic subunit when compared with control cells (Fig. 7A, lane 2 versus lane 1). The phosphotyrosine content of PP-2A in cells treated with (Sp)-cAMP alone (lane 3) was comparable with control cells. In contrast, cells treated with (Sp)-cAMP followed by insulin (lane 4) exhibited a reduction in the phosphotyrosine content of PP-2A. The observed alterations in PP-2A tyrosine phosphorylation seen in insulin and (Sp)-cAMP-treated cells were not due to variations in the amounts of immunoprecipitated proteins.

Next, we examined the phosphorylation status of PP-2A catalytic subunit in 32P-labeled cells that were exposed to insulin and (Sp)-cAMP. As shown in Fig. 8A, insulin treatment for 2, 5, 10, and 20 min (lanes 2, 5, 6, and 7) resulted in a rapid increase in the phosphorylation of the 36-kDa subunit of PP-2A.
shown. Similar results were obtained in four separate experiments.

Inactivation of PP-2A---Experimental Procedures. PP-2A was immunoprecipitated and activity measured as described under “Experimental Procedures.” Data are expressed as percentage of untreated control. Results are the mean ± S.E. of two independent experiments.

Graph showing Immunoprecipitation of PP-2A and the assay of PP-2A activity in the immunoprecipitates. L6 cells were treated with vanadate, (S)p-cAMP (Sp-AMP), and insulin as detailed as under “Experimental Procedures.” PP-2A was immunoprecipitated and activity measured as described under “Experimental Procedures.” Data are expressed as percentage of untreated control. Results are the mean ± S.E. of two independent experiments.

FIG. 5. Immunoprecipitation of PP-2A and the assay of PP-2A activity in the immunoprecipitates. L6 cells were treated with vanadate, (S)p-cAMP (Sp-AMP), and insulin as detailed as under “Experimental Procedures.” PP-2A was immunoprecipitated and activity measured as described under “Experimental Procedures.” Data are expressed as percentage of untreated control. Results are the mean ± S.E. of two independent experiments.

when compared with control cells (lane 1). Cells exposed to (S)p-cAMP for 20 min prior to (lane 3) or after 10 min of insulin treatment (lane 4) showed less phosphorylation of the PP-2A catalytic subunit when compared with insulin alone (lanes 5–7). Pretreatment with vanadate before (S)p-cAMP (lane 9) prevented the (S)p-cAMP effect on the phosphorylation status of the PP-2A catalytic subunit (compare lane 9 with lanes 3 and 4). Vanadate by itself (lane 8) caused a small increase in phosphorylation of PP-2A when compared with untreated control cells (lane 1). The extent of phosphorylation by (S)p-cAMP alone was comparable with control cells (data not shown). Western blot analysis of immunoprecipitates from an identical experiment in which cells were incubated with cold PI revealed similar amounts of PP-2A catalytic subunit in each treatment (Fig. 8B).

Effect of Wortmannin and Rapamycin on Insulin-induced Inactivation of PP-2A---To gain insight into the upstream signaling components that might mediate insulin inactivation of PP-2A, we incubated cells with wortmannin, a potent and selective inhibitor of PI-3 kinase, and rapamycin, an immunosuppressant that selectively blocks the phosphorylation and activation of 70-kDa S6 kinase. Both inhibitors blocked insulin’s effect on PP-2A inactivation and restored the enzyme activity to control levels (Table 1). Wortmannin alone had very little effect on the basal activity of PP-2A, but completely prevented insulin-mediated inactivation of the enzyme. In contrast, rapamycin alone had a stimulatory effect on PP-2A, and it completely abolished insulin-induced inactivation of PP-2A.

DISCUSSION

This study suggests a possible mechanism for the previously reported inhibition of PP-2A activity by insulin in rat skeletal muscle cells. The insulin-mediated inactivation is accompanied by increased tyrosine phosphorylation of the PP-2A catalytic subunit. (S)p-cAMP, a cAMP agonist, abrogates insulin inactivation of PP-2A by decreasing phosphorylation of the catalytic subunit and restores PP-2A enzyme activity to control levels. These results provide new evidence that PP-2A activity is regulated by phosphorylation. PP-2A was immunoprecipitated from lysates of cells treated with (S)p-cAMP (20 min) followed by insulin (10 min). The immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membrane, followed by immunoblot analysis with anti-phosphotyrosine antibody (Ab) (A) and PP-2A antibody (B). An autoradiogram from a representative experiment is shown. Lane 1, control; lane 2, insulin; lane 3, (S)p-cAMP; lane 4, (S)p-cAMP + insulin.

FIG. 6. Effect of insulin and (S)p-cAMP on contents of PP-2A catalytic subunit. Control, insulin-, and (S)p-cAMP-treated cells were immunoprecipitated with PP-2A antibody (Ab) as detailed under “Experimental Procedures.” The immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane, followed by immunoblotting with PP-2A C subunit antibody. Lane 1, positive control (purified rabbit skeletal muscle PP-2A); lane 2, control; lane 3, insulin-treated; lane 4, (S)p-cAMP; lane 5, (S)p-cAMP followed by insulin; lane 6, vanadate + (S)p-cAMP + insulin. A representative autoradiogram is shown. Similar results were obtained in four separate experiments.

FIG. 7. Insulin-mediated inactivation of PP-2A is accompanied by increased tyrosine phosphorylation of PP-2A catalytic subunit. (S)p-cAMP prevents insulin effect by decreasing tyrosine phosphorylation. PP-2A was immunoprecipitated from lysates of cells treated with (S)p-cAMP (20 min) followed by insulin (10 min). The immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membrane, followed by immunoblot analysis with anti-phosphotyrosine antibody (Ab) (A) and PP-2A antibody (B). An autoradiogram from a representative experiment is shown. Lane 1, control; lane 2, insulin; lane 3, (S)p-cAMP; lane 4, (S)p-cAMP + insulin.

FIG. 8. Effect of insulin and (S)p-cAMP on the phosphorylation status of PP-2A. 32P-Labeled cells were treated with (S)p-cAMP and insulin as detailed in Figs. 4 and 5. Cell lysates were immunoprecipitated with PP-2A antibody, followed by SDS-PAGE and autoradiography. Ab, antibody. A, an autoradiogram from a representative experiment. Lane 1, control; lane 2, 2-min insulin treatment; lane 3, (S)p-cAMP (20 min) followed by insulin (10 min); lane 4, insulin (10 min) followed by (S)p-cAMP (20 min); lanes 5–7, insulin treatment for 5, 10, and 20 min; lane 8, vanadate alone (20 min); lane 9, vanadate + (S)p-cAMP + insulin. B, a duplicate experiment on cold cells demonstrating equal amounts of PP-2A in the immunoprecipitates. Lane order is similar to A.
kinase cascade, due to dephosphorylation and inactivation of MAP kinase kinase and MAP kinase activities. In this respect, it is interesting to note that several previous studies have demonstrated an inhibition of insulin and growth factor-activated MAP kinase signaling by cAMP in various cell types including the rat adipose tissue (23–27). In contrast, cAMP did not block MAP kinase signaling in Swiss 3T3 fibroblasts and PC12 cells (28, 29). When observed, the cAMP inhibition was reported to be downstream of p21<sup>ras</sup> activation. However, these studies did not address the role of phosphatases in cAMP-mediated inhibition of MAP kinase signaling. We have recently demonstrated an inhibition of insulin-activated MAP kinase signaling by TNF-α in L6 cells (16). The inhibition was downstream of p21<sup>ras</sup> and Raf-1. This inhibitory effect of TNF-α on MAP kinase cascade was accompanied by elevations in PP-2A and MAP kinase signaling and restored insulin’s effect on MAP kinase activity. Therefore, PP-2A inactivation/reactivation may play an important role in insulin- and cAMP-mediated signal transduction.

It should be noted, however, that PP-2A activity measurements were performed using the conventional substrate phosphorylation assay. This may not be the ideal substrate for PP-2A in skeletal muscle cells. Future studies should be directed toward the identification of in vivo cellular protein substrates that undergo changes in phosphorylation level as a consequence of changes in PP-2A activity.

The prevention of the (S<sub>p</sub>)-cAMP effect on PP-2A activity with sodium orthovanadate suggests that the cAMP effect on PP-2A may be mediated either by self-autodephosphorylation of PP-2A as suggested by Chen et al. (9) or via the activation of a tyrosine phosphatase. In support of the latter observation, studies by Brautigan and Finault (30) indicate that activators of cAMP-dependent protein kinase increase the activity of phosphorylase phosphatase-1B (PTPase-1B) in intact cells. Although we have not directly measured tyrosine phosphatase activities in insulin- and (S<sub>p</sub>)-cAMP-treated L6 cells, results with sodium orthovanadate, an inhibitor of tyrosine and dual specificity phosphatases, suggest that cAMP agonists and insulin may regulate PP-2A activity by altering tyrosine phosphorylation of the enzyme. Thus the effect of insulin on PP-2A phosphorylation may be due to inhibition of PTPases or due to an activation of a cytoplasmic tyrosine kinase or both. cAMP agonist may abrogate insulin’s effect on PP-2A inactivation by activating a PTPase. An elevation of PTPase activity has been observed in insulin-resistant cells and in animal models of experimental diabetes (31).

In an attempt to identify the upstream signaling components that may participate in insulin-mediated inactivation of PP-2A, cells were treated with wortmannin, a potent and selective inhibitor of PI 3-kinase, and rapamycin, an inhibitor of 70-kDa S6 kinase (34). Both agents prevented insulin-mediated PP-2A inactivation and restored PP-2A activity to control values. These results suggest that (i) the insulin receptor may not directly phosphorylate PP-2A, and (ii) wortmannin-sensitive PI 3-kinase and/or S6 kinase pathway may be participating in insulin signaling leading to PP-2A inactivation. The present studies do not rule out the possibility that insulin inactivation of PP-2A may be mediated through the activation of the recently reported inhibitors of PP-2A.

In summary, the results of the present study indicate that insulin signaling involves a rapid inactivation of PP-2A by increased tyrosine phosphorylation on its catalytic subunit, and counter-regulatory hormones block the insulin effect on PP-2A by decreasing tyrosine phosphorylation.

| Treatment               | PP-2A activity | nmoi P<sub>i</sub> released/mg protein/min |
|-------------------------|----------------|--------------------------------------|
| None (control)          | 1.66 ± 0.162   | 0.100*                               |
| Insulin                 | 1.99 ± 0.230   | 0.205**                              |
| Rapamycin               | 1.48 ± 0.137   | 0.230                                |
| Rapamycin + insulin     | 1.42 ± 0.130** |                                     |

* N. Begum and L. Ragolia, unpublished results.

REFERENCES
1. Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508
2. Shendilkar, S., and Nairn, A. C. (1991) Adv. Second Messenger Phosphoprotein Res. 23, 1–31
3. Brautigan, D. L., Chen, J., and Thompson, P. (1993) Adv. Protein Phosphatases 7, 49–65
4. Mumber, M., and Walter, G. (1993) Physiol. Rev. 73, 673–699
5. Mayer-Jackel, R. E., and Hemmings, B. A. (1994) Trends Cell Biol. 4, 287–291
6. Mayer-Jackel, R. E., Okhura, H., Gomes, R., Sunkel, C. E., Baumgartner, S., Hemmings, B. A., and Glover, D. M. (1993) Cell 73, 601–611
7. Reudiger, R., Van Wart Hood, J. E., Mumber, M., and Walter, G. (1991) Mol. Cell. Biol. 11, 4282–4285
8. Kamibayashi, C., Eates, R., Slaughter, C., and Mumber, M. C. (1991) J. Biol. Chem. 266, 12521–12529
9. Chen, J., Martin, B. L., and Brautigan, D. L. (1992) Science 257, 1261–1264
10. Chen, J., Parsons, S., and Brautigan, D. L. (1994) J. Biol. Chem. 269, 4282–4285
11. Barnes, G. N., Slevin, J. T., and Vanaman, T. C. (1995) J. Neurochem. 64, 340–353
12. Guo, H., Reddy, S. A. G., and Damuni, Z. (1993) J. Biol. Chem. 268, 11193–11198
13. Guo, H., and Damuni, Z. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2500–2504
14. Xie, H., and Clarke, S. (1994) J. Biol. Chem. 269, 1981–1984
15. Srivivasan, M., and Begum, N. (1984) J. Biol. Chem. 269, 6091–6095
16. Begum, N., Ragolia, L., and Srivivasan, M. (1996) Eur. J. Biochem. 238, 214–220
17. Cohen, P., Klampf, S., and Schelling, D. L. (1989) FRSB Lett. 250, 596–600
PP-2A Regulation by Insulin and cAMP Agonists

18. Cohen, P. (1983) Methods Enzymol. 99, 243–250
19. Chajry, N., Martin, P. M., Cochet, C., and Berthois, Y. (1996) Eur. J. Biochem. 235, 97–102
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
22. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
23. Wu, J., Dest, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993) Science 262, 1065–1068
24. Cook, S. J., and McCormick, F. (1993) Science 262, 1069–1072
25. Burgering, B. M. Th., Pronk, G. J., Van Weeran, P. C., Chardin, P., and Bos, J. L. (1993) EMBO J. 12, 4211–4220
26. Stevenson, B. R., Kong, X., and Lawrence, J. C., Jr. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10305–10309
27. Graves, L. M., Bernfeldt, K. E., Raines, E. W., Potts, B. C., Macdonald, S. G., Ross, R., and Krebs, E. G. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10300–10304
28. Erhardt, P., Troppmair, J., Rapp, U. R., and Cooper, G. M. (1995) Mol. Cell. Biol. 15, 5524–5530
29. Petritsch, C., Woscholski, R., Edelmann, H. M. L., and Ballou, L. S. (1995) J. Biol. Chem. 270, 26619–26625
30. Brautigan, D. L., and Pinault, F. M. (1993) Mol. Cell. Biochem. 127/128, 121–129
31. Ahmad, F., and Goldstein, B. J. (1995) Am. J. Physiol. 268, E922–E940
32. Zhang, W. R., Li, P. M., Oswald, M. A., and Goldstein, B. J. (1996) Mol. Endocrinol. 10, 575–584
33. Mayerowitch, J., Backer, J. M., Csermely, P., Shoelson, Shoelson, S. E., and Kahn, C. R. (1992) Biochemistry 31, 10338–10344
34. Okada, T., Kawano, Y., Sakakibara, T., Hanoki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3568–3573