Interaction between cAMP-dependent and insulin-dependent signal pathways in tyrosine phosphorylation in primary cultures of rat hepatocytes

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The present studies were undertaken to determine whether the interaction between cAMP-dependent and insulin-dependent pathways in primary cultures of rat hepatocytes affects biological functions and tyrosine phosphorylation. Quiescent hepatocytes were pretreated with dibutyryl cAMP or cAMP-generating agents such as glucagon, and then treated or not with insulin. Preincubation for 6 h with dibutyryl cAMP or glucagon enhanced the effect of insulin on DNA synthesis, but not the effect of insulin on amino acid transport or glycogen and protein synthesis. Tyrosine phosphorylation of intracellular proteins was determined by immunoblot analysis using an anti-phosphotyrosine antibody. Maximum tyrosine phosphorylation of a 195 kDa protein, which may be a substrate of insulin receptor kinase, of 175–180 kDa proteins, including insulin receptor substrate (IRS)-1, and of 90–95 kDa proteins, including the insulin receptor β-subunit, was reached within 30 s of incubation with insulin. Pretreatment for about 3 h with dibutyryl cAMP or cAMP-generating agents clearly increased insulin-dependent tyrosine phosphorylation of the 195 kDa protein, but not IRS-1, IRS-2 or the insulin receptor β-subunit. Because dibutyryl cAMP and cAMP-generating agents did not increase insulin receptor number or its kinase activity, the effect of cAMP on this potentiation of tyrosine phosphorylation is assumed to be exerted at a step distal to insulin receptor kinase activation. The potentiation by cAMP pretreatment of insulin-stimulated tyrosine phosphorylation may in part be secondary to inhibition of phosphotyrosine phosphatase activity, because cAMP pretreatment blunted the effect of Na_3VO_4 on the net tyrosine phosphorylation of the 195 kDa protein as compared with cells pretreated with no additive. In summary, the interactions between cAMP-dependent and insulin-dependent pathways that lead to augmentation of DNA synthesis appear to parallel the changes in tyrosine phosphorylation. Further studies will be required to determine whether there is a causal relationship between these phenomena.

INTRODUCTION

Insulin is a major anabolic hormone that has a variety of biological functions including stimulation of amino acid and glucose transport, modification of the activities of various enzymes, such as glycogen synthetase, pyruvate dehydrogenase and S6 kinase, and further induction of proliferation in specific cell types (for a review, see [1]). These actions have been classified according to the time required for insulin to produce these effects and the mechanisms by which they are produced. Both the rapid metabolic effects and long-term growth effects of insulin are initiated by interaction with the α-subunit of the insulin receptor followed by stimulation of intrinsic tyrosine kinase activity on the receptor β-subunit (for a review, see [1–3]). Two of the major substrates for the receptor kinase, insulin receptor substrate (IRS)-1 and IRS-2, have been purified and act as 'docking proteins' that link insulin signalling to SH2-domain-containing proteins downstream in the phosphorylation cascade [4–6]. This includes activation of phosphatidylinositol 3-kinase and stimulation of the $p21^{ras}$ pathway through GRB-2 [7–11]. In addition, the insulin receptor kinase has been reported to phosphorylate Shc and then associate with the GRB-2–mSOS complex to activate the $p21^{ras}$ signal pathway [11,12].

The present study was prompted by our finding that cAMP-generating agents potentiate insulin-like growth factor I (IGF-I)-dependent growth in the rat thyroid cell line, FRTL-5 [13]. Insulin and IGF-I exhibit structural similarity both between the peptides themselves and between their receptors [1,14]. After IGF-I binds to the α-subunit of the IGF-I receptor, the tyrosine kinase of its β-subunit is stimulated in the same way as insulin receptor. The IGF-I receptor also phosphorylates IRS-1 at tyrosine residues, and this phosphoprotein is thought to be important for IGF-I-dependent signal transduction as well as insulin [15,16]. A number of studies using FRTL-5 cells showed that cAMP-generating agents potentiated IGF-I-dependent cell growth [13,17,18]. We have reported that cAMP-generating agents could potentiate tyrosine phosphorylation of intracellular proteins induced by IGF-I and increase IGF-I-independent tyrosine phosphorylation [19].

From this background, the present studies were undertaken to determine whether the interaction between cAMP-dependent and insulin-dependent pathways affects biological functions and tyrosine phosphorylation in primary cultures of rat hepatocytes.

EXPERIMENTAL

Materials

Williams' medium E was purchased from Flow Laboratories (Irvine, Scotland, U.K.) and Hanks balanced-salt solution

Abbreviations used: IGF-I, insulin-like growth factor I; IRS, insulin receptor substrate; HBSS, Hanks balanced salt solution; NCS, newborn calf serum; EGF, epidermal growth factor; KIU, kallikrein-inactivating units; PNPP, *p*-nitrophenyl phosphate; WGA, wheat germ agglutinin.

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(HBSS) from Nissui (Tokyo, Japan). Newborn calf serum (NCS) was obtained from Cell Culture Laboratories (Cleveland, OH, U.S.A.) or Summit Biotechnology (Greeley, CO, U.S.A.). Penicillin was obtained from Ban'yu Pharmaceutical Co. (Tokyo, Japan), streptomycin and kanamycin from Meiji Seika Co. (Tokyo, Japan) and amphotericin B (Fungizone) from Sankyo (Tokyo, Japan). All collagen-coated dishes (type I) were from Corning (Corning, NY, U.S.A.). Collagenase was from Nitta Gelatin Co. (Osaka, Japan) and trypsin inhibitor (chicken egg white and soya bean Kunitz type) was from Sigma (St. Louis, MO, U.S.A.). Anti-phosphotyrosine antibody was kindly provided by Dr. T. Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan) and anti-(human IRS-1) antibody for immunoprecipitation was supplied by Dr. M. Nishiyama (Jikei University, School of Medicine, Tokyo, Japan). Anti-(insulin receptor) antibody (AB-3) was obtained from Oncogene Science Co. (Uniondale, NY, U.S.A.) and anti-IRS-2 antibody (M-19) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Other chemicals were of reagent grade available commercially.

Primary cultures of rat liver parenchymal cells

Parenchymal cells were isolated from livers of male Wistar strain rats (body weight 150–300 g, Charles River Japan Inc., Tokyo, Japan) by perfusion with collagenase as described by Kato et al. [20]. The rats had been fed on laboratory pellets (Labobreeder MR, Nihon Nosanko Ltd., Yokohama, Japan) *ad libitum*.

The parenchymal cells were inoculated on to collagen-coated dishes at a density of 2×10^5 cells/24 wells or 1×10^6 cells/35 mm dish or 6×10^6 cells/100 mm dish and cultured for 2 h in Williams' medium E with 10 % NCS, 10^{-8} M bovine insulin and 10^{-6} M dexamethasone at 37 °C in an atmosphere of 95 % air/5 % CO_2 in a humidified incubator. All media contained penicillin (1×10^5 units/l), streptomycin (10 mg/l), kanamycin (10 mg/l) and amphotericin B (0.2 mg/l). The unattached cells were removed and the attached cells were incubated further for 22 h in the same medium. The medium was then discarded and the cells were washed three times with HBSS before various treatments as described below.

Cell treatments

The cultures of rat hepatocytes were continued for an additional 24 h in Williams' medium E containing 0.1 % BSA without NCS and various hormones to prevent their effects. The medium was then replaced by Williams' medium E without or with various concentrations of dibutyryl cAMP or cAMP-generating agents including glucagon, forskolin and cholera toxin for the indicated times. The cells were then washed three times with HBSS and then treated with various concentrations of insulin for the indicated times.

Thymidine incorporation into DNA [21]

For studies of [*methyl*-^{3}H]thymidine incorporation into DNA, hepatocytes were cultured as described above except that cells were sparsely seeded on to 24-well plates (1×10^5 cells/well). After reaching quiescence, the cells were pretreated without or with dibutyryl cAMP (10^{-3} M) or glucagon (5×10^{-8} M) for 6 h. At the end of the pretreatment, cells were washed three times with HBSS and treated with various concentrations of epidermal growth factor (EGF) or insulin for 24 h. [*methyl*-^{3}H]Thymidine (1 μCi/ml; Amersham Life Science Co.) was added to each dish 4 or 6 h before the termination of each experiment. Labelling was stopped by adding 1 M ascorbic acid, and 10 % (w/v) trichloroacetic acid-precipitable material was measured as thymidine incorporation into DNA [13]. In all experiments, each experimental point represents the mean from three replicate wells.

Immunoblotting using anti-phosphotyrosine antibody

After treatment of hepatocytes on a 35 mm dish, the cells were lysed at 0 °C in 0.25 ml of Tris/Triton X/lysis buffer solution containing 50 mM Tris/HCl, pH 7.4, 1 % Triton X-100, 0.5 mM Na_3VO_4, 5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml PMSF, 100 kallikrein-inactivating units (KIU)/ml aprotinin and 10 mg/ml *p*-nitrophenyl phosphate (PNPP). A 0.125 ml volume of concentrated 3 × Laemmli's sample buffer solution containing 9 % SDS and 6 % 2-mercaptoethanol was added to the lysates and the mixture was incubated for 5 min at 100 °C [22]. These samples were then stored at −80 °C until electrophoresis. A fraction of each sample that contained the same amount of protein was subjected to SDS/PAGE (8 % gel) using a Hoefer Standard Slab Gel Units SE600 apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). The proteins on the gel were transferred to a nitrocellulose membrane (BA-85; Schleicher & Schuell, Dassel, Germany) in buffer containing 15 mM Tris, 192 mM glycine, 20 % (v/v) methanol, 0.075 % SDS and 0.5 mM Na_3VO_4 using a Trans Blot Cell (Bio-Rad, Richmond, CA, U.S.A.). After transblotting to the nitrocellulose membrane, the membrane was washed with the rinsing buffer solution (50 mM NaCl, 1 mM EDTA and 10 mM Tris/HCl, pH 7.2) and further blocked at 4 °C overnight with the blocking buffer (the rinsing buffer plus 3 % BSA and 0.01 % NaN_3). Membrane was then incubated for 1.5 h at room temperature in the blocking buffer containing a monoclonal anti-phosphotyrosine antibody (1:1000). After incubation, the membrane was rinsed once for 10 min in large volumes of TBS-T buffer solution (20 mM Tris/HCl, pH 7.6, 137 mM NaCl and 0.01 % Tween 20), and twice more for 5 min in TBS-T. The blots were then probed for 1 h with anti-mouse IgG that had been conjugated with horseradish peroxidase. Phosphotyrosyl proteins were detected using an ECL kit, according to the manufacturer's directions (Amersham ECL Kit; Amersham Life Science Co.). The results were quantified using the NIH Image computer program. Protein was assayed by the method of Bradford [23].

Immunoprecipitation of IRS-1, IRS-2 and insulin receptor

Hepatocytes were seeded at 6×10^6 cells/100 mm dish. After various treatments, the cells were harvested in 0.5 ml of modified Tris/Triton X/lysis buffer solution consisting of 50 mM Tris/HCl pH 7.4, 1 % Triton X-100, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml PMSF and 100 KIU/ml aprotinin. The lysates were homogenized with a Dounce homogenizer (a tight pestle) using 20 strokes. The resulting homogenates were centrifuged at 12000 ***g*** for 10 min at 4 °C. The supernatant was diluted with Tris/Triton X/lysis buffer solution to 1 mg of protein/0.5 ml of lysis (final concentration). This lysate was incubated with anti-IRS-1 antibody (10 μl), anti-IRS-2 antibody (10 μl) or anti-(insulin receptor) antibody (1 μg) overnight at 4 °C; 30 μl of Protein G–Sepharose [50 % (v/v); Sigma] was then added and incubation was continued for 2 h. Immunoprecipitates were collected by centrifugation and washed three times with the modified Tris/Triton X/lysis buffer solution. In the case of IRS-1 immunoprecipitation, anti-phosphotyrosine antibody (10 μg) was added to the first supernatant and then the

above procedure was followed. The final immunoprecipitates were boiled for 5 min in 150 μl of modified Tris/Triton X/lysis buffer solution and 75 μl of 3 × Laemmli's sample buffer solution. Proteins in a 75 μl portion were separated by SDS/PAGE (8 % gel) and transferred to nitrocellulose. Immunoblotting analysis using anti-phosphotyrosine antibody was performed as described above.

Binding assay of insulin receptor

Insulin was labelled to a final specific radioactivity of 326 μCi/μg by a gentle chloramine-T method as previously described [24].

Binding studies were performed by the method of Tramontano et al. [25]. Rat hepatocytes were seeded on to 24-well plates and cultured as described above. The cells were treated without or with dibutyryl cAMP (10^{-3} M) or glucagon (5×10^{-8} M) for 6 h and then washed three times with binding assay buffer solution (Krebs–Ringer/Hepes containing 128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, 10 mM Na_2HPO_4, 20 mM Hepes, pH 7.6). ^{125}I-labelled insulin (36000 c.p.m.) and various concentrations of unlabelled insulin were added to the wells in 200 μl of the binding assay buffer containing 2 % BSA and 0.5 mM *N*-ethylmaleimide, and cells were incubated for a further 18 h at 4 °C. At the end of incubation, cells were washed three times with ice-cold binding assay buffer containing 0.1 % BSA, and the ^{125}I content of the cell lysates was measured after solubilizing the cells in 300 μl of 0.1 M NaOH. Non-specific binding was determined in the presence of 10^{-6} M unlabelled insulin.

Assay of tyrosine kinase activity [19]

Rat hepatocytes were seeded on to 100 mm dishes and cultured as described above. After reaching quiescence, the cells were pretreated without or with dibutyryl cAMP (10^{-3} M) or glucagon (5×10^{-8} M) for 6 h. At the end of the pretreatment, cells were washed three times with HBSS and treated with insulin for 30 s. Cells were lysed at 0 °C in 0.3 ml of Hepes/Triton X/lysis buffer containing 50 mM Hepes/NaOH, pH 7.4, 0.5 mM Na_3VO_4, 1 % Triton X-100, 5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml PMSF, 100 KIU/ml aprotinin and 10 mg/ml PNPP. The cell lysates were tumbled for 30 min at 4 °C. After centrifugation at 15000 ***g*** for 10 min, the supernatant was incubated with 100 μl of wheat germ agglutinin (WGA)–agarose [50 % (v/v); Seikagaku Co., Tokyo, Japan] for 1 h. WGA–agarose was then washed three times with lysis buffer and the adsorbed proteins were eluted with 100 μl of Hepes/Triton X/lysis buffer containing 0.3 M *N*-acetylglucosamine.

WGA-adsorbed fraction (50 μl) was preincubated for 10 min at 25 °C. A 20 min cell-free phosphorylation was initiated with 0.025 ml of reaction mixture to give a final concentration of 50 mM Hepes/NaOH, pH 7.6, 50 mM $MgCl_2$, 0.5 mM Na_3VO_4, 1 % Triton X-100, 10 μM [γ-^{32}P]ATP (6.7 Ci/mmol; Amersham Life Science Co.), 2 mg/ml poly(Glu-Tyr) (4:1). The reactions were terminated by applying 30 μl aliquots to Whatman no. 3MM filter paper. The papers were extensively washed at 4 °C in 10 % trichloroacetic acid containing 10 mM sodium pyrophosphate for 20 min followed by three 15 min 5 % trichloroacetic acid washes. Filters were rinsed in ethanol, dried, and counted in a liquid-scintillation counter. Tyrosine kinase activity was expressed as the phosphate incorporation into the poly(Glu-Tyr) (4:1) over 20 min. The data represent means from quadruplicate determinations.

RESULTS

Effect of cAMP-generating agents on biological action of insulin

Primary cultures of rat hepatocytes were pretreated for 6 h with dibutyryl cAMP or glucagon, and then DNA synthesis in the absence or presence of insulin was measured. cAMP by itself did not stimulate DNA synthesis, but cAMP pretreatment did potentiate the subsequent effect of insulin on DNA synthesis (Figure 1A). This potentiating effect of cAMP pretreatment on DNA synthesis was not observed when the cells were subsequently treated with EGF (Figure 1B). These results suggest that cAMP stimulation does not globally potentiate growth factor-dependent DNA synthesis in primary cultured rat hepatocytes.

Effect of cAMP-generating agents on tyrosine phosphorylation induced by insulin

Quiescent hepatocytes were pretreated for 6 h with dibutyryl cAMP, glucagon, forskolin or cholera toxin and then incubated for 30 s with insulin. Phosphotyrosyl proteins were analysed by immunoblotting using an anti-phosphotyrosine antibody. Insulin markedly induced the tyrosine phosphorylation of 195 kDa, 175–180 kDa and 90–95 kDa proteins (Figure 2). Extraction of rat hepatocytes with detergent-free buffer, Triton X/lysis solution or SDS lysis solution showed the existence of a few other species of insulin-dependent and insulin-independent phosphotyrosyl proteins around the 175–195 kDa, 90–95 kDa and 60–70 kDa regions (results not shown). We identified the 175 kDa protein as IRS-1 and the 95 kDa protein as the insulin receptor β-subunit by immunoprecipitation using anti-IRS-1 and anti-(insulin receptor) antibodies (Figure 3). The observation that the 195 kDa protein was not immunoprecipitated by anti-IRS-1 antibody showed that this protein may be distinct from IRS-1 (Figure 3A).

Dibutyryl cAMP and glucagon greatly potentiated insulin-dependent tyrosine phosphorylation of the 195 kDa protein (Figures 2 and 3A). In Figure 3(A), the 195 kDa band is faint after the sequential immunoprecipitation using anti-IRS-1 and anti-phosphotyrosine antibodies. Although the recovery of phosphotyrosyl proteins by this process is possibly low, it is very clear that this p195 band appears only in response to insulin in cultures that have been pretreated with glucagon or dibutyryl cAMP. Tyrosine phosphorylation of IRS-1 in response to cAMP was not potentiated; however, the band of tyrosine-phosphorylated IRS-1 was shifted upwards slightly by pretreatment with cAMP (Figure 3A). In addition, pretreatment with dibutyryl cAMP and glucagon did not affect insulin-dependent tyrosine phosphorylation of IRS-2 (Figure 3B). These results clearly indicate that the cAMP-dependent increase in tyrosine phosphorylation of the 195 kDa protein could not be explained by the changes in tyrosine phosphorylation of IRS-1 as well as IRS-2. Autophosphorylation of the insulin receptor was not affected by cAMP pretreatment either (Figure 3C). Pretreatment with other cAMP-generating agents such as cholera toxin and forskolin gave the same changes in tyrosine phosphorylation as pretreatment with dibutyryl cAMP or glucagon (results not shown). These results suggest that these changes in insulin-dependent tyrosine phosphorylation are mediated by a cAMP-dependent pathway.

Insulin-dependent stimulation of tyrosine phosphorylation

Tyrosine phosphorylation of the 195 kDa protein, 175–180 kDa proteins, including IRS-1, and 90–95 kDa proteins, including the

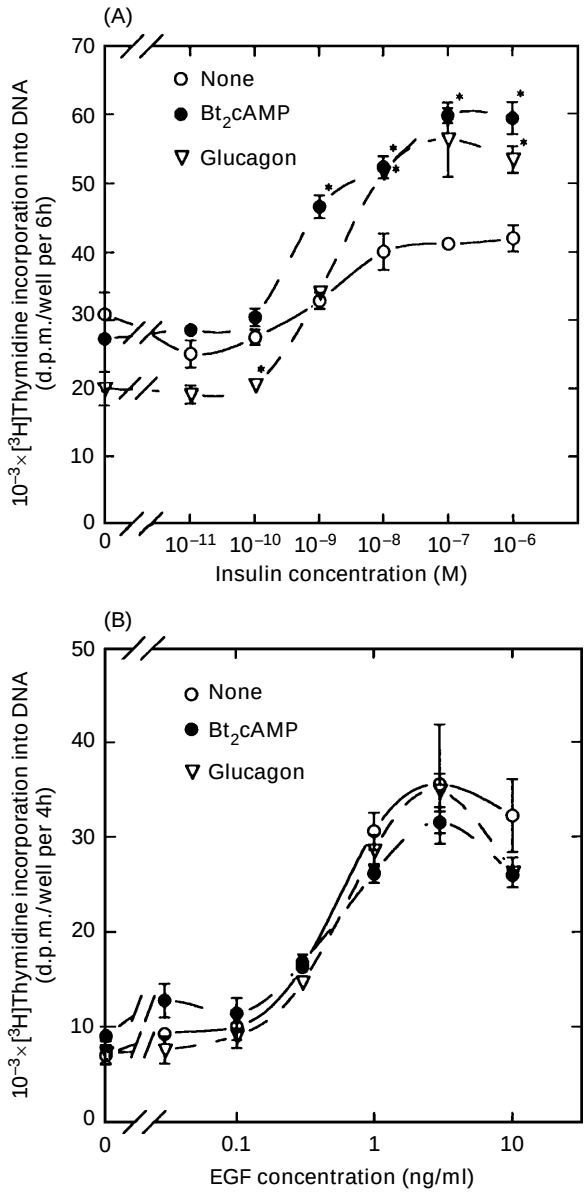

Figure 1 Effect of cAMP-generating agents on DNA synthesis induced by insulin (A) or EGF (B) in primary cultures of rat hepatocytes

Quiescent cells were pretreated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and incubated for 24 h with various concentrations of insulin (**A**) or EGF (**B**). Thymidine incorporation into DNA was measured over the last 6 or 4 h of the second 24 h period. The results shown are means ± S.E.M. from triplicate wells. *Significant difference ($P < 0.05$) between values obtained in the absence and presence of glucagon or Bt_2cAMP at the same insulin concentration (unpaired t test).

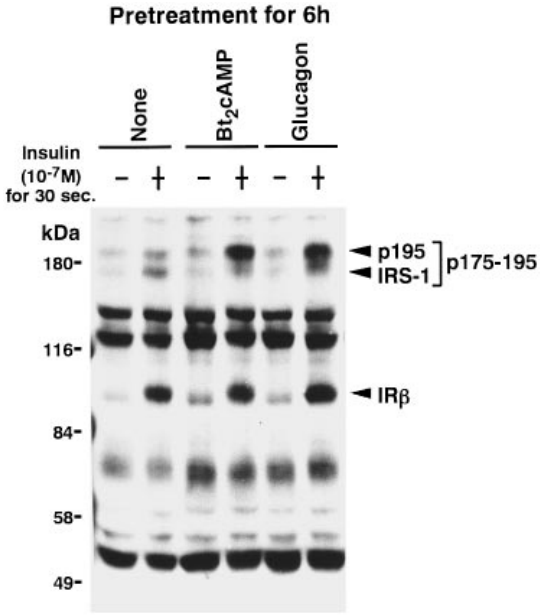

Figure 2 Effect of cAMP-generating agents on tyrosine phosphorylation induced by insulin in primary cultures of rat hepatocytes

Quiescent cells were pretreated for 6 h with no additives, dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M) and then treated with insulin (10^{-7} M) for 30 s. Lysates of the cells were subjected to SDS/PAGE (8% gel), and proteins containing phosphotyrosine were detected by immunoblotting as described in the Experimental section. A representative experiment is shown. IRβ; insulin receptor β-subunit.

insulin receptor β-subunit, was maximal within 30 s of exposure to insulin and decreased thereafter (Figure 4, top). The effect of insulin was concentration-dependent, being half-maximal at a concentration of around 10^{-8} M, and reached a plateau at 10^{-7} M (Figure 4, middle). These data suggest that both the 195 kDa protein and IRS-1 are substrates of insulin receptor kinase. Pretreatment with dibutyryl cAMP or glucagon for 6 h increased tyrosine phosphorylation of the 195 kDa protein without altering the time course (Figure 4, top). Tyrosine phosphorylation of 90–95 kDa proteins including the insulin receptor was stimulated within 15 s of exposure to insulin and remained stable for up to 5 min (Figure 4, top); however, potentiation of its tyrosine phosphorylation by cAMP-generating agents was not observed (Figures 3C and 4). Of the phosphotyrosyl proteins, the changes in tyrosine phosphorylation of the 195 kDa protein were well correlated with the increase in DNA synthesis as described above (Figures 1A and 4, bottom).

cAMP-dependent potentiation and enhancement of tyrosine phosphorylation

Pretreatment with dibutyryl cAMP or glucagon for over 3 h clearly caused potentiation of tyrosine phosphorylation of the 195 kDa protein induced by insulin, although this potentiation was not observed after a short pretreatment with cAMP-generating agents (Figure 5). Tyrosine phosphorylation of the 195 kDa protein stimulated by insulin was potentiated in a concentration-dependent manner by both dibutyryl cAMP and glucagon (results not shown). The effect of cAMP-generating agents on potentiation reached a plateau at 10^{-5} M with dibutyryl cAMP and 10^{-9} M with glucagon.

Effect of cAMP-generating agents and insulin on the insulin receptor

Dibutyryl cAMP or glucagon treatment for 6 h did not significantly affect the affinity for insulin nor the number of insulin receptors of these cells (Figure 6). To test the possibility

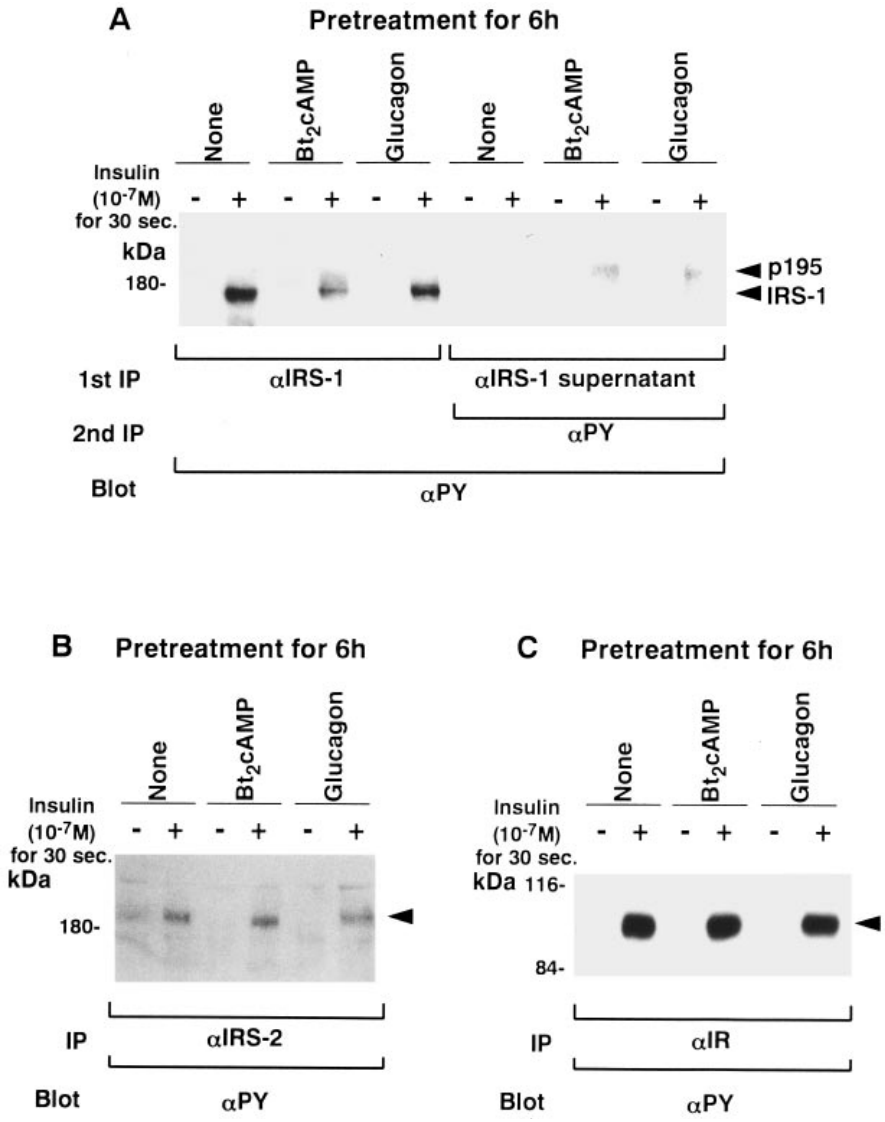

Figure 3 Effect of cAMP-generating agents on tyrosine phosphorylation of IRS-1, IRS-2 and the insulin receptor in primary cultures of rat hepatocytes

(**A**) Effect of dibutyryl cAMP or glucagon on tyrosine phosphorylation of IRS-1. Quiescent cells were pretreated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and incubated without or with insulin (10^{-7} M) for 30 s. After treatment, cells were lysed and IRS-1 was immunoprecipitated with anti-IRS-1 antibody. The supernatant obtained after immunoprecipitation with anti-IRS-1 antibody was re-immunoprecipitated with anti-phosphotyrosine antibody as described in the Experimental section. Proteins in immunoprecipitates (1st IP) with anti-IRS-1 antibody and in re-immunoprecipitates (2nd IP) with anti-phosphotyrosine antibody (αPY) were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. (**B**) Effect of dibutyryl cAMP or glucagon on tyrosine phosphorylation of IRS-2. Quiescent cells were pretreated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and incubated without or with insulin (10^{-7} M) for 30 s. After treatment, cells were lysed and IRS-2 was immunoprecipitated with anti-IRS-2 antibody. Proteins were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. (**C**) Effect of dibutyryl cAMP or glucagon on autophosphorylation of insulin receptor. Quiescent cells were pretreated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and incubated without or with insulin (10^{-7} M) for 30 s. After treatment, cells were lysed and the insulin receptor was immunoprecipitated with anti-(insulin receptor) antibody (αIR). Proteins were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody. Representative experiments are shown.

that cAMP-generating agents may have potentiated insulin receptor activity stimulated by insulin, we measured autophosphorylation of the insulin receptor (Figure 3C) and its intrinsic tyrosine kinase activity (Table 1). Neither dibutyryl cAMP nor glucagon changes autophosphorylation of the insulin receptor at all, but these reagents significantly depressed insulin-dependent receptor tyrosine kinase activity. These results suggested that cAMP-generating agents increase insulin-dependent tyrosine phosphorylation at a site distal to insulin receptor tyrosine kinase activation.

Effect of phosphotyrosine phosphatase inhibitor on tyrosine phosphorylation

Na_3VO_4, which is a known specific phosphotyrosine phosphatase inhibitor, increased the net tyrosine phosphorylation of the 195 kDa protein, the 175–180 kDa proteins, including IRS-1, and the 90–95 kDa proteins, including the insulin receptor β-subunit, induced by insulin in a concentration-dependent manner (Figure 7, top). However, pretreatment with dibutyryl cAMP or glucagon blunted the effect of Na_3VO_4 on tyrosine phos-

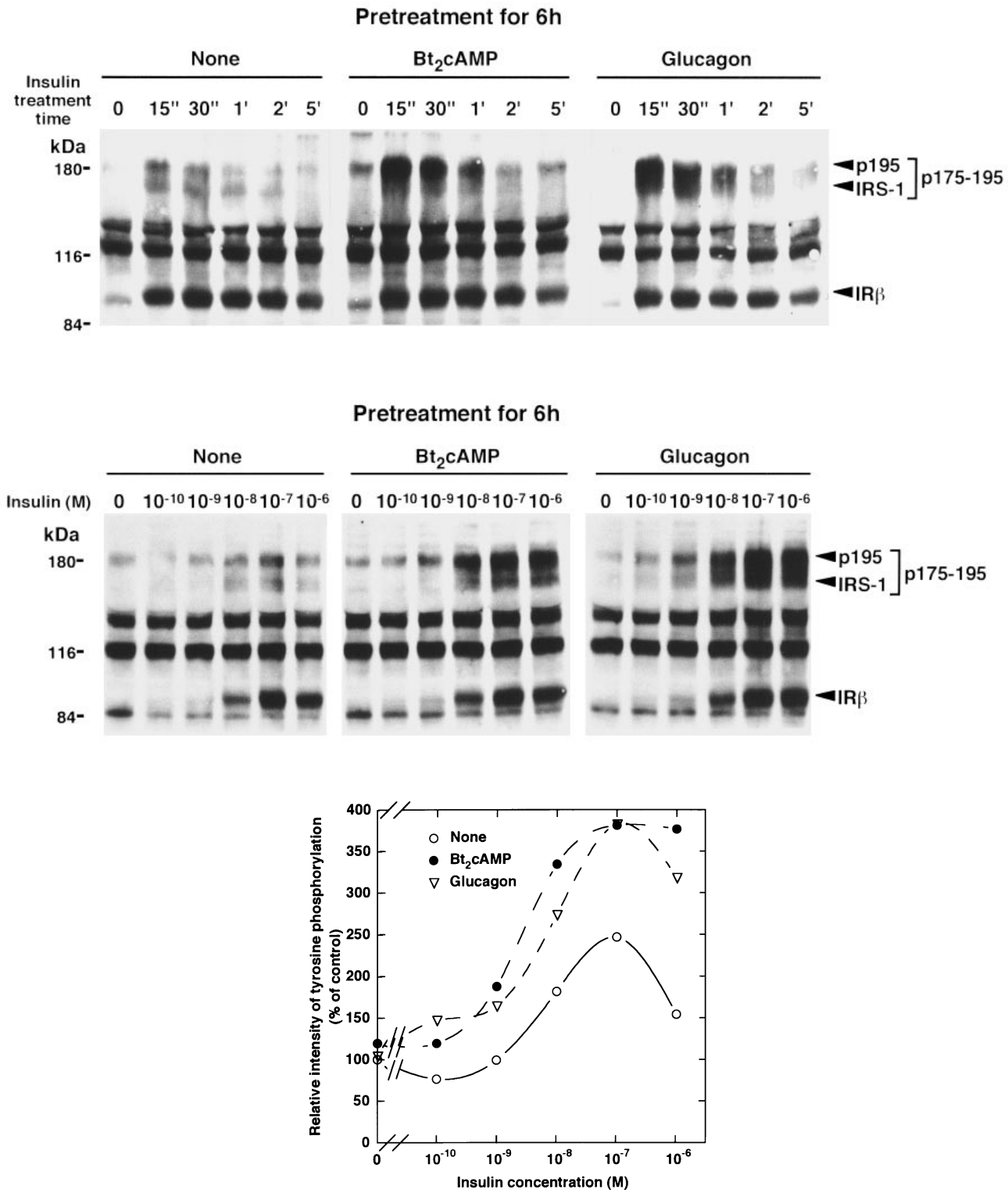

Figure 4 Effect of insulin on tyrosine phosphorylation in primary cultures of rat hepatocytes

Top, time course of insulin effect on tyrosine phosphorylation. Quiescent cells were pretreated for 6 h with no additives (None), dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M) and then treated with insulin (10^{-7} M) for the time indicated. A representative experiment is shown. IRβ, insulin receptor β subunit. Middle, concentration-dependence of effect of insulin on tyrosine phosphorylation. Quiescent cells were pretreated for 6 h with no additives (None), dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and treated with various concentrations of insulin for 30 s. After treatment, lysates of the cells were subjected to immunoblotting analysis using anti-phosphotyrosine antibody as described in the Experimental section. Bottom, quantification analysis of the middle panel. Since the band of the tyrosine-phosphorylated 195 kDa protein masked one of the tyrosine-phosphorylated IRS-1 bands, the regions of the 175–195 kDa proteins of the immunoblot were quantified using the NIH image program.

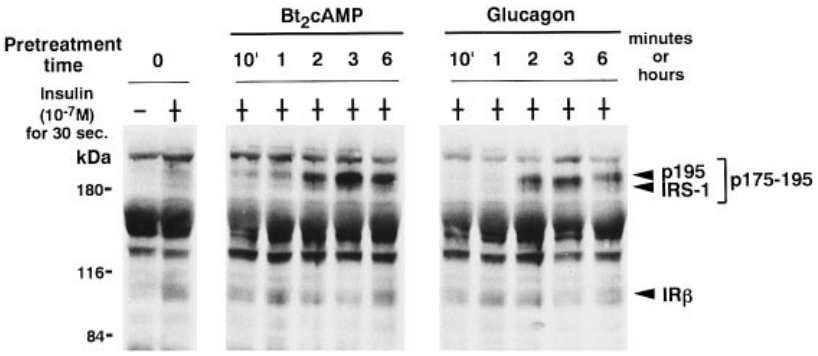

Figure 5 Time course of cAMP-dependent stimulation of tyrosine phosphorylation induced by insulin in primary cultures or rat hepatocytes

Quiescent cells were pretreated with no additives, dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M) for the time indicated, washed and treated with insulin (10^{-7} M) for 30 s. After treatment, lysates of the cells were subjected to immunoblotting analysis using anti-phosphotyrosine antibody as described in the Experimental section. A representative experiment is shown. IRβ, insulin receptor β-subunit.

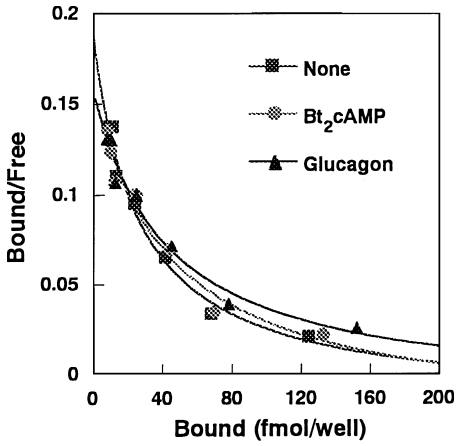

Figure 6 Effect of cAMP-generating agents on insulin binding to the insulin receptor in primary cultures of rat hepatocytes

The results of Scatchard analysis of insulin binding after dibutyryl cAMP or glucagon treatments are shown. Quiescent cells were treated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M). After treatment, binding assays were performed at 4 °C for 18 h, and the specific binding of insulin to its receptor was plotted by the method of Scatchard.

Table 1 Effect of cAMP-generating agents on tyrosine kinase activity of the insulin receptor in primary cultures of rat hepatocytes

Quiescent cells were pretreated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and incubated without or with insulin (10^{-7} M) for 30 s. Cells were solubilized in the Hepes/Triton X/lysis buffer. After centrifugation, the supernatant was incubated with WGA–agarose, and the adsorbed proteins were eluted with the lysis buffer containing 0.3 M *N*-acetylglucosamine. Tyrosine kinase activity of this fraction was measured using poly(Glu–Tyr) (4:1) and [γ-^{32}P]ATP as described in the Experimental section. Results are means ± S.E.M. from quadruplicate determinations. *Significant difference ($P < 0.05$) between values obtained in the absence and presence of glucagon or Bt_2cAMP without or with insulin treatment (unpaired *t* test).

	Phosphate incorporation into poly[Glu–Tyr] (pmol/20 min per 10 μg of protein)	
Pretreatment	− Insulin	+ Insulin
None	1.11 ± 0.12	45.80 ± 1.76
Bt_2cAMP	2.14 ± 0.15	36.02 ± 2.35*
Glucagon	1.68 ± 0.11	40.83 ± 2.65*

phorylation of the 195 kDa protein as compared with cells pretreated with no additive (Figure 7, top), i.e. when the cells were subsequently exposed to insulin in the presence of Na_3VO_4, an increase in intracellular cAMP decreased the blocking effect of Na_3VO_4 on dephosphorylation (Figure 7, bottom). These observations suggest that one mechanism by which cAMP-generating agents potentiate the action of insulin may be by decreasing phosphotyrosine phosphatase activity.

DISCUSSION

We tested the hypothesis that in primary cultures of rat hepatocytes the cAMP-dependent pathway affects insulin action by altering the phosphorylation of tyrosine kinase substrates. Our new findings indicate that activation of the cAMP-dependent pathway potentiates insulin-dependent tyrosine phosphorylation and also insulin-dependent DNA synthesis.

In rat hepatocytes, cAMP pretreatment did not affect insulin-dependent short-term metabolic effects significantly, i.e. protein synthesis (results not shown). In the case of amino acid transport and glycogen synthesis, cAMP pretreatment repressed rather than potentiated the insulin effect (results not shown). At present, the mechanism of this repression is unclear. In contrast, we observed that cAMP pretreatment potentiated insulin-dependent DNA synthesis. The results demonstrating that this pretreatment did not increase EGF-dependent DNA synthesis indicate that the cAMP-priming effect on the progression of the cell cycle to S-phase is specific for insulin in rat hepatocytes.

Insulin stimulated tyrosine phosphorylation of proteins with an estimated molecular mass of 175–195 kDa. Of the insulin-dependent phosphorylated proteins in these bands, only the 175 kDa band could be identified as IRS-1 with an anti-IRS-1 antibody. This antibody did not immunoprecipitate the phosphorylated proteins that migrated in the 195 kDa region. We found that cAMP-generating agents potentiated insulin-dependent tyrosine phosphorylation of the 195 kDa protein, but

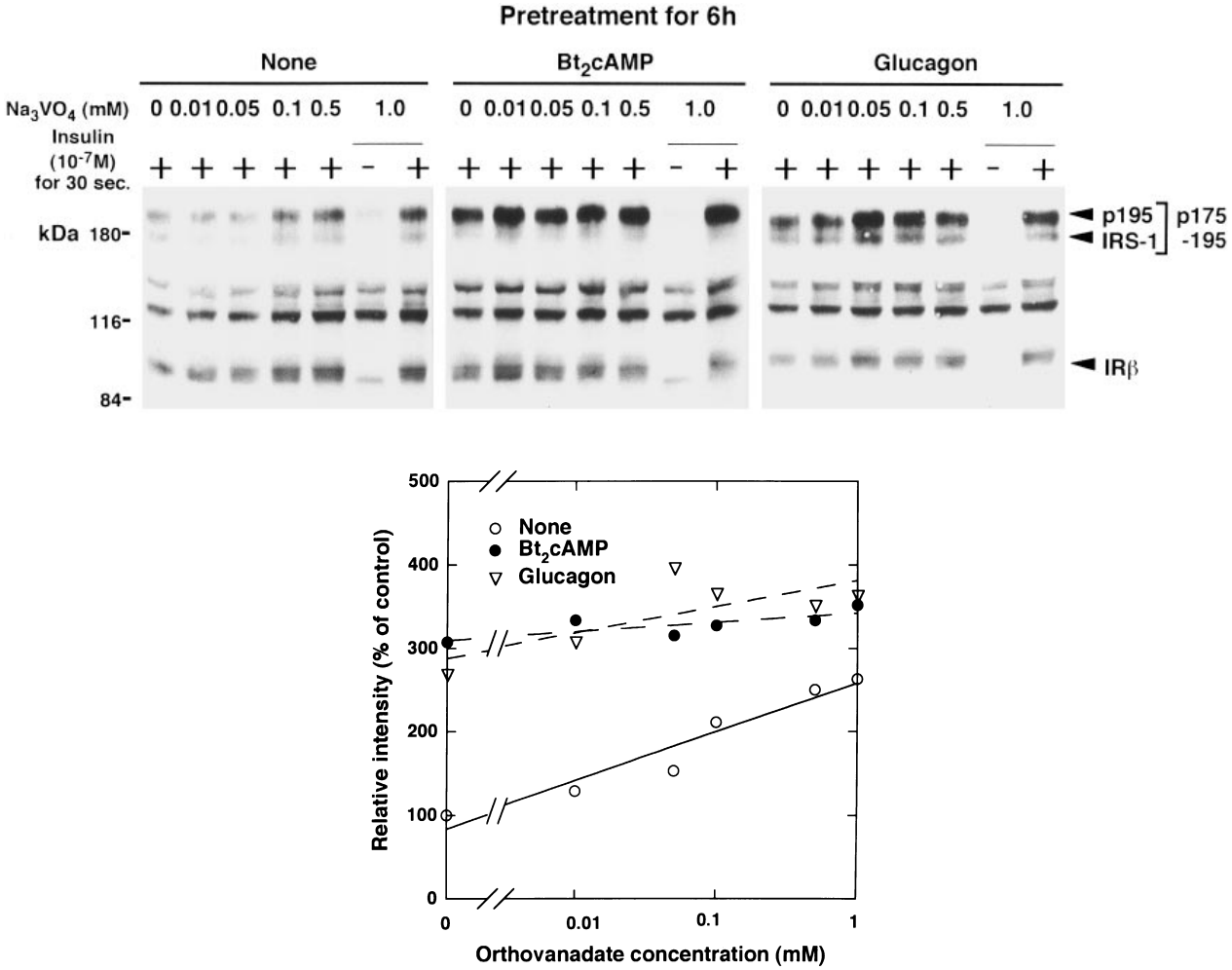

Figure 7 Effect of Na_3VO_4 on tyrosine phosphorylation induced by insulin in primary cultures of rat hepatocytes

Top, effect of Na_3VO_4 on tyrosine phosphorylation of 175–195 kDa and 90–95 kDa proteins. Quiescent cells were pretreated for 6 h without (None) or with dibutyryl cAMP (Bt_2cAMP, 10^{-3} M) or glucagon (5×10^{-8} M), washed, and incubated without or with insulin (10^{-7} M) in the absence or presence of various concentrations of Na_3VO_4 for 30 s. After treatment, lysates of the cells were subjected to immunoblotting analysis using anti-phosphotyrosine antibody as described in the Experimental section. A representative experiment is shown. IRβ, insulin receptor β-subunit. Bottom, quantification analysis of the top panel. The regions of the 175–195 kDa proteins of the immunoblot were quantified using the NIH image program.

not IRS-1. In addition, we observed insulin-dependent tyrosine phosphorylation of IRS-2, which also migrated in the 195 kDa region, suggesting that the 195 kDa band may include IRS-2; however, pretreatment with cAMP-generating agents did not affect insulin-dependent tyrosine phosphorylation of IRS-2. These results clearly indicate that potentiation of tyrosine phosphorylation of the 195 kDa protein was not due to the increase in tyrosine phosphorylation of IRS-2. The time course and concentration-dependent effects of insulin on tyrosine phosphorylation of 175–195 kDa proteins and insulin receptor suggest that the 195 kDa protein is a novel substrate of insulin receptor kinase.

Mechanisms by which the cAMP stimulus potentiates insulin-dependent tyrosine phosphorylation of the 195 kDa protein may involve (1) increased synthesis or activation of tyrosine kinase, (2) increased synthesis of substrates or alteration in substrate availability, (3) inhibition of phosphatases, or a combination of the above.

We first showed that cAMP-generating agents did not change the number or affinity of the insulin receptor. Pretreatment with cAMP-generating agents did not affect insulin-stimulated autophosphorylation but significantly decreased the tyrosine kinase activity of the insulin receptor in response to insulin. Others found that the insulin receptor can be phosphorylated at Ser/Thr residues by cAMP-dependent kinase and this phosphorylation decreases rather than increases the insulin-dependent activation of the insulin receptor kinase [26,27]. Taking these data together, we conclude that the effect of cAMP-generating agents on the potentiation of tyrosine phosphorylation of the 195 kDa protein induced by insulin is not dependent on the activity of the insulin receptor tyrosine kinase.

We have not excluded the possibility that cAMP-dependent stimulation increases the amount or availability of the substrates of the tyrosine kinase, such as the insulin receptor kinase, or decreases their availability to the phosphotyrosine phosphatases. IRS-1 is reported to contain many potential sites of phosphorylation by various kinases including cAMP-dependent kinase, Ca^{2+}-dependent kinase and casein kinase [4], and this phosphorylation may cause conformational changes in the protein followed by modulation of its affinity for tyrosine kinases or

phosphotyrosine phosphatases [28]. We observed that, after cAMP pretreatment, tyrosine phosphorylation of IRS-1 decreased slightly, while tyrosine phosphorylation of the 195 kDa protein markedly increased. The difference between tyrosine phosphorylation of IRS-1 and the 195 kDa protein may be explained by modulation of the substrates in a substrate-specific manner.

We then studied tyrosine phosphorylation stimulated by insulin in the presence of the potent phosphotyrosine phosphatase inhibitor, Na_3VO_4. In the presence of Na_3VO_4, pretreatment with cAMP-generating agents caused less net increase in tyrosine phosphorylation of the 195 kDa protein than after control pretreatment periods. This suggested that cAMP generation might decrease phosphotyrosine phosphatase activity and thus potentiate insulin-induced tyrosine phosphorylation. Recently, it was suggested that Syp might play a role in the dephosphorylation of kinase substrates including the insulin receptor and IRS-1 by binding to tyrosine-phosphorylated IRS-1 [29,30]. Furthermore, PTP-PEST, which is a novel cytosolic tyrosine phosphatase, is phosphorylated *in vitro* by both cAMP-dependent protein kinase and protein kinase C, and this modulation changes its phosphatase activity [31]. These enzymes could possibly be modulators of insulin-dependent tyrosine phosphorylation in response to an increase in the intracellular cAMP concentration. Again as tyrosine phosphorylation of IRS-1 was repressed and autophosphorylation of the insulin receptor was not affected by cAMP pretreatment, to answer these questions it will be necessary to measure phosphotyrosine phosphatase activity specific for the tyrosine-phosphorylated 195 kDa protein.

From our preliminary results, cycloheximide, a potent protein synthesis inhibitor, decreased the cAMP-dependent potentiation of tyrosine phosphorylation of the 195 kDa protein induced by insulin (results not shown). In view of the data showing that potentiation of tyrosine phosphorylation induced by cAMP-generating agents requires at least 3 h, it is likely that the potentiation of tyrosine phosphorylation is mediated by a process requiring protein synthesis. For example, tyrosine kinase substrates, or phosphatase inhibitors, or other autocrine growth factor(s) may be synthesized and their syntheses are necessary for the potentiation of the insulin signals.

We have previously shown in FRTL-5 cells that activation of the cAMP-dependent pathway acts in a synergistic manner to potentiate the actions of IGF-I on both tyrosine phosphorylation of certain substrates and cell growth [13,19]. A comparison of the present results for rat hepatocytes with our previous results for rat thyroid FRTL-5 cells indicates that the characteristics of the substrates are dependent on the cell or tissue type as well as on the nature of the agonist, i.e. insulin compared with IGF-I. In hepatocytes, cAMP-dependent potentiation of tyrosine phosphorylation was observed in only the 195 kDa protein and not IRS-1, whereas in FRTL-5 cells it was observed in IRS-1 and a 175 kDa protein distinct from IRS-1 (M. Ariga, Y. Ito, F. Hakuno, K. Onodera and S.-I. Takahashi, unpublished work). Furthermore, in hepatocytes, increasing cAMP affected maximal DNA synthesis without a shift in the concentration–response curve. This is also different from interaction between cAMP and IGF-I in FRTL-5 cells. In further studies it will be necessary to identify each protein to elucidate their physiological significance and to explain the mechanisms of this potentiation. Recently, we observed cAMP-dependent potentiation of tyrosine phosphorylation of the 195 kDa protein induced by insulin in rat hepatoma as well as human hepatoma cell lines (Y. Ito, A. Takenaka, S.-I. Takahashi and T. Noguchi, unpublished work). It suggested that this potentiation is generally important to the control of cell growth induced by insulin in hepatocytes. As the cAMP concentration in liver is known to be increased before liver regeneration [32], the interaction between cAMP-dependent and insulin-dependent pathways through tyrosine phosphorylation of the 195 kDa protein may play an important role in stimulating hepatocyte growth.

In summary, our data show that stimulation of the cAMP-dependent pathway regulates the steady-state level of tyrosine phosphorylation of several substrates in primary cultures of rat hepatocytes in a manner similar to that in FRTL-5 cells. In rat hepatocytes and FRTL-5 cells, we have demonstrated that cAMP-dependent and insulin- or IGF-I-dependent signals converge into a common pathway. The interactions between cAMP-dependent and insulin-dependent pathways that lead to augmentation of DNA synthesis appear to parallel the changes in tyrosine phosphorylation. Further studies will be required to determine whether there is a causal relationship between these phenomena.

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