ELSEVIER

European Journal of Pharmacology 409 (2000) 109–121

ejp

www.elsevier.nl/locate/ejphar

Insulin increased cAMP phosphodiesterase activity antagonizing metabolic actions of glucagon in rat hepatocytes cultured with herbimycin A

Kaname Ishibashi [a], Toshiyuki Fujioka [a], Michio Ui [a,b,*]

[a] *The Ui Laboratory, Institute of Physical and Chemical Research, Hirosawa 2-1, Wako 351-0198, Japan*
[b] *The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, Japan*

Received 31 July 2000; received in revised form 16 October 2000; accepted 18 October 2000

Abstract

The baseline activity of cyclic nucleotide phosphodiesterase 4 was markedly lowered by primary culture of rat hepatocytes with herbimycin A for 4 h [Eur. J. Biochem. 260 (1999) 398–408.]. We now report that insulin added to this preparation of hepatocytes, which had been completely freed of herbimycin, increased the thus lowered phosphodiesterase activity, consequently antagonizing glucagon-induced production of cAMP and activation of glycogen phosphorylase. The insulin receptor β-subunits and α-tubulin were tyrosine-phosphorylated upon the addition of insulin. The phosphorylation of α-tubulin afforded conditions unfavorable for microtubule assembly that is responsible for phosphodiesterase inhibition. These effects of insulin observed in herbimycin-pretreated hepatocytes were not inhibited by wortmannin that actually abolished insulin-induced activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase) under the same conditions. The physiological significance of the insulin action not mediated by PtdIns 3-kinase in herbimycin-pretreated hepatocytes is discussed.

Keywords: Insulin; Primary culture of hepatocyte; Tyrosine phosphorylation; cAMP phosphodiesterase; Microtubule; Wortmannin; Herbimycin A

1. Introduction

Cyclic AMP plays a key role as one of the second messengers that mediate intracellular signalling networks triggered by membrane receptor stimulation and eventually leading to alteration of cell functions including metabolic activities. The synthesis of cAMP is catalyzed by adenylate cyclase, which activity increases via activation of G_s coupled to typical heptahelical receptors, whereas the cyclic nucleotide undergoes breakdown through the sole route of hydrolysis to 5′-AMP by phosphodiesterases. Thus, the cellular concentration of cAMP can be regulated by changing the activity of either adenylate cyclase or phosphodiesterase (Houslay and Milligan, 1997). A number of hormones, neurotransmitters and autacoids are known to activate or inhibit (deactivate) adenylate cyclase via G_s or G_i (G_o), respectively, whereas there are much less cases in which these physiological extracellular signalling substances are involved in the regulation of phosphodiesterase. Exceptionally, attention has been focused on insulin as an activator of phosphodiesterase in view of its metabolic actions somehow antagonistic to adenylate-cyclase-activating hormones (Irvine et al., 1993; Manganiello et al., 1995; Rahn et al., 1996; Degerman et al., 1997; Helmsdorf and Dettmer, 1998; Castan et al., 1999; Van Harmelen et al., 1999).

Phosphodiesterase forms a multienzyme family in which eleven distinct classes, phosphodiesterase-1 to -11, have so far been identified depending on their differences in amino acid sequences, affinity for cyclic nucleotides, sensitivity to activators or inhibitors, subcellular localization and specific expression in various cell types. The individual members of each phosphodiesterase class are encoded by different genes, each of which, in turn, gives rise to variants by alternative splicing of mRNA or the use of alternate promoters (Beavo et al., 1994; Bolger, 1994;

* Corresponding author. The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, Japan. Tel.: +81-3-3823-2951; fax: +81-3-3823-2202.
E-mail address: ui@rinshoken.or.jp (M. Ui).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00803-7

Beavo, 1995; Manganiello et al., 1995; Fisher et al., 1998; Houslay et al., 1998; Soderling et al., 1998, 1999; Fujishige et al., 1999; Fawcett et al., 2000; Soderling and Beavo, 2000). Among these phosphodiesterase superfamilies, the cAMP-specific type-3 and the type-4 enzymes have been the subject of extensive studies, because changes in the cellular concentrations of cAMP resulting from regulation of their activities mediate a variety of cellular responses to hormonal, neuronal or paracrine stimuli as well as to drug additions. For instance, phosphodiesterase 3 (mostly 3B) is the enzyme selectively involved when insulin displays anti-lipolytic or other metabolic actions in adipocytes (Manganiello et al., 1995; Moberg et al., 1998; Enoksson et al., 1998; Wijkander et al., 1998; Castan et al., 1999; Kitamura et al., 1999; Tang et al., 1999; Van Harmelen et al., 1999; Rahn Landstrom et al., 2000) or when insulin secretion from pancreatic β-cells or related cell lines is regulated (Parker et al., 1997; Zhao et al., 1997, 1998; Ahmad et al., 2000). The phosphodiesterase-4 activity could be regulated by phosphorylation on serine residues (Sette and Conti, 1996; Hoffmann et al., 1998, 1999; Lim et al., 1999; Liu and Maurice, 1999) and could play an important role in immune, allergic, vascular or hormonal responses of various cells (Baroja et al., 1999; Brideau et al., 1999; Denis and Riendeau, 1999; Essayan, 1999; Fuhrmann et al., 1999; Liu and Maurice, 1999; Ma et al., 1999; Banner et al., 2000; Hansen et al., 2000; Liu et al., 2000; MacKenzie and Houslay, 2000; MacKenzie et al., 2000; Oki et al., 2000). The three-dimensional structure of phosphodiesterase-4 has recently been determined (Xu et al., 2000).

The phosphodiesterase-4 activity in hepatocytes is under regulation of a different mechanism. A short-term culture of rat hepatocytes with herbimycin A, an inhibitor of protein tyrosine kinases, gave rise to marked suppression of cellular rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone)-sensitive phosphodiesterase-4 activity as a result of assembly of microtubules whose tyrosine residues had been dephosphorylated (Ishibashi et al., 1999). Since there was no essential difference in the phosphodiesterase activity, if measured after microtubule disassembly or cell disruption, between herbimycin-treated and non-treated cells, the formation of microtubular networks connecting intracellular membranes would be likely to afford conditions unfavorable for full activation of phosphodiesterase due to conformational restraint of the protein structure or for other unknown reasons. It was then supposed that inhibition of tyrosine kinases in isolated hepatocytes would function as a means to restore the normal microtubular networks that has been impaired during the cell isolation by the collagenase-digestion method (Ishibashi et al., 1999).

The purpose of the present communication is to report that the thus lowered phosphodiesterase activity in herbimycin-treated hepatocytes increased upon the addition of insulin to the cultured cells from which the tyrosine kinase inhibitor had been well washed out. Glucagon-induced cAMP accumulation and accompanying phosphorylase activation was antagonized by insulin successfully under these conditions.

2. Materials and methods

2.1. Hepatocyte culture

Livers from male rats of the Wistar-derived Donryu strain, weighing 180–220 g, were perfused with collagenase to prepare parenchymal hepatocytes as described previously (Kajiyama and Ui, 1994). The hepatocytes obtained were routinely 95–99% viable as estimated by Trypan blue exclusion. Herbimycin A (1 μM) or dimethyl sulfoxide (DMSO, 0.01% as the vehicle) was added to the cell suspension of the isolated hepatocytes in Williams' E medium (ICN Flow) fortified with 10 units/ml of aprotinin (Hoechst, Japan), which was incubated for 10 min at 37°C before the start of culture. The cells were cultured for 4 h under atmosphere of 5% CO_2/95% air in the same medium containing 0.5% (v/v) fetal calf serum (Gibco), 1 nM dexamethasone (Sigma) and 1 nM insulin (Sigma) in collagen (type I)-coated 35-mm-diameter dishes (Iwaki, Japan), to which cells attached as monolayers within 1 h. A coverslip was placed on the bottom of the non-coated dish where indicated below. DMSO at the concentration used was essentially without effect on viability and phosphodiesterase activity of cultured cells and on their cAMP or phosphorylase responses during the subsequent incubation.

At the end of 4-h culture, the cell monolayers were washed thoroughly, under continuous pouring and suction, with Krebs–Ringer–HEPES (KR–HEPES) medium (composition in mM: NaCl 140, KCl 4.8, KH_2PO_4 1.2, $MgCl_2$ 1.4, $CaCl_2$ 2.5, $NaHCO_3$ 3.6, HEPES (pH 7.4) 10) before further short-term incubation at 37°C in the same medium supplemented with 0.1% bovine serum albumin (essentially free of fatty acids; Sigma) for analyses described below. In experiments in which phosphorylase assay was conducted, the hepatocyte culture was prolonged for additional 45 min with the addition of 40 mM glucose for the purpose of converting most of the enzyme into the inactive state (Okajima and Ui, 1982) before thorough washing under the same conditions as above.

2.2. Measurement of cellular cAMP

The hepatocytes cultured and thoroughly washed as described above were analyzed for cAMP after incubation at 37°C in KR–HEPES/0.1% bovine serum albumin under various conditions. After the incubation, cAMP was released to the supernatant by boiling the incubated cells in 0.1 M HCl to be measured radioimmunochemically as described in detail previously (Kajiyama and Ui, 1994). To

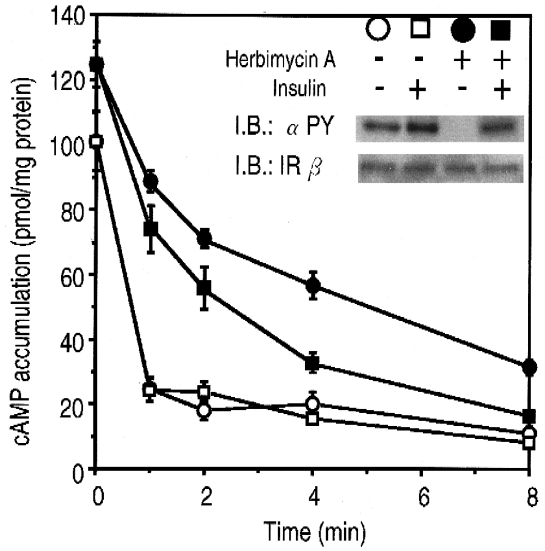

Fig. 1. Promotion by insulin of decomposition of cAMP during short-time incubation of hepatocytes precultured with herbimycin A. Rat hepatocyte monolayers cultured without (○, □) or with (●, ■) 1 μM herbimycin for 4 h were washed and incubated at 37°C with 1 mM IBMX for 10 min and with further addition of 100 μM forskolin for an additional period of 10 min to cause the accumulation of cAMP in the cells. After forskolin and IBMX were washed out quickly and completely, the accumulated cAMP was rendered to decompose by incubating the cells further with 100 μM 2′,3′-dideoxyadenosine, an inhibitor of adenylate cyclase, in the absence of IBMX for various length of time as shown on abscissa. Vehicle (○, ●) or 1 μM insulin (□, ■) was added together with 2′,3′-dideoxyadenosine. Each point represents the mean ± S.E.M. from triplicate experiments. The data shown in inset was obtained as follows. A fraction of the cultured cells, after quick washing, was incubated with or without 1 μM insulin for 10 min and was subjected to the procedure of cell lysis. The cell lysates were then subjected to immunoprecipitation with mouse monoclonal antibodies raised against the insulin receptor β-subunits, and the immunoprecipitates (5×10^6 cells equivalent per lane) were separated by 10.0% SDS-PAGE before being subjected to immunoblotting (I.B.) with anti-phosphotyrosine antibody (αPY) or with anti-insulin receptor β-subunits antibody (αIRβ). The protein bands corresponding to the insulin receptor β-subunit on SDS-PAGE are shown. Addition and non-addition are also indicated by "+" and "−", respectively.

estimate the breakdown of the once accumulated cAMP in Fig. 1, the cultured and washed cell monolayers were first incubated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min and then for additional 10 min with a further addition of 100 μM forskolin. These cells were washed quickly by pouring warmed KR–HEPES onto the cell monolayer under suction to remove forskolin and IBMX and finally incubated in KR–HEPES/0.1% bovine serum albumin containing 100 μM 2′,3′-dideoxyadenosine (an inhibitor of adenylate cyclase) for various length of time to follow decomposition of cAMP catalyzed by methylxanthine-susceptible phosphodiesterase.

In some experiments shown in Table 3, cAMP was measured in the presence of IBMX, a phosphodiesterase inhibitor with the high efficacy, to follow its generation due to adenylate cyclase activation solely by glucagon. In other experiments, however, measurement of cAMP was carried out with glucagon in the absence of phosphodiesterase inhibitor to see the effect of concurrently added insulin to antagonize, by inhibiting phosphodiesterase, the glucagon action.

2.3. Assay of glycogen phosphorylase in the active form

The hepatocyte monolayers exposed to 40 mM glucose at the final stage of the culture (see above) were, after thorough washing, incubated in KR–HEPES/0.1% bovine serum albumin at 37°C with 1 μM glucagon for various length of time in Fig. 2. In Tables 2 and 3, the cells were incubated with wortmannin, IBMX or insulin, as indicated, before 30-s incubation with glucagon. In either case, the incubation with glucagon was terminated by quick freezing in liquid nitrogen. A fraction of the frozen cells was subjected to cAMP assay by the above-described method after extraction with 0.1 N HCl. Another fraction of the frozen cells were disrupted and suspended in 100 mM Tris–HCl buffer (pH 7.4) containing 20 mM EDTA, 100 mM NaF, 1.5% glycogen and 15 mM [^{14}C]glucose 1-phosphate (2×10^4 cpm/tube) and 0.75 mM caffeine. Phosphorylase activity was measured as the incorporation of ^{14}C into glycogen during 30-min incubation at 37°C (Okajima and Ui, 1982).

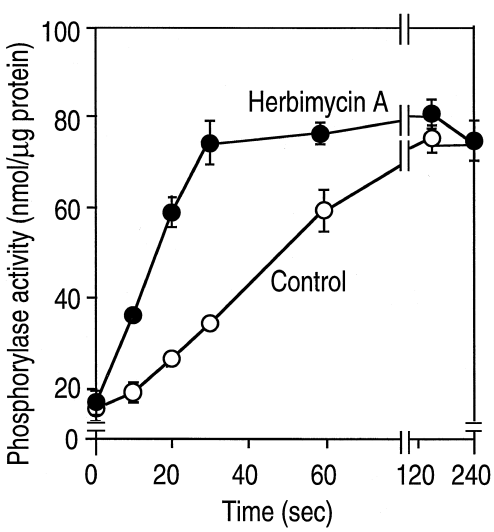

Fig. 2. Conversion of glycogen phosphorylase into the active form during short-term incubation with glucagon of hepatocytes cultured with or without herbimycin. Hepatocytes cultured for 4 h with (●) or without (○) 1 μM herbimycin were further cultured for 45 min with the addition of 40 mM glucose to maintain most of the phosphorylase enzyme in the inactive state. The cells, after thorough washing, were incubated with 1 μM glucagon for increasing period of time as shown on abscissa to be then frozen quickly at each time. The phosphorylase activity of the disrupted cells, measured as the incorporation of [U–^{14}C]glucose 1-phosphate into glycogen (nmol per mg of protein per min), is plotted as a function of incubation time with glucagon. Each plot represents mean ± S.E.M. from four different cell preparations. No error bar is shown when it was smaller than the diameter of the symbol. Similar results were obtained in additional two experiments.

2.4. Immunoprecipitation and immunoblotting

Hepatocytes cultured, washed and incubated for 10 min with or without insulin were washed with phosphate-buffered saline (PBS: 0.55 M NaCl, 0.1 M Na phosphate buffer; pH 7.4) three times and quickly lysed by immersing (2×10^7 cells/ml) in an ice-cold lysis buffer consisting of 1% Triton X-100, 1 mM Na_3VO_4 (Sigma), 100 mM NaF, 150 mM NaCl, 50 mM HEPES (pH 7.5), 100 units/ml of aprotinin, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Wako, Osaka), 2 mM dithiothreitol, 1 mM EGTA, 0.5% sodium deoxycholate and 0.1% bovine serum albumin. Routinely, the cell lysate was cleared by 1-h incubation with non-immune rabbit serum, which was followed by incubation with protein G-Sepharose. The cleared supernatants were subjected to immunoprecipitation with polyclonal rabbit anti-phosphotyrosine antibody (Chemicon) or with mouse monoclonal antibody raised against insulin receptor β-subunits (29B4, Santa Cruz) for longer than 1 h, and then with protein G-Sepharose for 1 h at 4°C. The immunoprecipitates, after washing with the ice-cold lysis buffer, were boiled for 3 min in 30 μl of sample buffer consisting of 1% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue and 62.5 mM Tris (pH 6.8). In Fig. 3, the incubated hepatocytes were directly added with 10% trichloroacetic acid (as indicated) and the resultant precipitates of denatured proteins were washed and boiled in the same sample buffer as that used for immunoprecipitates.

The thus solubilized peptides were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (10.0%) and transferred electrically to a nitrocellulose membrane at 2 mA/cm^2 for 40 min. After washing with Tris-buffered saline (TBS: 20 mM Tris (pH 7.8) and 0.1 M NaCl), the membrane was incubated at 4°C with TBS containing 3% bovine serum albumin (blocking buffer) for 1 h. The blot was then incubated at 4°C for 1 h with the same monoclonal anti-α-tubulin antibody (diluted 1:200 with the blocking buffer) as that used for the immunostaining (Fig. 3). In Fig. 1, immunoblotting was achieved with mouse monoclonal anti-phosphotyrosine antibody (PY20, Santa Cruz) or polyclonal anti-(insulin receptor β-subunit) antibodies (C-19, Santa Cruz). After several washings with TBS, the membrane was incubated further for 1 h with ^{125}I-labeled polyclonal rabbit anti-mouse immunoglobulin G (IgG) (NEN, at a 1:1000 dilution with the blocking buffer). The radioactive bands were visualized with a Fuji BAS2000 bioimaging analyzer.

2.5. Assay of PtdIns 3-kinase bound to tyrosine-phosphorylated proteins in response to insulin receptor stimulation

Phosphatidylinositol (PtdIns) 3-kinase activity was measured by the procedure reported previously (Matsuo et al., 1996). Briefly, the cultured cells further incubated with or without insulin for 10 min were subjected to the immunoprecipitation procedure (see above for detail) with anti-phosphotyrosine antibodies (PY20). The immunoprecipi-

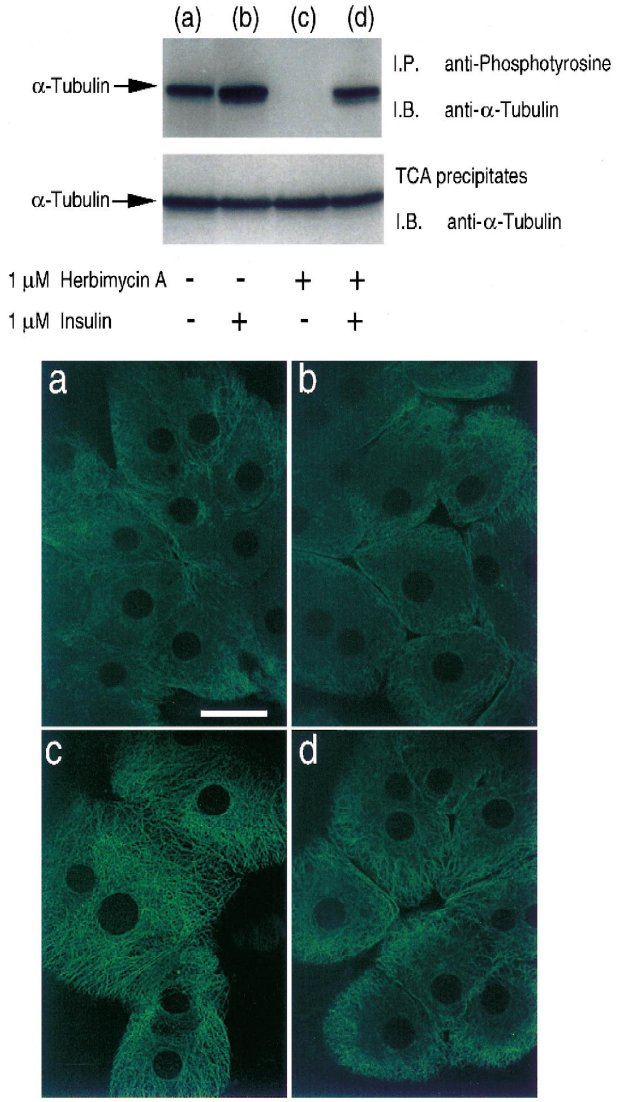

Fig. 3. Tyrosine phosphorylation of α-tubulin and cytoplasmic microtubular assembly in hepatocytes subjected to the 4-h culture with or without herbimycin followed by the 10-min incubation with or without insulin. Hepatocytes were cultured for 4 h with (c and d) or without (a and b) 1 μM herbimycin, and then, after quick washing, incubated for 10 min with (b and d) or without (a and c) 1 μM insulin. Addition and non-addition are also shown by "+" and "−", respectively. The lysates prepared from the thus cultured and incubated cells were subjected to immunoprecipitation (I.P.) with rabbit polyclonal anti-phosphotyrosine antibodies (αPY) or to total protein precipitation by means of 10% trichloroacetic acid (TCA). The immunoprecipitates (5×10^6 cells equivalent per lane) or the TCA precipitates (1×10^5 cells equivalent per lane) were separated by 10.0% SDS-PAGE before being subjected to immunoblotting with a mouse monoclonal anti-α-tubulin antibody. The protein bands corresponding to α-tubulin on SDS-PAGE are shown in the top two panels. Another fraction of the cultured and incubated cells were fixed and subjected to immunostaining with monoclonal anti-α-tubulin antibody (DM1A) and confocal microscopy as described in Section 2.6, and microscopic photographs are shown in the bottom panels a–d. The bar in panel a represents 25 μm.

tates were, after complete washing, suspended in the reaction mixture consisting of 40 mM Tris–HCl (pH 7.4), 0.5 mM EGTA, 0.1 mM [γ-^{32}P]ATP (5 μCi), 0.2 mM PtdIns and 0.2 mM phosphatidylserine in a total volume of 100 μl for 3×10^6 cell equivalent. The reaction, allowed to proceed at 37°C for 15 min, was terminated by the addition of 20 μl of 8% $HClO_4$ and 0.45 ml of chloroform/methanol (1:2). After vigorous stirring, the mixture was added with 0.15 ml of chloroform and 0.15 ml of 8% $HClO_4$ to separate the organic phase, which was washed with chloroform-saturated 0.5 M NaCl containing 1% $HClO_4$ and then evaporated to dryness. The extract was dissolved in 20 μl of chloroform/methanol (2:1) to be spotted on a silica gel plate (Silica Gel 60, Merck). The plate was developed in chloroform/methanol/28% NH_4OH/H_2O (70:100:25:15), dried and visualized for radioactivities in the PtdIns(3)P fraction with a Fuji BAS2000 bioimaging analyzer.

2.6. Fluorescence staining of microtubular structure in hepatocyte cytoplasm

Hepatocytes were cultured on coverslips coated with collagen gel that had been prepared from rat tail tendon as described previously (Dunn et al., 1989). The cells were fixed to the coverslips by 30-min exposure at 4°C to 4% paraformaldehyde in PBS and, after being washed three times with PBS, permeabilized by 1-h exposure at room temperature to 0.1% Triton X-100 in a blocking buffer which contains 10% normal goat serum (Vector) in PBS. The coverslips were then immersed for 1 h in the blocking buffer fortified with a mouse monoclonal antibody raised against α-tubulin (DM1A, Oncogene Science) at 1:100 dilution. After washing with PBS three times (5 min each), 1-h staining of the bound anti-α-tubulin antibodies was carried out in the dark with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (E-Y laboratories) at 1:100 dilution. Following 4-times washing with PBS, a drop of *p*-phenylenediamine (1 mg/ml, Sigma) in a 9:1 mixture (pH 8.5) of glycerol and PBS was added to each coverslip before placing it on a glass slide and sealing with nail polish as a specimen for microscopy.

2.7. Confocal microscopy

All specimens were examined with a Bio-Rad MRC 600 scanning confocal microscope outfitted with a krypton/argon laser. Confocal images were obtained with 100 × Plan-Neofluar objective (Zeiss), digitized using Bio-Rad COMOS software and stored as 768 × 512-pixel files with 8 bits (1 byte) per pixel. For three-dimensional reconstruction of stained hepatocytes, at least 20 images were recorded at 0.32- to 0.64-μm intervals. The images were compiled using the project Z series menu, converted to TIFF (Tagged Image File Format) images, and printed with an image scanner S3600-30 (Mitsubishi).

3. Results

3.1. Enhancement by insulin of spontaneous breakdown of cAMP in hepatocytes cultured with herbimycin A

Rat hepatocyte monolayers cultured for 4 h with or without 1 μM herbimycin were subjected to a 10-min incubation with IBMX, to cause total inhibition of phosphodiesterase, which was followed by additional 10-min incubation with further addition of an adenylate cyclase activator such as forskolin or glucagon to cause huge accumulation of cAMP in hepatocytes. After the phosphodiesterase inhibitor and adenylate cyclase activator were washed out quickly and completely, the cell monolayers were incubated in the fresh medium containing 2′,3′-dideoxyadenosine, an inhibitor of adenylate cyclase catalytic protein or a P-site inhibitor (Olaus et al., 1982) to follow breakdown of cAMP that was mostly dependent on hepatocyte phosphodiesterase activity. In fact, no breakdown was observed when the fresh medium was again fortified with IBMX (data not shown but see Ishibashi et al., 1999). A set of plots in Fig. 1 originates from the experiments with the use of forskolin as the adenylate cyclase activator. Plots in open and solid circles reproduced our recent results (Ishibashi et al., 1999); phosphodiesterase activity was so high as to cause prompt (i.e., within 1 min) breakdown of most of the once accumulated cAMP in hepatocytes cultured without herbimycin, whereas the decomposition of cAMP during incubation of the herbimycin-treated cells was much less prompt due to lowered phosphodiesterase activity (Fig. 1).

We tested the effect of insulin added to the phosphodiesterase assay medium containing 2′,3′-dideoxyadenosine but no IBMX (Fig. 1, plots in squares). The high phosphodiesterase activity of hepatocytes cultured without herbimycin was never susceptible to the insulin addition, which was, however, effective in increasing the rate of cAMP breakdown catalyzed by the lowered activity of phosphodiesterase in the herbimycin-treated cells. Thus, insulin proved to be an activator of phosphodiesterase, only when the baseline enzyme activity had been lowered by prior exposure of hepatocytes to an inhibitor of tyrosine kinase such as herbimycin, though the concentration (1 μM) of insulin used in Fig. 1 was much higher than its physiologic concentrations.

Hepatocytes cultured with or without herbimycin and then incubated with or without insulin were lysed and subjected to immunoprecipitation with the antibodies raised against insulin receptor β-subunits followed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 1, inset). Phosphorylation occurred on tyrosine residues of the insulin receptor β-subunits even if no insulin was added to the cells precultured without herbimycin, probably reflecting the "baseline" activity of the receptor or as a result of slight stimulation of the receptor by a low concentration (1 nM) of insulin that had been added to the culture medium.

Incubation of these cells with 1 μM insulin caused a significant increase in the magnitude of tyrosine phosphorylation of the receptor. In contrast, essentially no tyrosine phosphorylation occurred in the insulin receptor subunits of the herbimycin-treated cells due to the antibiotic-induced inhibition of protein tyrosine kinases (Ishibashi et al., 1999). The incubation of the herbimycin-pretreated cells with insulin brought about significant tyrosine phosphorylation of the receptor. The density of the receptor molecules in cells was not affected by any treatment during culture and incubation (lower autoradiograms in the inset of Fig. 1).

Since the cellular cAMP concentration during incubation of cells reflects balance between generation of the nucleotide by adenylate cyclase and its breakdown by phosphodiesterase (Houslay and Milligan, 1997), the herbimycin-induced inhibition of phosphodiesterase gave rise to enhanced production of cAMP during incubation of the herbimycin-treated hepatocytes with an adenylate cyclase activator such as forskolin, glucagon or β-adrenoceptor agonists, unless the incubation medium was supplemented with a strong phosphodiesterase inhibitor such as IBMX (Ishibashi et al., 1999). Likewise, insulin-induced activation of phosphodiesterase in the hepatocytes cultured with herbimycin could be readily observable as the hormone-induced antagonism (attenuation) of cAMP accumulation during 0.5- to 20-min incubation of the antibiotics-treated cells in the medium containing, in addition to insulin, any of these adenylate cyclase activators without addition of phosphodiesterase inhibitors. In Table 1, 5-min incubation with glucagon caused 5-to 6-fold more accumulation of cAMP in herbimycin-treated cells than in non-treated cells due to lower phosphodiesterase activities in the former cells. Insulin added to the herbimycin-treated cells at concentrations from 10 to 1000 nM gave rise to progressive inhibition of glucagon-induced accumulation of cAMP. No effect was exerted by insulin on hepatocytes cultured without herbimycin in agreement with the data in Fig. 1. The anti-insulin-receptor immunoprecipitates were prepared from these cells to be analyzed for their phosphotyrosine contents by immunoblotting with anti-phosphotyrosine antibodies. In the case of hepatocytes not treated with herbimycin, the relative degree of the tyrosine phosphorylation of insulin receptor β-subunits (as percentages of the "no insulin" value) was 100, 155, 200 and 195 for insulin concentrations 0, 10, 100 and 1000 nM. These values were 0, 15, 38 and 70 for cells precultured with herbimycin. Thus, the maximally effective concentration of insulin was less than 100 nM for hepatocytes cultured under physiologic conditions, whereas exposure of hepatocytes to 1 μM herbimycin for 4 h rendered the cellular insulin receptors much less responsive to the receptor agonist. Both potency and efficacy of insulin in phosphorylation of its own receptor was markedly reduced by 4-h pre-exposure of cells to a tyrosine kinase inhibitor, herbimycin.

Table 1
Concentration-dependent action of insulin to attenuate glucagon-induced cAMP accumulation in hepatocytes cultured with herbimycin A

Insulin (nM)	Cellular cAMP (pmol/mg protein) in	
	Non-treated hepatocytes	Herbimycin-treated hepatocytes
0	15.6 ± 1.45	85.2 ± 5.43
10	15.3 ± 2.13	76.0 ± 2.43
100	15.6 ± 1.75	50.6 ± 1.62[a]
1000	16.0 ± 1.10	41.5 ± 2.71[a]

Hepatocytes cultured for 4 h with 0 or 1 μM herbimycin were, after thorough washing, first incubated for 10 min with increasing concentrations of insulin, and then for 5 min with further addition of 1 μM glucagon. The incubation was terminated by adding HCl to make 0.1 M and the supernatant was used for cAMP analysis. The mean ± S.E.M. from four different cell preparations are shown. The results were reproduced in an additional experiment.

[a] The effect of insulin was significant ($P < 0.01$).

Although potency and efficacy of insulin was very low in herbimycin-treated hepatocytes, the effect of insulin to inhibit phosphodiesterase in the same cell preparation is very likely to have resulted from its own receptor stimulation, because the progressive effects of insulin added at concentrations from 10 to 1000 nM to lower the cellular cAMP level shown in Table 1 were well correlated with the degree of tyrosine phosphorylation of the receptor (shown above) under the same conditions. Since the prior exposure of hepatocytes to herbimycin afforded the conditions essential for the phosphodiesterase-decreasing effect of insulin to be observable in vitro, we further studied the effect of 1 μM insulin on herbimycin-treated cells in the subsequent experiments.

3.2. Antagonism by insulin of glucagon-induced activation of glycogen phosphorylase in hepatocytes cultured with herbimycin

Alterations of the metabolic rates determined by cAMP are included in the physiological actions of hormones, such as glucagon and β-adrenoceptor agonists, which raise the cellular concentration of the nucleotide in hepatocytes. For instance, cAMP-dependent protein kinase (or protein kinase A) phosphorylates two (β- and γ-subunits) of the four kinds of subunits of phosphorylase b kinase, thereby increasing the catalytic activity of the α-subunit in the presence of Ca^{2+} binding to the δ-subunit or calmodulin. Consequently, phosphorylase is activated or undergoes the b-kinase-catalyzed conversion from its inactive (b) into its active (a) form. Insulin, which interferes with the elevation of cellular cAMP induced by these adenylate-cyclase-activating hormones, may possibly antagonize the metabolic action of these hormones. For the purpose of addressing this possibility, we introduced a phosphorylase assay system in which activation of phosphorylase by glucagon was studied with cultured hepatocytes (Fig. 2).

Glycogen phosphorylase exists mostly in the active state in hepatocytes, when the cells have been isolated by the collagenase digestion technique, before or after primary culture (Okajima and Ui, 1982). Exposure of the isolated cells to glucose at a concentration as high as 40 mM for 30–45 min resulted in the conversion of phosphorylase into the inactive state, which is thus responsive to glucagon- or β-adrenoceptor agonist-induced activation (Okajima and Ui, 1982). Addition of glucagon to the thus treated 4-h cultured hepatocytes gave rise to prompt and progressive increase in the active phosphorylase within 2 min, with the initial rate of the increase being 3- to 5-fold higher in herbimycin-treated cells than in non-treated cells (Fig. 2). The difference between herbimycin-treated and non-treated cells in the glucagon-induced activation of phosphorylase was maximal at an incubation time as short as 30 s; the marked activation of the enzyme in herbimycin-treated cells tended to level off immediately afterwards. Thus, the effects of insulin on glucagon-induced activation of phosphorylase were studied in cells incubated with glucagon for a time as short as 30 s, as will be shown in Tables 2 and 3. The cellular level of cAMP was low before the onset of adenylate cyclase activation even in the presence of phosphodiesterase inhibitors, and the elevation following the cyclase activation was strictly time-dependent up to 5 min in the presence or absence of phosphodiesterase inhibition. The cAMP values recorded in Tables 2 and 3, in which the cells were incubated with glucagon for only 30 s, were hence lower than those in Table 1 or those shown in the previous paper (Ishibashi et al., 1999); however, these data would be good reflection of the turnover rates of cellular cAMP.

Insulin, added at 1 μM simultaneously with 1 μM glucagon, antagonized the action of glucagon to increase phosphorylase activity at 30 s of incubation, only if the hepatocytes had been cultured for 4 h with herbimycin (Table 2). The insulin-induced antagonism of the glucagon action regarding phosphorylase activation was accompanied by the antagonistic action of insulin on glucagon-induced cAMP accumulation. Failure of insulin to antagonize glucagon-induced phosphorylase activation in the hepatocytes not treated with herbimycin would thus be readily accounted for by the lack of difference in the cellular cAMP level between the insulin treatment and non-treatment under these conditions (Table 2). Insulin was more antagonistic to glucagon actions when it was added to the cultured cells 10 min prior to the glucagon addition than when both hormones were added at the same time (compare Experiment 2 with Experiment 1 in Table 2). This is the reason why insulin was added to cultured hepatocytes 10 min prior to the glucagon addition in experiments recorded in Tables 1 and 3.

Pre-incubation of cultured hepatocytes with 1 mM IBMX, an efficacious and non-selective inhibitor of many families of phosphodiesterase, for 10 min was adequate means to abolish the exaggerated cAMP-accumulating response of herbimycin-treated cells to adenylate cyclase activators (Ishibashi et al., 1999). When hepatocytes were incubated with IBMX together with glucagon or forskolin, huge accumulation of cAMP occurred in the same magnitude, regardless of whether the cells had been cultured with or without herbimycin (Ishibashi et al., 1999). In Table 3, cAMP accumulation was not so tremendous in magnitude in IBMX-added cells because glucagon recep-

Table 2
Effects of insulin to decrease glucagon-induced cAMP accumulation and to antagonize glycogen phosphorylase activation in wortmannin-insusceptible manner in herbimycin-treated hepatocytes

Herbimycin (μM)	Insulin (μM)	cAMP level (pmol/mg protein)	Phosphorylase activity (nmol/mg protein)
Experiment 1: insulin added at the time of glucagon addition			
0	0	3.5 ± 0.45	36.2 ± 2.93
0	1	3.7 ± 0.60	35.9 ± 3.66
1	0	11.1 ± 0.56	113.5 ± 5.22
1	1	7.7 ± 0.32[a]	74.0 ± 4.38[a]
Experiment 2: insulin added 10 min before glucagon addition			
0	0	3.2 ± 0.39 (3.4 ± 0.65)	38.6 ± 1.06 (36.1 ± 2.73)
0	1	3.5 ± 0.44 (3.3 ± 0.28)	36.6 ± 1.26 (38.2 ± 1.76)
1	0	10.9 ± 0.79 (11.0 ± 0.09)	115.1 ± 7.60 (112.6 ± 4.52)
1	1	5.5 ± 0.47[a] (5.7 ± 0.41[a])	54.8 ± 5.47[a] (57.0 ± 2.39[a])

Hepatocytes cultured for 4 h with 0 or 1 μM herbimycin were incubated in 40 mM glucose-containing medium for 45 min to maintain most of the phosphorylase enzyme in the inactive state. In Experiment 1, the cells, after washing, were incubated for 30 s with 0 or 1 μM insulin in the presence of 1 μM glucagon before being frozen quickly. In Experiment 2, the cells were first incubated for 10 min with 0 or 1 μM insulin and then for 30 s with further addition of glucagon. A fraction of the frozen and disrupted cells was analyzed for cAMP released by boiling in 0.1 N HCl and another fraction was subjected to the assay of phosphorylase activity, which is expressed as nmoles of [U–^{14}C]glucose 1-phosphate incorporated into glycogen (per mg of protein per min). In Experiment 2, the values in parentheses are those obtained for the cultured cells incubated for 10 min with 100 nM wortmannin before the further addition of insulin. The mean ± S.E.M. from four different cell preparations are shown. Essentially the same results were obtained in additional three experiments similarly designed.

[a]The effect of insulin was significant ($P < 0.01$).

Table 3
Failure of insulin to antagonize glucagon actions in the presence of IBMX in herbimycin-treated hepatocytes

Herbimycin (μM)	Insulin (μM)	cAMP level (pmol/mg protein)	Phosphorylase activity (nmol/mg protein)
Without addition of IBMX			
0	0	3.5 ± 0.65 (0.2 ± 0.03)	35.1 ± 2.3 (20.4 ± 2.0)
0	1	3.9 ± 0.48 (0.2 ± 0.03)	37.3 ± 2.1 (19.6 ± 6.4)
1	0	11.0 ± 1.44 (0.2 ± 0.01)	113.7 ± 1.4 (20.5 ± 3.2)
1	1	5.2 ± 0.39[a] (0.2 ± 0.03)	54.4 ± 4.3[a] (22.1 ± 1.9)
With addition of 1 mM IBMX			
0	0	15.2 ± 0.34 (0.3 ± 0.01)	131.8 ± 3.2 (25.6 ± 2.6)
0	1	16.6 ± 1.08 (0.3 ± 0.05)	136.6 ± 13.0 (28.3 ± 2.1)
1	0	15.6 ± 1.15 (0.3 ± 0.07)	134.7 ± 1.7 (27.3 ± 1.7)
1	1	15.7 ± 1.16 (0.35 ± 0.01)	138.8 ± 4.6 (24.8 ± 1.5)

The experimental conditions employed were exactly the same as those for Experiment 2 in Table 2 except for the replacement of 1 mM IBMX for 100 nM wortmannin as the addition into the 10-min incubation of cultured hepatocytes. The mean ± S.E.M. from four different cell preparations are shown. The values in parentheses are the data obtained without glucagon addition. The results were reproduced in an additional experiment.

[a] The effect of insulin was significant ($P < 0.01$).

tors were stimulated for a time as short as only 30 s. Nevertheless, neither herbimycin nor insulin exerted any detectable influence on cellular cAMP level in the presence of IBMX. In contrast, herbimycin and insulin were both effective in cells not treated with IBMX, strictly reproducing the data recorded in Table 2, i.e., the increase in cellular cAMP in herbimycin-treated cells and its attenuation by insulin, together with the same-directional changes in phosphorylase activities. The results are in good agreement with the notion that modifications of phosphorylase activity by insulin, just like those by herbimycin (Ishibashi et al., 1999), resulted from modification of the methylxanthine-susceptible phosphodiesterase that is responsible for alteration of the cellular concentration of cAMP, an activator of phosphorylase.

3.3. Tyrosine-phosphorylation of α-tubulin and inhibition of microtubular assembly by insulin in hepatocytes cultured with herbimycin

Intact-cell phosphodiesterase activity was lowered by 4-h culture of hepatocytes with herbimycin despite the fact that there was no change in the enzyme activity measured after cell disruption (Ishibashi et al., 1999). Such was the case with phosphodiesterase activation by insulin in the herbimycin-treated cells apparently shown in Fig. 1. We followed decomposition of [^{3}H]cAMP in the cell lysates prepared from cultured cells as a measure of cellular phosphodiesterase activity according to the routine method (Taylor et al., 1997) described recently in detail (Ishibashi et al., 1999). The phosphodiesterase activity (pmol cAMP breakdown per mg of cellular proteins per min) of cell lysates was 16.9 ± 0.17, 16.6 ± 0.16, 16.8 ± 0.35 and 16.7 ± 0.21, when the lysates were prepared from hepatocytes cultured for 4 h and incubated for 10 min without any addition, with 1 μM insulin, with 1 μM herbimycin and with both insulin and herbimycin, respectively (the mean ± S.E.M. from four different cell preparations showing no significant difference between them; insulin added into the incubation and herbimycin into the culture medium).

Since herbimycin-induced inhibition of intact-cell phosphodiesterase activity during hepatocyte culture was accounted for by microtubular assembly caused by inhibition of tyrosine phosphorylation of α-tubulin (Ishibashi et al., 1999), the effects of insulin in this regard were studied in Fig. 3. Although the cellular content of α-tubulin was not affected by any treatment of cells, there was no phosphorylation of α-tubulin on its tyrosine residues in herbimycin-treated hepatocytes in confirmation of our previous results (Ishibashi et al., 1999). Incubation of the cultured hepatocytes with insulin increased tyrosine phosphorylation of α-tubulin; the effect of the hormone was very marked in the cells precultured with herbimycin. Confocal microscopy of the cultured and incubated hepatocytes after immunostaining with anti-α-tubulin antibodies revealed formation of microtubular network in the cells cultured with herbimycin (Fig. 3, panel c). Incubation of the cultured cells with insulin tended to inhibit the microtubular assembly (Fig. 3, panel d), though this insulin effect was not so evident as the effect of vinblastine or an inhibitor of tyrosine phosphatase reported previously (Ishibashi et al., 1999). Thus, insulin was antagonistic to the actions of herbimycin to inhibit tyrosine-phosphorylation of α-tubulin, to favor microtubular assembly and to suppress phosphodiesterase activity in intact hepatocytes.

3.4. Failure of PtdIns 3-kinase to mediate insulin-induced activation of phosphodiesterase in herbimycin-treated hepatocytes

Cellular signaling cascades arising from membrane insulin receptors are mostly mediated by PtdIns 3-kinase, the

activity of which is efficiently inhibited by wortmannin at concentrations lower than 100 nM (Ui et al., 1995). One of the striking actions of insulin observable in rat adipocytes is activation of phosphodiesterase, which, through suppression of epinephrine-induced cAMP accumulation, is responsible for inhibition of the β-adrenoceptor agonist-induced lipolysis. Wortmannin was effective in inhibiting the anti-lipolytic action of insulin in adipocytes (Okada et al., 1994), affording evidence for essential functions of PtdIns 3-kinase upstream of phosphodiesterase in insulin signalling in this cell type. Unexpectedly, however, prior exposure of the herbimycin-treated hepatocytes to wortmannin at the highest concentration of 100 nM failed to exert any influence on insulin actions to decrease cAMP level and inhibit phosphorylase following glucagon treatment of cells (data in parentheses in Table 2). In fact, insulin increased the active PtdIns 3-kinase immunoprecipitated with anti-phosphotyrosine antibodies, when it was added to hepatocytes cultured either with or without herbimycin (Fig. 4). The insulin-induced activation of PtdIns 3-kinase was mostly abolished by wortmannin in either case (Fig. 4). The PtdIns 3-kinase activity of the same immunoprecipitates prepared from cells not treated with insulin was insusceptible to the wortmannin inhibition (data not shown). Thus, insulin was capable of activating phosphodiesterase in herbimycin-treated hepatocytes without mediation of wortmannin-susceptible PtdIns 3-kinase.

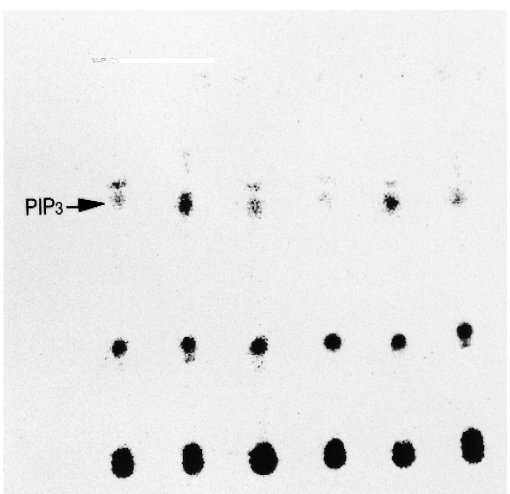

Fig. 4. Inhibition by wortmannin of insulin-induced activation of PtdIns 3-kinase in hepatocytes cultured with or without herbimycin. Hepatocytes cultured for 4 h with (+) or without (−) 1 μM herbimycin were incubated first for 10 min with (+) or without (−) 100 nM wortmannin and then for additional 10 min with (+) or without (−) further addition of insulin. The incubation was terminated by quick washing followed by the addition of cell-lysis buffer. The cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibody (PY-20) and the immunoprecipitates were analyzed for the bound PtdIns 3-kinase activity by 15-min incubation with [γ-^{32}P]ATP and PtdIns as substrates. The ^{32}P-labeled lipids extracted were applied to thin-layer chromatography; autoradiogram is shown with spots corresponding to PtdIns(3)P shown by an arrow. Similar results were obtained by an additional experiment with the same design.

4. Discussion

Since acute metabolic actions of insulin are, as a whole, antagonistic to the hormones and neurotransmitters whose actions depend on increases in cellular cAMP, it would be reasonable to assume that the insulin actions, at least in part, could be accounted for by its action to decrease cAMP. A typical example verifying this assumption is an anti-lipolytic action of insulin readily observable with rat adipocytes. A simultaneous exposure of isolated adipocytes to insulin and isoproterenol brings about attenuation of the β-adrenoceptor agonist-induced lipolysis as a result of enhanced breakdown of the cellular cAMP, which is responsible for the β-adrenoceptor agonist's action, via activation of insulin-sensitive phosphodiesterase 3 (Rahn et al., 1996; Enoksson et al., 1998; Wijkander et al., 1998; Kitamura et al., 1999; Castan et al., 1999; Van Harmelen et al., 1999).

Such would not exactly be the case with hepatocytes, in which glucagon and other cAMP-increasing agents exert glycogenolytic effects while insulin is conversely a glycogenic agent (Bollen et al., 1998). Although earlier papers reported that phosphodiesterase was activated upon direct addition of insulin to isolated rat hepatocytes (e.g., as cited in Irvine et al., 1993; Robles-Flores et al., 1995), hepatocyte-derived cell lines over-expressing insulin receptors (e.g., Sanchez-Margalet, 2000) or liver cells transformed with SV40 virus (Rother et al., 1998; Park et al., 1999) are employed, instead of hepatocytes freshly isolated from the rodent liver, in many recent publications. This might be probably because the collagenase digestion technique to isolate hepatocytes may afford the cell preparations whose cAMP-dependent sugar metabolisms are not promptly responsive to the addition of insulin, as mentioned in (Carlsen et al., 1997, 1999). In fact, confocal microscopic examination revealed that cellular microtubular networks in the hepatocytes isolated by the collagenase digestion technique were not so extensive as those in in-situ-fixed liver cells (Ishibashi et al., 1999). Cyclic AMP-degrading phosphodiesterase activity was so high, for unknown reasons, as to be insensitive to further activating stimuli in the hepatocytes with such poorer microtubular assembly (Ishibashi et al., 1999).

The inhibition of protein tyrosine kinases by exposure of hepatocytes to herbimycin A during primary culture afforded the cell preparations in which tyrosine phosphorylation of α-tubulin was reduced giving rise to dense microtubular assembly accompanied by a lower baseline activity of rolipram- and 4-(3-butyloxy-4-methoxyphenyl)-2-im-

idazolidinone (RO-20-1724)-sensitive phosphodiesterase 4 (Ishibashi et al., 1999). The inhibition of hepatocyte phosphodiesterase in these cell preparations was confirmed in Fig. 1, in which insulin reversed the inhibition thereby enhancing breakdown of cellular cAMP. Glucagon-induced increase in cellular cAMP was likewise diminished by the coaddition of insulin in herbimycin-pretreated cells (Tables 1–3). This action of insulin resulted from activation of phosphodiesterase, as evidenced by the lack of the insulin action in the presence of a phosphodiesterase inhibitor (Table 3), and led to interference with glucagon-induced activation of glycogen phosphorylase (Tables 2 and 3). Thus, insulin proved to actually antagonize the glycogenolytic effect of glucagon in herbimycin-pretreated hepatocytes in a manner solely dependent on changes in cellular cAMP.

Four-hour culture of rat hepatocytes with herbimycin was very effective in abolition of tyrosine phosphorylation of insulin receptor β-subunit (Fig. 1) and α-tubulin (Fig. 3). Both were tyrosine-phosphorylated markedly and rapidly upon addition of insulin to these hepatocytes completely freed of the tyrosine kinase inhibitor (Figs. 1 and 3). Insulin receptor substrates (IRS-1 and IRS-2) were also tyrosine-phosphorylated by insulin (data not shown). We have recently found that the regulatory subunit (p85) of PtdIns 3-kinase bound to the thus tyrosine-phosphorylated IRS-1 and IRS-2 leading to the activation of the associated catalytic subunit (p110) of the enzyme (Fujioka and Ui, submitted), in good agreement with other publications whose major subjects were identification of signaling substances located downstream PtdIns 3-kinase in the mammalian liver as Akt/PKB, p70 S6 kinase, MAP kinases, glycogen synthase, glycogen synthase kinase-3 (GSK-3) or phosphodiesterase 3 (Peak et al., 1998; Rother et al., 1998; Carlsen et al., 1999; Park et al., 1999; Benzeroual et al., 2000; Sanchez-Margalet, 2000; Zhao et al., 2000). Unexpectedly, the insulin action to antagonize glucagon-induced cAMP generation and phosphorylase activation was never inhibited by wortmannin, a selective inhibitor of PtdIns 3-kinase (Ui et al., 1995) (Table 2), despite the fact that insulin-induced activation of PtdIns 3-kinase was in fact abolished by wortmannin under the same conditions, i.e., in herbimycin-treated hepatocytes (Fig. 4). PtdIns 3-kinase is not likely to play any role in a signaling cascade connecting agonist-induced tyrosine phosphorylation of insulin receptors with phosphodiesterase 4 activation; the results appear to be at variance with current widely accepted view that PtdIns 3-kinase mediates most of cellular responses to insulin including phosphodiesterase 3 stimulation in rat adipocytes (Rahn et al., 1994).

The mechanism by which insulin receptor stimulation leads to activation of phosphodiesterase 4 in herbimycin-pretreated hepatocytes remains to be the subject of further investigation. The degree of microtubular assembly/disassembly in cells is suggested to play an essential role in the underlying mechanism. We have previously reported that the activity of membrane-bound receptor-G_s-adenylate cyclase complexes was strictly dependent on the state of microtubule polymerization in macrophages; the activity increased upon disassembly of microtubules induced by colchicine or vinblastine, and was strongly inhibited when the assembly was stimulated by taxol or D_2O (Hazeki et al., 1985). This finding was confirmed later in S49 lymphoma cells (Leiber et al., 1993). The formation and stabilization of microtubular networks connecting cellular membranes, as caused by dephosphorylation of tyrosine residues in α-tubulin molecules in herbimycin-treated cells, would afford conditions unfavorable for the membrane enzymes, such as phosphodiesterase 4, to display their full activities. Such unfavorable conditions could be reversed by insulin-induced tyrosine phosphorylation of α-tubulin (Fig. 3). Microtubule-interfering agents are currently known to trigger intracellular signaling cascades shared, under certain conditions, by insulin signaling as well (Lee et al., 1998; Schmid-Alliana et al., 1998; Wang et al., 1998, 1999; Subbaramaiah et al., 2000).

Finally, brief comments would be given to the possible physiological significance of the pharmacological action of insulin in vitro thus observed in the present communication. Herbimycin at 1 μM acted as such a toxic agent by inhibiting tyrosine kinases, in hepatocytes that responses of a number of cellular functions to extracellular stimuli as well as the functions themselves were impaired when the time of cell exposure to the antibiotics was prolonged beyond 6 h. This is the reason why we employed 4-h treated cells in which cAMP metabolisms and their responses to hormone additions were not apparently impaired. In this cell preparation, the insulin effect observed was only slight and statistically insignificant at 10 nM (Table 1), the concentration usually used to observe its effect in vitro on hepatocytes or related cell lines. Although the effective concentration of insulin in our hepatocyte preparation was as high as 100 nM, the lower concentration would be also effective when glucagon is added at much lower concentrations. It would be pointed out again that the degree of polymerization of microtubules is lower in isolated hepatocytes than cells in the liver fixed in situ (see above). Exposure of isolated hepatocytes to herbimycin affords an artificial and non-physiological preparation that is nevertheless mimicking the physiological situation in the state of microtubular assembly. The effect of insulin observed in such cell preparations may reproduce its physiological counterpart in certain respects.

Acknowledgements

We are grateful to Drs. Y. Hirabayashi and S. Furuya, the Laboratory for Cellular Glycobiology, Institute of Physical and Chemical Research, for their helpful advice and technical instruction on confocal fluorescence microscopy. This work was supported by a grant-in-aid for

scientific research on priority areas and other grants from the Ministry of Education, Science, Culture and Sports, Japan.

References

Ahmad, M., Abdel-Wahab, Y.H., Tate, R., Flatt, P.R., Pyne, N.J., Furman, B.L., 2000. Effect of type-selective inhibitors on cyclic nucleotide phosphodiesterase activity and insulin secretion in the clonal insulin secreting cell line BRIN-BD11. Br. J. Pharmacol. 129, 1228–1234.

Banner, K.H., Dimitriou, G., Kinali, M., Page, C.P., Greenough, A., 2000. Evidence to suggest that the phosphodiesterase 4 isoenzyme is present and involved in the proliferation of umbilical cord blood mononuclear cells. Clin. Exp. Allergy 30, 706–712.

Baroja, M.L., Cieslinski, L.B., Torphy, T.J., Wange, R.L., Madrenas, J., 1999. Specific CD3Œ association of a phosphodiesterase 4B isoform determines its selective tyrosine phosphorylation after CD3 ligation. J. Immunol. 162, 2016–2023.

Beavo, J.A., 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol. Rev. 75, 725–748.

Beavo, J.A., Conti, M., Heaslip, R.J., 1994. Multiple cyclic nucleotide phosphodiesterases. Mol. Pharmacol. 46, 399–405.

Benzeroual, K., Pandey, S.K., Srivastava, A.K., van de Werve, G., Haddad, P.S., 2000. Insulin-induced Ca(2+) entry in hepatocytes is important for PI 3-kinase activation, but not for insulin receptor and IRS-1 tyrosine phosphorylation. Biochim. Biophys. Acta 1495, 14–23.

Bolger, G.B., 1994. Molecular biology of the cyclic AMP-specific cyclic nucleotide phosphodiesterases: a diverse family of regulatory enzymes. Cell. Signalling 6, 851–859.

Bollen, M., Keppens, S., Stalmans, W., 1998. Specific features of glycogen metabolism in the liver. Biochem. J. 336, 19–31.

Brideau, C., Van Staden, C., Styhler, A., Rodger, I.W., Chan, C.C., 1999. The effects of phosphodiesterase type 4 inhibitors on tumour necrosis factor-alpha and leukotriene B4 in a novel human whole blood assay. Br. J. Pharmacol. 126, 979–988.

Carlsen, J., Christiansen, K., Vinten, J., 1997. Insulin stimulated glycogen synthesis in isolated rat hepatocytes: effect of protein kinase inhibitors. Cell. Signalling 9, 447–450.

Carlsen, J., Christiansen, K., Grunnet, N., Vinten, J., 1999. Involvement of PI 3-kinase and activated ERK in facilitating insulin-stimulated triacylglycerol synthesis in hepatocytes. Cell. Signalling 11, 713–717.

Castan, I., Wijkander, J., Manganiello, V., Degerman, E., 1999. Mechanisms of inhibition of lipolysis by insulin, vanadate and peroxovanadate in rat adipocytes. Biochem. J. 339, 281–289.

Degerman, E., Belfrage, P., Manganiello, V.C., 1997. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). J. Biol. Chem. 272, 6823–6826.

Denis, D., Riendeau, D., 1999. Phosphodiesterase 4-dependent regulation of cyclic AMP levels and leukotriene B4 biosynthesis in human polymorphonuclear leukocytes. Eur. J. Pharmacol. 367, 343–350.

Dunn, J.C.Y., Yarmush, M.L., Koebe, H.G., Tompkins, R.G., 1989. Hepatocyte function and extracellular matrix geometry long-term culture in a sandwich configuration. FASEB J. 3, 174–177.

Enoksson, S., Degerman, E., Hagstrom-Toft, E., Large, V., Arner, P., 1998. Various phosphodiesterase subtypes mediate the in vivo antilipolytic effect of insulin on adipose tissue and skeletal muscle in man. Diabetologia 41, 560–568.

Essayan, D.M., 1999. Cyclic nucleotide phosphodiesterase (PDE) inhibitors and immunomodulation. Biochem. Pharmacol. 57, 965–973.

Fawcett, L., Baxendale, R., Stacey, P., McGrouther, C., Harrow, I., Soderling, S., Beavo, J.A., Phillips, S.C., 2000. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. Proc. Natl. Acad. Sci. U. S. A. 97, 3702–3707.

Fisher, D.A., Smith, J.F., Pillar, J.S., St Denis, S.H., Cheng, J.B., 1998. Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. Biochem. Biophys. Res. Commun. 246, 570–577.

Fuhrmann, M., Jahn, H.U., Seybold, J., Neurohr, C., Barnes, P.J., Hippenstiel, S., Kraemer, H.J., Suttorp, N., 1999. Identification and function of cyclic nucleotide phosphodiesterase isoenzymes in airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 20, 292–302.

Fujishige, K., Kotera, J., Michibata, H., Yuasa, K., Takebayashi, S., Okumura, K., Omori, K., 1999. Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). J. Biol. Chem. 274, 18438–18445.

Hansen, G., Jin, S., Umetsu, D.T., Conti, M., 2000. Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. Proc. Natl. Acad. Sci. U. S. A. 97, 6751–6756.

Hazeki, K., Mori, Y., Ui, M., 1985. Prior exposure of macrophages to a low temperature enhances their cyclic AMP responses due to microtubule disruption. Arch. Biochem. Biophys. 237, 101–109.

Helmsdorf, T., Dettmer, D., 1998. Combined effects of insulin and dexamethasone on cAMP phosphodiesterase 3 and glycogen metabolism in cultured rat hepatocytes. Cell. Signalling 10, 629–635.

Hoffmann, R., Wilkinson, I.R., McCallum, J.F., Engels, P., Houslay, M.D., 1998. cAMP-specific phosphodiesterase HSPDE4D3 mutants which mimic activation and changes in rolipram inhibition triggered by protein kinase A phosphorylation of Ser-54: generation of a molecular model. Biochem. J. 333, 139–149.

Hoffmann, R., Baillie, G.S., MacKenzie, S.J., Yarwood, S.J., Houslay, M.D., 1999. The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. EMBO J. 18, 893–903.

Houslay, M.D., Milligan, G., 1997. Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem. Sci. 22, 217–224.

Houslay, M.D., Sullivan, M., Bolger, G.B., 1998. The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: intracellular targeting, regulation, and selective inhibition by compounds exerting anti-inflammatory and antidepressant actions. Adv. Pharmacol. 44, 225–342.

Irvine, F., Wallace, A.V., Sarawak, S.R., Houslay, M.D., 1993. Extracellular calcium modulates insulin's action on enzymes controlling cyclic AMP metabolism in intact hepatocytes. Biochem. J. 293, 249–253.

Ishibashi, K., Fujioka, T., Ui, M., 1999. Decreases in the cAMP phosphodiesterase activity in hepatocytes cultured with herbimycin A due to the cellular microtubule polymerization related to inhibition of tyrosine phosphorylation of α-tubulin. Eur. J. Biochem. 260, 398–408.

Kajiyama, Y., Ui, M., 1994. Switching from alpha(1)-to beta-subtypes in adrenergic response during primary culture of adult-rat hepatocytes as affected by the cell-to-cell interaction through plasma membranes. Biochem. J. 303, 313–321.

Kitamura, T., Kitamura, Y., Kuroda, S., Hino, Y., Ando, M., Kotani, K., Konishi, H., Matsuzaki, H., Kikkawa, U., Ogawa, W., Kasuga, M., 1999. Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. Mol. Cell. Biol. 19, 6286–6296.

Lee, L.F., Li, G., Templeton, D.J., Ting, J.P., 1998. Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). J. Biol. Chem. 273, 28253–28260.

Leiber, D., Jasper, J.R., Alousi, A.A., Martin, J., Bernstein, D., Insel, P.A., 1993. Alteration in Gs-mediated signal transduction in S49 lymphoma cells treated with inhibitors of microtubules. J. Biol. Chem. 268, 3833–3837.

Lim, J., Pahlke, G., Conti, M., 1999. Activation of the cAMP-specific phosphodiesterase PDE4D3 by phosphorylation-identification and function of an inhibitory domain. J. Biol. Chem. 274, 19677–19685.

Liu, H.G., Maurice, D.H., 1999. Phosphorylation-mediated activation and translocation of the cyclic AMP-specific phosphodiesterase PDE4D3

by cyclic AMP-dependent protein kinase and mitogen-activated protein kinases-a potential mechanism allowing for the co-ordinated regulation of PDE4D activity and targeting. J. Biol. Chem. 274, 10557–10565.

Liu, H., Palmer, D., Jimmo, S.L., Tilley, D.G., Dunkerley, H.A., Pang, S.C., Maurice, D.H., 2000. Expression of phosphodiesterase 4D (PDE4D) is regulated by both the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase signaling pathways: a potential mechanism allowing for the co-ordinated regulation of PDE4D activity and expression in cells. J. Biol. Chem. 275, 26615–26624.

Ma, D.M., Wu, P., Egan, R.W., Billah, M.M., Wang, P., 1999. Phosphodiesterase 4B gene transcription is activated by lipopolysaccharide and inhibited by interleukin-10 in human monocytes. Mol. Pharmacol. 55, 50–57.

MacKenzie, S.J., Houslay, M.D., 2000. Action of rolipram on specific PDE4 cAMP phosphodiesterase isoforms and on the phosphorylation of cAMP-response-element-binding protein (CREB) and p38 mitogen-activated protein (MAP) kinase in U937 monocytic cells. Biochem. J. 347, 571–578.

MacKenzie, S.J., Baillie, G.S., McPhee, I., Bolger, G.B., Houslay, M.D., 2000. ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases-the involvement of COOH-terminal docking sites and NH2-terminal UCR regions. J. Biol. Chem. 275, 16609–16617.

Manganiello, V.C., Taira, M., Degerman, E., Belfrage, P., 1995. Type III cGMP-inhibited cyclic nucleotide phosphodiesterases (PDE3 gene family). Cell. Signal. 7, 445–455.

Matsuo, T., Hazeki, K., Hazeki, O., Katada, T., Ui, M., 1996. Activation of phosphatidylinositol 3-kinase by concanavalin A through dual signalling pathways, G-protein-coupled and phosphotyrosine-related, and an essential role of the G-protein-coupled signals for the lectin-induced respiratory burst in human monocytic THP-1 cells. Biochem. J. 315, 505–512.

Moberg, E., Enoksson, S., Hagstrom-Toft, E., 1998. Importance of phosphodiesterase 3 for the lipolytic response in adipose tissue during insulin-induced hypoglycemia in normal man. Horm. Metab. Res. 30, 684–688.

Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., Ui, M., 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes—studies with a selective inhibitor wortmannin. J. Biol. Chem. 269, 3568–3573.

Okajima, F., Ui, M., 1982. Conversion of adrenergic regulation of glycogen phosphorylase and synthase from an alpha to a beta type during primary culture of rat hepatocytes. Arch. Biochem. Biophys. 213, 658–668.

Oki, N., Takahashi, S.I., Hidaka, H., Conti, M., 2000. Short term feedback regulation of cAMP in FRTL-5 thyroid cells. Role of PDE4D3 phosphodiesterase activation. J. Biol. Chem. 275, 10831–10837.

Olaus, T.H., Anand, S.M., Johnson, R.A., 1982. Regulation of hepatocyte cAMP and pyruvate kinase by site-specific analogs of adenosine. Mol. Cell. Endocrinol. 26, 269–279.

Park, B.C., Kido, Y., Accili, D., 1999. Differential signaling of insulin and IGF-1 receptors to glycogen synthesis in murine hepatocytes. Biochemistry 38, 7517–7523.

Parker, J.C., VanVolkenburg, M.A., Nardone, N.A., Hargrove, D.M., Andrews, K.M., 1997. Modulation of insulin secretion and glycemia by selective inhibition of cyclic AMP phosphodiesterase III. Biochem. Biophys. Res. Commun. 236, 665–669.

Peak, M., Rochford, J.J., Borthwick, A.C., Yeaman, S.J., Agius, L., 1998. Signalling pathways involved in the stimulation of glycogen synthesis by insulin in rat hepatocytes. Diabetologia 41, 16–25.

Rahn, T., Ridderstrale, M., Tornqvist, H., Manganiello, V., Fredrikson, G., Belfrage, P., Degerman, E., 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes. Studies using the selective inhibitor wortmannin. FEBS Lett. 350, 314–318.

Rahn, T., Ronnstrand, L., Leroy, M.J., Wernstedt, C., Tornqvist, H., Manganiello, V.C., Belfrage, P., Degerman, E., 1996. Identification of the site in the cGMP-inhibited phosphodiesterase phosphorylated in adipocytes in response to insulin and isoproterenol. J. Biol. Chem. 271, 11575–11580.

Rahn Landstrom, T., Mei, J., Karlsson, M., Manganiello, V., Degerman, E., 2000. Down-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumour necrosis factor alpha and cAMP. Biochem. J. 346, 337–343.

Robles-Flores, M., Allende, G., Pina, E., Garcia-Sainz, J.A., 1995. Cross-talk between glucagon- and adenosine-mediated signalling systems in rat hepatocytes: effects on cyclic AMP-phosphodiesterase activity. Biochem. J. 312, 763–767.

Rother, K.I., Imai, Y., Caruso, M., Beguinot, F., Formisano, P., Accili, D., 1998. Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. J. Biol. Chem. 273, 17491–17497.

Sanchez-Margalet, V., 2000. Stimulation of glycogen synthesis by insulin requires S6 kinase and phosphatidylinositol-3-kinase in HTC-IR cells. J. Cell. Physiol. 182, 182–188.

Schmid-Alliana, A., Menou, L., Manie, S., Schmid-Antomarchi, H., Millet, M.A., Giuriato, S., Ferrua, B., Rossi, B., 1998. Microtubule integrity regulates src-like and extracellular signal-regulated kinase activities in human pro-monocytic cells. Importance for interleukin-1 production. J. Biol. Chem. 273, 3394–3400.

Sette, C., Conti, M., 1996. Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. J. Biol. Chem. 271, 16526–16534.

Soderling, S.H., Beavo, J.A., 2000. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr. Opin. Cell Biol. 12, 174–179.

Soderling, S.H., Bayuga, S.J., Beavo, J.A., 1998. Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. J. Biol. Chem. 273, 15553–15558.

Soderling, S.H., Bayuga, S.J., Beavo, J.A., 1999. Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. Proc. Natl. Acad. Sci. U. S. A. 96, 7071–7076.

Subbaramaiah, K., Hart, J.C., Norton, L., Dannenberg, A.J., 2000. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2-evidence for involvement of ERK1/2 and p38 mitogen-activated protein kinase pathways. J. Biol. Chem. 275, 14838–14845.

Tang, Y., Osawa, H., Onuma, H., Nishimiya, T., Ochi, M., Makino, H., 1999. Improvement in insulin resistance and the restoration of reduced phosphodiesterase 3B gene expression by pioglitazone in adipose tissue of obese diabetic KKAy mice. Diabetes 48, 1830–1835.

Taylor, C.C., Limback, D., Terranova, P.F., 1997. Src tyrosine kinase activity in rat thecal-interstitial cells and mouse TM3 Leydig cells is positively associated with cAMP-specific phosphodiesterase activity. Mol. Cell. Endocrinol. 126, 91–100.

Ui, M., Okada, T., Hazeki, K., Hazeki, O., 1995. Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. Trends Biochem. Sci. 20, 303–307.

Van Harmelen, V., Reynisdottir, S., Cianflone, K., Degerman, E., Hoffstedt, J., Nilsell, K., Sniderman, A., Arner, P., 1999. Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. J. Biol. Chem. 274, 18243–18251.

Wang, T.H., Wang, H.S., Ichijo, H., Giannakakou, P., Foster, J.S., Fojo, T., Wimalasena, J., 1998. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J. Biol. Chem. 273, 4928–4936.

Wang, T.H., Popp, D.M., Wang, H.S., Saitoh, M., Mural, J.G., Henley, D.C., Ichijo, H., Wimalasena, J., 1999. Microtubule dysfunction

induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and-independent pathways in ovarian cancer cells. J. Biol. Chem. 274, 8208–8216.

Wijkander, J., Landstrom, T.R., Manganiello, V., Belfrage, P., Degerman, E., 1998. Insulin-induced phosphorylation and activation of phosphodiesterase 3B in rat adipocytes: possible role for protein kinase B but not mitogen-activated protein kinase or p70 S6 kinase. Endocrinology 139, 219–227.

Xu, R.X., Hassell, A.M., Vanderwall, D., Lambert, M.H., Holmes, W.D., Luther, M.A., Rocque, W.J., Milburn, M.V., Zhao, Y.D., Ke, H.M., Nolte, R.T., 2000. Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. Science 288, 1822–1825.

Zhao, A.Z., Zhao, H., Teague, J., Fujimoto, W., Beavo, J.A., 1997. Attenuation of insulin secretion by insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. Proc. Natl. Acad. Sci. U. S. A. 94, 3223–3228.

Zhao, A.Z., Bornfeldt, K.E., Beavo, J.A., 1998. Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. J. Clin. Invest. 102, 869–873.

Zhao, A.Z., Shinohara, M.M., Huang, D., Shimizu, M., Eldar-Finkelman, H., Krebs, E.G., Beavo, J.A., Bornfeldt, K.E., 2000. Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. J. Biol. Chem. 275, 11348–11354.