Evidence That Growth Hormone Stimulates Protein Kinase C Activity in Isolated Rat Hepatocytes

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The mechanism of action of growth hormone (GH) is not known, although indirect evidence suggests that protein kinase C (PKC) might play an important role in the insulin-like actions of GH. In this investigation, we directly examined the effects of GH relative to those of insulin on PKC activity in isolated rat hepatocytes. Human GH (10^{-7} mol/L) significantly increased the activity of PKC in both cytosolic and particulate fractions. The effect was maximal at 1 minute, disappeared at 5 minutes, and then increased again at 30 minutes in both fractions. At 1 minute, maximal and half-maximal stimulation of PKC activity occurred at hGH concentrations of 10^{-7} and 5×10^{-9} mol/L, respectively. Insulin (10^{-7} mol/L) also induced a significant and transient increase in enzyme activity at 2 minutes in cytosolic and particulate fractions; at 30 minutes, PKC activity was decreased in the soluble fraction (−17%) and increased in the particulate fraction (+65%). Measurement of specific [^{3}H]-phorbol dibutyrate (PDBu) binding suggested translocation of PKC from the cytosol to the membrane fraction after 30 minutes of incubation, only after insulin treatment. The early effects of GH and insulin on PKC activity were additive in both the particulate and cytosolic fractions. Although the later effects of GH and insulin on PKC were quite different, both hormones rapidly activated PKC in isolated hepatocytes, suggesting that PKC might be involved in triggering the insulin-like actions of GH.

THE EFFECTS OF GROWTH HORMONE (GH) on the metabolism of carbohydrates and lipids have been classified as insulin-like and insulin-antagonistic.[1] Insulin-like effects, which occur early after GH treatment, notably include stimulation of glucose oxidation, lipogenesis, and antilipolysis.

The mechanism involved in these insulin-like effects of GH is not known, but various investigators have obtained indirect evidence that protein kinase C (PKC) might play an important role. Smal and De Meyts have shown that 12-O-tetradecanoyl 4-β-phorbol-13-acetate (TPA), an activator of PKC, induces an increase in lipogenesis in isolated adipocytes, as do GH and insulin.[2] They also reported that GH failed to stimulate lipogenesis in rat adipocytes depleted of PKC[2] or treated with inhibitors of PKC.[3,4] Moreover, in various cell types, GH increases the production of diacylglycerols,[5-9] which are physiologic activators of PKC. This kinase is involved in various pathways of signal transduction, hormone action, and cell regulation,[10] and may also participate in the action of insulin.[11-16]

The aim of this study was to examine directly the effects of GH relative to those of insulin on PKC activity in isolated hepatocytes, in which GH and insulin induce rapid effects.[17,18]

MATERIALS AND METHODS

Animals and Chemicals

Male Sprague–Dawley rats weighing 180 to 220 g were obtained from Iffa Credo (Lyon, France); they were maintained in a 12-hour light/dark cycle and fed ad libitum.

DEAE-cellulose (DE-52) was obtained from Biorad (Richmond, CA). DEAE-sephacel was from Pharmacia (Uppsala, Sweden). [γ-^{32}P]Adenosine triphosphate (ATP) (specific activity, 185 TBq/mmol) and [^{3}H]-phorbol 12,13-dibutyrate (specific activity, 370 to 740 GBq/mmol) were from Amersham (Bucks, UK). [1-^{14}C]-Pyruvic acid (9.8 mCi/mmol) was from New England Nuclear (Boston, MA). Collagenase was from Boehringer (Mannheim, France). Biosynthetic human GH (hGH) was from Lilly (Windlesham, UK), and porcine insulin was from Novo Research Institute (Bagsvaerd, Denmark). All other chemicals were from Sigma (St Louis, MO).

Isolation and Treatment of Hepatocytes

Isolated hepatocytes were prepared by sequential Ca^{2+} chelation and enzymatic treatment as described by Seglen[19] with minor modifications.[17] The viability of the cell suspension as estimated by the trypan blue exclusion method ranged between 85% and 95%.

After incubation at 37°C with hGH, insulin, or TPA, cell suspensions were centrifuged for 10 seconds. The supernatant was removed and the pellet ($\sim 20 \times 10^6$ cells) was stored in liquid nitrogen for later determination of PKC activity. Frozen cell pellets were resuspended in 2.5 mL buffer A (20 mmol/L Tris hydrochloride, pH 7.5, 0.25 mmol/L sucrose, 0.4 mmol/L EGTA, 1.6 mmol/L EDTA, 20 mmol/L 2-mercaptoethanol, 0.1 mmol/L benzamidin, 2 mmol/L phenyl methyl sulfonyl fluoride, 5 mg/L leupeptin, 50 mg/L trypsin inhibitor, 5 mg/L aprotinin, and 2.5 mg/L pepstatin) and then sonicated twice for 10 seconds at 40W in an ice bath. Homogenates were centrifuged at 200,000 × *g* for 30 minutes, and the supernatants were used as the cytosolic (soluble) fraction. The pellets were suspended in buffer A supplemented with 1% (vol/vol) Nonidet NP-40 and then sonicated and stirred at 4°C for 30 minutes before centrifugation for 20 minutes at 200,000 × *g*. The resulting supernatants were used as membrane (particulate) fractions.

PKC Assay

The fractions were partly purified with DEAE-cellulose chromatography in buffer B (Tris hydrochloride 20 mmol/L, pH 7.5, EGTA 0.4 mmol/L, EDTA 1.6 mmol/L, and 2-mercaptoethanol 20 mmol/L).[20] More than 90% of the total PKC activity was recovered in buffer B supplemented with 100 mmol/L NaCl. PKC activity was measured in 96-well dishes, started and stopped row by row using a

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multipipette,[21] by transfer of the γ-phosphoryl group of radioactive ATP to histone H1-IIIs. A brain extract (85% of phosphatidylserine), 1,2-dioleoyl-*sn*-glycerol (diolein), and Ca^{2+} were used as activators; blanks lacked these activators. The final volume was 100 μL 25 mmol/L Tris hydrochloride, pH 7.5, 5 mmol/L $MgCl_2$, 0.6 mmol/L EDTA, 0.15 mmol/L EGTA, 5 mmol/L 2-mercaptoethanol, 1.5 mmol/L $CaCl_2$, 40 μmol/L ATP (0.2 to 0.4 μCi [γ-^{32}P]ATP per incubation), 0.5 g/L histone H1-IIIs, 100 mg/L brain extract, and 10 mg/L diolein. Reactions were terminated by addition of trichloroacetic acid (TCA). Acid-precipitable materials in eight wells (one row) were simultaneously aspirated and collected in a cell harvester (Nunc, Roskilde, Denmark) on small glass-fiber disks. The filters were then extensively washed with 5% TCA, and their radioactivity was measured in Picofluor 40 (Packard Instruments, Downers Grove, IL).

Phorbol 12,13-Dibutyrate Binding

This was assayed in 20 mmol/L Tris hydrochloride, pH 7.5, 4 mmol/L $CaCl_2$, mixed micelles of Triton X-100 containing 0.3% Triton X-100 and 1 mg/mL brain extract, 50 nmol/L [^{3}H]-phorbol 12,13-dibutyrate ([^{3}H]-PDBu), and enzyme extract (50 μL DEAE extract) in a final volume of 0.1 mL.[21] PDBu-PKC complexes were adsorbed on DEAE-Sephacel and separated from free PDBu by filtering in the cell harvester.

Pyruvate Dehydrogenase Assay

Pyruvate dehydrogenase (PDH) activity was measured as the release of $^{14}CO_2$ from [1-^{14}C]-pyruvic acid[17] using hepatocyte extracts preincubated at 37°C for 15 minutes with hGH or insulin.

Protein Determination

Protein content was determined using Coomassie blue G-250.[22]

Statistical Analysis

Significant differences were identified with Student's paired *t* test.

RESULTS

We evaluated the capacity of sphingosine, a PKC inhibitor, to block PDH activation induced by hGH or insulin (Table 1); hGH and insulin stimulated enzyme activity by 18% and 44%, respectively. Preincubation with sphingosine (40 μmol/L) decreased control enzyme activity and partially blocked the effects of both hormones.

In our experimental conditions, PKC activity increased linearly with the time of incubation up to 15 minutes, and was proportional to the amount of protein added (up to 15 μg partly purified protein; results not shown).

Exposure of hepatocytes to TPA decreased cytosolic PKC activity by about 90% within 1 minute, with a corresponding increase in particulate enzyme activity (Fig 1); this intracellular redistribution persisted for 10 minutes. TPA caused a progressive loss of total enzyme activity, which reached approximately 40% after 10 minutes (Table 2).

Table 1. Effect of Sphingosine on PDH Activity Stimulated by hGH or Insulin

Sphingosine	Control	hGH	Insulin
−	2.33	2.76	3.37
+	1.48	1.50	1.77

NOTE. Hepatocytes (5 × 10^6 cells/mL) were preincubated with 40 μmol/L sphingosine for 6 minutes at 37°C and then incubated for 15 minutes with hGH or insulin (10^{-7} mol/L). Results are expressed as pmol/min/mg of protein and represent the mean of three separate experiments.

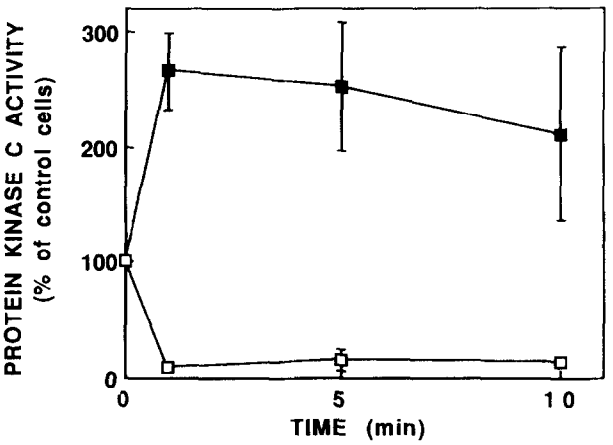

Fig 1. Effect of TPA on hepatocyte PKC activity. Cells were incubated for various times with 10^{-6} mol/L TPA and results were compared with control values. Data are expressed as the percentage of control activity for the soluble (□) and particulate (■) fractions, and represent the mean ± SEM of three experiments.

Figure 2 shows the time course of PKC activation by hGH (10^{-7} mol/L) in isolated hepatocytes. At 1 minute, hGH significantly increased the activity of PKC in both the cytosolic and particulate fractions; this effect disappeared at 5 minutes, but enzyme activity again increased in the soluble fraction at 30 minutes, the last time interval tested.

The effects of increasing concentrations of hGH on PKC activity of isolated hepatocytes at 1 minute are shown in Fig 3. Maximal stimulation of PKC activity occurred at a hGH concentration of 10^{-7} mol/L with both the soluble and particulate fractions. Half-maximal stimulation occurred at a hGH concentration of about 5 × 10^{-9} mol/L in both fractions.

The effects of insulin (10^{-7} mol/L) on PKC activity in isolated rat hepatocytes appeared to be biphasic (Fig 4). There was first a rapid (2 minutes) and transient increase in activity in both the cytosolic and particulate fractions. At 30 minutes, insulin decreased PKC activity in the soluble

Table 2. Effect of TPA on Total PKC Activity in Isolated Hepatocytes

Cells Fractions	Incubation Time (min) 1	5	10
Control			
Soluble	18.1	21.5	17.8
Particulate	5.6	8.9	8.3
Total activity	23.7	30.4	26.1
TPA-treated			
Soluble	1.9	2.2	2.5
Particulate	17.8	20.3	13.9
Total activity	19.7	22.5	16.4

NOTE. Cells were incubated for 1 to 10 minutes with 10^{-6} mol/L TPA. Results are expressed as nmol/min/10^6 cells (soluble and particulate fractions) and represent the mean of three separate experiments.

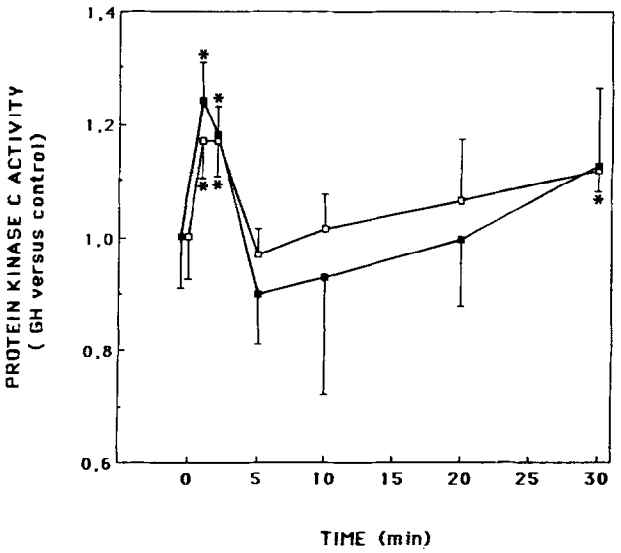

Fig 2. Effect of hGH on hepatocyte PKC activity. Cells were incubated for various times with hGH (10^{-7} mol/L). Results are expressed as PKC activity (GH *v* control cells) and represent the mean ± SEM of five to eight experiments. PKC activity in control cells was 964 and 1,108 pmol/min/mg for soluble (□) and particulate (■) fractions, respectively; *$P < .05$.

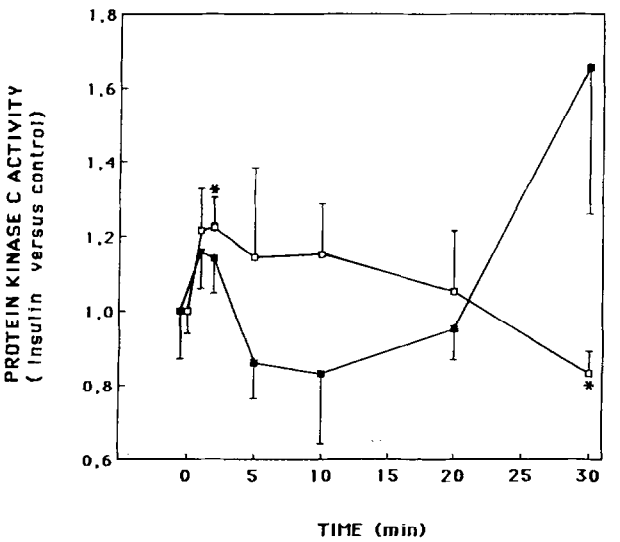

Fig 4. Effect of insulin on hepatocyte PKC activity. Cells were incubated for various times with insulin (10^{-7} mol/L). Results are expressed as PKC activity (insulin *v* control) and represent the mean ± SEM of four experiments. PKC activity in control cells was 1,109 and 1,512 pmol/min/mg for the soluble (□) and particulate (■) fractions, respectively; *$P < .05$.

fraction by 17% ($P < .05$) and increased it in the particulate fraction by 65%.

hGH, insulin (10^{-7} mol/L), and both hormones together increased particulate PKC activity by 18%, 25%, and 47%, respectively, after a 1-minute incubation (mean of three experiments); similar results were obtained with the cytosolic fraction. Thus in our experimental conditions, the effects of hGH and insulin on PKC activity appeared to be additive.

To evaluate a possible translocation of PKC between the cytosol and membranes, we measured the specific binding of PDBu in selected cytosol fractions containing sufficient

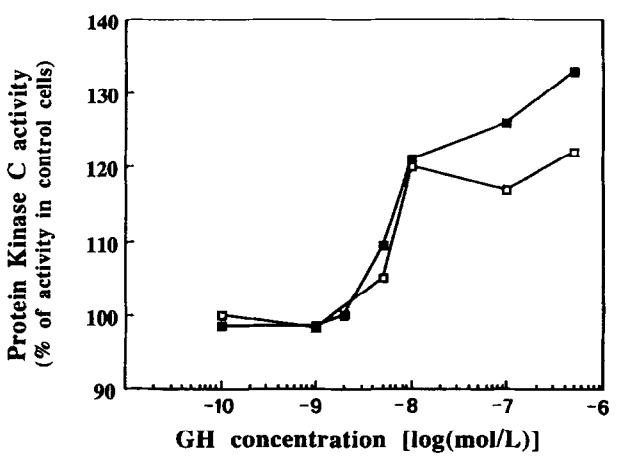

Fig 3. Effect of various hGH concentrations on hepatocyte PKC activity. Cells were incubated for 1 minute in the presence of increasing concentrations of hGH. Results are expressed as the percentage of control activity and represent the mean of three experiments. PKC activity in control cells was 794 and 1,003 pmol/min/mg for the soluble (□) and particulate (■) fractions, respectively.

levels of PKC. Incubation for 1 minute with hGH did not change specific PDBu binding to cytosolic proteins (1.68 and 1.60 pmol/mg protein in control and GH-treated cells, respectively; means of three experiments), whereas incubation for 30 minutes with insulin led to a marked decrease (1.44 and 0.82 pmol/mg protein in control and insulin-treated cells, respectively; means of three experiments).

DISCUSSION

We evaluated the early effects of GH on PKC activity, since it has been suggested that this kinase could be involved in the insulin-like actions of GH. We first verified that the freshly isolated hepatocyte model was suited to detect a PKC-dependent metabolic effect of hGH and insulin. Using the same model, we have previously shown that hGH and insulin increase PDH activity.[17] Here, we found that sphingosine, a potent inhibitor of PKC,[4] markedly blocked the effects of both hormones on PDH activity, suggesting that PKC might be a physiologic mediator of some metabolic effects of hGH or insulin in liver cells. Insulin is known to activate PKC in various cell types, and in some cases it induces a translocation of PKC from the cytosol to the membranes. Before evaluating a possible translocation of hepatic PKC in response to these hormones, we tested the effects of TPA, a well-known PKC activator that induces translocation. TPA effectively provoked an intracellular redistribution of PKC, with a rapid decrease in the cytosolic fraction and a corresponding increase in the particulate fraction. Incubation with TPA also induced a progressive loss of enzyme activity, confirming previous data.[22,23]

The effects of insulin on PKC activity vary with the cell type or tissue used and with the experimental method. Many studies have been performed with the BC3H-1 cell

line, adipocytes, and muscle tissue, but few with liver tissue.[22,24] Using freshly isolated hepatocytes, we found that insulin provoked an early activation (2 minutes) of PKC in both the cytosolic and particulate fractions, and later (30 minutes) seemed to induce translocation of the enzyme from the cytosol to the membranes, as reflected by enzyme activity and PDBu binding. The rapid effect of insulin has previously been reported by Cooper et al,[24] but late translocation was not observed by these investigators, probably because the incubation time was too short. By contrast, other investigators have found no effect of insulin on PKC activity[22]; however, it was only estimated in the soluble fraction and for only 20 minutes. Moreover, the cells were not preincubated before insulin treatment, in contrast to the study of Cooper et al[24] and the present study.

An important role of PKC in the insulin-like actions of GH and its involvement in the putative common pathway shared by insulin and GH is suggested by indirect evidence. In isolated fat cells, TPA mimicked some insulin-like actions, and inhibition or downregulation of PKC largely blocked the rapid effects of GH.[2-4] Moreover, in the present study, the activation of liver PDH by hGH or insulin was partially blocked by a PKC inhibitor. Finally, many investigators have reported that GH induces a marked and early increase in levels of diacylglycerol (DAG),[5-9] a physiologic activator of PKC. We found that GH induced a rapid (1 to 2 minutes) and transient activation of PKC in both the cytosolic and membrane fractions, apparently without modifying specific PDBu binding. This hormonal effect could be due to the early accumulation of DAG in isolated hepatocytes, as reported by Johnson et al.[8] Moreover, the PKC activation we observed and the reported DAG accumulation[8] were seen at physiologic concentrations of GH. The observation that the early effects of hGH and insulin on PKC activity were additive suggests that different transduction pathways are involved in the effects of these hormones. At later times, hGH did not seem to induce a redistribution of PKC activity, in contrast to insulin. This difference might be explained by the late production of different types of DAG, by a different location of DAG in subcellular compartments, or by activation of different PKCs.

hGH specifically interacts with both somatogenic and lactogenic receptors. In female rat hepatocytes, which possess both receptor types, prolactin, like hGH, increases DAG levels.[8] However, hepatocytes freshly isolated from adult male Sprague–Dawley rats contain only somatogenic binding sites,[25] and thus the hGH effect we observed is of the somatogenic type.

In conclusion, GH, like insulin, rapidly activates PKC in isolated hepatocytes. Since PKC is a large family of various isotypes (probably located in specific subcellular fractions), it is possible that the effect of GH involves mainly one isozyme or one subcellular compartment. Whether PKC activation is the triggering event that follows GH binding to its receptor remains to be determined.

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