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Effect of protein kinase C activation and depletion on insulin stimulation of glycogen synthesis in cultured hepatoma cells

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Summary. Insulin stimulation of glycogen synthesis was nearly abolished in hepatoma cells shortly treated with 4 β-phorbol 12 β-myristate, 13 α-acetate (protein kinase C activation) but remained unmodified in cells chronically treated with the phorbol ester (protein kinase C depletion). Thus, although exogenous activation of protein kinase C results in an inhibition of insulin action, protein kinase C depletion has no influence on this process. The results suggest that, in hepatoma cells, no endogenous activation of protein kinase C may occur in response to the signal triggered by insulin.
Key words. Glycogen synthesis; insulin; protein kinase C; Zajdela hepatoma cells.

Calcium-activated, phospholipid-dependent protein kinase (protein kinase C, PKC) has recently emerged as a key cellular enzyme which appears to be involved in both the transduction and the modulation of the signals triggered by several growth factors and hormones including insulin[1]. Supporting this dual role of PKC is the finding that 4 β-phorbol 12 β-myristate, 13 α-acetate (PMA), a potent tumor promoter which directly activates PKC, exhibited in rat adipocytes insulin-like as well as insulin-antagonizing effects on metabolic processes such as glucose transport and lipogenesis[2-4]. However, when tested on rat liver glycogen synthesis, the effect of PMA proved quite different, since the phorbol ester was reported either to decrease the basal activity of glycogen synthase[5-7] or to be ineffective[8, 9]. In any case, PMA was found to antagonize insulin stimulation of glycogen synthase in Fao hepatoma cells, indicating that PKC is able to counteract the hormone bioactivity on glycogen synthesis when exogenously activated by the phorbol ester.

The present study was designed to determine whether, in Zajdela hepatoma cultured cells (ZHC cells), the signal triggered by insulin for stimulation of glycogen synthesis might be modulated through a mechanism involving endogenous activation of PKC. Thus, we measured the hormone stimulation of this process in ZHC cells where PKC had been either exogenously activated by a short exposure to PMA or largely depleted by a chronic exposure to the phorbol ester, in comparison to the stimulation observed in untreated cells.

Materials and methods. Stock cultures of ZHC cells, derived from the Zajdela rat ascite hepatoma (strain D), were subcultured into 25-ml flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum and glucose (45 mM final concentration)[11]. Down-modulation of PKC was obtained by preincubating confluent ZHC cells for 17 h in serum-free DMEM either in the absence (control cells) or the presence of 500 ng/ml of PMA (PKC-deficient cells). After 3 extensive washings, cells were used for the determination of either glycogen synthesis from [U-^{14}C] glucose, or insulin binding or PKC activity. Cell viability was estimated by the Trypan blue exclusion test.

Incorporation of [U-^{14}C] glucose into glycogen was measured as described previously[12]. Briefly, control and PKC-deficient ZHC cells, once treated as described in the legends of the figure and table 2, were incubated for 1 h at 37 °C in 2 ml serum-free DMEM containing 45 mM glucose and a tracer amount of 2.5 μCi of [U-^{14}C] glucose (240 mCi/mmol, Commissariat à l'Energie Atomique, France). Glycogen was then extracted and its radioactivity was determined as described[12]. Data are given as nanomoles of glucose incorporated into glycogen/mg protein/h. Protein content was evaluated by the Bradford dye method, with the use of Bio-Rad reagent and bovine serum albumin as the standard.

Insulin binding was measured by incubating (17 h at 4 °C) control or PKC-deficient ZHC cells in suspension (6×10^5 cells/ml) with 100 pM [^{125}I]-labeled insulin (80–100 μCi/μg, New England Nuclear Corp., USA) in the presence of increasing concentrations (0–10^4 ng/ml) of native insulin, as previously described[12].

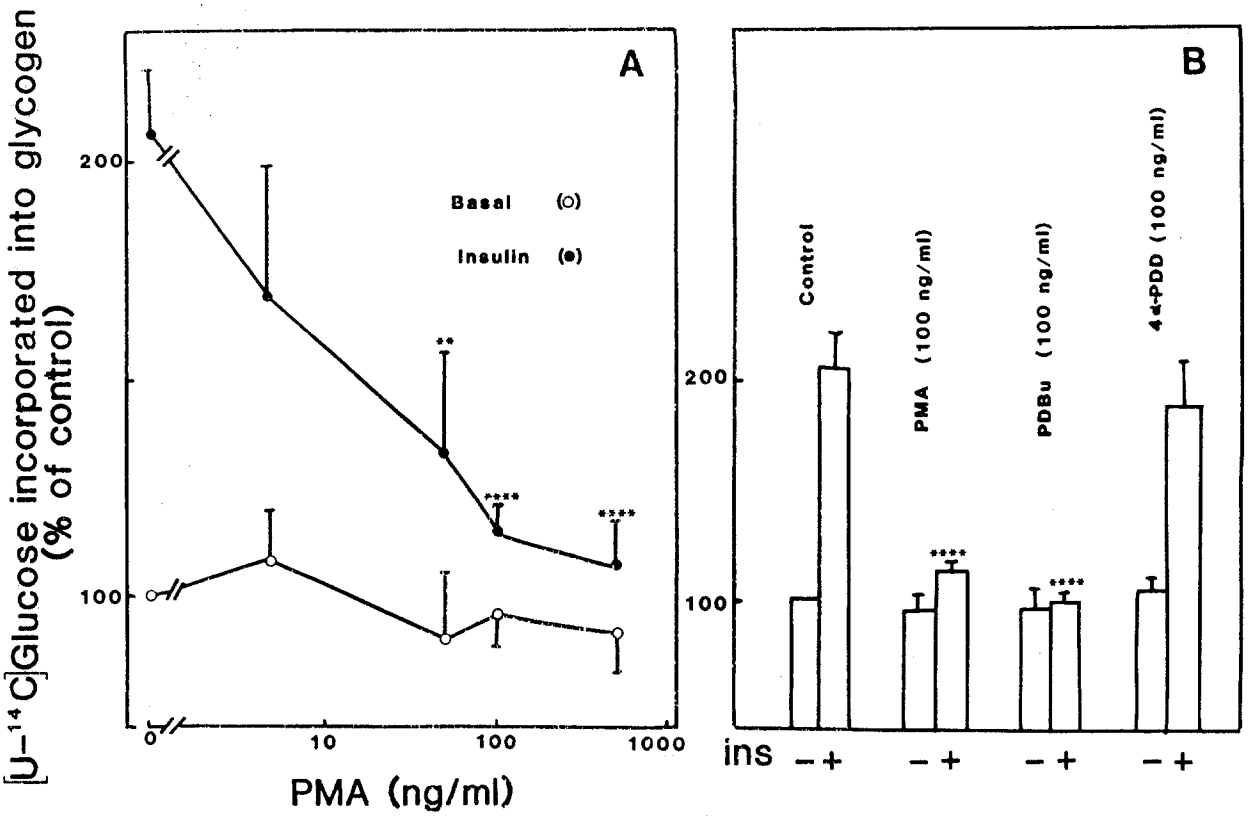
Protein kinase C activity was measured in total cellular extracts. In short, control or PKC-deficient ZHC cells were suspended in 2 ml of extraction buffer (Tris-HCl 20 mM pH 7.5, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 50 mM β-mercaptoethanol, 0.2 mM PMSF, 0.1% Triton X 100) and

briefly sonicated. After 1 h at 4 °C, the homogenate was centrifuged at 105,000 × g for 1 h. The resulting supernatant was immediately assayed for PKC activity which was determined as previously reported [13] by measuring the incorporation of ^{32}P from [$\gamma^{32}P$]-ATP (10 Ci/mmole, New England Nuclear Corp., USA) into histone III-S (Sigma). The standard reaction mixture contained 20 mM Tris-HCl pH 7.4, 200 µg/ml of histone, 20 µM ATP (700 cpm/pmol), 10 mM Mg-acetate, 0.8 mM $CaCl_2$, 20 µg/ml phosphatidylserine, 4 µg/ml diolein and the enzyme solution (10 µg of protein) to be assayed in a final volume of 250 µl. After 5 min at 30 °C, the reaction was terminated as indicated by Skoglund et al. [14]. PKC activity, which was determined by subtracting the activity measured in the absence of Ca^{++} and lipids from that measured in their presence, is expressed in picomoles of ^{32}P transferred to histone/mg protein/min.

All results are given as the means ± SEM for the indicated numbers (n) of independently performed experiments. Differences between the mean values were evaluated by Student's t-test.

Results. Insulin stimulation of glycogen synthesis in ZHC cells briefly treated with PMA. Figure A shows that, in the absence of PMA, the stimulation of glycogen synthesis elicited by 100 nM insulin reached 207.0 ± 15.0% of the basal level, which is in agreement with our previous results [12]. When ZHC cells were preincubated in the presence of increasing concentrations (5–500 ng/ml) of PMA, a dose-dependent decrease in the hormone stimulation of glycogen synthesis was observed, with no modification of the basal level of this process (fig. A). The concentrations of PMA which produced the half-maximal and the maximal inhibitions of insulin action were 10 and 100 ng/ml respectively. Similar results were obtained when ZHC cells were first incubated with insulin (30 min) and then with PMA (additional 30 min) before assays for glycogen synthesis were initiated. Figure B shows that 4 β-phorbol 12,13 dibutyrate (PDBu, 100 ng/ml), another active phorbol ester (PE), also markedly decreased insulin action, whereas 4 α-phorbol 12,13 didecanoate (4 α-PDD, 100 ng/ml) an inactive PE, was ineffective. Thus, PMA exerted specific insulin antagonizing effects in ZHC cells, which therefore appear to be mediated through a direct activation of PKC. On the other hand, we observed that PMA inhibition of insulin action on glycogen synthesis resulted from an effect distal to the inital step of insulin binding; indeed, neither the amount of [^{125}I]-labeled insulin bound nor the B_{50} value (the concentration of native insulin which displaced 50% of the bound [^{125}I]-labeled insulin) were significantly modified in the PMA-treated ZHC cells (12.5 ± 2.3 pg/10^6 cells and 1 nM, respectively) as compared to the control ones (13.5 ± 0.8 pg/10^6 cells and 1 nM, respectively, n = 5). Similarly, a lack of PMA effect on insulin binding was observed in H 35 hepatoma cells [10].

Insulin stimulation of glycogen synthesis in ZHC cells chronically treated with PMA. It has been reported that active phorbol esters such as PMA and PDBu are able to induce a down-modulation of PKC when chronically maintained in the incubation cell medium [13, 15, 16]. In the present study, we exploited this property of PMA to develop a model of PKC-deficient cells. In preliminary assays in which several concentrations (100–500 ng/ml) of PMA were tested, we determined that a 17-h pretreatment of ZHC cells with 500 ng/ml PMA efficiently down-modulated PKC since



Effect of a short treatment of ZHC cells with active (PMA, PDBu) or inactive (4 α-PDD phorbol esters on basal and insulin-stimulated glycogen synthesis. ZHC cells were incubated for 30 min at 37 °C without or with: *A* increasing concentrations of PMA; *B* 100 ng/ml of either PMA, PDBu or 4 α-PDD. After a further incubation in the absence or in the presence of 100 nM insulin, glycogen synthesis from [U-^{14}C] glucose was measured as described in 'Materials and methods'. The control value (100%) of [U-^{14}C] glucose incorporated into glycogen was 29.0 ± 4.0 nmol/mg protein/h. Results are the means ± SEM of 4–9 experiments performed in triplicate. Significant differences between phorbol ester-treated and untreated cells are indicated: ** $p < 0.02$, **** $p < 0.001$.

Table 1. Loss of PKC activity in ZHC cells chronically treated with PMA

Treatment	PKC activity (pmol ^{32}P transferred to histone III-S/mg protein/min)	
None	106.4 ± 22.3	(100)
PMA (500 ng/ml)	12.7 ± 2.2****	(12)
4α-PDD (500 ng/ml)	99.6 ± 20.0	(94)

ZHC cells were incubated for 17 h at 37 °C in serum-free DMEM, in the absence or in the presence of either PMA or 4α-PDD. After 3 extensive washings with ice-cold phosphate buffered saline over 45 min, whole cell extracts were prepared as described in 'Materials and methods' and assayed for PKC activity. Results are the means ± SEM of 6 experiments. The numbers in parentheses indicate the percent of activity. **** $p < 0.001$ (vs control value, i.e. 100%).

it resulted in a 88% decrease in the enzyme catalytic activity (table 1). This treatment was found not to modify either the cell viability or the total cellular protein content (3.38 ± 0.05 mg/flask for control cells, vs 3.43 ± 0.07 mg/flask for PMA-treated cells, n = 12). By contrast, a 17-h pretreatment of ZHC cells with 500 ng/ml of 4α-PDD was ineffective (table 1), which assesses for the specificity of the PMA-induced down-modulation of PKC. Further support for the hypothesis that ZHC cells which were chronically treated with PMA were made effectively PKC-deficient is provided by the finding that subsequent exposure of these cells to PMA (100 ng/ml) for 30 min no longer inhibited insulin stimulation of glycogen synthesis (table 2). Thus, these cells proved useful in examining the incidence of PKC deficiency on insulin stimulation of glycogen synthesis.

As shown in table 2, the basal level of glycogen synthesis in PKC-deficient ZHC cells was not significantly modified as compared to that measured in the control ones. Table 2 also shows that insulin stimulation of glycogen synthesis failed to be modified in PKC-deficient ZHC cells as compared to that observed in the control ones, indicating that the loss of PKC has not any incidence on the hormone signal, as discussed elsewhere.

Discussion. In the present study, we took advantage of the ability of PMA, on the one hand, to activate PKC (short exposure) and, on the other hand, to almost completely deplete the enzyme activity (chronic exposure), in order to investigate the role of PKC in the regulation of the signal triggered by insulin for the activation of glycogen synthesis in ZHC cells.

Under conditions where PKC was exogenously activated by a short exposure of ZHC cells to PMA, insulin stimulation of glycogen synthesis was markedly inhibited, with no modification of the basal level of this process. These data, which provide evidence for the ability of PMA to selectively antagonize the action of insulin on glycogen synthesis in ZHC cells indicate an indirect effect of PKC via the insulin effector system, rather than a direct effect on the enzymatic system responsible for glycogen synthesis. Two observations support this hypothesis: a) PKC has been reported to antagonize, both in vivo [10, 17] and in vitro [18], the insulin stimulation of the autophosphorylation of the insulin receptor on tyrosine residues, a process which has recently been shown to be involved in the hormone bioactivity [19]; ii) although PKC has been found to directly phosphorylate glycogen synthase, the finding that this is concomitant with an inactivation of the enzyme remains controversial in the case of the liver [8, 9]. Under conditions where ZHC cells were chronically treated with PMA, PKC was almost completely lost, as assessed by the dramatic decrease in the enzyme catalytic activity as well as by the inability of the phorbol ester to subsequently inhibit insulin activation of glycogen synthesis. These data, which agree with results we have reported elsewhere [13], do not exclude other effects of the PMA preincubation on cellular processes. However, neither cell viability nor the basal level of glycogen synthesis were found to be altered in ZHC cells which were chronically treated with PMA, further validating the model of PKC-deficient cells used in this study. Our results show that the loss of PKC in these cells failed to modify the insulin stimulation of glycogen synthesis. However, one might have expected an increase in the hormone effect in the PKC-deficient cells as compared to the effect observed in the control ones. Indeed, the role of PKC, which was evidenced through exogenous activation with PMA, consists in inhibiting insulin stimulation of glycogen synthesis. Thus, PKC does not appear to antagonize insulin action in control cells, suggesting that the enzyme fails to be endogenously activated in response to the hormone. Of interest in this regard is the finding that, in contrast to that which has been reported for fat cells [20, 21], insulin proved unable to increase the phosphoinositide turnover in hepatocytes [22, 23] which is considered as a primary event in the release of diacylglycerol, the endogenous activator of PKC. In any case, we cannot completely rule out the possibility that, although the modulation of insulin bioactivity on glycogen synthesis through endogenously activated PKC may be effective in vivo, it is too weak to have been evidenced with our methodological design.

In conclusion, the present results suggest that although protein kinase C, when exogenously activated, is potent to antagonize insulin stimulation of glycogen synthesis, in ZHC cells, it fails to be endogenously activated in response to the hormone.

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Table 2. Insulin stimulation of glycogen synthesis in control and PKC-deficient ZHC cells

PMA treatment	[U-^{14}C]glucose incorporated into glycogen (nmol/mg protein/h)		
	Basal	Insulin (100 nM)	PMA (100 ng/ml) + insulin (100 nM)
(−)	26.9 ± 4.0 (100)	55.8 ± 7.9*** (207)	29.5 ± 2.5 (109)
(+)	30.4 ± 4.0 (113)	56.2 ± 7.6*** (209)	56.9 ± 6.6*** (211)

ZHC cells were preincubated for 17 h at 37 °C in serum-free DMEM in the absence or in the presence of PMA (500 ng/ml). After 3 extensive washings in serum-free DMEM over 45 min, cells were treated for 30 min at 37 °C without or with PMA and further incubated for 30 min in the absence or in the presence of insulin. Glycogen synthesis assays were then conducted as described in 'Materials and methods'. Results are the means ± SEM of 8–13 experiments performed in triplicate. The number in parentheses indicate the percent of activity. *** $p < j0.01$ (vs control value, i.e. 100%).

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The binding of FITC-insulin to ANAE-positive cells in rat thymus

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Summary. To determine if thymic macrophages have insulin receptors, alternate sections of rat thymus were stained with FITC-insulin and examined for nonspecific esterase (ANAE) activity. Cells showing a diffuse ANAE staining pattern also bound FITC-insulin. These cells were concentrated in the cortico-medullary border and increased in number following administration of cortisol. Thymic macrophages may be insulin-dependent and therefore could be malfunctional in diabetes.
Key words. Thymus; thymic macrophages; ANAE; insulin; FITC-insulin; diabetes mellitus.

T-cell mediated function has been reported to be abnormal in diabetes mellitus [1], and one of the characteristic features of experimental diabetes is an impairment of thymic lymphocyte maturation [2]. These findings suggest that insulin may play a role in T-cell development in the thymus. Although binding of ^{125}I-insulin to a mixture of thymic cells has been demonstrated [3], the cellular subtype to which the hormone binds has yet to be identified. Our studies of the pattern of binding of monofluoresceinthiocarbamyl-insulin (FITC-insulin) to frozen or fixed sections of thymus indicate that the cells in the thymus which bind insulin stain diffusely with nonspecific esterase (ANAE) suggesting that macrophages in the thymus are the cells which have insulin receptors. These cells may be important in thymic T-cell maturation and may function suboptimally in diabetes resulting in depressed T-cell development.

Materials and methods. Monofluoresceinthiocarbamyl-insulin (FITC-insulin) was prepared essentially as described [4] using crystalline bovine insulin, (23.6 IU; Lot 49C-0197) and fluorescein-isothiocyanate, isomer I on Celite obtained from the Sigma Chemical Co. Following the coupling reaction, the mono FITC-insulin was purified by chromatography on Sephadex G-25 (eluted with 0.9% NaCl-0.01 M phosphate buffer, pH 7.4), isoelectric precipitation, followed by chromatography on DEAE-Sephadex A-25 (eluted with 7 M urea in 0.01 M Tris-HCl, pH 7.6, with a 0–1 M NaCl gradient). In two separate studies, FITC-insulin was shown to have 40 or 51% of the bioassayable potency of native insulin [4, 5].

Rats (SASCO, Omaha, NE) were decapitated and the thymuses quickly frozen in O.C.T. compound (Lab-Tek Products) using 2-methylbutane and dry ice. Cryostat sections were cut at 4 μm. The mounted slices were washed 2 times with phosphate-buffered saline (PBS) (0.01 M sodium phosphate, pH 7.8) and treated for 30 min at 37 °C with 1×10^{-6} M FITC-insulin in PBS containing 1% bovine albumin. The sections were washed 2 times with PBS and photographed using a Wild fluorescence microscope. Fluoresceinisothiocyanated-bovin albumin (Sigma Chemical Co.) was dissolved in PBS and used as a negative control stain. Alpha-naphthylacetate esterase (ANAE) staining of thymus sections was performed as described [6].

Results. FITC-insulin was found to bind to a subpopulation of cells in all sections of rat thymus studied, but the number of reactive cells was significantly greater in the thymus of animals that had been treated with a lymphocytolytic steroid (e.g. 5 mg cortisol/100 g b. wt, s.c., 44 h prior to sacrifice). The figure shows a typical fluorescent micrograph of a frozen FITC-insulin-stained thymus section from a cortisol-treated rat. FITC-insulin fluorescent cells were found with the highest frequency at the cortico-medullary junction. No fluorescent cells were found in control studies using FITC-albumin as the fluorescent probe. Preincubation of the frozen tissue with unlabeled insulin (3×10^{-5} M) for 30 min at 37 °C decreased the number of fluorescent cells stained with FITC-insulin by 50–60%. Prior fixation of the tissue in 95% methanol decreased the number of cells found to stain with FITC-insulin, but a significant number of cells re-