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Phorbol esters modulate insulin receptor phosphorylation and insulin action in cultured hepatoma cells

(protein kinase C/tyrosine aminotransferase/glycogen synthase)

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ABSTRACT **The effect of the tumor-promoting agent phorbol 12-myristate 13-acetate (PMA) on insulin receptors and insulin action was studied in rat hepatoma cells in culture. PMA (0.1–1.0 μg/ml) did not affect insulin binding either acutely or chronically but inhibited insulin stimulation of glycogen synthase and tyrosine aminotransferase. PMA (1 μg/ml) stimulated the phosphorylation of the β subunit of insulin receptor purified from [^{32}P]phosphate-labeled Fao cells by 1.3-fold in the absence of insulin. In contrast, insulin-stimulated phosphorylation in the presence of PMA was reduced. Phosphoamino acid analysis of the β subunit after PMA stimulation revealed an increase of both phosphoserine and phosphothreonine residues, whereas insulin stimulated primarily phosphorylation of tyrosine and serine residues. Insulin stimulation of cells after PMA treatment revealed a decrease in phosphotyrosine when compared to cells stimulated by insulin alone. Tryptic peptide mapping of the β subunit by a two-dimensional chromatographic/electrophoretic separation revealed nine phosphopeptides from the cells treated with PMA. Insulin stimulated phosphorylation at six new sites in the receptor, three of which appeared to be similar to those in PMA-treated cells. This report shows that phorbol esters stimulate insulin receptor phosphorylation, inhibit insulin-induced receptor phosphorylation and insulin action, and suggest a physiologic relation between insulin action and the calcium-activated and phospholipid-dependent protein kinase C.**

Phorbol diesters are potent tumor promotors and have effects on cellular differentiation and proliferation in a variety of different cell types (1, 2). Phorbol diesters also have been shown to induce the phosphorylation of several cellular proteins. Protein phosphorylation is believed to be increased through a direct activation by phorbol esters of a calcium-activated and phospholipid-dependent protein kinase (protein kinase C). This kinase appears to be the cellular receptor for phorbol esters (3). Protein kinase C is found in many tissues, and although its physiologic role is not known it has been suggested, based on these studies and the many effects of phorbol esters, that protein kinase C may be involved in regulation of cellular proliferation and other metabolic processes.

Recently, Cochet *et al.* (4) reported that protein kinase C phosphorylates the epidermal growth factor (EGF) receptor and reduces the tyrosine kinase activity of the EGF receptor. Studies have also shown that phorbol ester treatment of lymphoid cells may alter insulin binding (5). Recently, phorbol esters have been shown to stimulate phosphorylation of insulin receptors in cultured lymphocytes (6) and to induce phosphorylation of the EGF receptor in A431 cells (7).

In this study, we examined the effect of phorbol 12-myristate 13-acetate (PMA), the most potent tumor promotor, on insulin receptors and insulin action in intact hepatoma cells. We find that PMA stimulates insulin receptor phosphorylation at specific serine and threonine sites without altering insulin binding. This effect is accompanied by a decrease in insulin stimulation of tyrosine phoshorylation of the insulin receptor and a decrease in insulin-stimulated glycogen synthase and tyrosine aminotransferase (TyrATase). Our results suggest that phorbol esters may modulate early events in the pathway of insulin action.

MATERIALS AND METHODS

Materials. Porcine insulin was purchased from Elanco (Indianapolis, IN), [^{32}P]orthophosphate was from Amersham, and ^{125}I-labeled insulin (^{125}I-insulin) and uridine-5′-diphospho[^{14}C]glucose were from New England Nuclear. PMA, aprotinin, and phenylethylsulfonyl fluoride were obtained from Sigma. Wheat germ agglutinin coupled to agarose was from Vector Laboratories (Burlingame, CA). L-1-tosylamido-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin was from Worthington (lot no. 32N798, 182 units/mg). All reagents for NaDodSO$_4$/PAGE were from Bio-Rad. Fao cells, a clonal cell line derived from the well-differentiated Reuber H35 rat hepatoma, are maintained in culture as described (8).

Methods. *Insulin binding.* For the binding experiments, Fao cells were subcultured into six-well tissue culture dishes 2 days prior to the experiment. After the cells were incubated with PMA as indicated, insulin binding was measured as described (9).

Glycogen synthase and TyrATase assays. Fao cells were grown to confluence in 100-mm dishes, after which they were incubated with or without PMA as indicated. Just prior to the insulin stimulation, the cells were incubated in 4 ml of buffered F-12 medium (pH 7.4) for 3 hr at 37°C in a humidified incubator, and then insulin was added at the indicated concentrations for 30 min at 37°C. The cells were washed at 4°C with 3 ml of 100 mM NaF/10 mM EDTA. Then the monolayer was scraped from the plates, collected by centrifugation, and disrupted by sonication. The particulate fraction was removed by centrifugation, and the glycogen synthase in the supernatant was assayed as described (9).

For the assay of TyrATase, confluent Fao cells in six-well tissue culture dishes were incubated in 1 ml of buffered F-12 medium with and without PMA at 1 μg/ml for 30 min. Then various concentrations of insulin were added, and the incubation was continued for 4 hr at 37°C in a humidified incubator. Cells were extracted and TyrATase was assayed as described by Diamondstone (10). One unit of TyrATase is defined as the formation of 1 μmol of *P*-hydroxyphenylpyruvate per min at 37°C.

[^{32}P]Phosphate labeling of the Fao cells and immunoprecipitation of the insulin receptor. Fao cells were cultured in RPMI 1640 medium containing 10% fetal calf serum in a 100-mm plastic dish. Confluent cells were equilibrated with

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; TyrATase, tyrosine aminotransferase; EGF, epidermal growth factor.

[^{32}P]phosphate as described (11, 12). After 120 min, the cells were exposed to PMA and/or insulin as indicated for 15 min. The reaction was stopped with phosphate-buffered saline at 4°C containing 10 mM pyrophosphate, 10 mM NaF, 4 mM EDTA, and 1 mM sodium vanadate. The labeled cells were solubilized with 1% Triton X-100 containing 2 mM phenylmethylsulfonyl fluoride, aprotinin (12 units/ml), 10 mM pyrophosphate, 10 mM NaF, 4 mM EDTA, and 1 mM sodium vanadate for 10 min. The elapsed times between solubilization and ^{32}P-labeling were the same in all treatments. The insulin receptor was purified by affinity chromatography on immobilized wheat germ agglutinin-agarose and immunoprecipitation with anti-insulin receptor serum (12). The immunoprecipitates were analyzed by NaDodSO$_4$/PAGE, after which the gels were autoradiographed with Kodak X-Omat film for 2–4 days.

Identification of phosphoamino acids and two-dimensional peptide mapping. Phosphoproteins contained in gel fragments excised from NaDodSO$_4$/polyacrylamide gel were digested with trypsin as described (13). This digest was used for peptide mapping and phosphoamino acid analysis. The phosphopeptides were separated in two dimensions by electrophoresis on cellulose thin-layer plates sprayed with 30% formic acid (pH 1.9) and ascending chromatography in *n*-butanol:acetic acid:pyridine:H$_2$O, 60:12:40:48 (vol/vol), as described by Ellis *et al.* (14). Autoradiographs were prepared by exposure of the thin-layer plates to Kodak X-Omat film for 2 wk. For phosphoamino acid analysis, samples of phosphopeptides were lyophilized, subjected to acid hydrolysis, and the phosphoamino acids were separated by electrophoresis (13, 15).

RESULTS

Insulin Binding. Insulin binding to hepatoma cells was measured before exposure to PMA or after the cells were incubated with PMA for 30 min or 48 hr. ^{125}I-insulin (10^{-11} M) binding was unaffected by a 30-min incubation with several concentrations of PMA (0.1–10 μg/ml); however, insulin binding increased slightly at the highest PMA concentration after 48 hr of exposure. Insulin binding competition curves were almost superimposable in the PMA-treated and control cells (Fig. 1). Therefore, in contrast to the finding that PMA alters insulin binding to IM-9 lymphocytes (5), PMA has no detectable effect on insulin bindin to Fao cells.

Glycogen Synthase and TyrATase Activity. We have shown previously that Fao cells possess many insulin responses that are similar to those of normal liver, including activation

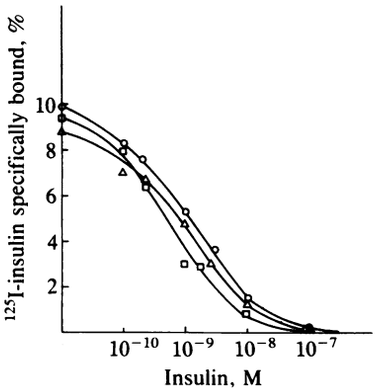

FIG. 1. Effect of PMA on insulin binding to its receptor on Fao cells: Competition curves. Confluent Fao cells were incubated in the absence of PMA (○) or with PMA at 0.1 μg/ml for 30 min (△) and 48 hr (□), after which the cells were incubated with medium containing ^{125}I-insulin and various concentrations of unlabeled insulin for 3 hr at 15°C. The insulin binding was measured as described.

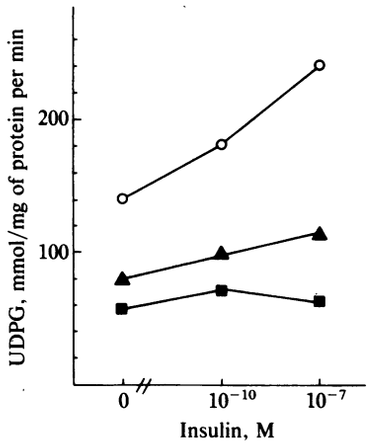

FIG. 2. Effect of PMA on insulin stimulation of glycogen synthase. Cells were incubated with 0.1 μg/ml (▲) or 1.0 μg/ml (■) of PMA for 30 min. Glycogen synthase activity in the presence of various concentrations of insulin was then measured as described. The activity is expressed as the amount of glucose 6-phosphate-independent (I) form. ○, Control; UDPG, uridine-5′-diphosphoglucose.

of the enzyme glycogen synthase and TyrATase (9). After 30 min of incubation with PMA (0.1 μg/ml), there was a marked inhibition of insulin-stimulated glycogen synthase, and this effect was dependent on the concentration of PMA used (Fig. 2). In some experiments, this also was associated with a decrease in basal levels of the I form of the enzyme. Upon continued incubation with PMA for 48 hr, this inhibitory effect was lost and insulin stimulation returned to normal. Insulin stimulation of TyrATase also was inhibited significantly after incubation of cells with PMA for 30 min (Fig. 3). PMA did not inhibit the basal level of TyrATase activity but reduced insulin stimulation at all hormone concentrations.

The Phosphorylation of Insulin Receptor. The phosphorylation of the insulin receptor was examined after 30 min and 48 hr of incubation of hepatoma cells with 0.1 and 1 μg/ml of PMA. In the absence of insulin, densitometric scanning of the corresponding autoradiograph showed that phosphorylation of the β subunit of the insulin receptor was stimulated 1.3-fold by PMA and 2.8-fold by insulin when compared with the control (Fig. 4, lanes A, B, and C). In cells preincubated for 30 min with PMA, insulin-stimulated receptor phosphorylation was reduced, both in absolute terms and with respect to the control (by a factor of 1.7 vs. 2.8) (Fig. 4, lanes C and D).

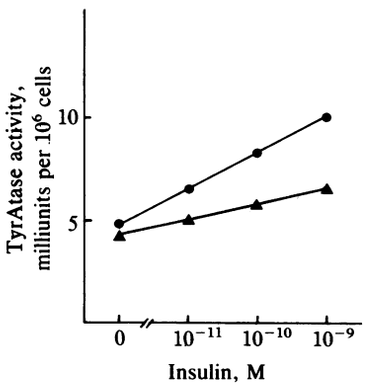

FIG. 3. Effect of PMA on insulin stimulation of TyrATase. Fao cells were incubated with 1.0 μg/ml of PMA (▲) or without PMA (●) for 30 min, and then various concentrations of insulin were added for 4 hr at 37°C. TyrATase activity was measured on cell extracts as described. This is a representative experiment.

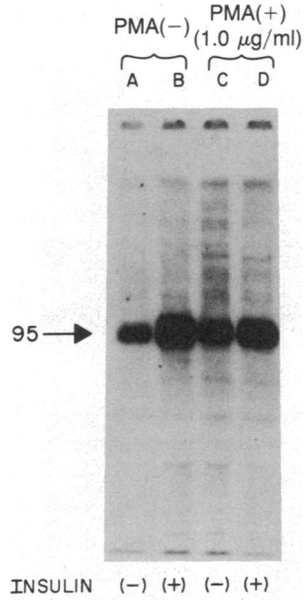

FIG. 4. Effect of PMA on insulin receptor phosphorylation. Confluent cells were incubated with [^{32}P]orthophosphate for 120 min and then exposed to PMA at 1.0 μg/ml for 30 min and/or to 0.1 μM insulin for 15 min as indicated. The labeled cells were solubilized, the extract was purified partially on immobilized wheat germ agglutinin, and the insulin receptor was immunoprecipitated with anti-insulin receptor antibody (B-9). The immunoprecipitates were analyzed by NaDodSO$_4$/PAGE and autoradiography. Lanes: A, basal phosphorylation; B, with 0.1 μM insulin; C, with PMA at 1 μg/ml; D, with PMA and insulin. Molecular weight is shown $\times 10^{-3}$.

After 48 hr of incubation with PMA, no effect of the phorbol ester on insulin receptor phosphorylation could be detected (Fig. 5, compare lanes F and H with B and D). In addition, a band of M_r 110,000 appeared in PMA-treated cells in both the control and anti-receptor antibody immunoprecipitates. The nature of this phosphoprotein remains to be determined. It is unlikely to be the α subunit of the insulin receptor because of its molecular weight, its phosphorylation was unaffected by insulin, and it was immunoprecipitated with control serum. Two unidentified low-molecular-weight phosphoproteins (M_r = 60,000 and 30,000) also appeared after prolonged PMA treatment.

Phosphoamino Acid Determinations. The insulin receptor in intact hepatoma cells is phosphorylated constitutively on serine residues of the β subunit (8). After insulin stimulation for 15 min, phosphoserine increased and phosphotyrosine appeared (Fig. 6, lanes A and B). With PMA treatment alone, phosphoserine increased relative to the basal state (Fig. 6, lanes A and C), and a trace of phosphothreonine was also detected. Insulin stimulation of PMA-treated cells also revealed an increase in phosphoserine; however, the insulin-stimulated increase in phosphotyrosine was less than in cells treated with insulin alone (Fig. 6, compare lanes D and B). The relative amounts of the phosphorylated amino acids were determined by densitometric scanning. In cells treated with insulin alone, the phosphotyrosine spot was twice the density of that of cells stimulated by insulin in the presence of PMA (13.6 vs. 6.6 arbitrary units). The ratio of phosphoserine to phosphotyrosine calculated from these scans was 1.5 in the cells with insulin alone and 3.91 in the cells treated with both insulin and PMA.

Phosphopeptide Mapping. To identify the sites of phosphorylation after insulin stimulation and PMA treatment, the M_r 95,000 band was hydrolyzed with trypsin and the phosphopeptides were examined by using 2-dimensional peptide mapping. In the basal state, three phosphopeptides were seen (Fig. 7*A*). Six additional spots (I_1 to I_6) were detected in

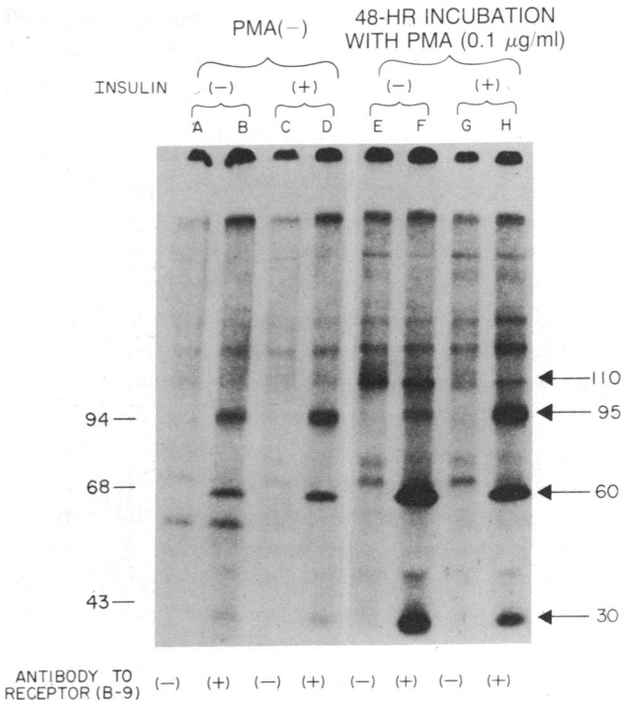

FIG. 5. Effect of long-term treatment with PMA on insulin receptor phosphorylation. Confluent cells were labeled with [^{32}P]orthophosphate as described, then exposed to PMA at 0.1 μg/ml for 48 hr (lanes E, F, G, and H) and/or 0.1 μM insulin for 15 min (lanes C, D, G, and H). The labeled cells were solubilized, and insulin receptor was purified on wheat germ agglutinin-agarose and immunoprecipitated by anti-insulin receptor antibody (B-9) (lanes B, D, F, and H) or by control pooled serum (lanes A, C, E, and G). The immunoprecipitates were analyzed by NaDodSO$_4$/PAGE and autoradiography. Molecular weights are shown $\times 10^{-3}$.

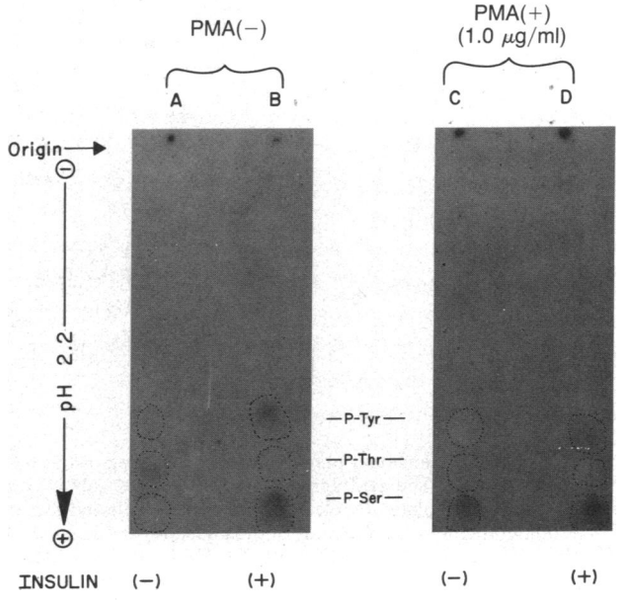

FIG. 6. Phosphoamino acid analysis of the insulin receptor. The immunoprecipitated M_r 95,000 bands (β subunit) were excised from the NaDodSO$_4$/polyacrylamide gel. After removing NaDodSO$_4$ with 10% methanol, the gel fragments were trypsinized to elute the labeled peptides and hydrolyzed with 6 M HCl as described. Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine were added to the samples and separated by electrophoresis on cellulose thin-layer plates at pH 2.2. Lanes: A, from cells in the basal state; B, with 0.1 μM insulin; C, with PMA at 1.0 μg/ml; D, with 0.1 μM insulin and PMA at 1.0 μg/ml.

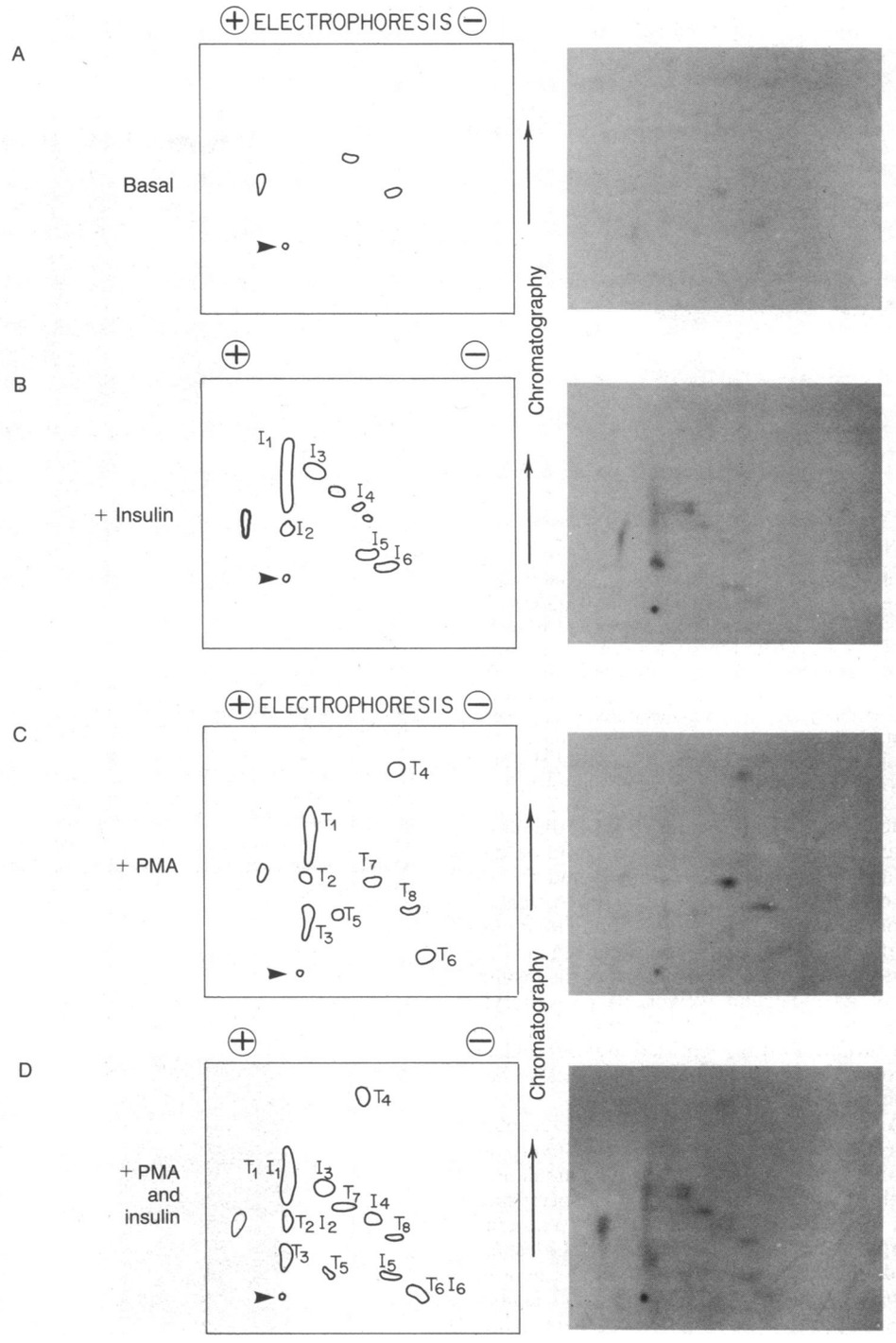

FIG. 7. Two-dimensional phosphopeptide mapping of the β subunit of insulin receptor. The phosphorylated β subunit was separated by $NaDodSO_4$/PAGE. The gel fragment containing the β subunit was incubated with trypsin, and the eluted phosphopeptides were separated on cellulose thin-layer plates by electrophoresis (pH 1.9) and ascending chromatography (pH 3.5) as described. (*Right*) Autoradiograms. (*Left*) Corresponding schematic diagrams of the phosphopeptide spots. The origin is identified with an arrowhead. (A) Basal. (B) With 0.1 μM insulin. (C) With PMA at 1.0 μg/ml. (D) With 0.1 μM insulin and PMA at 1.0 μg/ml.

cells after stimulation with insulin for 15 min (Fig. 7*B*), the major spots being identified as I_1 and I_2. PMA treatment also resulted in six new spots (T_1 through T_6) as well as a marked increase in two spots with similar mobility to those in the basal state (T_7 and T_8) (Fig. 7*C*). Three of the PMA-stimulated spots appeared to be similar or identical to those in insulin-stimulated cells (T_1, T_2, T_6 and I_1, I_2, I_6) (Fig. 7*D*); however, at least three sites were clearly different from those found after insulin stimulation. Insulin stimulation of PMA-treated cells revealed a combination of the two patterns, although there appeared to be a decrease in the major insulin-stimulated phosphopeptides (I_1 and I_2) when compared to insulin alone.

DISCUSSION

Phorbol diesters, such as PMA, are potent tumor promotors that alter cellular function in a variety of ways (1, 16, 17). Phorbol diesters are believed to exert their effects by activating a serine-threonine protein kinase, referred to as protein kinase C (18). PMA stimulates directly this kinase in the

presence of calcium and phospholipid. Niedel *et al.* (3) have shown that protein kinase C copurifies with the receptor of phorbol diester in rat brain. Nishizuka and coworkers also have presented a large body of data suggesting that this kinase mediates protein phosphorylation and induces the release of serotonin from platelets (18). Although the physiological role of protein kinase C in most cells is not known, this kinase may be regulated by phospholipid degradation and phosphatidylinositol turnover (19, 20). Since many growth factors (21) and hormones (22, 23) stimulate phosphatidylinositol turnover, it is possible that the signals of these hormones and growth factors that are initiated from their membrane receptors can be transmitted to the cytosol through a protein kinase C pathway.

The relation between PMA and growth factors such as EGF, insulin, and the insulin-like growth factors has received some study (5, 24, 25). In a monocyte-like cell U-937 and IM-9 lymphocytes (5), PMA inhibits insulin binding to its receptor, apparently by altering the affinity of the receptor for insulin. Grunberger and Gordon (5) also found an effect of PMA on insulin binding to human fibroblasts, but this effect was much smaller than that observed with the cultured white cells. Phorbol esters stimulate phosphorylation of insulin receptors in IM-9 lymphocytes (6). Likewise, PMA alters EGF binding to a variety of cell types and has been shown to alter EGF receptor phosphorylation (4, 7).

In the present study, we have characterized the effect of PMA on insulin receptors and insulin action in rat hepatoma cells. In contrast to the findings with fibroblasts and white cells, insulin binding to the receptor was not affected by PMA either acutely or chronically. However, PMA inhibited insulin activation of glycogen synthase and TyrATase. Roach and Goldman have reported that PMA stimulates the phosphorylation of glycogen synthase in hepatocytes; thus, glycogen synthase itself may be a substrate for protein kinase C (26). Thus, the finding of reduced glycogen synthase activity in treated cells could reflect a direct effect on the enzyme or an indirect effect via the insulin receptor. In either case, the finding that PMA inhibited insulin stimulation of TyrATase activity suggests a more generalized state of insulin resistance. This insulin-resistant state was produced within 30 min by PMA and occurred in a dose-dependent manner.

We also found that PMA stimulated the phosphorylation of insulin receptors in intact hepatoma cells at serine and threonine residues. This phosphorylation occurred at as many as nine sites in the β subunit, as detected by two-dimensional peptide mapping. Insulin also stimulates phosphorylation of the insulin receptor at multiple sites; however, this occurs at tyrosine and serine residues. Pretreatment of cells with PMA resulted in a decrease in the insulin-induced phosphorylation of receptor, particularly that which occurred at tyrosine residues. Identification of the similarities and differences by two dimensional peptide mapping of insulin receptor is difficult because of the complexity of the patterns and the unavailability of a simultaneous mixed sample. However, our results suggest that cells treated with both insulin and PMA exhibit a peptide map that contains several phosphorylated peptides (I_1, I_2, I_6, and T_1, T_2, T_6) that are similar to the combination of peptides obtained from cells treated separately with insulin and PMA. Thus, we conclude that insulin and PMA stimulate the phosphorylation of insulin receptor on different sites but may phosphorylate some amino acids that are present on the same peptides. These findings are similar to those of Cochet *et al.* (4) and Iwashita and Fox (7) who reported that PMA stimulated the phosphorylation of EGF receptor and reduced the tyrosine phosphorylation by EGF in A431 cells. In the former case, the same effect was observed when EGF receptor purified from A431 cells was incubated *in vitro* with EGF and protein kinase C. Whether purified protein kinase C can phosphorylate the insulin receptor remains to be determined.

In conclusion, our data indicate that PMA, probably acting through protein kinase C, can stimulate serine and threonine phosphorylation of the insulin receptor, altering insulin receptor autophosphorylation and insulin action. Since the initial description by Kasuga *et al.* (8) that the insulin receptor is a tyrosine-specific protein kinase, there has been considerable speculation as to the role of this activity in insulin action. Our results show that altering the tyrosine phosphorylation is associated with a decrease in insulin action. Thus, these data provide additional evidence that tyrosine phosphorylation of insulin receptor may be involved in the insulin action, and protein kinase C may modulate insulin action by altering receptor phosphorylation.

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