Reversible and Phorbol Ester-specific Defect of Protein Kinase C Translocation in Hepatocytes Isolated from Phenobarbital-treated Rats

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ABSTRACT

Phorbol ester-induced translocation of the calcium/phospholipid-dependent protein kinase, protein kinase C (PKC), from soluble to particulate cell fractions was inhibited in primary cultures of hepatocytes isolated from rats chronically exposed to the liver tumor promoter phenobarbital (PB). Inhibition of translocation (34%) was significant after a 15-min treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA, 500 nM); an 85% inhibition was observed after 60 min. In contrast, the translocation responses to two non-phorbol ester activators of PKC, ATP (1 mM) and arginine-vasopressin (0.1 nM), were not significantly impaired. Assessment of total PKC specific activity revealed that translocation induced by TPA and the two nonphorbol activators was not associated with PKC degradation in hepatocytes from either control or PB-exposed rats. The defect in TPA-induced translocation was correlated with an impaired down-regulation of the hepatocyte surface receptor for epidermal growth factor in hepatocytes from PB-exposed rats. Chronic exposure to PB did not affect the total content or specific activity of PKC in whole liver, nor did it affect the distribution of PKC activity between soluble and particulate fractions in unstimulated liver or hepatocytes. However, both the diminished epidermal growth factor receptor response and the inhibition of TPA-induced PKC translocation were reversed by withdrawal of PB for 2 to 4 weeks. Hepatocytes isolated from female rats were found to contain a 3-fold greater PKC specific activity and content than hepatocytes from male rats. However, no sex-related differences were observed in PKC distribution or in the modulation of translocation by chronic PB exposure and withdrawal. Immunoblotting of partially purified liver extracts revealed that the defect in phorbol ester-induced translocation was not caused by altered expression of PKC isozymes. PKC isozymes II and III, but not I, were detected, and their amounts were unaffected by PB exposure, although higher levels were detected in female relative to male livers. These data demonstrate reversible inhibition of phorbol ester-induced PKC activation by the liver tumor promoter, PB, and suggest that PB alters a component of the PKC-signaling pathway other than the expression of PKC isozymes.

INTRODUCTION

In multistage models of experimental rat liver carcinogenesis, tumor promotion occurs in an environment which selectively stimulates the growth of initiated cells relative to that of normal cells (1). In recent years we have accumulated evidence that chronic long-term exposure of rats to the liver tumor promoter PB (2) confers a selective growth disadvantage on normal hepatocytes. We have previously reported that chronic PB exposure causes profound alterations in hepatocyte response to the mitogen EGF, reducing the mitotic response to EGF at physiological calcium concentrations and decreasing expression of the EGF receptor at the cell surface (2).

PKC is a family of calcium, phospholipid-dependent kinases which have been identified as the phorbol ester receptor (3–5). Phorbol esters activate PKC by binding to the regulatory site, thus substituting for endogenous 1,2-diacylglycerols which can be produced by several pathways in the cell, including receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (6). In its inactive state, PKC is partitioned between the cytosol and a membrane-associated compartment which is resistant to extraction with chelators; but in the presence of phorbol esters and calcium, most of the cytosolic enzyme is translocated to the membrane compartment (7). This translocation is believed to be a critical step in PKC activation (8), allowing access to membrane protein substrates including the receptors for EGF, insulin, insulin-like growth factor I, interleukin 2, transferrin, somatomedin C, and β-adrenergic agonists (9). In the case of the EGF receptor, activation of PKC results in its phosphorylation which by an unknown mechanism leads to down-regulation of the receptor. Recently, we have reported that the ability of phorbol esters to down-regulate the hepatocyte EGF receptor is compromised in rats chronically exposed to PB (10). Because PKC modulates the mitotic response to EGF and because EGF-dependent mitogenesis is compromised in hepatocytes isolated from rats chronically exposed to PB (2), we interpreted this effect on EGF receptor down regulation to suggest that PKC activation could be altered by chronic PB exposure. In the present work, we have directly tested for this alteration by comparing the activation of PKC in hepatocytes isolated from livers of control and PB-exposed rats. The results of these studies demonstrate that chronic exposure to PB significantly inhibits phorbol ester-induced PKC translocation in rat hepatocytes.

MATERIALS AND METHODS

Materials. Sprague-Dawley and Fischer 344 rats were obtained from Charles River Laboratories (Wilmington, MA). Sodium phenobarbital was purchased from Mallinckrodt, Inc. (Paris, KY). Minimal essential medium (Earle’s salts) plus nonessential amino acids was purchased from GIBCO (Grand Island, NY). Collagenase D was from Boehringer Mannheim, Inc. (Indianapolis, IN). Receptor grade EGF was from Collaborative Research (Bedford, MA). Leupeptin was from United States Biochemical Corp. (Cleveland, OH). [γ-32P]ATP (2–10 Ci/mmol) was from New England Nuclear/DuPont (Boston, MA). Rainbow visible molecular weight markers and [125I]-labeled mouse EGF were from Amersham (Arlington Heights, IL). P-81 ion exchange chromatography paper was from Whatman, Ltd. (Maidstone, Kent, England). Tween 20 was from Bio-Rad Laboratories (Richmond, CA). Electrophoresis grade acrylamide and bisacrylamide were from Fisher Scientific (Fair Lawn, NJ). All other chemicals including benzamidine hydrochloride, 12-O-tetradecanoylphorbol-13-acetate, arginine-vasopressin, ATP, lysine-rich histone type III-S, soybean trypsin inhibitor type I-S, L-α-phosphatidylserine, bovine serum albumin fraction V, carrier-free glycine, Nonidet P-40, DEAE-cellulose, Sephadex G-25, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO).
Animals. Age-matched male and female Fischer 344 rats (80 to 100 g) were maintained on either normal water (control), water containing 0.1% (w/v) sodium phenobarbital for 8 to 16 weeks (PB-exposed), or 0.1% sodium phenobarbital for 8 weeks followed by normal drinking water for 2 to 4 weeks (PB-withdrawn). Food (Purina 5010 rat chow) and water (plus or minus sodium phenobarbital) were supplied ad libitum.

Hepatocyte Isolation and Culture. Primary cultures of rat hepatocytes were isolated as described previously (11) and plated at a density of 10^7 cells/cm^2 on collagen-coated 100-mm plastic dishes for PKC determination or collagen-coated 24-well plates for 125I-EGF binding. Culture medium consisted of minimum essential medium with nonessential amino acids supplemented with 1 mM pyruvate, 0.2 mM aspartate, 0.2 mM serine, 0.1 mM insulin, and 50 μg/mL gentamicin. Plated hepatocytes were incubated at 37°C in an atmosphere of 5% CO2 for 80 min to allow for attachment before application of treatment media.

125I-EGF Binding Studies. 125I-EGF binding to isolated hepatocytes was carried out as described previously (2, 12, 13). Briefly, plated hepatocytes were treated with varying concentrations of TPA (37°C, 1 h) and then were incubated in buffer containing 5 ng/ml 125I-EGF (100 μCi/μg) for 2 h at 4°C. Binding was terminated by aspiration of the binding medium and three washings with buffer (4°C). Cells were solubilized in 0.33 N NaOH and counted in a LKB/Wallace 1272 gamma counter. All values were corrected for nonspecific binding (<3% of total binding), determined in the presence of a 750-fold excess of unlabeled EGF. Maximal responses and ID_{50} for TPA inhibition of EGF binding were estimated from dose-response curves that were fitted to the EGF Scatchard model using the LIGAND program as modified by McPherson (14). Values determined by fitting data from each hepatocyte preparation were statistically analyzed using unpaired t tests.

Protein Kinase C Analysis. Culture medium was replaced with fresh medium containing the indicated additions and incubated at 37°C. The medium was then removed and the cells washed twice rapidly with Dulbecco’s phosphate-buffered saline (4°C), and once with Solution A (20 mM Tris-Cl, pH 7.0-2 mM EDTA-1 mM benzamidine hydrochloride-1 mM phenylmethylsulfonyl fluoride-0.1% (w/v) soybean trypsin inhibitor-0.01% (w/v) leupeptin-50 mM 2-mercaptoethanol) (15), supplemented with 0.25 M sucrose. All subsequent procedures were performed at 4°C.

Cells from each 100-mm plate were scraped into 1 ml of Solution A. Cells from 2 plates were pooled for each experimental condition. Scraped cells were then disrupted by sonication (three 5-s bursts) and centrifuged at 15,000 x g for 15 min. The supernatant was saved as the soluble fraction. The pellets were washed in 1 ml Solution A to remove contaminating supernatant before being extracted in 0.8 ml of Solution A supplemented with 10 mM EGTA and 1% (v/v) Nonidet P-40 for 60 min with frequent vortexing. The pellet extract was centrifuged (15,000 x g, 10 min) and the supernatant was saved as particulate fraction. Excess detergent was removed by application of 0.3 ml of the supernatant to Sephadex G-25 columns (17 x 1 cm) and elution in Solution A minus leupeptin. Fractions were assayed for protein using the Bio-Rad kit and bovine serum albumin as a standard. Fractions containing protein were pooled for the assay of PKC activity.

A modification of this procedure was used to isolate PKC in fractions of whole liver. Rats were sacrificed by cervical dislocation and the liver was quickly removed, rinsed in 0.9% NaCl solution (4°C), and homogenized in Solution A (40 ml/g wet weight) using a motorized Teflon/glass homogenizer. Incompletely disrupted tissue was removed by centrifugation at 1,000 x g for 3 min. The supernatant was saved and centrifuged at 100,000 x g for 60 min in a Beckman L7 ultracentrifuge. The high speed supernatant was saved as soluble fraction. The pellet was washed in Solution A and particulate PKC was extracted for 60 min in Solution A supplemented with 10 mM EGTA and 1% Nonidet P-40. Both soluble and particulate fractions were partially purified by DEAE-cellulose chromatography on Bio-Rad minicolumns (1.8-mL bed volume) (16). The columns were preequilibrated in Solution A prior to loading 1 mg of sample protein in a 2-mL volume. Columns were then washed with 20 ml of Solution A and eluted with stepwise additions of Solution A containing 120 mM NaCl (two 5-mL volumes) and 250 mM NaCl (one 5-mL volume). All three volumes were assayed for PKC activity.

Kinase activity was measured in the presence and absence of 0.5 mM CaCl2 and 25 μg/mL phosphatidylserine using histone as a substrate as described previously (12, 13). Statistical analysis was performed using Student’s unpaired t test.

Electrophoresis and Western Blotting. Tissue extracts were prepared for Western blotting by homogenization in Solution A (7 ml/g wet weight) using a Brinkmann homogenizer. The homogenates were centrifuged at 15,000 x g, and the supernatants were filtered through glass wool and brought to 150 ml with Solution A. Samples were loaded onto DEAE-cellulose columns (17 x 3 cm), washed with 1000 ml of Solution A, and eluted in a 400 ml gradient of 0 to 0.3 M NaCl in Solution A. Fractions containing PKC were pooled and concentrated in an Amicon ultrafiltration cell fitted with a PM10 membrane. The concentrate was dialyzed overnight against 62.5 mM Tris-Cl, pH 6.8, and concentrated in a Savant Speed-Vac. Protein content was determined using the Bio-Rad assay with bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (17) using 8% polyacrylamide gels. After samples were electrophoresed at 35 W for 3 h, the gels were equilibrated for 15 min in 25 mM Tris, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. Electrophoresed proteins were transferred to nitrocellulose paper (Schleicher and Schuell BA 85) using a Buchler Instruments Semi-dry blotter run for 2 h at 180 mA. Nitrocellulose papers were incubated at 4°C overnight in blocking buffer containing 100 mM Tris-Cl, pH 7.5, supplemented with 0.9% (w/v) NaCl, 0% (v/v) Tween 20, 3% (w/v) bovine serum albumin fraction V, and 10% (v/v) milk diluted blocking solution concentrate (Kirkegaard and Perry Labs, Gaithersburg, MD). Monoclonal antibodies to isozymes of PKC from rabbit brain were obtained as ascites fluid of BALB/c mice and were purchased from Seikagaku America, Inc. (Rockville, MD) (18). Stock solutions of antibodies [100 μg/ml in distilled water] were diluted 1:100 in antibody binding buffer [blocking buffer diluted 1:2 with blotting buffer (100 mM Tris-Cl, pH 7.5, containing 0.9% (w/v) NaCl and 0.1% (v/v) Tween-20]). Nonspecific binding was determined using duplicate nitrocellulose blots incubated with control ascites fluid (Sigma; 1:100) instead of primary antibodies. Incubations were 2.5 h at room temperature. The immunoblots were then washed three times in 200 ml of blotting buffer and stained using the Vectastain Elite ABC Kit for mouse IgG (Vector Laboratories, Burlingame, CA).

RESULTS

The effect of chronic PB exposure on PKC distribution in whole liver is shown in Fig. 1. A total specific activity (calculated from the sum of soluble and particulate activities) of 4192 ± 288 (SE) pmol/min/g tissue was measured in DEAE-purified...
extracts of liver from male control rats, with 32.9 ± 2.4% of total liver PKC recovered in the particulate fraction. Exposure of rats to PB for 14 weeks had no significant effect (P > 0.2) on either the specific activity of PKC in whole liver (3639 ± 322 pmol/min/g tissue) or the percentage of total enzyme activity recovered in the particulate fraction (32.4 ± 3.4%).

We have shown previously that both TPA and PB applied in vitro inhibit EGF binding in untreated hepatocytes, although only TPA induces PKC translocation (12, 13). In order to determine whether chronic PB exposure affected the responsiveness to phorbol esters and PB in vitro, we prepared primary cultures of hepatocytes from livers of male control (Fig. 2A) and PB-exposed (Fig. 2B) rats and treated them with TPA or PB. Total PKC specific activity was 83.5 ± 7.0 pmol/min/mg protein in hepatocytes isolated from control rats. Exposure in vitro to either TPA or PB for 60 min had no effect on the total specific activity recovered (89.0 ± 9.0 and 86.1 ± 7.9 pmol/min/mg protein, respectively). In hepatocytes isolated from PB-exposed rats, total specific activity was reduced (51.6 ± 2.6 pmol/min/mg), most likely because of the generalized anabolic effect of PB on proteins. However, in vitro treatment with TPA and PB again did not affect the total specific activity of PKC recovered after 60 min (51.3 ± 1.4 and 45.9 ± 2.2 pmol/min/mg protein, respectively). Therefore, neither TPA nor PB given in vitro caused any detectable PKC degradation. Particulate PKC activity in unstimulated hepatocytes was approximately 15% of total in both control and PB-exposed rats. Therefore, chronic exposure to PB also had no effect on the distribution of PKC in unstimulated hepatocytes. Addition of 5 mM PB in vitro also had no effect on enzyme translocation in either group. In contrast, addition of 500 nM TPA for 60 min caused a 381 ± 16% increase in particulate PKC activity (P < 0.001) in hepatocytes from control liver (Fig. 2A) but stimulated translocation by only 56 ± 5% (P < 0.05) in hepatocytes from PB-exposed rats (Fig. 2B). This decreased ability of TPA to cause PKC translocation in PB-exposed versus control hepatocytes was highly significant (P < 0.001).

The effect of in vivo PB-exposure on TPA-induced translocation of PKC to the cell membrane was also tested for reversibility and phorbol ester specificity (Fig. 3). Hepatocytes were prepared from three groups of rats: (a) control; (b) PB-exposed; and (c) PB-withdrawn. Control hepatocytes were first screened for their responsiveness to non-phorbol ester agonists known to activate phosphatidylinositol 4,5-bisphosphate hydrolysis in hepatocytes. In order to directly compare the effects of TPA with those of shorter-acting agonists, all treatment times were reduced from 60 min (Fig. 2) to 15 min. In these preliminary experiments we found the ability of the various agonists to induce PKC translocation (1 mM ATP > 0.1 μM VP > 0.1 μM angiotensin II > 10 μM epinephrine; data not shown) correlated with their reported abilities to generate production of 1,2-diacylglycerols in hepatocytes (19). In additional preliminary experiments it was found that the PKC specific activity of hepatocytes prepared from female rat livers was 3 times higher than in those from males (Table 1) although the distribution

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![Image](https://cancerres.aacrjournals.org)
between soluble and particulate fractions was not different (Fig. 3A versus Fig. 3B, control, ■). The following experiments were therefore carried out in both males and females to determine if there were also any sex-related differences in the translocation response.

In both male (Fig. 3A) and female (Fig. 3B) animals, approximately 15% of total PKC activity was recovered in the particulate fraction in all three groups. As in the previous experiments (Fig. 2) the total specific activity of PKC was unchanged by treatments (data not shown). Hepatocytes from male control rats responded to 500 nM TPA, 1 mM ATP, and 0.1 μM VP (15 min, 37°C) with increases of particulate PKC to 77.8, 41.4, and 33.9% of total, respectively (Fig. 3A). Response of hepatocytes from PB-exposed rats to 1 mM ATP and 0.1 μM VP was slightly diminished when compared to control hepatocytes (35.7 and 30.8% of total, respectively), but this difference was not significant (P > 0.03). However, the response to TPA (49.4% of total) was significantly reduced (P < 0.02) compared to that of hepatocytes from control rats although the magnitude of this reduction was not as great as was observed at 60 min (Fig. 2B). When hepatocytes were isolated from rats withdrawn from PB for 2 to 4 weeks, the PKC translocation response to TPA (70.9% of total) was not significantly different (P > 0.4) from that of control rat liver hepatocytes. Responses to ATP and VP in PB-withdrawn hepatocytes were also not different from those of control (P > 0.05). The results of these experiments with hepatocytes from female rats are shown in Fig. 3B. Again, exposure to PB yielded hepatocytes with a significantly reduced (P < 0.05) translocation response to TPA, but not to ATP or VP, when compared to that in control animals, and withdrawal of PB restored responsiveness to TPA (P > 0.2 compared to control).

We and others have previously reported that TPA causes a down-regulation of hepatic EGF receptors (10, 12, 13, 20). TPA-induced down regulation of EGF receptors in several systems has been shown to be mediated through PKC (21, 22). Therefore, we also examined the effect of chronic PB exposure on this TPA response in hepatocytes as a bioassay of PKC activation. Dose-response curves for hepatocytes from control, PB-exposed, and PB-withdrawn female rats are presented in Fig. 4. As we have shown previously (12), EGF receptor down-regulation was saturated at TPA concentrations > 500 nM in hepatocytes from control rats. Maximal inhibition of EGF binding and the ID50 were estimated as 71.8 ± 3.9% and 52.7 ± 9.9 nM TPA, respectively. Although saturation of EGF receptor down-regulation was not achieved in hepatocytes from PB-exposed rats, a minimal estimate of the ID50 derived from the curve that best fitted these data was 331 ± 54 nM (Fig. 4). This ID50 significantly differed from that of control hepatocytes (P < 0.01). In contrast, TPA-induced down regulation of the EGF receptor of hepatocytes from PB-withdrawn rats was saturable with maximal inhibition of 62.2 ± 5.8% and an ID50 of 50.9 ± 18.7 nM. Neither the maximal response or ID50 of PB-withdrawn hepatocytes differed significantly from those of control hepatocytes (P > 0.20 and P > 0.90, respectively). Parallel studies performed with hepatocytes isolated from male rats gave similar results (data not shown).

Since PKC exists as a family of discrete isoforms with slight differences in their requirements for activation, we examined PKC isozyme expression in partially purified liver extracts by immunoblotting with monoclonal antibodies specific for rabbit brain PKC I, II, and III (Fig. 5). Partially purified brain extracts from Sprague-Dawley rats were also examined as a positive control for all three isozymes. Although PKC I was clearly detectable in liver extracts from either control male, control female, or PB-exposed female rats (Fig. 5A). Other bands staining at M, 75,000 and 130,000 in all 4 extracts were also observed when immunoblotting was repeated with control as-

### Table 1 Specific activity of PKC in hepatocytes from male and female rats

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<tr>
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<th>Soluble</th>
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<tr>
<td>Males</td>
<td>23.0 ± 1.9</td>
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<tr>
<td>Females</td>
<td>75.8 ± 7.2</td>
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### DISCUSSION

The translocation of PKC from the cytosol to the plasma membrane has now been characterized in many cell types using both biochemical (7, 8) and immunocytochemical (23-25) methods. This redistribution allows the enzyme access to several membrane protein substrates, many of which are involved...
the same range reported by Kuo et al. (26) using similar techniques. In addition, our finding that liver PKC activity is due to the expression of the isoforms PKC II and PKC III (but not PKC I) also corroborates previous reports (5, 27). Kikkawa et al. (28) estimated that liver particulate PKC activity was 22% of total. The slightly higher value reported in this study (33% of total) may reflect slight differences in the particulate extraction procedure or result from partial purification of extracts on DEAE-cellulose. Similarly, our recovery of approximately 15% of total activity in the particulate fraction of plated hepatocytes agrees well with the value (15.7% of total) obtained by Houweling et al. (29) in suspended hepatocytes.

The functional significance of the 3- to 4-fold increase in PKC specific activity and content in female relative to male hepatocytes cannot yet be elucidated since female hepatocytes responded to PKC activators and modulation by PB in the same manner as male hepatocytes. However, this relative deficit in male liver PKC activity was apparently due to decreased expression of both the PKC II and PKC III isoforms (Fig. 5). It is therefore possible that liver PKC expression may be at least partly regulated by estrogens and/or androgens. In this regard it is interesting to note that estrogens are also effective liver tumor promoters (30). These findings raise another interesting point concerning the modulation of EGF receptors by PKC and its effect on EGF-dependent mitogenesis. Although hepatocytes isolated from male rats have 3 times more surface EGF receptors than hepatocytes from female rats (31), these extra receptors are subject to regulation by less than 30% of the PKC found in the female cells. A rigorous comparison of the effects of PKC activators on EGF binding and EGF-stimulated mitogenesis in male and female hepatocytes might provide insight into this important regulatory pathway.

In one hypothesis for the decreased EGF receptor expression and defective EGF-dependent mitogenesis in PB-exposed rats, PB causes a constitutive activation of PKC that maintains the kinase in its membrane-associated form and causes a tonic down-regulation of the EGF receptor. The present study provides two new pieces of evidence that mitigate against this idea: (a) under conditions which inhibit EGF binding PKC distribution is not altered in whole liver or hepatocytes isolated from the liver of PB-exposed rats, (b) we have previously reported that in vitro PB does not alter PKC distribution in control rat hepatocytes (12, 13) and we now report that in vitro PB also has no effect on PKC translocation in hepatocytes isolated from in vivo PB-exposed rats.

A possible mechanism of resistance to TPA-induced PKC translocation could involve altered gene expression in response to chronic PB exposure. PB induces expression of specific isoforms of cytochrome P-450 in the liver (32) and may additionally regulate the expression of PKC isoforms. Both PKC II and PKC III are expressed in liver, and the PKC II isoform is reported to be resistant to phorbol ester-induced translocation (27). Thus, if chronic PB exposure preferentially increased the expression of PKC II at the expense of PKC III, the ability of TPA to induce translocation would be impaired even though the total PKC activity was unaltered. However, immunoblot analysis of partially purified liver extracts revealed no changes in PKC isozyme expression following 6 months of chronic PB exposure. We have also found no differences in control and PB-exposed rat liver PKC expression using hydroxyapatite chromatography to separate PKC isoforms (data not shown). These findings mitigate against the hypothesis that the trans-

in the signal transduction of factors influencing mitogenesis and differentiation. One of the most thoroughly studied of these interactions is the phosphorylation of the EGF receptor by PKC (21, 22). We have shown previously that PB administered in vitro and in vivo causes decreased expression of hepatocyte EGF receptors and inhibits EGF-dependent mitogenesis (2, 12, 13). In the present study we report that PB exposure in vivo also causes an inhibition of TPA-induced PKC translocation which is accompanied by a decreased sensitivity of hepatocyte EGF receptors to down-regulation by TPA. Since exposure to TPA (as well as to ATP and VP) caused no detectable decrease in the total calcium and phospholipid-dependent kinase specific activity recovered, these changes occur in the absence of PKC degradation or “down-regulation.” The restoration of TPA-induced PKC translocation and EGF receptor sensitivity to TPA following PB withdrawal is consistent with the reversible nature of the promotinal phase of multistage carcinogenesis (1).

The present study corroborates several previous reports of PKC distribution, content, and isozyme expression in unstimulated liver and hepatocytes. Our estimates of the specific activity of PKC in DEAE-purified extracts of whole liver are in
location defect is due to altered PKC isozyme expression in response to PB. PKC translocation has often been described as part of the pleiotropic response to mitogens and tumor promoters (9, 33). In liver, translocation of PKC precedes the proliferative burst induced by partial hepatectomy (34, 35). In contrast, translocation in the opposite direction (from membrane to cytosol) has recently been correlated with the commitment of keratinocytes to terminal differentiation (36). Several examples of resistance to a variety of phorbol ester-induced effects are also known, in both cultured cells (37–42) and whole animals (43, 44). The mechanisms of resistance, however, appear to vary. Studies comparing phorbol ester-resistant versus sensitive systems have found: (a) no differences in TPA-induced PKC translocation (37, 39, 41, 42); (b) impaired translocation from the cytosol to the membrane (38, 43); and (c) translocation of PKC from the cytosol to non-plasma membrane region (25, 40) in resistant cells. Recently the nature of the PKC-membrane association has been examined in detail by Bazzi and Nelsen et al. (45). An initial calcium-dependent PKC-membrane complex is formed which dissociates upon calcium chelation. However, after long incubations in the presence of phorbol esters, PKC inserts into the phospholipid bilayer and can only be released with detergents. This model is further supported by recent freeze fracture studies of the distribution of [3H]TPA in membranes showing a strong association of TPA with integral membrane proteins and lipids (46). Unlike synthetic diacylglycerols and other more “physiological” activators of PKC, phorbol ester-induced translocation is prolonged, sustaining PKC-membrane association for periods of 1 h or more, possibly due to the resistance of phorbol esters to metabolism. Furthermore, diacylglycerols are unable to induce the reversible insertion of PKC into membranes caused by phorbol esters (45). The lack of any effect of chronic in vivo PB on ATP- or VP-stimulated PKC translocation suggests that the translocation defect is phorbol ester specific. This may indicate that PB exposure alters the integrity of the hepatocyte plasma membrane (47) in such a way that TPA-induced insertion of PKC cannot occur. In addition, the finding that inhibition was relatively slight at 15 min after TPA application and almost complete at 60 min is consistent with the requirement for prolonged incubation necessary for TPA-induced insertion of PKC into the plasma membrane.

In conclusion, these findings provide additional evidence for an overall inhibitory effect of chronic PB exposure on the growth of normal (uninitiated) hepatocytes. If initiated or putative preneoplastic cells are refractory to these growth-inhibitory effects, then chronic PB exposure may lead to the clonal expansion of preneoplastic liver cells and to the formation of liver tumors by differential inhibition of cell proliferation (1).

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PROTEIN KINASE C AND LIVER TUMOR PROMOTION


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