Differential translocation of protein kinase C isozymes by phorbol esters, EGF, and ANG II in rat liver WB cells

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Maloney, Judith A., Oxana Tsygankova, Agnieszka Szot, Lijun Yang, Quiyang Li, and John R. Williamson. Differential translocation of protein kinase C isozymes by phorbol esters, EGF, and ANG II in rat liver WB cells. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C974–C982, 1998.—The protein kinase C (PKC) family represents an important group of enzymes whose activation is associated with their translocation from the cytosol to different cellular membranes. In this study, the spatial distribution of PKC-α, -δ and -ϵ in rat liver epithelial (WB) cells has been examined by Western blot analysis after subcellular fractionation. Cytosolic, membrane, nuclear, and cytoskeletal fractions were obtained from cells stimulated with phorbol 12-myristate 13-acetate (PMA), angiotensin II (ANG II), or epidermal growth factor (EGF). PMA caused most of the PKC-α, -δ and -ϵ initially present in the cytosol to be transported to the membrane and nuclear fractions. In contrast, both ANG II and EGF induced only a minor translocation of PKC-α to the membrane fraction but caused a statistically significant membrane-directed movement of PKC-δ and -ϵ. Translocation of PKC-δ and -ϵ to the nucleus induced by ANG II and EGF was transient and quantitatively smaller than that induced by PMA. PKC-δ and -ϵ were present in the cytoskeleton of resting cells, but although PMA, ANG II, and EGF caused some changes in their content, these were variable, suggesting that the cytoskeleton fraction was heterogeneous. PKC depletion inhibited ANG II-induced mitogenesis and the sustained activation of Raf-1 and extracellular regulated protein kinase (ERK). However, although PKC depletion inhibited EGF-induced mitogenesis, the maximum EGF-induced activation of the ERK pathway was only slightly retarded. We hypothesize that PKC-δ and -ϵ are involved in mitogenesis via both ERK-dependent and ERK-independent mechanisms. These results support the notion that specific PKC isozymes exert spatially defined effects by virtue of their directed translocation to distinct intracellular sites.

mitogen-activated protein kinase; extracellular regulated protein kinase; Raf-1; mitogenesis; angiotensin II; epidermal growth factor

PROTEIN KINASE C (PKC) is composed of a family of serine/threonine kinases that modulate the function of a variety of signal transduction pathways leading to gene expression, cell proliferation, and differentiation. PKC isoforms can be classified into three subgroups. The conventional PKC isozymes, namely, α, βI, βII, and γ, are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), or phorbol esters [phorbol 12-myristate 13-acetate (PMA)]. The novel PKC isozymes, consisting of PKC-δ, -ϵ, -η, -θ, and -μ, are activated by PS and DAG or PMA but are insensitive to Ca^{2+}. The atypical isoforms, ζ and ι/λ, are not affected by Ca^{2+}, DAG, or PMA but are dependent on PS for activation (for review, see Ref. 33).

The evolution of numerous isoforms of PKC and their differential expression in various tissues implies that they may possess specific, possibly unique functions. Furthermore, various agonists induce the translocation of different PKC isoforms to distinct subcellular locations. For example, by overexpressing the different isoforms of PKC into NIH/3T3 cells, which contain insignificant levels of isoforms other than PKC-α, Goodnight et al. (14) were able to show that each isoform translocates to a unique subcellular location after stimulation with phorbol esters. These results indicate that the different isoforms may have distinct roles in signal transduction pathways. This suggestion is supported by evidence from studies in which selected isoforms were either overexpressed or underexpressed using antisense technology. In these studies, PKC-δ was demonstrated to be involved in differentiation and decreased cell proliferation (27, 30, 40), whereas PKC-ϵ and -α have been implicated in increased cell proliferation (24, 30). However, although different isoforms appear to be involved in different aspects of cell growth and differentiation, their specific roles in signal transduction pathways have not been elucidated.

The translocation of PKC from the cytosol to membranes has been used as an indication of its activation. Early studies concerning PKC translocation were performed with crude membrane fractions that consisted of both plasma membrane and nuclear components. However, with the consideration of its role in proliferation and differentiation, an involvement of PKC, either directly or indirectly, in nuclear events is indicated (9). In addition, activation of PKC induces cytoskeletal reorganization (6, 32), and several PKC binding proteins have been shown to associate with the actin cytoskeleton (1, 25). PKC has also been shown to be involved in the activation of extracellular regulated protein kinase (ERK), a major signaling pathway leading to cellular proliferation in many cell types. Therefore, it was of interest to investigate the role of PKC in the activation of ERK and one of its upstream activators, Raf-1, by angiotensin II (ANG II) and epidermal growth factor (EGF) in a nontransformed rat liver epithelial WB cell line.

In this study, we have shown that although PMA induced the translocation of PKC-α, -δ, and -ϵ to the membrane and nuclear fractions in WB cells, there was a significant translocation of only the δ- and ϵ-isoforms to these sites after ANG II or EGF stimulation. Furthermore, phorbol ester-sensitive PKC isoforms are shown to play a role when the ERK pathway is stimulated by

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ANG II, but they have a limited effect on EGF-induced ERK activation. Therefore, it appears that PKC-δ and/or PKC-ϵ affect ANG II-induced mitogenesis via an ERK-dependent pathway, whereas the effect of PKC on EGF-induced mitogenesis is essentially ERK independent.

MATERIALS AND METHODS

Materials. EGF was purchased from UBI (Lake Placid, NY). ANG II, PMA, and myelin basic protein (MBP) were obtained from Sigma Chemical (St. Louis, MO). Rabbit polyclonal antibodies to PKC-δ, PKC-ϵ, and Raf-1 were from Santa Cruz Biotechnology (Santa Cruz, CA), whereas monoclonal antibodies to PKC-α were obtained from Transduction Laboratories (Lexington, KY). A plasmid encoding an inactive glutathione-*S*-transferase-coupled ERK kinase (GST-MEK-1) was provided by Michael J. Weber (University of Virginia, Charlottesville, VA). [γ-^{32}P]ATP was purchased from Amersham Life Sciences (Arlington Heights, IL).

Cell culture. WB cells are an epithelial cell that was originally isolated from the liver of an adult Fischer rat (38). The cells were plated onto 100-mm tissue culture plates and incubated in Richter's improved essential medium containing L-glutamine and insulin (Irvine Scientific, Santa Ana, CA) plus 10% fetal bovine serum until confluent. Cells were incubated overnight in Richter's medium without serum before the start of the experiment. Cells were used between *passages 20* and *40*.

Subcellular fractionation. Serum-starved WB cells were treated with agonist for 0–60 min as indicated. Cells were washed twice with ice-cold PBS and scraped into homogenization buffer containing 25 mM Tris·HCl, pH 7.4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol, 10% glycerol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells were allowed to swell for 10 min and then homogenized with 30 strokes of a Dounce homogenizer using a tight-fitting pestle. This produced complete lysis of the cells as determined by phase-contrast microscopy. Nuclei were pelleted by centrifugation at 500 *g* for 5 min, and the low-speed supernatant was centrifuged at 100,000 *g* for 30 min. The high-speed supernatant constituted the cytosolic fraction. The pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 30–60 min. The Triton-soluble component (membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at 100,000 *g* for 15 min. The cytoskeletal fraction was washed three times with homogenization buffer, resuspended in the same buffer, and dispersed by sonication.

Nuclei (low-speed pellet) were resuspended in nuclear buffer containing 25 mM Tris·HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM PMSF, 10 mM β-mercaptoethanol, and 0.05% Triton X-100 and homogenized with 10 strokes of a Dounce homogenizer to remove contaminating membrane components. They were centrifuged for 5 min at 500 *g*, resuspended in nuclear buffer without Triton X-100, layered over 45% sucrose, and centrifuged at 1,900 *g* for 30 min. The purified nuclei, which were visually free of cytoplasmic/cytoskeletal attachments as assessed by phase-contrast microscopy, were resuspended in homogenization buffer containing 1% Triton X-100 and incubated for 30–60 min. The small amount of insoluble material was removed by centrifugation at 100,000 *g* for 15 min at 4°C. Protein concentration was measured by the method of Bradford (4) using BSA as a standard.

The purity of the subcellular fractions was assessed biochemically by measuring lactate dehydrogenase (LDH) as a cytosolic marker and oubain-sensitive Na^+-K^+-ATPase as a measure of plasma membrane contamination (13, 34). LDH activity was 4.9 ± 0.3 U/mg protein in the cytosolic fraction, as compared with 0.2 ± 0.03 and 0.5 ± 0.08 U/mg protein in the nuclear and membrane fractions, respectively. The specific activity of Na^+-K^+-ATPase was 18 $nmol \cdot mg^{-1} \cdot min^{-1}$ in the membrane fraction, whereas it was 0.7 $nmol \cdot mg^{-1} \cdot min^{-1}$ in the nuclear fraction.

Western blot. Ten to thirty micrograms of protein were applied to a 10% SDS-polyacrylamide gel and electrophoresed. The amount of protein applied was routinely within the linear range for densitometric studies. The proteins were transferred to nitrocellulose membranes. Equal protein loading and the efficiency of protein transfer were assessed by staining the nitrocellulose membranes with Ponseau S. Membranes were blocked with 5% BSA in phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 1 h and were then incubated with isoform-specific anti-PKC antibodies for 1 h at room temperature, or overnight at 4°C. Nitrocellulose membranes were washed three times with PBST and then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Protein bands were visualized by enhanced chemiluminescence (ECL; Amersham). The results were analyzed by densitometry, which was kept in the linear range of exposures, using a Hewlett Packard scanner and SigmaGel software. In some experiments, the nitrocellulose membrane was stripped by incubation for 1–2 h in Immunopure Elution Buffer (Pierce, Rockford, IL), washed twice with PBST, and then reblotted with a different antibody as described above.

Raf-1 assay. After stimulation with ANG II or EGF, WB cells were lysed for 30 min on ice with lysis buffer [10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin]. Precleared cell lysates were incubated with antibody against Raf-1 for 1 h on ice followed by incubation with protein A-agarose with rotation for 1 h at 4°C. An irrelevant rabbit antibody was used as a negative control. The agarose beads were washed three times with the lysis buffer and twice with kinase buffer (10 mM PIPES, pH 7.0, and 10 mM $MgCl_2$). The reactions were carried out by addition of 5 μCi [γ-^{32}P]ATP and 10 μg/ml GST-MEK to the kinase buffer at 30°C for 10 min, and stopped by heating at 95°C for 5 min after the addition of Laemmli sample buffer. The kinase assay samples were subjected to 10% SDS-PAGE followed by gel drying and exposure to X-ray film at −86°C. The results were analyzed by densitometry of the autoradiograms, which was kept in the linear range of exposures, using a Hewlett Packard scanner and SigmaGel software.

ERK assay. Stimulation of WB cells with ANG II or EGF and immunoprecipitation of ERK were carried out as described above using anti-ERK-2 antiserum. Immunoprecipitates were washed once with lysis buffer, twice with modified RIPA buffer (10 mM MOPS, pH 7.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, and 1 mM Na_3VO_4) and twice with kinase buffer. MBP (5 μg/ml) was used as substrate for the ERK assay. The reaction was initiated by addition of 5 μCi [γ-^{32}P]ATP and carried out at 30°C for 15 min. The kinase assay samples were subjected to 15% SDS-PAGE and analyzed as described for the Raf-1 assay.

DNA synthesis. DNA synthesis was determined by [^{3}H]thymidine incorporation into DNA. WB cells were serum starved for 48 h and incubated with ANG II, EGF, or PMA for 24 h. [^{3}H]thymidine (2.5 μCi/ml) was added 16 h before the end of the incubation. The cells were quickly washed three times with ice-cold phosphate-buffered saline, incubated for 10 min

with 2 ml of 10% (wt/vol) trichloracetic acid (TCA), and washed twice with 2 ml of 10% TCA and three times with 2 ml of 95% ethanol. The acid-insoluble precipitate was incubated for 60 min in 800 µl of 0.2 N NaOH, and the solution was neutralized with HCl. The radioactivity was determined by liquid scintillation counting.

Statistical analysis. Data are expressed as means ± SE. Comparison of the effect of various hormonal treatments was performed by Student's *t*-test. Differences with a *P* value of <0.05 were considered statistically significant.

RESULTS

Distribution of PKC-α in WB cells. In unstimulated cells, PKC-α appeared as a single band at 80 kDa and was present mainly in the cytosolic fraction (Fig. 1*A*). Similar findings were observed in insulinoma β-cells (21) and vascular smooth muscle cells (15). With longer times of ECL development, however, PKC-α could be detected as a faint band in the membrane fraction of control cells, suggesting that it is present in very low abundance in the membrane. Stimulation with 1 µM PMA induced a rapid translocation of PKC-α from the cytosol to the membrane and nuclear fractions (Fig. 1). Translocation of PKC-α was evident at 30 s, maximal at 15 min, and subsequently declined over the next 45 min. This decline in membrane- and nuclear-bound PKC-α from 15 to 60 min is probably because of proteolytic degradation. Very little PKC-α was detected

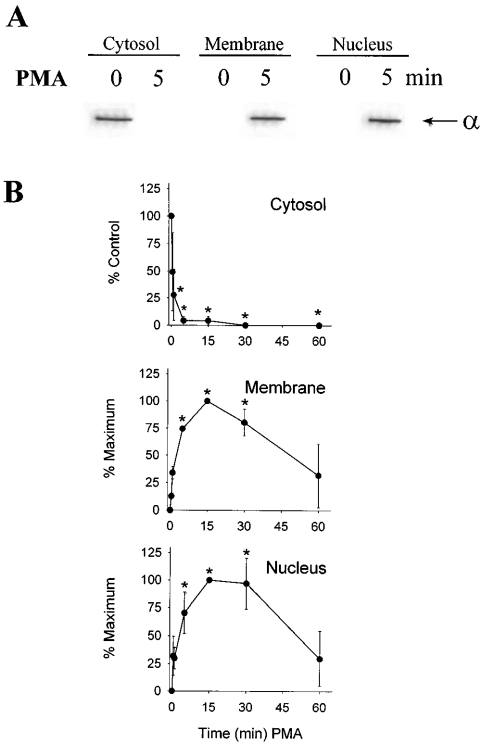

Fig. 1. Phorbol 12-myristate 13-acetate (PMA)-induced translocation of protein kinase C (PKC)-α to membrane and nuclear fraction of WB cells. Serum-starved WB cells were stimulated with 1 µM PMA for 0–60 min. *A*: subcellular fractions were subjected to SDS-PAGE and immunoblotted with anti-PKC-α antibodies. *B*: Western blot analysis of subcellular fractions immunoblotted with anti-PKC-α antibody. Western blots were quantitated by densitometric scanning. Results are means ± SE from 3 independent experiments. $*P < 0.05$.

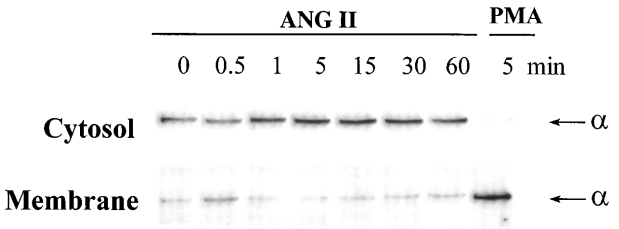

Fig. 2. Angiotensin II (ANG II)-induced translocation of PKC-α to membrane fraction of WB cells. Serum-starved WB cells were stimulated with 1 µM ANG II for 0–60 min. Cytosolic and membrane fractions were subjected to SDS-PAGE and Western blotting with anti-PKC-α antibodies. Similar results were obtained in 2 separate experiments.

in the cytoskeleton fraction in control cells, and this was not increased by treatment with PMA (data not shown). After stimulation with ANG II, cytosolic levels of PKC-α were 76 ± 4% of control after 30 s but returned to control levels by 1 min (Fig. 2). This corresponded to a transient fourfold increase of PKC-α in the membrane fraction compared with a >10-fold increase 5 min after PMA treatment. Similar results were obtained with EGF (data not shown). Neither ANG II nor EGF induced the translocation of PKC-α to the nuclear or cytoskeleton fractions.

Distribution of PKC-δ in WB cells. In unstimulated cells, PKC-δ was present in the cytosolic, membrane, and cytoskeletal fractions but was not detectable in the nuclear fraction (Fig. 3*A*). These findings are in agreement with other studies in which PKC-δ has been shown to be constitutively associated with the membrane component of unstimulated cells (21, 37). PMA induced the translocation of PKC-δ from the cytosol to the membrane, nuclear, and cytoskeleton fractions, but with different kinetics. By 5 min, PKC-δ was undetectable in the cytosolic fraction and within 1 min appeared in the membrane, nuclear, and cytoskeleton fractions. Thereafter, there was an additional PMA-induced translocation of PKC-δ to the membrane and nuclear fractions, whereas that in the cytoskeleton became depleted (Fig. 3*B*). From 30 to 60 min after PMA addition, there was a loss of PKC-δ from the membrane and nuclear fractions, as observed for PKC-α (Fig. 1).

In the cytosol, membrane, and cytoskeletal fractions, PKC-δ appeared as a doublet of ~76 and 78 kDa. In the nucleus, however, PKC-δ appeared as a single band with an apparent molecular mass of 78 kDa. In the membrane and cytoskeletal fractions, PMA induced a time-dependent mobility shift of PKC-δ. When WB cells were treated with PMA, immunoprecipitated with anti-phosphotyrosine antibodies, and immunoblotted with anti-PKC-δ antibodies, there was an increased tyrosine phosphorylation of PKC-δ (data not shown). This finding is consistent with previous observations demonstrating tyrosine phosphorylation of PKC-δ after PMA stimulation (26, 37).

Both ANG II and EGF caused a rapid, statistically significant ($P < 0.05$) translocation of PKC-δ from the cytosol to the membrane fraction, where it remained elevated for the 1-h duration of the experiment (Fig. 4*B*). With ANG II stimulation, there was also a rapid,

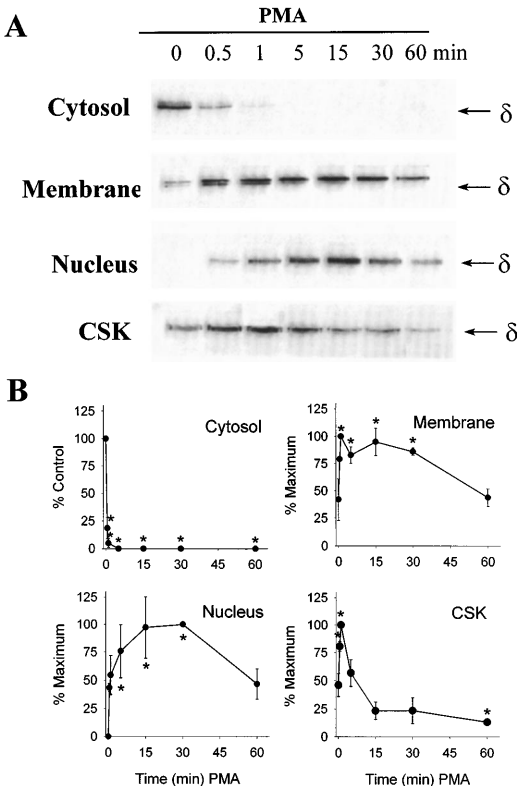

Fig. 3. PMA-induced translocation of PKC-δ to membrane, nucleus, and cytoskeletal (CSK) fractions of WB cells. Serum-starved WB cells were stimulated with 1 μM PMA for 0–60 min. Subcellular fractions were subjected to SDS-PAGE and immunoblotted with anti-PKC-δ antibodies. *A*: Western blot analysis of subcellular fractions immunoblotted with anti-PKC-δ antibodies. *B*: Western blots were quantitated by densitometric scanning. Results are means ± SE of 3 or 4 independent experiments. $*P < 0.05$.

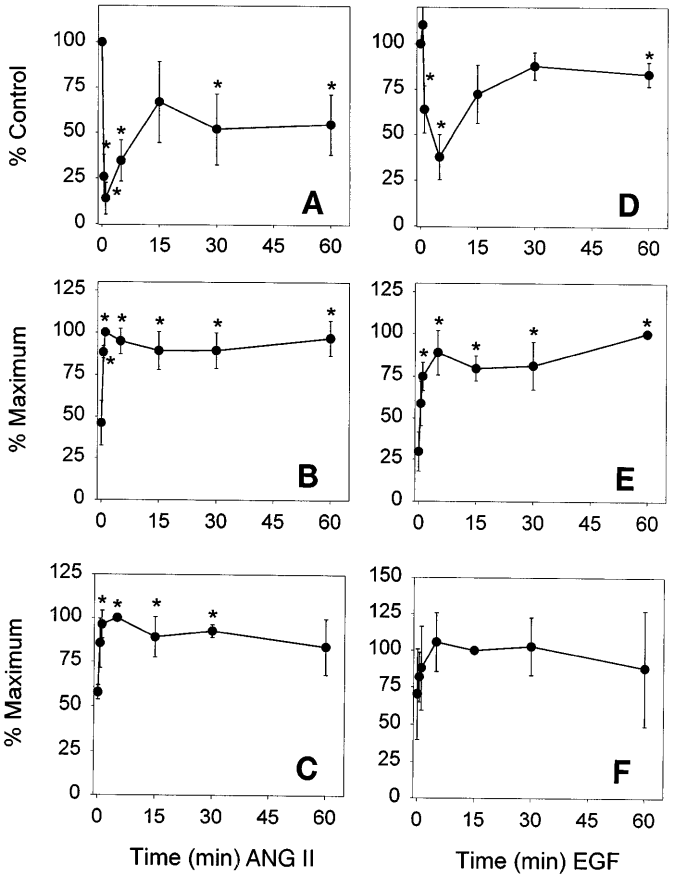

Fig. 4. ANG II- and epidermal growth factor (EGF)-induced translocation of PKC-δ to membrane and cytoskeletal fractions of WB cells. Serum-starved WB cells were stimulated with either 1 μM ANG II (*A–C*) or 200 ng/ml EGF (*D–F*) for 0–60 min. Subcellular fractions were subjected to SDS-PAGE and immunoblotted with anti-PKC-δ antibodies. Western blots were quantitated by densitometric scanning and expressed either as percent of control (cytosol, *A* and *D*) or percent of maximal response [membrane (*B* and *E*) and cytoskeleton (*C* and *F*) fractions]. Results are means ± SE of 3–5 independent experiments. $*P < 0.05$.

sustained translocation of PKC-δ to the cytoskeleton fraction, with a peak at 1 min (Fig. 4*C*), but with EGF stimulation the changes of PKC-δ in the cytoskeleton fraction were not statistically significant (Fig. 4*F*). As seen from the PKC-δ immunoblot in Fig. 5*A*, translocation of PKC-δ to the nuclear fraction occurred more quickly under the influence of ANG II than with EGF, but the amount translocated was much less than that induced by PMA. This kinetic difference was confirmed using an immunofluorescence approach with primary antibodies to PKC-δ and secondary antibodies labeled with Texas red. Again, translocation induced by EGF was slower than that with ANG II (data not shown).

Distribution of PKC-ε in WB cells. As previously observed in mouse neuroblastoma × rat glioma (NG 108–15) cells (3) and insulinoma β-cells (21), PKC-ε ran as a doublet in immunoblots of the cytosol and cytoskeletal fractions (Fig. 6). However, in the nuclear and membrane fractions, PKC-ε appeared as a single band. Additionally, there was another 130-kDa band recognized by the PKC-ε antibody that did not change after PMA or hormonal stimulation (data not shown). This band has been previously detected in insulinoma β-cells (21), but its identity remains unknown.

As with PKC-α and -δ, PMA caused a complete loss of PKC-ε from the cytosol within 1 min (Fig. 6*B*, *top left*). Translocation of PKC-ε to both the membrane and nuclear fractions was observed after 30 s, increased to a peak at 15 min, and remained elevated for up to 60 min (Fig. 6*B*, *top right* and *bottom left*). PKC-ε was present in the cytoskeleton of resting cells, fell by 90% after 1

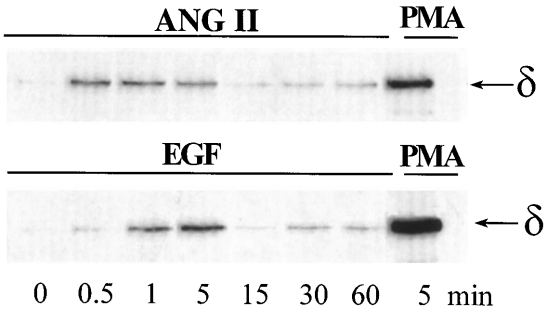

Fig. 5. ANG II- and EGF-induced translocation of PKC-δ to nuclear fraction of WB cells. Serum-starved WB cells were stimulated with either 1 μM ANG II or 200 ng/ml EGF for 0–60 min. Nuclear fractions were subjected to SDS-PAGE and immunoblotted with anti-PKC-δ antibodies. Similar results were obtained in 3 separate experiments.

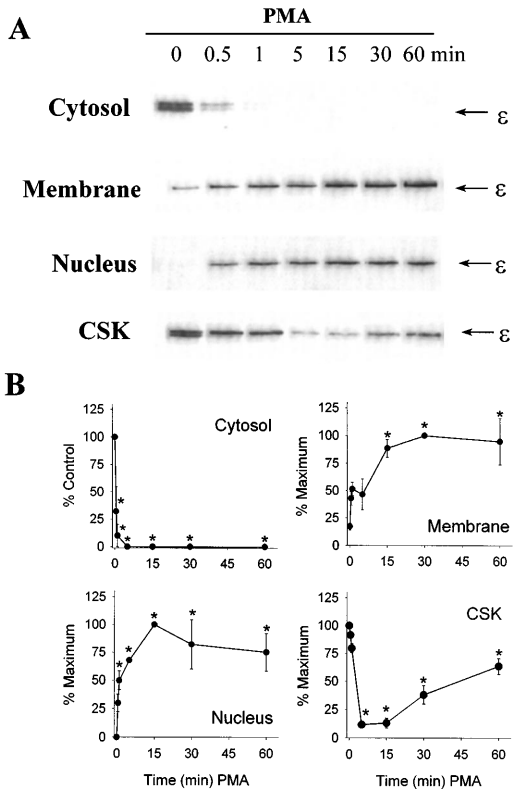

Fig. 6. PMA-induced translocation of PKC-ϵ to membrane, nucleus, and cytoskeletal fractions of WB cells. Serum-starved WB cells were stimulated with 1 μM PMA for 0–60 min. Subcellular fractions were subjected to SDS-PAGE and immunoblotted with anti-PKC-ϵ antibodies. *A*: Western blot analysis of subcellular fractions immunoblotted with anti-PKC-ϵ antibodies. *B*: Western blots were quantitated by densitometric scanning. Results are means ± SE of 3 or 4 independent experiments. $*P < 0.05$.

min, and subsequently increased gradually to 60% of control values after 1 h (Fig. 6*B*, *bottom right*). Interestingly, the most marked difference between the translocations of PKC-δ and PKC-ϵ, as affected by PMA, was to the cytoskeleton fraction where the initial increase observed with PKC-δ was not apparent with PKC-ϵ.

After stimulation of the cells with ANG II, PKC-ϵ decreased rapidly in the cytosol, and like PKC-δ subsequently returned partially to basal levels (cf. Figs. 4*A* and 7*A*), whereas the decrease of PKC-ϵ observed with EGF was slower and completely reversible (cf. Figs. 4*D* and 7*D*). Removal of PKC-ϵ from the cytosol induced by both EGF and ANG II was associated with translocation of PKC-ϵ to the membrane fraction, where it increased twofold (Fig. 7, *B* and *E*). In contrast, the hormone-induced translocation of PKC-ϵ to the cytoskeletal fraction was small and did not reach statistical significance. As observed for PKC-δ, ANG II and EGF both caused a small transient increase of PKC-ϵ in the nucleus, with the EGF-induced translocation being slightly more delayed than that induced by ANG II (data not shown). The major effect of hormonal stimulation in WB cells, therefore, was to elicit a rapid, substantial translocation of both PKC-δ and PKC-ϵ from the cytosol to the membrane fraction.

Effect of PKC downregulation on ANG II- and EGF-induced stimulation of DNA synthesis and activation of Raf-1 and ERK2 in WB cells. As shown in Figs. 1, 3, and 6, PMA acutely administered induced a rapid translocation of PKC-α, -δ, and -ϵ to the nucleus, indicating that PKC may exert a direct regulation of nuclear events. Alternatively, PKC may be translocated and activated at the plasma membrane with subsequent phosphorylation of substrates that convey signals to the nucleus through the mitogen-activated protein (MAP) kinase cascade (5). This latter possibility may be the primary one by which signals are transmitted from activated receptors, since the effects of EGF and ANG II on translocation of PKC isoforms directly to the nucleus were small and transient (Fig. 5).

To investigate the effects of PKC activation on cell function in WB cells, conventional and novel isoforms of PKC (which include PKC-α, -δ and -ϵ) were downregulated by prolonged treatment of the cells with PMA. Figure 8 shows the results of an experiment that illustrates this phenomenon. The disappearance of

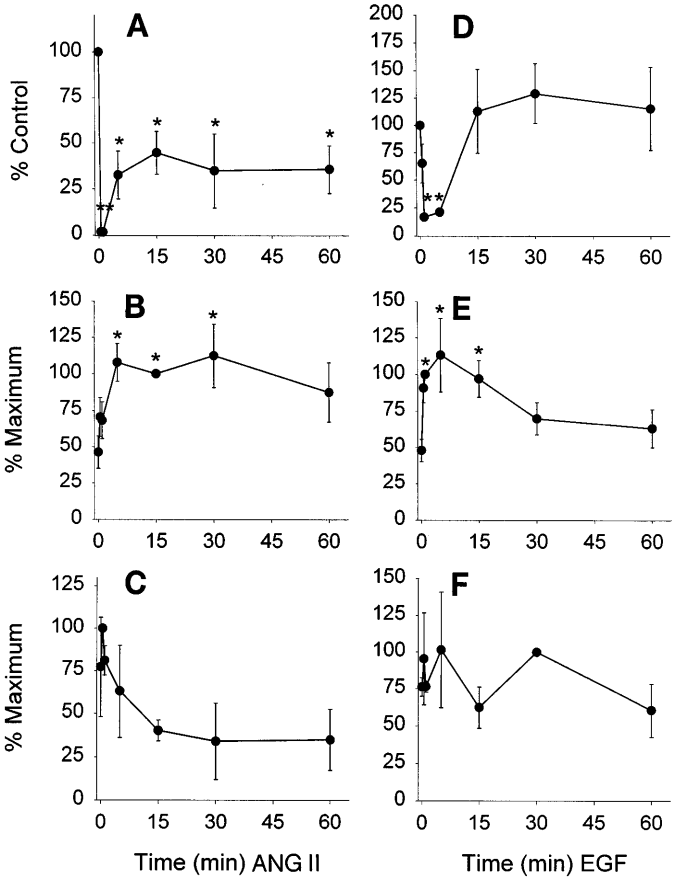

Fig. 7. ANG II- and EGF-induced translocation of PKC-ϵ to membrane and cytoskeletal fractions of WB cells. Serum-starved WB cells were stimulated with either 1 μM ANG II (*A–C*) or 200 ng/ml EGF (*D–F*) for 0–60 min. Subcellular fractions were subjected to SDS-PAGE and immunoblotted with anti-PKC-ϵ antibodies. Western blots were quantitated by densitometric scanning, and results are expressed either as percent of control (cytosol, *A* and *D*) or percent of maximal response [membrane (*B* and *E*) and cytoskeleton (*C* and *F*) fractions]. Results are means ± SE of 4 or 5 independent experiments. $*P < 0.05$.

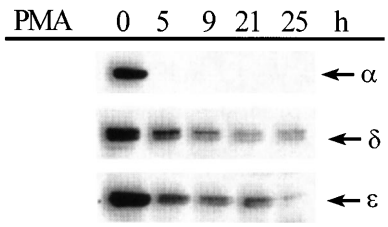

Fig. 8. Downregulation of PKC-α, -δ, and -ϵ by prolonged PMA treatment of WB cells. Serum-starved WB cells were treated with 1 μM PMA for 0–25 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotted with anti-PKC-α, -δ, and -ϵ antibodies. Film was overexposed in regard to control lane to adequately visualize PKC isoforms at later time points.

PKC-α, -δ and -ϵ isoforms, as determined by immunoblotting, was followed over a 25-h period. Immunoreactive PKC-α was no longer observed at the first assayed time point after 5 h, whereas PKC-δ and -ϵ exhibited slower kinetics of downregulation.

In further experiments, these PKC isoforms were downregulated by pretreatment of the WB cells with PMA for 24 h to assess the potential role of hormone-stimulated PKC on cell proliferation and activation of the MAP kinase pathway. The cells were subsequently stimulated with either 1 μM ANG II or 200 ng/ml EGF for various times, and the activities of Raf-1 and ERK were measured. In untreated cells, both hormones stimulated a three- to sixfold increase in the activities of Raf-1 and ERK, and these kinases remained activated for the 90 min of the experiment (Figs. 9 and 10). In PMA-pretreated cells, there was little effect of PKC depletion on the ANG II-induced activations of both Raf-1 and ERK within the first 5 min, but after 60 and 90 min, both kinases were significantly inhibited (Fig. 9). These data suggest that the ANG II-induced stimulation of the ERK cascade is at least partially PKC dependent, possibly at the level of Raf-1. However, after 15 min of ANG II stimulation, ERK activity was significantly decreased in PKC-depleted cells, although there was no effect on Raf-1 activity. Additional data will be needed to verify this point, which suggests that ANG II may also activate ERK in a Raf-1-independent manner. Similarly, ERK activity was significantly decreased by EGF in PKC-depleted cells after 5 min, whereas Raf-1 activity was not significantly affected. The major point of interest is that there was no effect of PKC depletion on the EGF-induced sustained activation of Raf-1 or ERK (Fig. 10). These results indicate that the EGF-induced activation of the ERK pathway is minimally affected by those PKC isoforms that can be downregulated by PMA. PKC-ζ was shown by immunoblotting experiments to be present in both the soluble and particulate fractions prepared from WB cells, but their relative amounts were not affected by acute or prolonged addition of PMA (data not shown).

Stimulation of the ERK pathway in many cells is known to be correlated with increased mitogenesis. Consequently, it was of interest to determine the effects of PKC downregulation on ANG II- and EGF-stimulated proliferation in WB cells. Figure 11 shows that addition of 10 nM PMA, 1 μM ANG II, and 100 ng/ml EGF to WB cells produced increasing percentage effects on [^{3}H]thymidine incorporation into DNA. Pretreatment of the cells for 24 h with 500 nM PMA completely inhibited the effects of ANG II and PMA and greatly diminished the effect of EGF on DNA synthesis. These results indicate that activation of PKC-α, -δ, or -ϵ is an important component of the mitogenic pathway in WB cells.

DISCUSSION

The role of different PKC isoforms in signal transduction pathways remains unclear despite extensive studies. It has been suggested, however, that each isoform may perform distinct functions via its translocation to discrete regions within the cell. The present study was initiated to investigate this possibility by examining the translocation of PKC-α, -δ, and -ϵ to different subcellular fractions after stimulation of WB cells with PMA, EGF, or ANG II. PMA induced the translocation of all three isoforms from the cytosol to the membrane and nuclear fractions. PKC-δ and -ϵ were present in the cytoskeleton fraction of resting cells, but the major effect of PMA was to cause a depletion of these PKC isoforms in the cytoskeleton. PKC-δ and -ϵ were also the major isoforms translocated from the cytosol to the

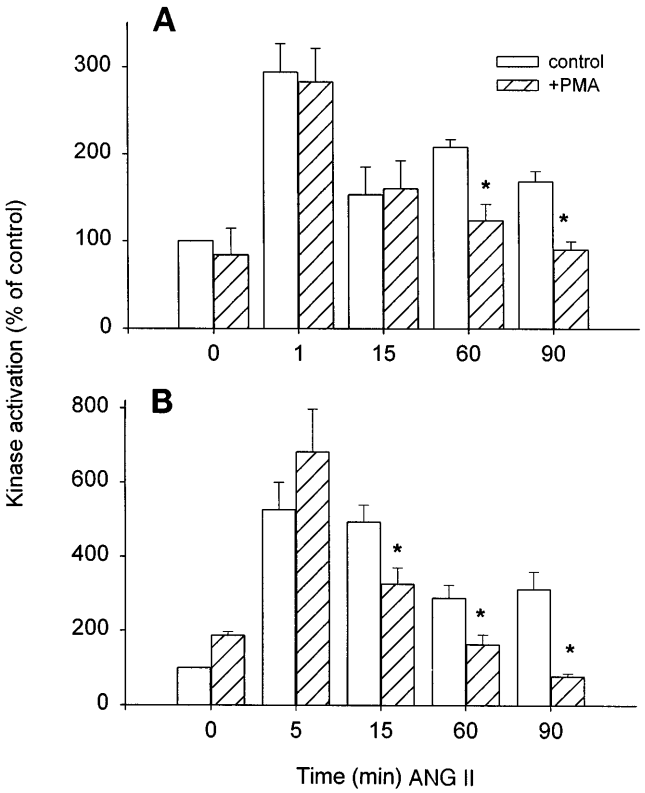

Fig. 9. ANG II-stimulated activation of Raf-1 (*A*) and extracellular regulated protein kinase (ERK) (*B*) in control and PKC-depleted cells. WB cells were pretreated with DMSO alone (open bars) or with 1 μM PMA (hatched bars) for 18 h, and then stimulated with 1 μM ANG II for various time intervals up to 90 min. Immune complex kinase assays for Raf-1 and ERK activities were performed as described in MATERIALS AND METHODS. Protein kinase activity in cells treated with DMSO alone was taken as 100%. Results are means ± SE of 4 independent experiments. $*P < 0.05$.

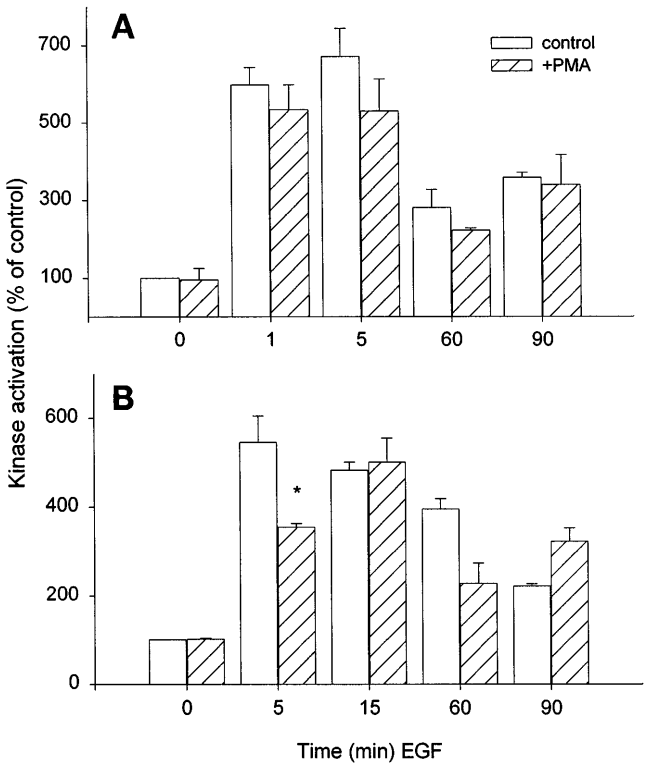

Fig. 10. EGF-stimulated activation of Raf-1 (*A*) and ERK (*B*) in control and PKC-depleted cells. WB cells were pretreated with DMSO alone (open bars) or with 1 μM PMA (hatched bars) for 18 h and then stimulated with 200 ng/ml EGF for various time intervals. Immune complex kinase assays for Raf-1 and ERK activities were performed as described in MATERIALS AND METHODS. Protein kinase activity in cells treated with DMSO alone was taken as 100%. Results are means ± SE of 3 independent experiments. $*P < 0.05$.

membrane fraction after ANG II and EGF stimulation. However, unlike the changes induced by PMA, ANG II and EGF caused very little translocation of PKC-δ and -ϵ to the nucleus. On the other hand, hormonal stimulation of WB cells was essentially ineffective in causing a translocation of PKC-α to membrane or nuclear fractions, suggesting that it may play a relatively minor role in the signal transduction pathway induced by these two agonists in WB cells. This finding contrasts with the fact that PKC-α is ubiquitously expressed in diverse cells types. However, its translocation induced by various hormones and growth factors is variable (15, 17), although PKC-α translocation to the nucleus has been described (15). Interestingly, arachidonic acid has been shown to induce the translocation of PKC-α to the particulate fraction in WB cells (16). This indicates that PKC-α may respond to signaling through the phospholipase A_2 pathway in WB cells.

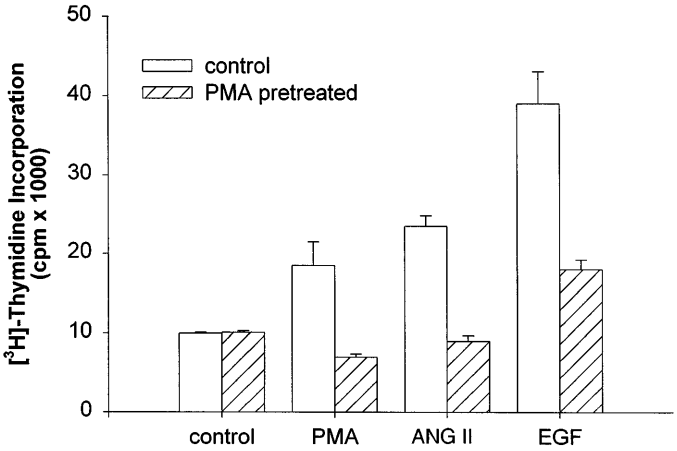

Fig. 11. Effects of PKC depletion on EGF- and ANG II-induced [^{3}H]thymidine incorporation into DNA in WB cells. Serum-starved WB cells were pretreated for 24 h with 500 nM PMA before stimulation with 10 nM PMA, 1 μM ANG II, or 100 ng/ml EGF and [^{3}H]thymidine (2.5 μCi/ml) for an additional 16 h. Control experiments (open bars) were pretreated with DMSO alone, whereas PMA pretreatment is shown by hatched bars. Results are means ± SE of 6 independent experiments.

A number of reports have described the translocation of PKC-α, -δ, and -ϵ to the Triton X-100-insoluble cytoskeletal fraction (19, 20, 35). In the present study, some differences were observed in the PMA- or hormone-stimulated movement of PKC-δ and -ϵ to this fraction. Phorbol ester and ANG II induced a rapid translocation of PKC-δ to the cytoskeleton, which was transient with PMA but sustained with ANG II. On the other hand, there was no apparent effect of EGF in causing a translocation of PKC-δ to the cytoskeleton fraction. Interestingly, there was a decrease in the amount of PKC-ϵ bound to the cytoskeleton after both PMA and ANG II treatment, but again EGF failed to give a statistically significant movement of PKC-ϵ to the cytoskeleton. Taken together, these results suggest a differential activation and/or subcellular targeting of PKC-δ and -ϵ after stimulation with PMA, ANG II, and EGF.

In recent years, several PKC binding proteins have been identified that associate with the membrane and the Triton X-100-insoluble cytoskeletal fraction. These include receptors for activated C kinase (RACKS) (31), myristoylated alanine-rich C-kinase substrate (MARCKS) (1), and the adducins (10). Although there has been no definitive evidence that a particular PKC isoform preferentially associates with a specific binding protein in vivo, a peptide based on the RACK binding site of PKC-β was shown to inhibit the translocation of PKC-α and -β after phorbol ester addition to cardiac myocytes or glucose stimulation of pancreatic β-cells (36, 42). Further work showed that a peptide based on the RACK binding site for PKC-ϵ behaved similarly (18, 42). These studies lend credence to the idea that the differential movement of PKC isoforms to the cytoskeleton and other subcellular structures may be at least partially because of a differential targeting to distinct binding proteins within the cell.

In this study, the phorbol ester-sensitive PKC isoforms were shown to play a major role in the EGF- and ANG II-induced [^{3}H]thymidine incorporation in WB cells. Hence, the selective translocation of PKC-δ and -ϵ by these agents supports the view that they may be involved in the mitogenic pathway initiated by both G protein-coupled and tyrosine kinase receptors. To explore this possibility in greater detail, we investigated the involvement of PKC in ERK activation.

The relative importance of PKC in activation of the ERK pathway has been shown to depend on the agonist and the cell type (11, 17, 43). Moreover, there has been conflicting data with regard to the role of PKC in ANG II and EGF stimulation of the ERK pathway (2, 12, 23, 28, 29). Here, we demonstrate that PKC plays a role in the ANG II-induced activation of ERK and Raf-1, especially in the later phase of their activation. This is an interesting finding in light of the fact that a sustained activation of ERK is believed to be necessary for mitogenesis (29). Consistent with this hypothesis, PKC depletion inhibited [^{3}H]thymidine incorporation into DNA induced by ANG II. Although the mechanism by which Raf-1 is activated remains unresolved, it is believed to occur via the translocation of Raf-1 to the plasma membrane, where it is subsequently phosphorylated by kinases such as PKC (8). Recently, PKC-α and -ε have been shown to phosphorylate and activate Raf-1 (7, 22). Because we observed a sustained translocation of PKC-ε to the membrane fraction after ANG II stimulation, this isoform is a possible candidate for Raf-1 activation in WB cells. Similarly, in cardiac myocytes and rat aortic smooth muscle cells, it was suggested that PKC-ε is involved in endothelin- and ANG II-induced activation of ERK, respectively (17, 28). However, PKC-δ has also been implicated in activation of the ERK pathway (39, 43). A prolonged activation of PKC-δ and/or -ε may, therefore, be involved in the mitogenic action of ANG II via promoting a sustained activation of ERK in at least a partially Raf-1-dependent manner.

In contrast to the effects of ANG II, EGF-induced activation of ERK was minimally affected in PKC-depleted cells. It should be noted, however, that EGF produced a sustained activation of ERK, in accordance with the well-established mitogenic effect of growth factors. Although PKC depletion had no effect on this sustained phase of ERK activation, it did decrease the EGF-induced [^{3}H]thymidine incorporation into DNA. This implicates an additional PKC-dependent pathway that is required for DNA synthesis after EGF stimulation and is consistent with the notion that ERK activation is required but alone is insufficient for mitogenesis (41). Alternativelly, PKC isoforms that are insensitive to PMA-induced downregulation may be involved in ERK activation in WB cells. Therefore, although the phorbol ester-sensitive PKC isoforms may have a minimal role in the ERK pathway induced by EGF, they clearly play a major role in mitogenesis in these cells.

In conclusion, we observed the translocation of PKC-δ and -ε mostly to the membrane fraction after ANG II and EGF stimulation. This indicates that both isoforms are important for signaling events initiated at the plasma membrane by each hormone. PKC depletion appears to inhibit ANG II-induced mitogenesis via an ERK-dependent pathway, whereas it inhibits EGF-induced mitogenesis in an ERK-independent pathway. The mechanism by which PKC-δ or -ε is involved in hormone stimulation of the ERK pathway and cellular proliferation remains to be determined. Interestingly, translocation of PKC-δ but not PKC-ε to the cytoskeleton was enhanced by ANG II, whereas EGF caused no significant translocation of either PKC isoform to the cytoskeleton. These data suggest PKC isozyme and hormone-directed specificity of functions in liver epithelial WB cells.

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