Protein Kinase C Isozyme Expression and Down-Modulation in Growing, Quiescent, and Transformed Renal Proximal Tubule Epithelial Cells

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Abstract

Renal α-protein kinase C (PKC) is rapidly down-modulated in animals treated with the renal toxin and tumor promoter, folic acid (Dong et al., Cancer Res., 53: 4542–4549, 1993). To further explore the role of PKC isozymes in renal growth and carcinogenesis, we compared phorbol ester receptor and PKC isozyme content, distribution, and regulation in primary and oncogene-altered rat renal proximal tubule epithelial cells (RPTE) in culture. Immunoblot analysis and RNase protection assays indicated that RPTE expressed at least four PKC isozymes, α, δ, ε, and ζ. Total phorbol ester receptors were decreased in primary proliferating, ElA-immortalized, and SV40-transformed RPTE compared to primary quiescent RPTE. The decrease in PDBu binding was largely due to a specific decrease in α-PKC protein content to ~50% of the level in quiescent RPTE. Degradation rates and message levels were compared to determine the mechanism for the decrease in α-PKC. Whereas α-PKC message levels in quiescent and proliferating primary RPTE were comparable, α-PKC degradation was increased in proliferating cells. These results indicate that the decreased α-PKC content was due largely to increased turnover. Phorbol ester stimulated the rate of degradation, thus demonstrating a link between degradation rate and PKC activation. These results suggest that the increased basal degradation rate in proliferating and oncogene-altered cells reflects an increase in activity of PKC in these cells.

Introduction

PKC plays a pivotal role in transducing signals for cell growth, differentiation, and transformation (1, 2). PKC is a gene family including at least ten isozymes, α, βI, βII, γ, δ, ε, ζ, η/λ, θ, and λ. Differences among PKC isozymes in tissue distribution and substrate specificity suggest that PKCs have unique functional roles. Furthermore, overexpression of individual isozymes in fibroblasts results in distinct changes in growth regulation and signaling pathways (3–5). However, the role of individual isozymes in cell transformation has not been studied in detail.

Phorbol esters are potent mouse skin tumor promoters (6) and specific activators of PKC. Although these results indicate that PKC is involved in tumorigenesis, a role for PKCs in non-phorbol ester-mediated carcinogenesis in skin and other tissues has been a more difficult question to address. We have used the renal-specific tumor promoter, folic acid, to study the role of PKCs in chemical damage and compensatory hyperplasia (7). Folic acid treatment rapidly down-modulated renal phorbol ester receptors in general and α-PKC in particular. Since down-modulation has been associated with increased activation and proteolysis of PKCs (8, 9), we proposed that the folic acid-induced down-modulation of renal α-PKC in vivo was a consequence of increased PKC activation in the regenerating, hyperplastic renal tissue.

In vitro studies with phorbol esters have established that transient activation of PKC signaling pathways is associated with rapid redistribution of PKCs from soluble to particulate fractions. In contrast, prolonged activation causes down-modulation of PKCs, a process attributed to increased proteolytic degradation of active versus inactive conformations (10, 11). Thus, it is likely that, in some cases, decreased PKC content is a reflection of chronic PKC activation. The initial purpose of our present study was to use cultured RPTE to determine if the PKC down-modulation observed in vivo in regenerating tissue also occurred in proliferating cells in culture and to investigate the mechanism of down-modulation.

Since folic acid is a renal tumor promoter, an additional goal of these studies was to determine if activation and down-modulation of PKCs could play a role in non-phorbol ester tumor promotion. Several lines of evidence suggest that changes in expression and/or activation of PKCs may occur in oncogene-transformed cells. Certain oncogenes (ras and src) may directly regulate PKC activity by increasing levels of DAG (12–17), the endogenous mediator of PKC activity. The increased DAG levels correlated with decreased PKC levels (12, 15, 18), indicating that these DAG pools may cause chronic activation and down-modulation of PKCs. Thus, PKCs cooperate with oncogenes to produce a more fully transformed cell. The roles of individual PKCs in this process have not yet been defined.

To further define the role of PKCs in renal growth and carcinogenesis, we compared PKC isozyme content, activation, and down-modulation in primary cultures of normal RPTE with oncogene-altered RPTE-expressing adenoviral E1A (E1A-RPTE) or SV40 large T (SV-RPTE). RPTE were used because renal cell carcinomas, the predominant form of kidney tumor, are derived from this cell type. E1A-RPTE are immortalized but do not express properties associated with
transformation, such as growth in soft agar.

Results

Stimulation of Primary RPTE Growth by Phorbol Ester. To determine if PKC activation is directly involved in RPTE mitogenesis, we studied the effects of PDBu on RPTE growth. As in other cells (20–23), PDBu alone did not stimulate $[^3H]$thymidine incorporation in primary RPTE (Fig. 1). However, in combination with insulin, PDBu was a potent mitogen. bFGF is also a potent RPTE mitogen in combination with insulin (24). In the presence of insulin, PDBu was as potent a mitogen as bFGF. The combination of PDBu and bFGF (in the presence of insulin) did not further stimulate $[^3H]$thymidine incorporation. These results indicate that activation of PKC stimulates the RPTE mitogenic program.

Phorbol Ester Receptors in Primary and Oncogene-altered RPTE. As a first step in investigating the role of PKC in RPTE growth and oncogenic transformation, we compared total content and subcellular distribution of PKC in normal and oncogene-altered RPTE. PDBu binding activity rather than kinase activity was used to estimate PKC content because quantitation of PKC catalytic activity is complicated by the presence of PKC inhibitors in crude samples (25) and by the differences in substrate specificity among the isozymes (26). Since $\zeta$-PKC does not bind phorbol esters (27–29), it is not quantitated by this assay. Total PDBu receptors were reduced in primary growing, E1A-immortalized, and SV40-transformed RPTE compared to primary confluent RPTE (Table 1). The decrease was due mainly to a decrease in soluble receptors, whereas particulate-associated PDBu receptors remained relatively constant. Cell density did not influence PDBu receptors in E1A- and SV40-RPTE, which, unlike the primary cultures, are not growth arrested at high cell density (data not shown). Since down-modulation of phorbol ester receptors/PKC appears to be associated with rapid cell proliferation characteristic of either growing normal cells or oncogene-altered populations, we further characterized the isozyme distribution and activation in the various cell lines.

Comparison of PKC Isozyme Content by Immunoblot Analysis. To determine if the measured decrease in phorbol ester receptors was due to a general or specific decrease in PKC isozymes, we compared the PKC isozyme content in cultured primary RPTE and their oncogene-altered derivatives. Four isozymes, $\alpha$, $\delta$, $\epsilon$, and $\zeta$, were detected in primary cultured RPTE as well as their E1A- and SV40-transformants (Fig. 2A). $\beta$, $\gamma$, and $\eta$-PKCs were not detected in any of these cells (data not shown). $\beta$- and $\lambda$-PKCs were not tested due to the lack of the appropriate reagents. The pattern of isozyme expression in the primary cells was similar to the pattern observed from rat kidney cortex (7), which is composed mainly of proximal tubules. These data indicate that expression of PKC isozymes was not modified by adapting proximal tubule cells to culture.

PKC isozyme content was quantitated by densitometric scanning (Fig. 2B). $\alpha$-, $\delta$-, and $\epsilon$-PKCs were reduced in primary growing RPTE to about 60% of the levels in primary confluent RPTE (Fig. 2). In contrast, $\zeta$-PKC, which is not a phorbol ester receptor (27–29), was increased. These data suggest that down-modulation of both calcium-dependent and -independent PKCs contributes to the decreased PDBu binding in growing RPTE.

In both E1A-immortalized and SV40-transformed RPTE, $\alpha$-PKC was decreased relative to primary confluent RPTE. $\delta$-PKC was not changed significantly in either of the trans-
formants. α-PKC was increased in E1A-RPTE but not in SV-RPTE. Since ζ-PKC is not a phorbol ester receptor (27, 28), the decrease in ζ-PKC in E1A- and SV-RPTE does not contribute to the measured decrease in phorbol ester receptors in these cells (Table 1). Thus, the decrease in α-PKC in particular appears to account for most of the PDBu receptor down-modulation in E1A- and SV-RPTE.

Comparison of PKC Isozyme Messages by RNase Protection Assay. To address the mechanism(s) responsible for the decreased α-PKC, α-PKC message levels and turnover rates were compared. The effects of growth and/or oncogenic transformation on expression of PKCs was determined by measuring PKC message levels in primary RPTE and their oncogene transformants by RNase protection assay with isozyme-specific riboprobes (Fig. 3). α, δ, ε, and ζ-PKC messages were detected in all of the RPTE. Primary growing and confluent RPTE expressed similar amounts of α-PKC message. Thus, the decrease in α-PKC protein in the growing RPTE (Fig. 1) cannot be accounted for by differences in mRNA levels. However, in E1A- and SV-RPTE, α-PKC mRNA was reduced to about 30% of the amount in primary cultures. These data suggest that decreased α-PKC protein content in E1A- and SV-RPTE may be due, at least partially, to reduced α-PKC message.

No consistent effect of E1A and SV40 transfection on the various messages was found. δ, ε, and ζ-PKC messages were increased in E1A-RPTE but not in growing primary and SV40-transformed RPTE (Fig. 2). Thus, these increases are specific to E1A transfection and are not directly related to growth.

Fig. 2. Immunoblot analysis of PKC isozymes. A, cell lysate proteins (0.1 mg) were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with PKC isozyme-specific antibodies. Molecular weight standards are indicated on the left in kilodaltons. B, immunoblots were quantitated by densitometric scanning. The data were normalized to the amount of each isozyme in confluent culture (100). Bars, SD from five independent experiments. * P < 0.05 as compared to confluent cultures by two-tailed, unpaired Student's t test.

Fig. 3. RNase protection assay of PKC isozymes. A, aliquots (15 μg) of total RNA were hybridized with pairs of PKC isozyme-specific riboprobes, α plus ε or δ plus ζ. The approximate size of protected fragments from predicted sequences and from [32P]-labeled multienzyme digested SK(+)/vector or SK(-) sequence reactions are indicated on the left. B, the autoradiographs were quantitated by densitometric scanning. Two sets of doublet bands were quantitated for ε-PKC since the same pattern was obtained when this probe was hybridized to in vitro-synthesized sense RNA from the same region. The data were normalized to the amount of each isozyme in confluent cultures (100). Bars, SD from three independent experiments. * P < 0.05 as compared to confluent cultures by two-tailed, unpaired Student's t test.
\(\zeta\)-PKC mRNA was overexpressed in E1A-RPTE but was underexpressed in SV-RPTE when detected with \(\zeta\)172 probe (Fig. 2). However, \(\zeta\)-PKC protein levels in E1A- and SV-RPTE were similar (Fig. 2). To rule out the possibility that the lower amount of \(\zeta\)-PKC message detected in SV-RPTE was due to low affinity hybridization, we prepared two more \(\zeta\)-PKC specific probes: \(\zeta\)159, which includes the coding region for the ATP binding domain; and \(\zeta\)295, which includes the COOH-terminal coding region against which antisera was generated. The same results were observed with these two probes as with the \(\zeta\)172 probe (not shown). It has been reported that incomplete \(\zeta\)-PKC clones were isolated with high frequency (27, 30). Thus, the lower level of \(\zeta\)-PKC message detected in SV-RPTE may reflect message instability. These results indicate a complicated relationship between measured levels of \(\zeta\)-PKC message and protein.

**Degradation Rate of \(\alpha\)-PKC.** Down-modulation has been associated with increased degradation of catalytically active PKC conformations (10, 11, 31). To determine if the decreased \(\alpha\)-PKC content in rapidly growing RPTE (including primary growing, E1A-, and SV-RPTE) was associated with increased degradation, we compared \(\alpha\)-PKC degradation rate (half-life) by immunoprecipitating \(\alpha\)-PKC in pulse-chase experiments. A representative experiment with SV-RPTE is shown in Fig. 4, and a summary of the data is shown in Table 2. In the absence of PDBu, the \(\alpha\)-PKC degradation rate in growing cells was almost twice the degradation rate in confluent cells. Since message levels were similar (Fig. 3), it is likely that the increased degradation is a major factor in decreasing the cellular contents of \(\alpha\)-PKC in growing RPTE. \(\alpha\)-PKC degradation rates in SV-RPTE (Table 2) and E1A-RPTE (data not shown) were also faster than quiescent RPTE. Since \(\alpha\)-PKC message levels are reduced in these cells, it appears that both transcription and proteolysis contribute to decreased \(\alpha\)-PKC content in transformed RPTE.

PDBu increased the degradation rate in all the cells, consistent with other reports that increased PKC activity is associated with increased susceptibility to proteolysis (10, 31). In vitro studies have indicated that this is due to the fact that the membrane-associated, phospholipid-bound form is a better substrate for calpain (11). It appears that increased basal turnover in proliferating and oncogene-altered RPTE also reflects increased basal \(\alpha\)-PKC activity.

**Effect of Cell Growth on PKC Isozyme Subcellular Distribution.** Transient activation of PKCs with phorbol esters correlates with their redistribution from the soluble to the particulate fractions. In contrast, prolonged activation correlates with PKC down-modulation. To determine if cell growth (in the absence of phorbol esters) influenced RPTE isoform subcellular distribution, soluble and particulate fractions were prepared from proliferating and quiescent RPTE and analyzed by immunoblotting (Fig. 5). The amounts of soluble \(\alpha\), \(\delta\), and \(\epsilon\)-PKCs were reduced in the proliferating cells, whereas the amounts of particulate \(\alpha\), \(\delta\), and \(\epsilon\)-PKCs were similar or slightly increased. Consequently, there is an increase in the ratio of particulate to soluble PKC. These data are in accordance with the PDBu binding data shown in Table 1. The slight increase in \(\zeta\)-PKC content in proliferating cells (Fig. 2) was associated with an increase in the particulate fraction with no apparent change in the soluble fraction. The significance of these changes is unknown at the present time since endogenous activators of \(\zeta\)-PKC have not yet been defined.

In all of the cells, \(\alpha\) and \(\epsilon\)-PKCs in particulate fractions and \(\delta\) and \(\zeta\)-PKCs in both soluble and particulate fractions were resolved into doublets. Doublets reflect the presence of multiple phosphorylated forms of PKCs and have been reported previously (5, 32). No consistent effect of either growth or oncogenic transformation on doublet patterns was noted.

**Effects of Cell Growth and Transformation on PKC Redistribution and Down-Modulation.** At this point, the data indicated that down-modulation of \(\alpha\)-PKC is due to re-
Concentration and degradation which accompanies persistent activation. To better understand the relationship between PKC activation, subcellular distribution, and increased turnover, the effect of PDBu on PDBu receptors and PKC isozymes was studied. Brief PDBu treatment caused redistribution of PDBu receptors in all RPTEs to a similar extent (Fig. 6). Prolonged treatment caused down-modulation of PDBu receptors in primary growing, E1A-, and SV-RPTE but not in primary confluent RPTE (Fig. 6). Down-modulation was apparent by 90 min (data not shown) and was complete by 16 h. Down-modulation was due to a preferential loss of receptors from the soluble fraction. Particulate receptor concentrations were relatively constant. Decreases were noted only in experiments in which down-modulation was most pronounced. These results indicate that, after severe depletion of the soluble pools, the particulate pools are also depleted.

Immunoblot analysis demonstrated that each of the PKCs (except α) redistributed with brief (0.2 h) PDBu treatment in each of the RPTE populations as noted by decreases in soluble fractions and increases in membrane fractions (Figs. 7–9). Increased phosphorylation of PKCs which can be observed as retarded gel mobilities has also been shown to correlate with PKC activation. PDBu did increase the amount of the upper bands of the α- and ε-PKC doublets that were resolved in our gel system. However, in the absence of PDBu, differences in doublet patterns between quiescent and proliferating or oncogene-altered cells were not reproducibly evident (Fig. 2).

Prolonged PDBu treatment caused down-modulation of α-, δ-, and ε-PKCs in growing, E1A-, and SV40-RPTE but not in quiescent primary cultures. In some cases in which partial down-modulation was apparent in the 1.5-h treatment groups, the major effect of PDBu was the loss of soluble PKCs rather than an increase in particulate PKCs. Rapid depletion of particulate PKCs due to proteolysis could potentially contribute to the absence of measurable increases in particulate PKCs.

The lack of down-modulation of PDBu receptors in quiescent RPTEs (Fig. 6) was confirmed by immunoblot analysis of individual isozymes (Figs. 7–9). In contrast to the growing RPTEs, prolonged PDBu treatment did not deplete α-, δ-, or ε-PKC contents in the confluent, quiescent RPTE. The general lack of down-modulation in confluent RPTE suggests that the block is a common event in the down-modulation pathways for all of these isozymes.

Discussion

Although PKCs have been implicated in tumor promotion and progression, the roles of individual isozymes have not been defined. In this study, we have compared PKC isozyme content among various cultured RPTEs in a proximal tubule tumor progression model to determine the involvement of individual PKCs in renal cell growth and transformation. Isozyme analysis demonstrated that four PKC isozymes, α, δ, ε, and ζ, are expressed in primary RPTE in agreement with data from whole kidney cortex (7). PDBu receptor content was down-modulated in the growing and transformed cells compared to confluent cells. This down-modulation correlated with decreased α-PKC content in rapidly growing cells (including primary growing, E1A-, and SV-RPTE). α-PKC message levels in primary growing and confluent cells were similar, indicating that down-modulation in growing cells was due primarily to a change in posttranslational regulation of α-PKC protein. Additional studies demonstrated that in fact, α-PKC degradation rate was twice as rapid in growing versus confluent cells. Thus, increased degradation of α-PKC accounts for α-PKC down-modulation in growing versus confluent RPTE. α-PKC degradation rates were also increased in E1A- and SV-RPTE, although in these cells, decreased message content may also contribute to the α-PKC down-modulation.

In previous work, we demonstrated that of the four PKC isozymes (α, δ, ε, and ζ) expressed in renal cortex, only α-PKC was down-modulated in kidneys in which proliferation had increased markedly after treatment with large doses of folic acid (7), a renal toxin and tumor promoter (33). Down-modulation preceded the folic acid-induced hyperplastic response. These results suggested that partial down-modulation of α-PKC was an early event in the RPTE response to the tumor promoter. We proposed that partial down-modulation was actually a reflection of increased α-PKC activation associated with the acute toxicity and regenerative response. Biochemical studies in the cultured...
RPTE strengthen this proposal since increased degradation rate and partial down-modulation of α-PKC were also observed in mitogenically active RPTE. Taken together, the evidence for increased α-PKC activation and the finding that PDBu stimulated growth of primary RPTEs suggest that sustained activation of α-PKC may be of importance for RPTE growth in vivo and in culture. Sustained activation of PKC has also been suggested to be essential for other PKC-mediated responses, including HL-60 cell differentiation (34) and T-cell activation (35). Since sustained (rather than transient) activation is required for PKC mitogenic effects, providing evidence that down-modulation occurs in grow-

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**Fig. 6.** Effects of cell growth and transformation on phorbol ester receptor redistribution and down-modulation. Cells were treated with 200 nM PDBu at 37°C for the indicated times. After the 0.2-h treatments, samples were prepared as described in "Materials and Methods." After the 16-h treatments, PDBu was removed by washing and incubating cells in a defined medium containing 0.5% bovine serum albumin at 37°C for 20 min before cell lysates were prepared. Soluble and particulate fractions were separated by centrifugation at 100,000 × g. Aliquots of proteins (0.1 mg) were used to measure phorbol ester receptors as described in "Materials and Methods." The data are means from three independent experiments for primary confluent and growing RPTE and four experiments for E1A- and SV-RPTE in which samples were assayed in triplicate; bars, SD. * P < 0.05 as compared to untreated cultures by two-tailed, unpaired Student's t test.

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**Fig. 7.** α-PKC isozyme redistribution and down-modulation. Cells were treated with 200 nM PDBu for the indicated times before scraping into lysis buffer. Soluble and particulate fractions were prepared by centrifugation at 100,000 × g. Aliquots of proteins (0.1 mg) were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with α-PKC-specific monoclonal antibody M6 as described in "Materials and Methods." The blots shown are representative of three independent experiments. For these experiments, lysates and soluble and particulate fractions were run on separate blots which were stained at different times. Therefore, comparisons among cell lines or fractions are not as accurate as for experiments shown in Figs. 2 and 5.
Fig. 8. \( \delta \)-PKC isozyme redistribution and down-modulation. Samples were prepared as described in the legend to Fig. 7. Blots were stained with \( \delta \)-PKC-specific antibody as described in "Materials and Methods."

Fig. 9. \( \varepsilon \)-PKC isozyme redistribution and down-modulation. Samples were prepared as described in the legend to Fig. 7. Blots were stained with \( \varepsilon \)-PKC specific antibody as described in "Materials and Methods."

...ing cells may be a useful approach toward demonstrating PKC isozyme involvement in vivo. In this regard, it is worthwhile to compare the PDBu effects on isozyme content in vitro with the physiological effects of growth in response to folic acid in vivo. Whereas phorbol esters caused down-modulation of \( \alpha \)-, \( \delta \)-, and \( \varepsilon \)-PKC, folic acid caused selective down-modulation of \( \alpha \)-PKC. These results emphasize that physiological agonists, such as those produced in the regenerating kidney, can selectively influence the activity of one isozyme.

The effects of E1A and SV40 transformation on \( \delta \)-, \( \varepsilon \)-, and \( \zeta \)-PKC message and protein levels were compared to determine if isozyme-specific changes could be associated with RPTE growth and/or transformation. The data suggest that the relationship between message and protein levels is complicated. For example, although \( \delta \)-, \( \varepsilon \)-, and \( \zeta \)-PKC messages were increased in E1A-RPTE, only the \( \varepsilon \)-PKC protein level was increased. In contrast, although \( \varepsilon \)- and \( \zeta \)-PKC message levels in SV-RPTE were very low, \( \varepsilon \)- and \( \zeta \)-PKC protein levels were similar to levels in the other RPTE. These comparisons suggest that straightforward measurements of steady state levels of PKC message and protein levels may not sufficiently address the complexity of the system. In contrast, measurements of dynamic processes such as isozyme degradation rates may more directly address PKC isozyme activation state since the only common change observed among the growing and oncogene-altered cells compared to quiescent RPTE was decreased content of \( \alpha \)-PKC. This decrease was at least partially attributable to increased turnover of \( \alpha \)-PKC in each case. These results further emphasize the potential role of \( \alpha \)-PKC in RPTE growth and transformation.

The resistance to down-modulation in quiescent RPTE was an interesting and relatively unique observation. This effect could be linked to the relatively minor (1.5-fold) PDBu-mediated increase in \( \alpha \)-PKC degradation rate. In contrast, PDBu caused a 3-4-fold increase in \( \alpha \)-PKC degradation rate in primary growing and SV-RPTE (Table 2). In one experiment with E1A-RPTE, PDBu increased the \( \alpha \)-PKC degradation rate 6.7-fold. Thus, the larger increases in degradation rates in growing and oncogene-altered RPTE correlated with PDBu-mediated down-modulation, whereas the smaller effect in the confluent cells correlated with the relative lack of down-modulation. These results further demonstrate that degradation rates regulate steady state \( \alpha \)-PKC levels.

The significance of the increased basal turnover of PKCs in the growing and oncogene-altered cells is not yet clear.
However, it seems that increased turnover is associated with increased activity since PDBu stimulates both PKC activity and turnover. Additional evidence, such as increased phosphorylation of target proteins, will be needed to resolve this question. In this regard, we have compared phosphorylation of one substrate cloned as a PKC-binding protein to a immunoprecipitation (formalin-fixed Staph A cells), fetal bovine serum, DMEM, Ham’s F-12 medium, and methionine-free DMEM were from Gibco-BRL (Grand Island, NY). [3H]PDBu (19.1 Ci/mmol) and [α-32P]UTP (3000 Ci/mmol) from were from Du-Pont-New England Nuclear (Boston, MA). [35S]Methionine (1200 Ci/mmol) was from INC (Irvine, CA). All restriction enzymes and alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG were from Promega (Madison, WI). The *in vitro* transfection kit was from Ambion (Austin, TX). A Bio-Image densitometer scanner was purchased from Millipore (Ann Arbor, MI).

**Cell Culture.** Primary RPTE cultures were prepared from rat kidney proximal tubules and were grown on collagen type I-coated plastic culture dishes as described (24,37). Primary RPTE were infected with adenovirus type 5, which contains only E1A 12S and is E1A 135 defective (38), provided by Dr. M. P. Quinlan (University of Tennessee, Memphis, TN) or were transfected with replication-defective SV40 viral genome in a pX-B vector (39) provided by Dr. K. Reznikoff (University of Wisconsin, Madison, WI). E1A-RPTE and SV-RPTE (19) were selected by growing them on uncoated plastic culture dishes, which does not support growth of normal primary cells. Immunofluorescence studies demonstrated that E1A-RPTE expressed the adenovirus E1A 125 gene product and SV-RPTE expressed SV40 large T-antigen (19). Both E1A- and SV-RPTE (19) expressed low levels of proximal tubule differentiation markers as well as keratin and vimentin. E1A- and SV-RPTE could be maintained in culture for over 50 passages (higher passages were not tested). Passages 15–50 were used for all experiments. Cells were cultured under optimal conditions for each cell type. Primary and SV-RPTE were cultured in a 1:1 DMEM and Ham’s F-12 medium supplemented with 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 10 ng/ml cholera toxin, 50 μM dithiothreitol, 30 μg/ml penicillin, 70 μg/ml streptomycin, and 0.5 mg/ml bovine serum albumin. E1A-RPTE were cultured in the same medium with the addition of 1% fetal bovine serum. Cultures were maintained at 37°C in an atmosphere of 5% CO2-95% air and fed every other day. Primary cultures were grown for 6 days (subconfluent growing RPTE) or for 12 days (confluent differentiated RPTE) before harvesting. Subconfluent E1A- and SV-RPTE cultures were used in experiments shown. Increased cell density did not influence cell properties and PKC properties in E1A- and SV-RPTE.

**Preparation of Cell Lysates and Subcellular Fractions.** Cells were washed and scraped into lysis buffer which was 50 mM Tris–Cl (pH 7.4), 0.25 M sucrose, 2.5 mM EGTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotonin, and 1 mM phenylmethylsulfonyl fluoride. Cells lysates were homogenized by sonication and centrifuged at 100,000 × g to separate soluble and particulate fractions. The particulate fractions were resuspended in lysis buffer and sonicated. Protein concentrations were determined according to the method of Bradford (40) using reagents from Bio-Rad (Richmond, CA).

**Phorbol Ester Binding Assay.** Phorbol ester binding assays were carried out as described previously (41). Aliquots of proteins (0.1 mg) were assayed using 20 nM [3H]PDBu ± 20 μM unlabeled PDBu in the presence of 100 μg/ml phosphatidylserine and 0.5 mM calcium chloride in excess of EGTA. Reactions were performed at 37°C for 15 min. Samples were precipitated with 14% polyethylene glycol. The

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*5* L. Dong, C. Chapline, J. L. Stevens, and S. Jaken. Protein kinase C activity regulates the association of the novel protein kinase C substrate, 35H, with the cytoskeleton, submitted for publication.
amount of \(^{1}H\)PDBu in the precipitate was measured by liquid scintillation counting.

**Immunoblot Analysis.** Proteins were separated on denaturing polyacrylamide gels (7.5%) and transferred to nitrocellulose paper. Blots were blocked with 5% nonfat dry milk in TBS (50 mm Tris, pH 7.4, containing 0.5 m sodium chloride) and stained sequentially with PKC isozyme-specific antibodies and alkaline-phosphatase conjugated goat anti-mouse or anti-rabbit antibodies. Antibodies were diluted in TBS containing 1% bovine serum albumin. TBS washes were included between antibody incubations. α-PKC was detected with a mixture of anti-α-PKC catalytic domain and regulatory domain monoclonal antibodies (M6 and M9) raised against purified α-PKC (42). Anti-β- and -γ-PKC monoclonal antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-δ-, ε-, and ζ-PKC antibodies were raised against synthetic peptides corresponding to the δ-, ε-, and ζ-PKC carboxy terminal sequences. Anti-η-PKC antibody was a gift from Dr. P. J. Parker (ICRF, London, United Kingdom). Immunoreactive bands on immunoblots were quantitated by densitometry. Each blot was developed to maximal color density. A standard curve was made by blotting and quantitating a series of known amounts of purified PKC. Quantitation was performed in the linear density range.

**Preparation of PKC Isozyme-specific Riboprobes.** The following DNA restriction fragments were prepared from rat PKC complementary DNAs for synthesizing riboprobes (anti-sense RNA) used in RNase protection assays: α360, BamHI-ClaI 360-bp fragment of α-PKC within the C2-V3 (C, constant; V, variable) (2) regions; 8279, PstI-PstI 279-bp fragment of δ-PKC within the V1 region; 160, XbaI-Apal 160-bp fragment of ε-PKC within the C3 region; 172, PstI-ClaI 172-bp fragment of ζ-PKC within the C1-V3 regions; 159, Clal-SacI 159-bp fragment of η-PKC within the V3-C3 regions; and 295, 3'-end of PAM 295-bp fragment of ζ-PKC within the C4-V5 regions. All DNA fragments were subcloned into pBluescript SK(+) plasmid and confirmed by complete DNA sequencing. Riboprobes (anti-sense RNA) were synthesized in vitro using an Ambion transcription kit in the presence of [α-\(^{32}\)P]UTP.

**RNase Protection Assay.** RNA was isolated from cells by extraction with 4 m guanidine isothiocyanate followed by centrifugation through cesium chloride (43). Total RNA (15 μg) was hybridized with PKC isozyme-specific riboprobes at 50°C for 16 h. RNA alone was used as a negative control. Single-stranded RNA was digested with RNase A and RNase T1. Protected fragments were separated on 5% polyacrylamide/urea gels. Autoradiographs were quantitated by densitometry; multiple exposures of gels were used to ensure linearity.

**Metabolic Labeling with \(^{35}\)SMethionine.** Cells grown on 35-mm dishes were washed twice with PBS, incubated for 1 h in methionine-free DMEM with supplements as described under "Cell Culture," and then labeled with 100 μCi/ml \(^{35}\)SMethionine in the same medium for 4 h at 37°C. After labeling, cells were washed twice with PBS and placed in normal culture medium in the presence or absence of 200 μM PDBu for the indicated times. After washing the monolayers with PBS, cells were scraped into 1 ml of RIPA, which was 50 mm Tris-Cl (pH 7.4), 0.15 m sodium chloride, 1 mm EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mm phenylmethylsulfonyl fluoride. Samples were homogenized by repeated passages through a 25-gauge needle.

**Immunoprecipitation of α-PKC.** Samples (1 ml) in RIPA were preincubated with 150 μl of formalin-fixed Staph A cells (10% v/v) for 1 h. Precleared supernatants were collected and incubated with 1 μg of purified anti-α-PKC monoclonal antibody M4 (42) or nonimmune mouse IgG. Protein A-Sepharose (40 μl; 50% v/v) preequilibrated with rabbit anti-mouse IgG was added, and the incubation continued for another h. All incubations were performed at 4°C on a shaker. Immunocomplexes were collected by centrifugation, washed twice in RIPA, once in low-salt buffer (50 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 0.15 m sodium chloride, 1 mm EDTA, and 0.1% Nonidet P-40), once in high-salt buffer (50 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 0.5 m sodium chloride, 1 mm EDTA, and 0.1% Nonidet P-40), and once in low-salt buffer again. Immunoprecipitated proteins were eluted from protein A-Sepharose by boiling for 5 min in Laemmli buffer (44) and then separated on denaturing polyacrylamide gels. Immunoprecipitated radiolabeled α-PKC was visualized by fluorography. The corresponding bands were quantitated by densitometry.

**Determination of DNA Synthesis in Cultured Cells.** Cell growth was determined by incorporation of \(^{3}H\)thymidine into cells. Primary RPTE were cultured in basal medium supplemented with insulin, epidermal growth factor, and cholera toxin as described under "Cell Culture" for 6 days (subconfluence). Growth factors were depleted by incubating for 48 h in medium without added factors. At this time, individual factors were added along with 0.15 μCi/ml \(^{3}H\)thymidine for 24 h. Incorporation of radioactivity into the 10% trichloroacetic acid insoluble fraction was determined by liquid scintillation counting.

**References**


