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SUPPRESSION OF PHORBOL ESTER-ENHANCED RADIATION-INDUCED MALIGNANCY *IN VITRO* BY PROTEASE INHIBITORS IS INDEPENDENT OF PROTEIN KINASE C*

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X-irradiation and the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) act in a synergistic manner to increase the yield of transformed C3H10T1/2 cells *in vitro*. TPA modulated both translocation from the cytosol to the plasma membrane, and down regulation of protein kinase C (PKC) after prolonged (48 h) TPA exposure. N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), antipain, and soybean-derived Bowman-Birk inhibitor, protease inhibitors that supress transformation of C3H10T1/2 cells, had no effect on these TPA-mediated alterations of PKC activity, suggesting that protease inhibitors suppress TPA-stimulated promotion *in vitro via* a PKC-independent pathway. Several experiments were performed to determine whether non-toxic concentrations of the PKC inhibitors, N-p-tosyl-L-lysine chloromethyl ketone (TLCK), TPCK, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), or 1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine (H-7), modulated the movement of cells from a quiescent state into the cell cycle. TPCK and the combination of H-7 and W-7 lowered DNA synthesis when cells were stimulated to divide by TPA. Because other protease inhibitors that slow transformation *in vitro* did not have the same suppressive effect on DNA synthesis, the inhibitory pathway that suppresses carcinogenic activity is likely to be different from the suppression of DNA synthesis.

Nishizuka and his colleagues first described PKC as a protein kinase that could be activated *in vitro* by partial proteolysis (for a review, see 1). It was subsequently shown that PKC activity could be stimulated by Ca^{2+}, and acidic phospholipids could stimulate PKC in the absence of proteolysis. A physiological role for PKC became apparent with the discovery that DAG stimulated PKC activity by reducing the dependence of the enzyme on Ca^{2+} and phospholipids (2). In addition to a role for PKC as a regulator of cellular functions, the enzyme was implicated in tumorigenesis when it was found that PKC is a primary receptor for tumor promoting phorbol esters (3), This finding allowed the biochemical actions underlying tumor promotion to be studied in detail.

We have previously reported that the tumor promoting agent TPA enhances radiation-induced transformation (4, 5), whereas several protease inhibitors suppress transformation *in vitro* (6-9). Our past experiments have indicated that these agents, which modify transformation *in vitro*, are effective during the proliferation of "initiated" cells. In the present study, we examined whether TPA and anti-carcinogenic protease inhibitors are capable of

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Abbreviations Used: Protein kinase C (PKC); 12-O-tetradecanoylphorbol-13-acetate (TPA); N-tosyl-L-phenylalanine chloromethylketone (TPCK); N-p-tosyl-L-lysine chloromethyl ketone (TLCK); Diacylglycerol (DAG); N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7); 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H-7); phenylmethylsulfonylfluoride (PMSF); dimethylsulfoxide (DMSO); Bowman-Birk protease inhibitor (BBI).

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affecting specific steps in the PKC signal transduction pathway. Specifically, we studied i) the subcellular distribution of PKC activity in normal and irradiated C3H10T1/2 cells in the absence and presence of protease inhibitors, PKC inhibitors, or both; ii) whether inhibitors of PKC or proteases affect DNA synthesis in plateau phase cultures in which the cells have been stimulated to divide by treatment with either TPA or serum. The present study was designed to determine whether the PKC signal transduction pathway is involved in the modification of radiation-induced transformation by TPA and anti-carcinogenic protease inhibitors. The protease inhibitors used in this study, TPCK, antipain, and BBI from soybeans all suppress radiation-induced transformation in C3H10T1/2 cells at non-toxic levels *in vitro*, and inhibit carcinogenesis *in vivo* (10).

MATERIALS AND METHODS

Cell Culture - The C3H10T1/2 stock cultures were maintained in 60 mm Petri dishes and were subcultured at a 1:20 dilution every 7 days. The cells used were in passages 9-14. They were grown in a humidified 5% CO_2 atmosphere at 37 ^{o}C in Eagle's basal medium supplemented with 10% heat-inactivated fetal bovine serum and gentamycin. Quiescent cultures were obtained by incubating confluent cultures in medium containing 0.5% serum for 24-48 h. after they became confluent.

Chemicals - The concentrations and sources of chemicals used in these studies were: TPA (lot O31, Consolidated Midland Co., Brewster, NY), 100 ng/ml TPCK, TLCK, phosphatidyl-serine, diolein, histone H1 (type III-S), antipain, PMSF, DMSO, and leupeptin were from Sigma Chemical Co. (St. Louis, MO); H-7 and W-7 were from Cal Biochem (LaJolla, CA). [γ-^{32}P] ATP, specific activity = 2-10 Ci/mmol,) and [methyl-^{3}H] thymidine were purchased from New England Nuclear (Boston, MA). The purified BBI was prepared in our laboratory as previously described (9). TPA was dissolved in acetone, TPCK was dissolved in DMSO, TLCK, H-7, W-7, antipain, and BBI were dissolved in EBSS.

Protein Kinase C Assay - Confluent cultures exposed to either TPA, or TPA and protease inhibitors, were washed three times with 150 mM NaCl. The cells were harvested in cold buffer A (20 mM Tris-HCl, pH 7.5/ 6 mM EDTA/250 mM sucrose/0.5 mM dithiothreitol/0.5 mM PMSF) by scraping with a rubber policeman. Cells were broken by several bursts in a bath sonicator. The cytosolic fraction was separated from membranes by centrifugation at 100,000 x g for 1 h. Membrane-bound PKC was solubilized by extracting the pellet from the centrifugation step in Buffer A containing 1% Triton X-100 for 45 mins. on ice, with vortexing every 10 min. The detergent-solubilized fraction was separated from membranes by centrifugation at 100,000 x g for 1 h. The PKC activity in the cytosol and detergent-solubilized fractions was separated from phosphatases and other cellular constituents by anion exchange chromatography on DE-52 cellulose (Whatman). Columns containing 0.2 ml of DE-52 were equilibrated with buffer A. After the sample was applied to the column, a 2-ml wash with buffer A removed much of the protein but essentially no PKC activity. PKC was eluted with 1-ml of buffer A containing 40 mM NaCl. Enzyme activity was assayed by measuring incorporation of ^{32}P into histone H1 using [γ-^{32}P] ATP as the substrate, as previously described (11).

^{3}H-Thymidine incorporation into macromolecules - The measurement of ^{3}H-thymidine incorporation was performed after quiescent cells had been treated with either TPA or serum (20%) in the presence or absence of PKC inhibitors. The rate of DNA synthesis in quiescent cells after TPA or serum stimulation was determined by measuring the amount of radioactivity incorporated into the cells after a 30 min. pulse with [methyl-^{3}H]-thymidine, at various time intervals of from 6 to 36 h after stimulation. At each time point, cells were incubated for 30 min. with [methyl-^{3}H] thymidine (10 μCi/ml), washed three-times with ice-cold EBSS, once with 1 mM EDTA, and heated for 1 min. at 95 ^{o}C in 1 mM EDTA. The cell extract was collected, placed on ice, precipitated with ice-cold trichloroacetic acid, collected on GF/A filter paper, and the radioactivity was measured by liquid scintillation counting.

RESULTS

The subcellular distribution of PKC activity between the particulate and soluble fractions was dependent on cell density and the growth rate in normal C3H10T1/2 cells. At low cell density, rapidly growing cultures exhibited high levels of particulate PKC, but little PKC activity was found in the cytosol (Fig. 1). With increasing cell density, and as cultures began to slow in growth rate, a progressive decrease in particulate PKC activity was observed, whereas the cytosolic PKC activity increased. In resting, serum-deprived quiescent cultures, approximately 90% of PKC activity was located in the cytosol (data not shown).

When quiescent cells were stimulated to divide with either TPA or serum, PKC activity was rapidly translocated from the cytosol to the membrane fraction. After prolonged exposure of the cells to TPA, total PKC activity was diminished (Fig 2). The effects of several protease inhibitors on TPA-mediated translocation of PKC

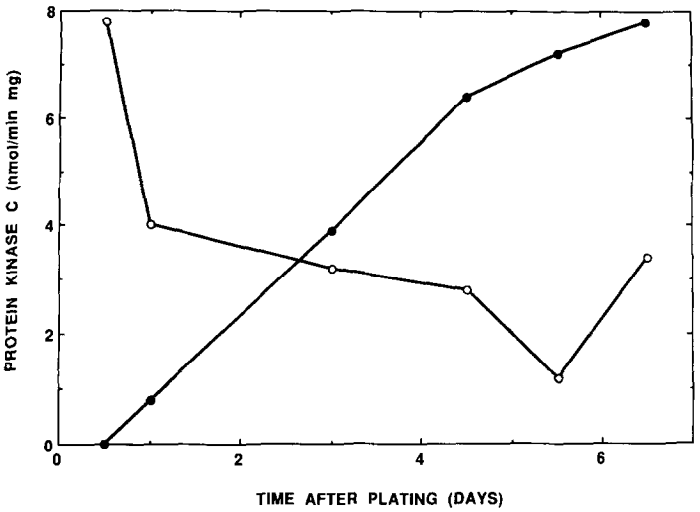
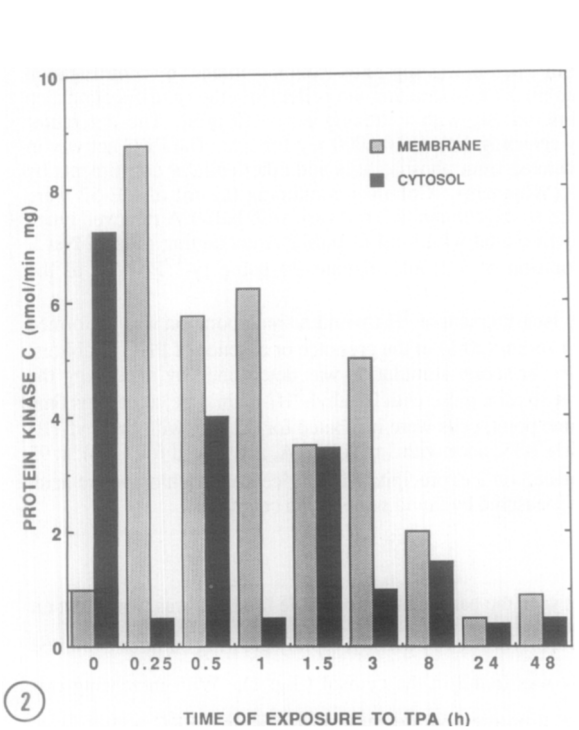


Figure 1. Cytosol and membrane- associated protein kinase C activity in C3H10T1/2 cells as a function of increasing cell density. C3H10T1/2 were plated at high cell density in 150cm^2 dishes at 1x10^6 cells per dish; cells were then grown at 37°C in BME containing 10% fetal calf serum. At the times indicated, cells were harvested; kinase activity was measured in the soluble (□-□) and particulate (◆-◆) fractions and expressed as nmol^{32}P incorporated per min/mg of total cellular protein. The figure shows the distribution of PKC activity as the cell population progressed from an actively growing to a density-inhibited growth state.



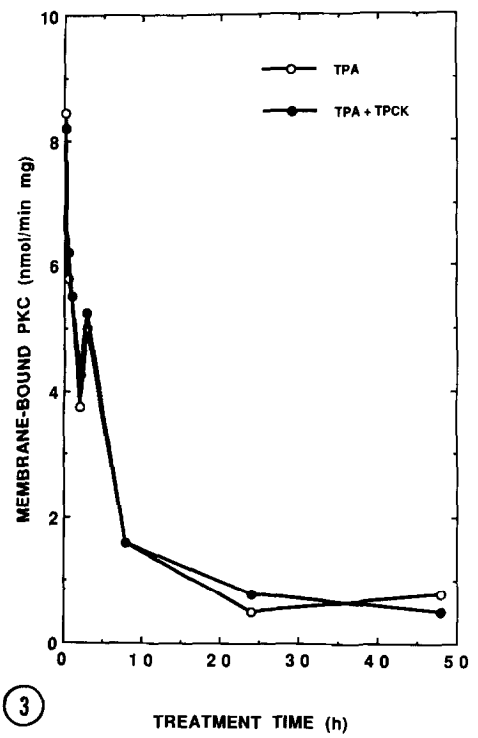


Figure 2. Time course for the changes in the distribution of PKC activity in the cytosol and membrane-associated forms. Within 15 minutes after TPA treatment, there was a rapid translocation of PKC activity from the cytosol to the membrane fraction.

Figure 3. Changes in membrane-associated (particulate) protein kinase C activity after 20% serum stimulation of cells in the presense and absence of TPCK. Cells were seeded and allowed to grow to confluence, the growth medium was replaced with medium containing 0.5% FCS for 2 days. The cells were then stimulated with fresh medium containing 20% FCS, with or without TPCK. Kinase activities were measured in the particulate fraction; symbols used are as follows: (□-□) , TPA, (◆-◆), TPA + TPCK.

were examined. The protease inhibitors and the concentrations used were: TPCK (10 ng/ml; 2.8 nM), antipain (50 μg/ml; 85 μM), and BBI (300 μg/ml; 38 μM). These concentrations are all non-toxic to C3H10T1/2 cells, and have been previously shown to suppress radiation-induced transformation *in vitro* (12). None of the protease inhibitors altered either the translocation of PKC from cytosol to the membrane, or the observed down regulation of PKC. A representative experiment for TPCK is given in Fig. 3.

We examined whether the levels of PKC activity could be modified by x-ray treatment of the cells prior to exposure to TPA. Radiation-induced transformation *in vitro* is enhanced by TPA; we reported previously that a synergistic interaction occurs between radiation and TPA that ultimately leads to higher levels of transformed cells (4, 5). Two separate experiments were performed to determine the effect of different doses of radiation (prior to TPA treatment) on membrane-associated PKC activity. Membrane-associated PKC activity in irradiated cells did not show a synergistic increase in TPA-mediated translocation of PKC, therefore, several experiments were carried out to evaluate the actions of compounds that modify transformation in C3H10T1/2 cells on the mitogenic response in stimulated plateau-phase cultures. TPA is a potent mitogen for C3H10T1/2 cells in plateau phase cultures (6), we therefore measured ^{3}H-thymidine uptake in confluent cultures stimulated to divide with either TPA or serum.

To determine whether PKC inhibitors affect cell growth at the highest non-toxic concentrations, incorporation of ^{3}H-thymidine into DNA was measured in quiescent cultures that had been stimulated to divide with

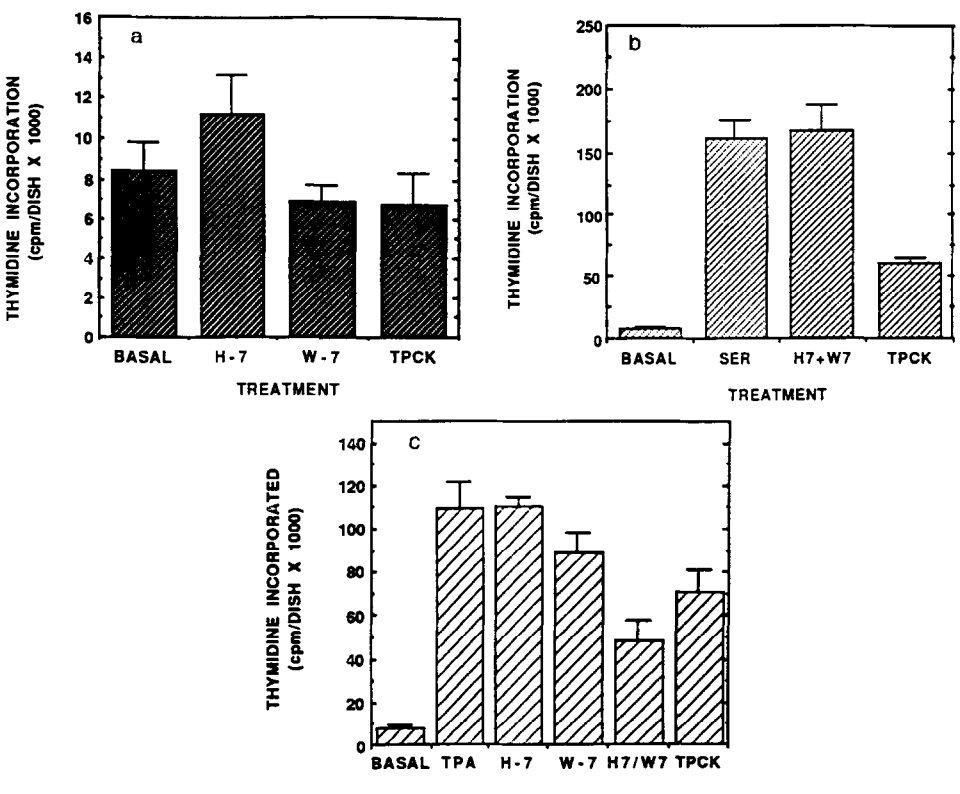


Figure 4. DNA synthesis induced by treatment of cells with serum or TPA is inhibited by TPCK or W-7 + H-7. Results are presented as of histograms: (a) control cultures; (b) serum-treated cultures; (c) TPA-treated cultures. [^{3}H]-thymidine incorporation was measured after quiscent cells had been stimulated with fresh medium containing 20% FCS, or TPA with or without TPCK and TLCK, or W-7 and H-7 (3 μM each). At each time point chosen, cells were incubated for 30 min in ^{3}H-thymidine, washed three times with ice-cold EBSS, precipitated with ice-cold trichloroacetic acid (TCA), collected on Whatman filter paper and counted by liquid scintillation.

either TPA or serum. The actions of several established PKC inhibitors are shown in Fig. 4. Of the inhibitors used, only TPCK decreased DNA synthesis (70% in serum stimulated, and 30% in TPA-stimulated cultures). Neither H-7 or W-7 (both at 3 μM) alone inhibited the mitogenic effects of either serum or TPA, but when H-7 and W-7 were combined with TPA treatment, a 50% decrease in DNA synthesis was observed (Fig. 4). The highest non-toxic concentrations of these compounds were well below the levels that inhibit PKC in other cell types. The changes over time in ^{3}H-thymidine incorporation in the presence or absence of TPCK were examined. In a dose-response experiment, TPCK had the same suppressive effect on DNA synthesis over the range of concentrations studied (~ 30 % from 5-100 ng/ml TPCK). At the concentrations of TPCK and TLCK used in these experiments, there was no effect on cell growth in a logarithmically growing cell population (data not shown). None of the other protease inhibitors used in these studies had any effect on DNA synthesis (data not shown).

DISCUSSION

The Ca^{2+}-phosphatidylserine-stimulated enzyme PKC exists as both an inactive soluble, and an active membrane-bound form. It has been proposed that changes in the bimodal distribution of PKC induced by DAG, or tumor-promoting phorbol esters, regulate the activity of PKC *in vivo*. It is thought that the translocation of PKC from the cytoplasm to membrane plays a major role in the regulation of cell growth (1, 13, 14). We have observed that cell density and growth rate influence the distribution of PKC between the particulate and soluble fractions in C3H10T1/2 cells, as has been reported for many other cell types (13). Specifically, we observed that PKC was primarily cytosolic in resting quiescent cells and associated with membrane in actively growing cells.

In the plateau phase cultures used for many of these studies, addition of TPA to the growth medium caused a rapid (~15 min) translocation of PKC from the cytosol to the membrane. Subsequent down-regulation of total PKC activity occurred after prolonged (up to at least 48 h) exposure to TPA . A similar time sequence for the translocation of PKC from cytosol to membrane followed by down-regulation of the enzyme has been observed in many other cell types (see 15 for a review). This response of cells to TPA is thought to be involved in the mechanism of tumor promotion (13). Alterations in PKC activity are often associated with cancer. For example, high levels of PKC activity are found in the membrane fractions of several types of transformed cells (16, 17). Thus, a possible mechanism for the protease inhibitor suppression of carcinogenesis *in vivo* and *in vitro* could involve an effect on PKC. In these studies three different anticarcinogenic protease inhibitors, TPCK, antipain, and BBI, failed to affect the alterations in PKC activity brought about by TPA treatment, suggesting that these anticarcinogenic protease inhibitors do not affect promotion *in vitro* through effects on PKC activity.

It has been reported previously that TPCK and certain other protease inhibitors could suppress the TPA-induced down-regulation of PKC in a fetal rat keratinocyte cell line (18). In those studies, however, the concentration of TPCK used is quite toxic to C3H10T1/2 cells. In our studies, we considered it important to use non-toxic concentrations of protease inhibitors; the protease inhibitors at non-toxic concentrations did not affect PKC activity. A few anticarcinogenic protease inhibitors (TPCK and leupeptin) have been reported to suppress TPA-induced down regulation of PKC; other protease inhibitors, however, showed no correlation with anticarcinogenic activity (18). For example, some anticarcinogenic protease inhibitors (antipain and chymostatin) were ineffective even at high concentrations tested and some protease inhibitors lacking anticarcinogenic activity, such as TLCK, were effective in the suppression of down-regulation.

It is reasonable to expect that protease inhibitors could affect PKC. The binding of TPA to PKC at the DAG binding site results in activation of the enzyme (1). It has been reported that limited proteolysis of PKC with calpain converts the enzyme to a permanently active form known as protein kinase M (19, 20). This form of the

enzyme is soluble and does not require other effectors for activity. The calpain activity can be inhibited in vitro by high concentrations of several different thiol protease inhibitors that have anticarcinogenic activity (21). A complete correlation does not exist, however, between anticarcinogenic activity and the ability of a compound to inhibit calpain. Some protease inhibitors lacking anticarcinogenic activity, such as TLCK, inhibit this protease while several different protease inhibitors known to have anti-carcinogenic activity, such as antipain and BBI, do not affect calpain, even at very high concentrations (22).

We examined whether specific inhibitors of PKC could affect movement of cells from G_0 to G_1, and into the S phase of the cell cycle. Cell cycle progression was studied by determining the rate of incorporation of ^3H thymidine into cells after stimulation of quiescent cells with either TPA or serum in the presence of various PKC inhibitors. The mechanism of action of the PKC inhibitors used in this study have been reported to act at substrate sites (TPCK and TLCK), phospholipid cofactor site (H-7), or Ca^{2+}-calmodulin sites (W-7). In addition to inhibiting PKC, TPCK and TLCK are protease inhibitors.

TLCK, H-7, and W-7 alone did not affect DNA synthesis, but TPCK, and H-7 in combination with W-7 were capable of inhibiting DNA synthesis. In these studies, exposing the cells to a combination of W-7 and H-7 was about as effective as TPCK, when the cells were stimulated to divide by TPA. The combination of H-7 and W-7 had no effect on DNA synthesis in serum-stimulated cells. This is not surprising considering the many different growth factors present in serum. It is quite likely that one of the many factors in serum could affect the parameters involved in cell growth somewhat differently from TPA. TPCK had a major suppressive effect on DNA synthesis whether serum or TPA was used as the mitogenic agent. Our results suggest that the mechanisms involved in the movement of cells from G_0 into the cell cycle are subject to controls that do not function in an actively growing cell population because the concentration of TPCK that effectively suppressed DNA synthesis had no effect on cell growth in exponentially growing cultures. The study of mechanisms involved in regulation of the cell cycle is an active area of investigation. Much recent research has focused on a specific protein kinase, the activation of which is thought to be the signal that initiates the cellular changes necessary for cells to divide (see (23) for review). An effect of TPCK on such a kinase that functions at the initiation of the cell cycle, but not in exponentially growing cells, could explain the actions of TPCK observed in the present study.

The mechanism by which TPCK affects DNA synthesis is not known. TPCK and TLCK inhibit both PKC and proteases. TPCK has anticarcinogenic activity that is thought to be related to its ability to inhibit chymotrypsin (12), whereas TLCK lacks anticarcinogenic activity. In the present study TLCK had no effect on DNA synthesis under conditions where TPCK was a potent inhibitor of DNA synthesis, it is therefore possible that the anticarcinogenic property of TPCK is related to its ability to inhibit DNA synthesis. Other anticarcinogenic protease inhibitors, such as antipain or BBI, did not have the same effect on DNA synthesis. Thus, it seems unlikely that the mechanism by which TPCK inhibits DNA synthesis is related to the mechanism by which other protease inhibitors suppress carcinogenesis.

We examined whether pre-treatment of cells with X-radiation could modify the actions of TPA on PKC. We observed no effect of various doses of X-radiation on TPA-induced translocation of PKC from cytosol to membrane. These results suggest that the synergism observed between radiation and TPA as mediators of transformation in C3H10T1/2 cells occurs *via* a pathway independent of PKC. Similarly, the suppressive actions of protease inhibitors on promotion in C3H10T1/2 cells does not appear to be caused by alteration of PKC activity. Further studies are needed to determine whether PKC plays a role in radiation transformation occurring in the absence of promotion.

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