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Overexpression of protein kinase C β1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts

(phosphatidylcholine hydrolysis/phosphatidylethanol formation)

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ABSTRACT **We are using a Rat-6 fibroblast cell line that stably overexpresses the β1 isozyme of protein kinase C (PKC) to study regulation of phospholipid hydrolysis by PKC. Stimulation of control (R6-C1) or overexpressing (R6-PKC3) cells with phorbol ester results in an increase in diacylglycerol (DAG) mass with no increase in inositol phosphates, indicating that DAG is not formed by inositol phospholipid breakdown. A more dramatic DAG increase occurs in R6-PKC3 cells (4.0-fold over basal) compared to R6-C1 cells (1.5-fold over basal). To further define the source of DAG, phosphatidylcholine (PC) pools were labeled with [^{3}H]myristic acid or with [^{3}H]- or [^{32}P]alkyllyso-PC and formation of labeled phosphatidylethanol, an unambiguous marker of phospholipase D activation, was monitored. Phorbol ester-stimulated phosphatidylethanol formation is 5-fold greater in the R6-PKC3 cell line. Formation of radiolabeled phosphatidic acid (PA) is also enhanced by PKC overexpression. In cells double-labeled with [^{3}H]- and [^{32}P]-alkyl-lysoPC, the ^{3}H/^{32}P ratio of PA and PC are identical 15 min after stimulation, suggesting that a phospholipase D mechanism predominates. In support of this, the PA phosphohydrolase inhibitor propranolol decreased phorbol 12-myristate 13-acetate-stimulated DAG formation by 72%. Increases in DAG and phosphatidylethanol were inhibited by the PKC inhibitors K252a and staurosporine. These results indicate that phospholipase D is regulated by the action of PKC. Enhanced phospholipase D activity may contribute to the growth abnormalities seen in PKC-overexpressing cells.**

Many growth factors, hormones, and neurotransmitters exert their biological effects by inducing the breakdown of cellular phospholipids. The role of the inositol phospholipid-specific phospholipase C family in production of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) second messengers has been extensively investigated (reviewed in ref. 1). $InsP_3$ mobilizes calcium from intracellular stores, while DAG activates protein kinase C (PKC).

Recently, hydrolysis of phosphatidylcholine (PC) has been demonstrated to occur in a variety of agonist-stimulated cells (reviewed in ref. 2) and may, in some cases, be the primary route of DAG second messenger formation (e.g., see refs. 3 and 4). PC hydrolysis to DAG has been reported to occur by the action of a PC-specific phospholipase C (e.g., see refs. 5 and 6) or by the sequential action of a phospholipase D [producing phosphatidic acid (PA) and choline] and PA phosphohydrolase (e.g., see refs. 7 and 8).

To understand the mechanisms controlling these pathways, it is essential to clarify how PKC regulates phospholipid hydrolysis. Short-term incubation of cells with PKC activators (e.g., phorbol esters) (e.g., see refs. 9 and 10) or inhibitors (11), or down-regulation of PKC by chronic exposure to phorbol esters (12) prior to agonist stimulation indicates that inositol phospholipid hydrolysis is negatively regulated by PKC.

In contrast, PC hydrolysis appears to be positively regulated by PKC. In 1981, Mufson *et al.* (13) reported that phorbol esters stimulate PC hydrolysis in mouse fibroblasts. Other investigators have since reported that phorbol ester treatment of cells can induce PC hydrolysis by either phospholipase C (5, 14, 15) or phospholipase D (3, 4, 16–20). Phorbol ester-induced PC hydrolysis is abolished or attenuated by PKC down-regulation (4, 5, 14, 17, 18) or by pretreatment with PKC inhibitors (16–18). However, failure to achieve complete inhibition with PKC inhibitors has led to the suggestion that phorbol esters may act in part through a PKC-independent mechanism (see ref. 16).

PC hydrolysis is also induced by agonists acting at cell-surface receptors. Receptor-mediated activation of PC hydrolysis by phospholipase C (5, 6, 14, 21) and phospholipase D (e.g., see refs. 7, 8, 17, 19, 20, and 22) has been reported. In some cases, activation of both hydrolytic pathways was observed (4, 23). The mechanism by which PC hydrolysis is coupled to receptor activation remains to be clarified. A role for PKC is suggested by the finding that down-regulation of PKC blocks or attenuates agonist-stimulated PC hydrolysis (4, 14, 19, 22, 24). However, in most cases, PKC inhibitors were found to have little or no effect (e.g., see refs. 6, 16, and 19). Studies in cell-free systems in which nonhydrolyzable GTP analogs were used suggest that PC hydrolysis may also be regulated by a guanine nucleotide binding protein (25–28).

To further explore how PKC affects phospholipid hydrolytic pathways, we are using a rat fibroblast line that stably overexpresses the β1 isozyme of PKC. The Rat-6 PKC-3 (R6-PKC3) cell line overexpresses kinase activity 53-fold relative to controls lacking the PKC cDNA insert (R6-C1) (29). These cells exhibit multiple growth abnormalities (29) and are more susceptible to transformation by an activated Ha-*ras* oncogene (30). In this paper, we report that overexpression of PKC dramatically increases phospholipase D activity in phorbol 12-myristate 13-acetate (PMA)-stimulated fibroblasts, leading to enhanced production of DAG. This strongly supports a direct role for PKC in the regulation of phospholipase D.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Alkyl lyso-PC (92 Ci/mmol; 1 Ci = 37 GBq), *myo*-[2-^{3}H]inositol and [9,10(n)-^{3}H]myristic acid were from Amersham. [^{32}P]Alkyl lyso-PC (2–4 Ci/mmol) was prepared as described (8). [γ-^{32}P]ATP was purchased from New En-

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Abbreviations: DAG, diacylglycerol; DMSO, dimethyl sulfoxide; $InsP_3$, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PEt, phosphatidylethanol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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gland Nuclear. Lipids were from Avanti Polar Lipids. K252a and staurosporine were from Kyowa Hakko USA (New York). PMA and 4α-PMA were from LC Services (Boston).

Maintenance and Labeling of Cell Cultures. For most experiments, R6-C1 and R6-PKC3 cells were grown to confluency on 100-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine, and G418 (50 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were then serum deprived for 24 hr in DMEM supplemented as described above, except the serum content was reduced to 0.5% and G418 was omitted.

To label PC pools, [^{3}H]myristic acid, [^{3}H]alkyl lyso-PC, or alkyl [^{32}P]lyso-PC was dried under nitrogen, dissolved in 20 μl of dimethyl sulfoxide (DMSO) and DMEM was added to achieve final specific activities of 5, 1, and 3 μCi/ml, respectively. Labeling was for 2 hr with [^{3}H]myristate and for 3 hr with alkyl lyso-PCs. Thin-layer chromatography analysis of lipid extracts indicated that 85% of the alkyl lyso-PC was incorporated into PC, with the remainder being unmodified. The extractable myristate label was recovered as PC (89%), phosphatidylinositol (3%), phosphatidylethanolamine (5%), and lyso-PC.

For analysis of inositol phosphates, cells were plated at 3 × 10^5 cells per 35-mm dish, grown overnight in DMEM containing 10% fetal calf serum, and labeled for 2 days in inositol-free DMEM containing 0.5% fetal calf serum and 5 μCi of *myo*-[2-^{3}H]inositol per ml.

Assay of Phorbol Ester-Stimulated Phospholipase D. After labeling, cell monolayers were washed twice with phosphate-buffered saline and fresh medium was added. In some experiments, cells were preincubated for 5 min with the indicated concentration of staurosporine or K252a added from DMSO stocks. Cells were then stimulated with PMA (100 nM) in the presence of 0.5% ethanol. The final concentration of DMSO was kept at or below 0.1%. Control dishes received DMSO alone. At the indicated time, the medium was aspirated and cells were scraped into 1 ml of ice-cold methanol and transferred into tubes containing 1 ml of chloroform. Dishes were washed once with an additional 1 ml of methanol. Lipids were extracted by the method of Bligh and Dyer (31). Standard samples of PA, phosphatidylethanol (PEt), and DAG (10 μg of each) were added to each extract prior to drying under nitrogen and spotting on Silica gel G plates. Thin-layer chromatograms were run as described (3). Lipids were visualized by iodine staining of the standards or by autoradiography. Areas corresponding to PEt and PA were scraped and counted by liquid scintillation spectrometry.

Analysis of DAG Formation. Serum-deprived confluent monolayers were stimulated with PMA in the presence or absence of the PKC inhibitors as described above, except that ethanol was omitted from the reaction mixture. At the indicated time, cells were extracted as described above except that 1 M NaCl was used in place of perchloric acid as the aqueous extraction phase. DAG mass was determined by conversion to [^{32}P]PA as described (11, 32). DAG data were normalized to phospholipid phosphate determined by the method of Ames and Dubin (33) and expressed either as a mol % or as a percent of the basal (unstimulated) level.

Inositol Phosphate Analysis. [^{3}H]Inositol-labeled cells were washed twice with 1 ml of serum-free DMEM and incubated for 15 min with 10 mM LiCl prior to stimulation with PMA. Reactions were terminated by adding 1 ml of ice-cold 10% perchloric acid containing 3 mM EDTA and 1 mM diethyltriaminepentaacetic acid. After 20 min on ice, solutions were neutralized with 6 M KOH and centrifuged (1000 rpm for 1 min) to remove salt. The supernatant was diluted with 3 ml of water and applied to Dowex AG 1-X8 anion-exchange columns. Inositol phosphates were separated according to Berridge *et al.* (34) with modification to separate inositol trisphosphates from inositol tetrakisphosphates (35).

RESULTS

PKC Overexpressing Cells Have Decreased Basal DAG Levels. Initially, we examined the effect of PKC overexpression on fibroblast DAG mass levels. R6-PKC3 cells were found to have lower basal DAG levels than control cells at all stages of growth examined (Fig. 1). The largest difference was observed in preconfluent cultures. DAG levels in both cell lines began to decrease upon reaching confluency. The rate of this decrease was accelerated slightly by serum deprivation. In most experiments, cells were used 24–48 hr after transfer to low serum, at which time the mean DAG level was 0.25 ± 0.08 mol % (n = 9) for control cells and 0.11 ± 0.02 mol % (n = 8) for R6-PKC3 cells.

Overexpression of PKC Enhances DAG Formation in Response to Phorbol Ester. PMA treatment increased DAG mass levels in a dose-dependent manner in both cell lines (Fig. 2). In the R6-PKC3 cells, DAG formation was maximal at 200 nM PMA and a half-maximal response was observed at 30 nM. The inactive derivative, 4α-PMA, was ineffective up to 200 nM (data not shown).

DAG levels peaked 15 min after PMA addition and remained elevated above basal throughout the 60-min time course (Fig. 3*A*). In the experiment shown, DAG levels in control cells peaked at 0.46 mol % or ≈170% of the basal value. The mean increase in control cells was 152.0% ± 8.3% basal (n = 8 independent experiments). A more pronounced increase in DAG was consistently observed in PKC overexpressing cells. In the experiment shown, DAG levels peaked at 0.67 mol %, representing a 7.5-fold increase over basal levels. The mean increase in overexpressing cells was 406.2% ± 51.6% (n = 8 independent experiments). The PKC overexpressing cells must hydrolyze at least 2.5- to 3-fold more phospholipid per cell to achieve the observed DAG increase.

As shown in Fig. 3*B*, 200 nM PMA had little or no stimulatory effect on InsP_3 levels in R6-C1 cells and clearly did not significantly stimulate InsP_3 levels in R6-PKC3 cells. Simi-

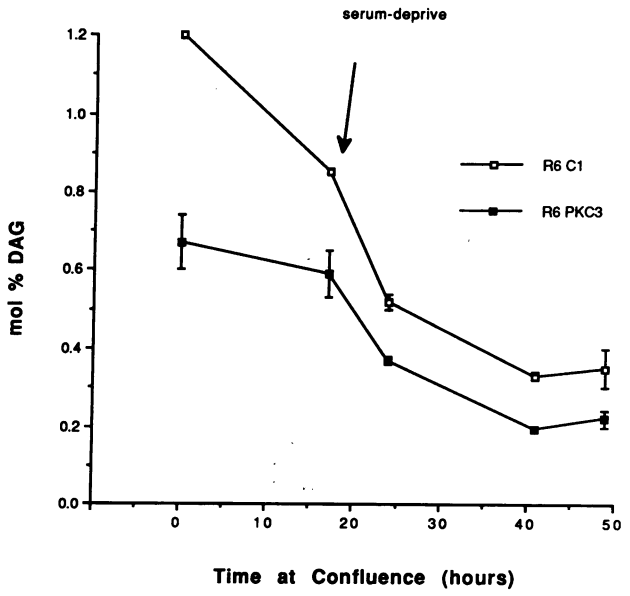

FIG. 1. Basal DAG mass levels in control and R6-PKC3 fibroblasts. Control (R6-C1) or PKC-overexpressing (R6-PKC3) fibroblasts were grown to confluency in 100-mm dishes (t = 0). Cultures were serum deprived after 18 hr at confluency. Lipids were extracted at the indicated times and DAG and phospholipid content of the extracts were determined. Values represent the means ± SE of duplicate determinations. Similar results were obtained in two experiments.

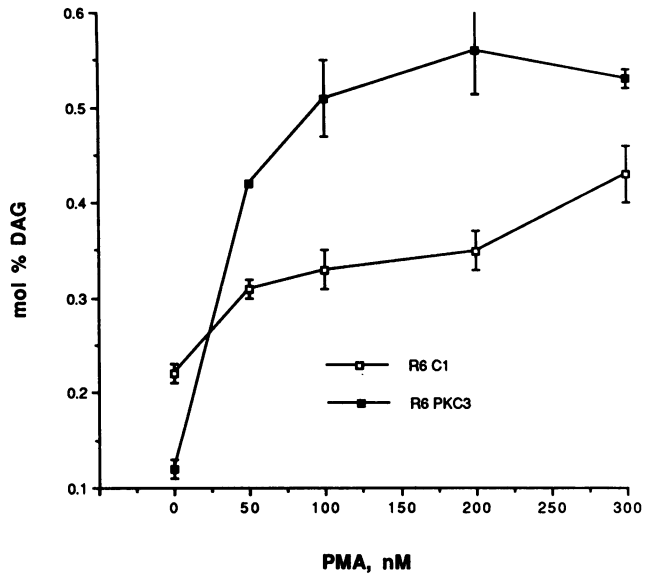

FIG. 2. Effect of PMA on DAG levels. Confluent cultures of R6-C1 or R6-PKC3 cells were serum-deprived for 24 hr. Cells were stimulated with the indicated concentration of PMA for 10 min, lipids were extracted, and DAG and phospholipid analyses were performed. Data shown are the means ± SE of duplicate determinations. Similar results were seen in at least five independent experiments.

larly, PMA did not affect inositol mono-, bis-, or tetrakisphosphates in either cell type in the presence or absence of LiCl (data not shown). In nine separate experiments under similar conditions, thrombin (10 nM) stimulated InsP_3 levels 3- to 11-fold within 20 sec, demonstrating that inositol polyphosphate levels in these cells respond to other stimuli (data not shown). These results indicate that DAG formed in response to PMA does not result from inositol phospholipid hydrolysis.

Overexpression of PKC Enhances PEt Formation in Response to Phorbol Ester. To determine whether phospholipase D hydrolysis of PC contributes to the increase in DAG, PC pools were labeled with [^{3}H]myristic acid. PEt, formed in the presence of ethanol by phospholipase D-catalyzed transphosphatidylation, was used as an unambiguous marker of phospholipase D activity (7, 8). Stimulation with PMA in the presence of ethanol induced the generation of PEt (Fig. 4). Unlike DAG, PEt continued to accumulate for up to 60 min after stimulation, due to a lack of further PEt metabolism. PMA induced formation of [^{3}H]PEt in a dose-dependent manner, while the inactive analog, 4α-PMA was ineffective (data not shown).

PMA was much more effective in stimulating [^{3}H]PEt formation in the PKC overexpressing cells as compared to the control cells (Fig. 4). [^{3}H]PEt formation at 60 min was 5-fold greater in R6-PKC3 cells. PMA-induced [^{3}H]PA formation (due to phospholipase D catalyzed hydrolysis of [^{3}H]PC) was also enhanced in the overexpressing cells (data not shown). These results indicate that PKC overexpression enhances the phospholipase D response.

Relative Contribution of Phospholipases C and D to Formation of [^{3}H]PA and DAG. Formation of radiolabeled PA from PC could occur by the action of phospholipase D or by the sequential action of phospholipase C and DAG kinase. To estimate the relative contribution of these two pathways, PC pools of R6-PKC3 cells were double-labeled with [^{3}H]alkyl lyso-PC and alkyl [^{32}P]lyso-PC. PMA induced the formation of alkyl PA containing both ^{3}H and ^{32}P (Table 1). At 15 min after stimulation, the ^{3}H/^{32}P ratio of alkyl PA (and of alkyl PEt) was very similar to that of alkyl PC. This is consistent with phospholipase D being the predominant pathway of PA formation since PA formed by the phospholipase C–DAG

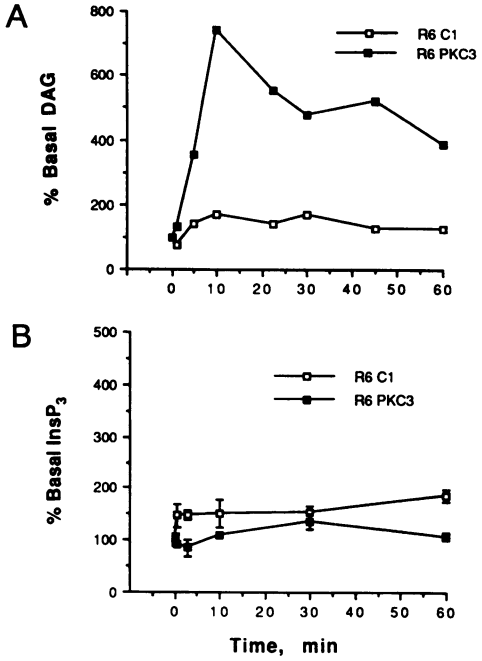

FIG. 3. Effect of PMA on DAG and inositol trisphosphate levels. Time course. (*A*) This experiment was performed as described in the legend to Fig. 2, except that cells were stimulated for various times with 200 nM PMA. Data are normalized to the mol % DAG present prior to PMA stimulation for each cell line (0.27 mol % for R6C1 and 0.09 mol % for R6PKC3). Data shown are representative of three independent experiments. (*B*) Serum-deprived confluent cultures of R6-C1 or R6-PKC3 cells were labeled with [^{3}H]inositol for 48 hr and exposed to 200 nM PMA in the presence of 10 mM LiCl for the indicated time. Data are expressed as % of [^{3}H]IP_3 levels in untreated cells. Basal [^{3}H]IP_3 levels were 0.128% ± 0.011% and 0.277% ± 0.032% of total [^{3}H]inositol lipid for R6-C1 and R6-PKC3 cells, respectively. Values represent means ± SE of triplicate determinations and are typical of two independent experiments.

kinase pathway would have an increased ^{3}H/^{32}P ratio. At 60 min after PMA treatment, the ^{3}H/^{32}P ratio in PA is elevated slightly while, as expected, the PEt ratio remains similar to that of PC. The slight loss of ^{32}P label from PA could be due to dephosphorylation of PA formed by phospholipase D or it could be due to some hydrolysis of PC by a phospholipase C mechanism (with DAG formed by either mechanism being rephosphorylated by DAG kinase).

The effect of propranolol, an inhibitor of PA phosphohydrolase (e.g., see ref. 3) was examined to determine the contribution of the phospholipase D pathway to DAG formation. As expected, pretreatment of cells with 200 μM propranolol increased PMA-stimulated [^{3}H]PA formation in R6-PKC3 cells (data not shown). Propranolol (200 μM) greatly diminished PMA-stimulated DAG mass formation in both PKC-overexpressing (72.6% ± 4.0% inhibition; n = 4) and control (64.6% ± 8.9% inhibition; n = 3) cells. The remaining DAG formation may be due to incomplete inhibition of PA phosphohydrolase or, alternatively, some contribution of phospholipase C action on PC.

PKC Inhibitors Attenuate the Phorbol Ester Response in PKC-Overexpressing Cells. To further examine the role of PKC in the response to PMA we examined the effect of the kinase inhibitors staurosporine and K252a on both DAG mass and [^{3}H]myristate PEt formation. Production of [^{3}H]PEt in R6-PKC3 and R6-C1 cells was inhibited by both compounds in a dose-dependent manner (Fig. 5). Inhibition was maximal at 10 μM with both inhibitors (in R6-PKC3 cells, 53% and 58% inhibition with K252a or staurosporine, respectively).

Treatment of the R6-PKC3 cells with staurosporine also inhibited the PMA-stimulated increase in DAG mass (Fig. 6).

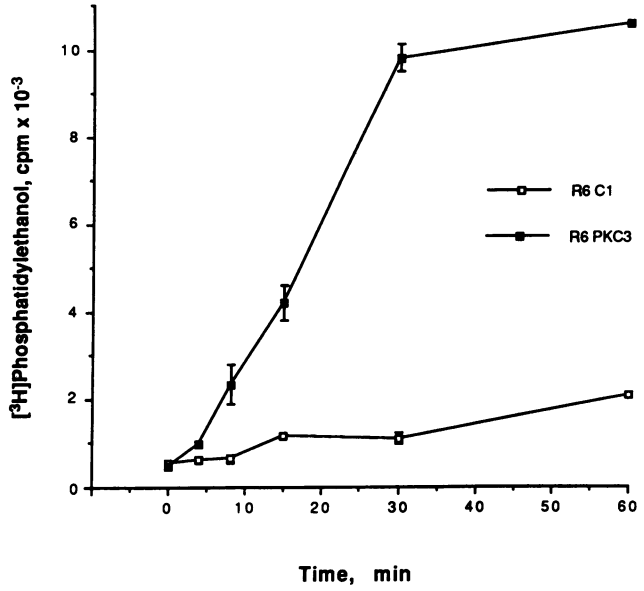

FIG. 4. Formation of [^{3}H]PEt in PMA-stimulated fibroblasts. Serum-deprived confluent cells were labeled with [^{3}H]myristic acid and stimulated with 100 nM PMA in the presence of 0.5% ethanol for various times. [^{3}H]PEt was separated by thin-layer chromatography and quantified. Data presented are means ± SE of duplicate determinations. Similar results were obtained in at least five independent experiments.

Maximal inhibition of 85% and 91% was observed in two independent experiments and occurred between 5 and 10 μM. Staurosporine also inhibited PMA-stimulated DAG formation in R6-C1 cells (71% and 83% in two independent experiments; data not shown). K252a was less effective than staurosporine, only inhibiting PMA-stimulated DAG by ≈40% (data not shown).

DISCUSSION

The PKC overexpression system used here (28) allows the role of PKC in cellular processes to be evaluated by comparing the response in rat fibroblasts containing different amounts of a specific PKC isozyme. Overexpression of PKC β1 was shown to enhance PMA-induced formation of the phospholipase D products PEt and PA as well as the formation of DAG. Although PA and DAG may be formed by several pathways, increased synthesis of PEt, an unambiguous phospholipase D marker, strongly supports a direct role for PKC in the regulation of phospholipase D.

The overproduction of DAG in PMA-stimulated R6-PKC3 cells also is likely due to enhanced phospholipase D activity. Since there is no change in inositol phosphates after PMA stimulation, inositol phospholipid hydrolysis can be ruled out as a DAG source. This is not surprising in light of earlier reports that inositol phospholipid hydrolysis is suppressed by PKC activation (9–12). The finding that the ^{3}H/^{32}P ratios of

Table 1. ^{3}H/^{32}P ratio of phospholipids from double-labeled R6-PKC3 fibroblasts stimulated with PMA

Phospholipid	15 min			60 min		
	^{3}H	^{32}P	Ratio	^{3}H	^{32}P	Ratio
Alkyl PC	233.7	1699.6	0.14	247.5	1723.6	0.14
Alkyl PA	0.8	5.6	0.14	1.1	6.4	0.17
Alkyl PEt	0.8	6.3	0.13	7.8	58.3	0.13

R6-PKC3 cells were double-labeled with [^{3}H]alkyl lyso-PC and alkyl [^{32}P]lyso-PC as described and treated with PMA (100 nM) for 15 or 60 min. After separation of lipids by thin-layer chromatography, the ^{3}H/^{32}P ratios were determined. Values (cpm × 10^{-3}) are the averages of two determinations, which were within 5% of the mean.

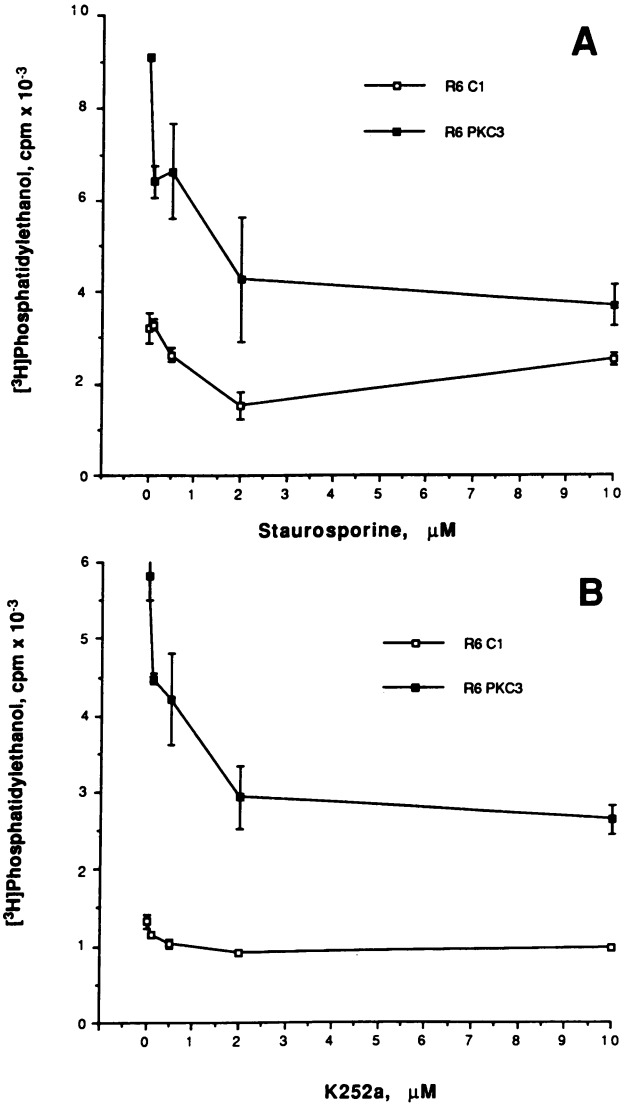

FIG. 5. Inhibition of PEt formation by PKC inhibitors. [^{3}H]Myristic acid-labeled cells were preincubated with the indicated concentration of staurosporine (*A*) or K252a (*B*) for 5 min prior to stimulation with 20 nM PMA in the presence of 0.5% ethanol for 15 min. [^{3}H]PEt formation was determined as described.

PA and PC are similar at early times after PMA stimulation suggests that a phospholipase D mechanism predominates in the formation of [^{3}H]PA. Results with propranolol indicate that the phospholipase D–PA phosphohydrolase pathway is the predominant route of DAG formation, although a minor role for PC hydrolysis by phospholipase C is not ruled out.

Down-regulation of cellular PKC abolishes or attenuates PMA-stimulated PC turnover (e.g., see refs. 4, 5, 14, 18, and 19). We have now demonstrated that when PKC levels are increased (by stable expression of a cDNA clone) PMA-induced PC turnover is enhanced, supporting the hypothesis that phospholipase D is directly regulated by PKC. PKC may regulate both inositol phospholipid hydrolysis and phospholipase D by direct phosphorylation of the phospholipase(s) or by phosphorylation of regulatory proteins. Thus, PKC may act as a molecular switch, turning off inositol phospholipid hydrolysis and turning on PC hydrolysis.

Failure to abolish PMA-stimulated PEt formation with staurosporine or K252a in either cell line is a surprising result. Incomplete inhibition of phorbol ester-stimulated PC hydrolysis has been observed (e.g., see ref. 3). Several possible explanations for these observations exist. First, PMA may

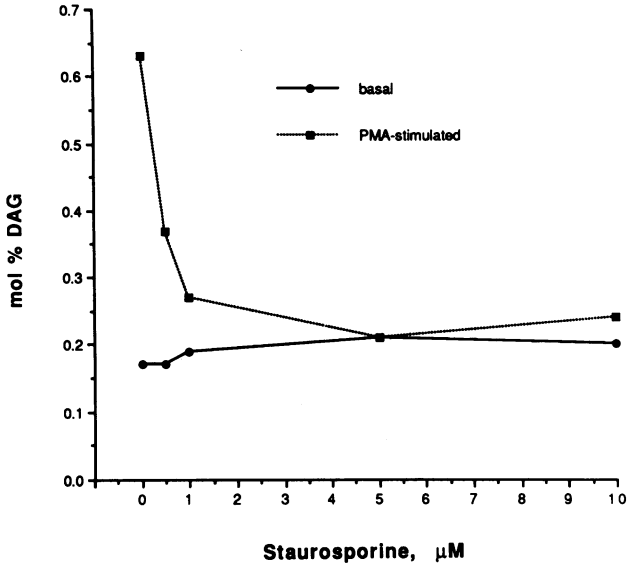

FIG. 6. Inhibition of DAG formation by staurosporine. Confluent, serum-deprived R6-PKC3 cells were preincubated with the indicated concentration of staurosporine for 5 min prior to addition of either PMA or DMSO (200 nM) (solvent control; basal) for 15 min. DAG formation was analyzed as described.

also have PKC-independent effects on phospholipase D activity (16). Other PKC-independent effects of PMA have been suggested (e.g., see refs. 36 and 37). Second, the inhibitors used may not completely inhibit the relevant PKC isozyme or PKC in a particular subcellular compartment. In this regard, it was recently reported that although K252a is a potent *in vitro* inhibitor of calcium-dependent PKC isoforms (e.g., α, β, and γ), a calcium-independent isoform from spleen is relatively insensitive to inhibition by this compound (38). The finding that DAG formation in response to PMA is more sensitive to staurosporine than is PEt formation may be due to the stability of PEt. Since DAG is rapidly metabolized to other products, PEt is a more sensitive marker for residual phospholipase D activity.

During the course of establishing basal conditions to examine the effect of PKC overexpression on phospholipid hydrolysis, we noted that the overexpressing cells had lower basal DAG content. This was reproducibly observed at all growth states. There are several possible explanations for this. First, it may simply be a property of the clonal isolate used for this work. An alternative explanation is that overexpression of PKC lowers basal DAG by decreasing inositol phospholipid turnover in these cells. It is not known how much of the cellular DAG present during growth in serum is due to *de novo* synthesis and how much is due to turnover of inositol phospholipids, PC, or other phospholipids.

Earlier studies have demonstrated numerous alterations in growth properties and enhanced susceptibility to transformation in fibroblasts overexpressing PKC β1 (28, 29) and PKC γ (39). The enhanced phospholipase D activity in the PKC β1 overexpressers may contribute to these properties, especially in light of the possible second messenger role of PA in regulation of cell growth (e.g., see refs. 40 and 41). In addition, since DAG formed by the phospholipase D–PA phosphohydrolase pathway may activate PKC, PKC and phospholipase D may comprise a feed forward loop of cell stimulation as suggested by Exton (2). Some inhibitory control must be exerted over this loop since upon PMA stimulation DAG reaches a maximum and begins to decline after 15 min. It will be important to identify these negative regulatory factors and to examine how overexpression of other PKC isozymes affects these signal transduction pathways.

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