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Protein Kinase C Isoform Expression and Function in Transformed and Non-transformed Pancreatic Acinar Cell Lines

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Members of the protein kinase C (PKC) family of multifunctional serine/threonine phosphorylating enzymes are believed to play a role in regulating cellular differentiation and proliferation in many cell types. In the present study, we examined the expression of PKC isoforms in non-transformed (BMRPA.430) and transformed (TUC3) rat pancreatic acinar cell lines and compared this to PKC expression in freshly dispersed acini from rat pancreas. BMRPA.430 cells maintain characteristics of normal acini and are not tumorigenic, whereas TUC3 cells do not express tight junctions or polygonal morphology and are tumorigenic. As reported previously, PKC α, δ, ϵ, and ζ are expressed in freshly prepared acini. Likewise, these isoforms were detected in both the BMRPA.430 and TUC3 cell lines. In addition, PKCθ, a novel isoform, was detected in all three cell types at low levels. We used two PKC inhibitors to examine the role of PKC in acinar cell proliferation. CGP 41 251, a selective PKC inhibitor, and Go 6976, an agent which specifically inhibits calcium-dependent PKC isoforms, inhibited cell proliferation of both cell lines. Translocation of PKCα to the membrane was not observed in either cell line. Hence, our data indicate that ras-induced transformation does not alter PKC isoform expression in pancreatic acinar cells and that activation of PKCα is involved with acinar cell growth. © 1998 Academic Press

Protein kinase C (PKC) plays a major role in receptor-mediated signal transduction and regulates many cellular processes including proliferation, differentiation and secretion [1,2]. Biochemical and molecular cloning studies have revealed more than 10 lipid-dependent serine/threonine kinases belonging to the PKC family. These include conventional, novel and atypical PKC isoforms which vary with respect to their structure, ability to bind calcium and phorbol esters, intracellular localization and substrate specificity [3,4]. For example, conventional PKC isoforms are activated by calcium, diacylglycerol (DAG) and phospholipids, whereas novel and atypical PKC isoforms are calcium-independent. Many PKC isoforms are expressed in brain and most tissues express two or more isoforms. Nevertheless, the functional relevance of differential PKC isoform expression in various tissues is unclear.

A rat pancreatic acinar cell line, BMRPA.430, has been established that exhibits many morphological and functional characteristics of normal acinar cells including formation of tight junctions, cell polarization, polygonal morphology and the ability to synthesize and secrete acinar cell enzymes such as lipase [5]. BMRPA.430 cells perpetuate at a normal growth rate (64-70 hrs) and do not form tumors *in vivo*. However, it is not clear why these cells continue to grow beyond passage 50. A transformed acinar cell line, TUC3, was established by transfection of BMRPA.430 cells with plasmids containing an activated human c-Ki-ras promoter and minigene that possesses a single point mutation on codon 12. After transfection, TUC3 cells lost normal characteristics of acinar cells and acquired a growth rate of 20 hrs (Bao, Zheng, Thelmo, Somnay-Wadjonkar, Madahar and Michl, submitted for publication). The BMRPA.430 and TUC3 cell lines serve as useful *in vitro* models in studies designed to elucidate the molecular and biochemical events that accompany malignant transformation in pancreatic acinar cells.

The c-Ki-ras gene is mutated in many cancers including pancreas, lung, colon and bladder [6]. The Ras protein, a guanosine triphosphatase, regulates complex signal transduction pathways involving many protein kinases that control cell growth and differentiation [7]. For example, activation of Ras leads to an increase in the activity of two MAP kinases, Erk-1 and Erk-2,

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which translocate to the nucleus where they phosphorylate transcription factors [8]. It has also been demonstrated that Ras also serves to localize c-Raf, a MAP kinase kinase, to the plasma membrane [9]. In addition, there is evidence that Ras-induced transformation involves activation of PKC at some point downstream [10]. In fibroblasts, Ras activation results in the phosphorylation of PKC substrates [11] and, in oocytes, Ras-induced maturation can be blocked by PKC inhibitors [12,13]. In contrast, other Ras-mediated functions do not appear to involve PKC [10].

The effect of Ras-induced transformation on PKC expression has not been examined. Differential expression of PKC isoforms in transformed and non-transformed acinar cells may indicate a role for specific isoforms in Ras-induced transformation. Four PKC isoforms (α, δ, ϵ and ζ) have been detected in dispersed pancreatic acini from rat [14], whereas only one isoform (ζ) was detected in hamster pancreas and pancreatic carcinoma cells [15]. In the present study, we examined PKC isoform expression in transformed and non-transformed acinar cell lines derived from rat, as well as dispersed acini prepared from rat pancreas. To determine the role of PKC in acinar cell proliferation, the effects of PKC inhibitors on the growth of transformed and non-transformed acinar cells were also examined.

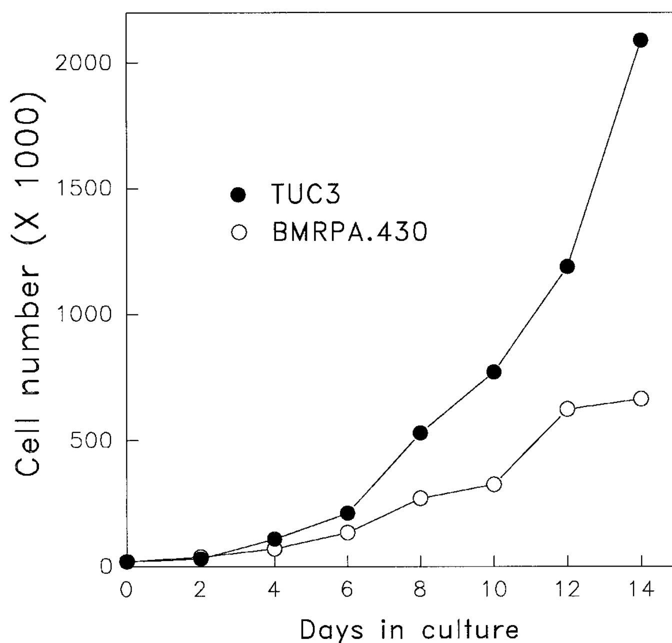


FIG. 1. BMRPA.430 (hollow circles)or TUC3 (filled circles) cells were seeded in 60 mm culture dishes at 2 X 10^4 cells per dish. On even days, up until day 14, cells from three dishes were collected and counted, and the mean number of cells per dish was determined.

MATERIALS AND METHODS

Materials. Antibodies specific for PKC isoforms α, β, γ, δ, ϵ and ζ, 1640 medium, 0.2% trypsin-EDTA, penicillin, streptomycin and HEPES were from GIBCO BRL (MD); fetal bovine serum from Atlanta biologicals (GA); antibodies specific for PKC isoforms η, θ and μ from Santa Cruz Laboratories (CA); alkaline phosphatase-conjugated antibodies, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, diaminobenzidine tetrahydrochloride, Dulbecco's PBS and trace elements for complete medium from Sigma (MO); nitrocellulose blotting membrane from Schleicher and Schuell (NH) and Go 6976 from Calbiochem (CA). CGP41 251 was a generous gift from Dr. Karl Scheibli (Basel, Switzerland).

Cell culture and growth assays. BMRPA.430 and TUC3 cells were maintained in 75-ml flasks in RPMI 1640 medium supplemented with 20 mM glutamine, 30 mM HEPES, 0.02 mg/ml bovine zinc insulin, 0.1 μg/ml hydrocortisone, 0.5 μM $ZnSO_4$, 0.1 nM $NiSO_4$, 10 nM $CuSO_4$, 1 μM $FeSO_4$, 1 nM $MnSO_4$, 0.1 μM Mo_7O_2, 0.5 μg/ml Na_2SeO_3, 50 μM $SnCl_2$, 10 μM carbacholamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin. Cells were maintained at 37°C (95% air-5% CO_2) and medium was replaced every 2-3 days.

To examine the growth characteristics of the two acinar cell lines, cells were seeded in 60- mm culture dishes at 2 X 10^4 cells per dish and fed every other day. On even days, up until day 14, cells from three dishes were collected and counted, and the mean number of cells per dish was determined.

To examine the effects of PKC inhibitors on cell growth, BMRPA.430 and TUC3 cells were seeded as above. On day 4, cells were fed media containing DMSO alone or various concentrations of CGP 41 251 or Go 6976. The cells were fed on day 6 with media containing DMSO or the appropriate concentration of the PKC inhibitor. Cells were collected and counted on day 8.

Preparation of cell lysates and subcellular fractions. Dispersed pancreatic acini were prepared from rat as previously described [16]. Dispersed acini were washed twice with PBS, then resuspended in lysing buffer (50 mM Tris-HCl, 0.15 M NaCl, 1 mM EGTA, 1% Triton X-100, 2 μg/ml leupeptin and 5 μg/ml aprotinin). Subconfluent or confluent BMRPA.430 and TUC3 cells were washed twice with PBS, then scraped into lysing buffer with a rubber policeman. Lysates were agitated at 4°C for 30 min, then sonicated (2 X 10 sec bursts) and mixed with an equal volume of SDS-gel electrophoresis buffer.

To obtain cytosolic and membrane fractions, cells or acini were suspended in lysing buffer without Triton X-100 and sonicated as above. Cell lysates were centrifuged for 40 min at 100,000 X g. The supernatant was removed and represented the cytosolic fraction. The membranes were resuspended in an equal volume of lysing buffer. Protein concentrations were determined using the BioRad protein assay (Bio-Rad, CA).

Western blotting. Approximately 100-200 μg of cell protein/lane was applied to 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose sheets and immunoblotted with antibodies specific for PKC isoforms α, β, γ, δ, ϵ, η, θ, and ζ. Nitrocellulose strips were incubated with 1% BSA-TBST (50 mM Tris-HCl [pH 7.5], 0.15 NaCl, 0.05% Tween-20) for 2 hours to block non-specific binding. The blots were incubated overnight with TBST + BSA containing PKC isoform-specific antibodies (2 μg/ml). Blots were washed 3 X 30 min with TBST + BSA and incubated with alkaline phosphatase-conjugated anti-rabbit IgG. Following extensive washing with TBST, bands were visualized with developing buffer [0.1 mM Tris (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.4 mg/ml nitroblue tetrazolium, and 0.2 mg/ml 5-bromo-4-chloro-3-indolylphosphate]. For quantitative analysis, band intensity was determined by densitometry using a XRS 6cx Scanner (Omnimedia, CA) and National Institutes of Health Image software. Immunoblots presented are representative of at least four similar experiments.

RESULTS

Growth patterns of BMRPA.430 and TUC3 cells. The growth of the two cell lines in culture was monitored for 14 days. As shown in Fig. 1, TUC3 cells grew at a

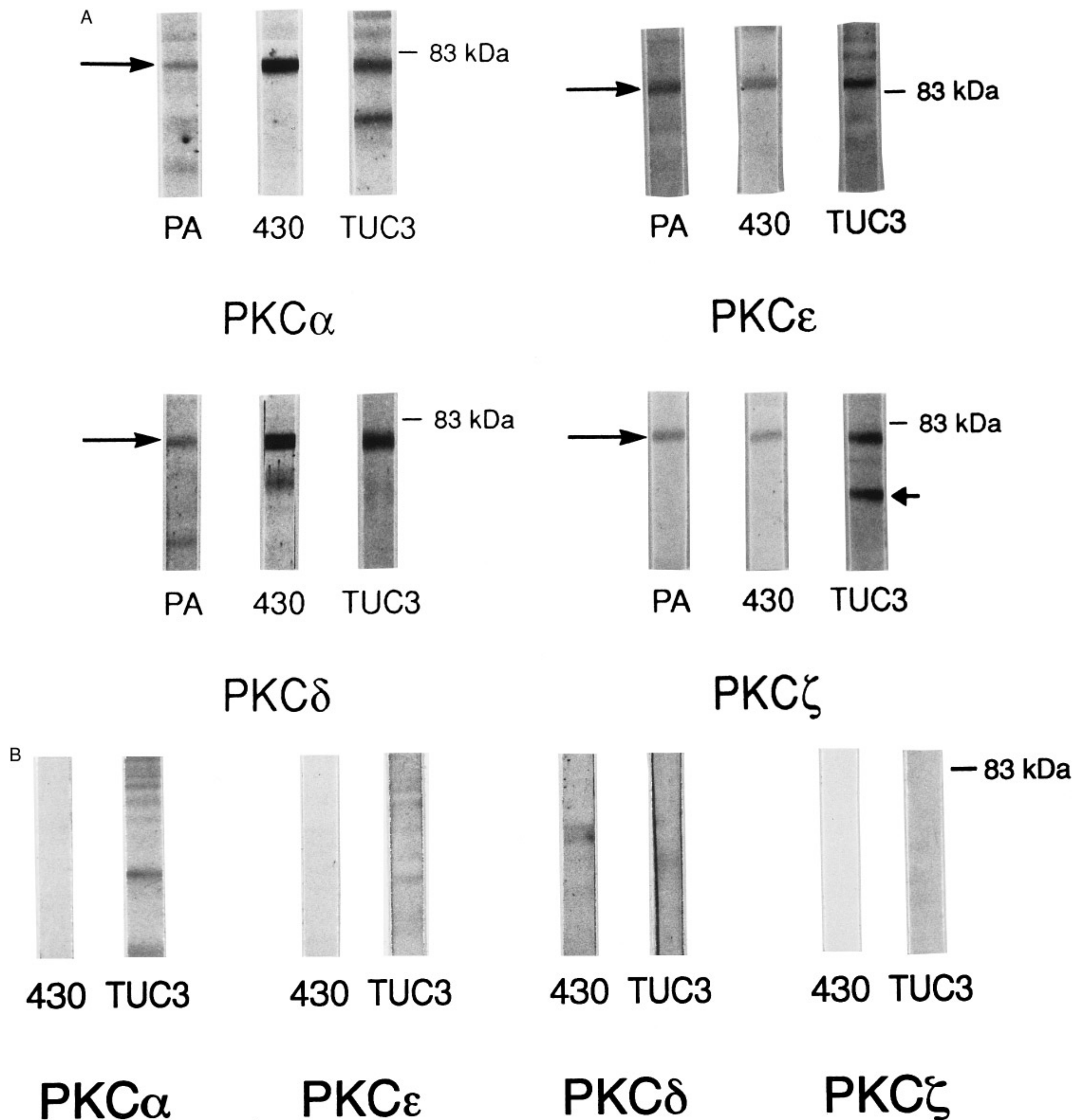


FIG. 2. (A) Immunoblotting of PKC isoforms in dispersed pancreatic acini, BMRPA.430 and TUC3 cells. Cell lysates (100 μg protein) prepared from pancreatic acini (PA), BMRPA.430 cells (430) and TUC3 cells were resolved by SDS-PAGE, electroblotted to nitrocellulose and immunostained with antibodies specific for PKC isoforms α, δ, ϵ and ζ as described in Methods. Arrows on left indicate PKC isoforms. A 51-kDa band was frequently detected in all three lysates with the PKCζ-specific antibodies and is visible here in the TUC3 lysate (short arrow on right). (B) Immunostaining of BMRPA.430 (430) and TUC3 cell lysates with PKC isoform specific antibodies preincubated with their corresponding antigenic peptide.

faster rate than BMRPA.430 cells and by day 8 TUC3 cells reached confluency, whereas BMRPA.430 cells were confluent at day 12. The doubling rates for BMRPA.430 and TUC3 cells were approx. 66 and 40 hours, respectively. TUC3 cells grew in layers and cell numbers exceeded 4-times the maximum density capacity of the culture dish by day 14, indicative of their lack of contact inhibition. TUC3 cells also possessed nuclei of varying shapes and sizes. In contrast, BMRPA.430 cells: 1) demonstrated contact inhibition; 2) did not grow past confluency; 3) retained a uniform cobblestone pattern and 4) grew as a single layer of cells.

Western blotting. The expression of PKC isoforms in the acinar cell lines as well as dispersed pancreatic acini

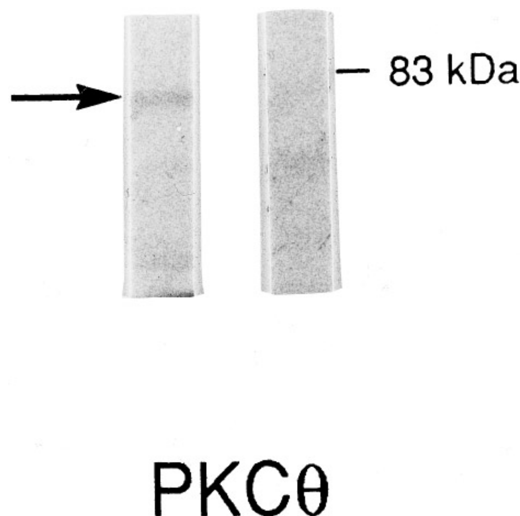


FIG. 3. Immunoblotting of PKCθ in dispersed pancreatic acini. Lysates prepared from pancreatic acini were immunoblotted for PKCθ in the presence (right) or absence (left) of its antigenic peptide as described in Methods. An approximately 80-kDa band was detected (arrow).

from rat were examined by Western blotting. As shown in Fig. 2A, a conventional PKC isoform (α), two novel isoforms (δ and ϵ) and an atypical isoform (ζ) were detected in dispersed acini, BMRPA.430 and TUC3 cell lines. Staining was not observed when antibodies were preincubated with their respective antigenic peptides, indicating specificity (Fig 2B). The approximate molecular masses for isoforms α, δ, ϵ and ζ were 80, 78, 86 and 78 kDa, respectively. The molecular masses of each of the four PKC isoforms detected did not vary among the three lysates. In addition to the 78-kDa band stained with PKCζ-antisera, a 51-kDa fragment was detected. Although it is visible only in the TUC3 lysate on the immunoblots presented in Fig. 2A (right arrow), this fragment was also detected in lysates prepared from BMRPA.430 cells and dispersed acini. Staining of the 51-kDa band detected with PKCζ-specific antiserum was blocked when the antiserum was preincubated with its antigenic peptide, indicating that it is a specific band. A low molecular weight band was also detected with PKCα-specific antisera in TUC3 cells (Fig. 2A).

In addition to the four isoforms previously detected in pancreatic acini, PKCθ, a novel isoform, was detected in dispersed acini (Fig. 3). This isoform was also detected at low levels in BMRPA.430 and TUC3 lysates (not shown).

Effect of PKC inhibitors on BMRPA.430 and TUC3 cell proliferation. We examined the role of PKC in the growth of the acinar cell lines using two PKC inhibitors. CGP 41 251, a staurosporine derivative, is a relatively selective inhibitor of PKC and inhibits PKC-dependent cellular functions *in vivo* [17-19]. Go 6976 is also a staurosporine analogue which specifically inhibits calcium-dependent or conventional PKC isoforms [20]. As described above, PKCα was the only conventional PKC isoform detected in BMRPA.430 and TUC3 cells. Hence, the use of these two PKC inhibitors in cell proliferation studies may indicate a role for one or more PKC isoform(s) in acinar cell growth and transformation.

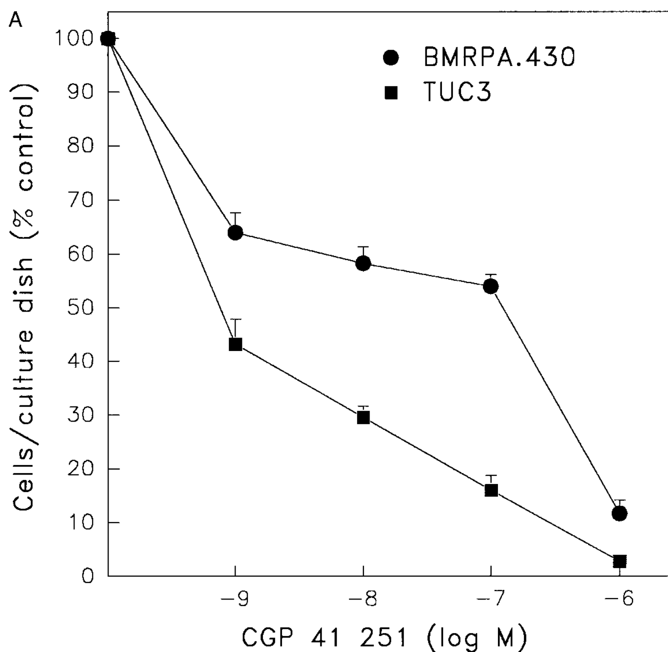
CGP 41 251 inhibited TUC3 cell growth (IC_{50} approx. 0.8 nM) with much greater potency than BMRPA.430 cells (IC_{50} >100 nM) (Fig. 4A). In fact, the dose-dependent effects of this inhibitor on BMRPA.430 cell growth appeared to be biphasic. A sharp decrease in cell number was observed at 1 nM and then at 1 μM. As shown in Fig. 4B, Go 6976 was slightly more potent in inhibiting TUC3 cell growth compared with BMRPA.430 cell growth (IC_{50} 2 nM vs. 8 nM, respectively). Moreover, inhibition of TUC3 cell growth was nearly maximal (94% inhibition) at 100 nM Go 6976, whereas BMRPA.430 cell growth was inhibited 60% at this concentration. Since in these cells the actions of Go 6976 are probably due to its inhibitory effect on PKCα, these data indicate a role for PKCα in cell proliferation of BMRPA.430 and TUC3 cells.

Subcellular localization of PKCα in dispersed pancreatic acini, BMRPA.430 and TUC3 cells. Activation of PKCα is often accompanied by its translocation from the cytosol to the membrane. To examine PKCα activation, we determined the relative levels of this isoform in cytosolic and membrane fractions prepared from dispersed pancreatic acini, BMRPA.430 and TUC3 cells. As shown in Fig. 5, the subcellular localization of this isoform was not different in dispersed acini and the two cell lines.

DISCUSSION

The results of the present study indicate that PKC isoforms α, δ, ϵ and ζ are expressed in dispersed acini prepared from rat pancreas, as well as non-transformed and transformed acinar cell lines derived from rat. In addition, a novel isoform, PKCθ, was detected at low levels in dispersed acini and both cell lines. In a previous study, the presence of PKC isoforms α and δ in dispersed acini was attributed to contamination of the preparation with pancreatic islets, since immunohistochemical studies revealed that these two isoforms were not present in pancreatic duct or acinar cells [14, 21]. Our results suggests that acinar cells do express α and δ isoforms, since these isoforms were detected in both acinar cell lines by immunoblotting. Moreover, it is unlikely that the detection of these isoforms in the acinar cell lines results from contamination with other cell types since neither insulin nor somatostatin were detected in BMRPA.430 cells by immunohistochemical methods, indicating that the cell line is not contaminated with islet cells (Bao and Michl, unpublished observations). Hence, these findings indicate that, in pancreatic acinar cells, Ras-induced transformation does not alter PKC isoform expression.

The low molecular mass band detected with PKCζ-antisera has been observed in other tissues and may result from protease activity during preparation of cell lysates, despite the presence of protease inhibitors [22, 23]. Alternately, this fragment may represent a constitutively active proteolytic fragment of PKCζ, termed PK**M**ζ, lacking the N-terminal or regulatory domain of the kinase [22]. In hamster pancreatic tissue, hamster pancreatic carcinoma cells (PC-1) and human pancreatic carcinoma cells (PANC-1), all of which express PKCζ, this fragment was not detected. However, it was detected in hamster brain lysates [15]. Clearly, further studies are required to determine the relevance, if any, of the PKCζ fragment in the growth of pancreatic acinar cells.



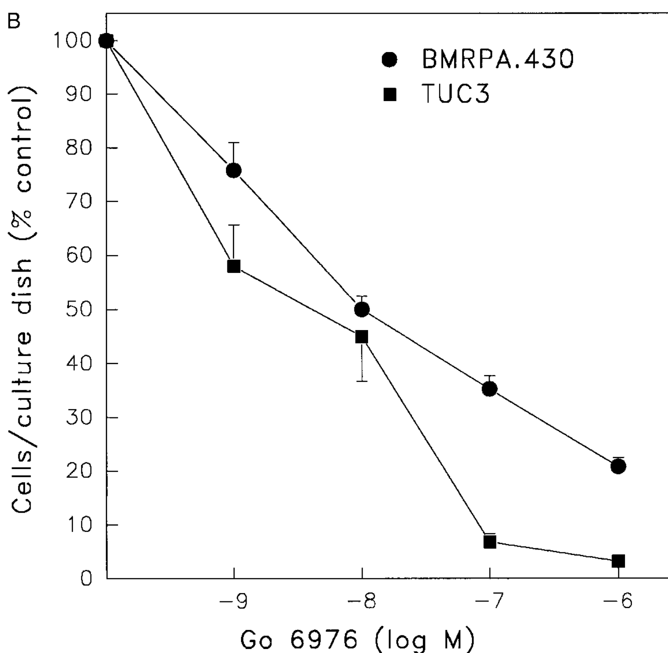


FIG. 4. Effect of PKC inhibitors on BMRPA.430 and TUC3 cell proliferation. BMRPA.430 or TUC3 cells were seeded in 60 mm culture dishes at 2 X 10^4 cells per dish. On day 4, cells were fed media containing DMSO alone or the indicated concentration of CGP 41 251 (A) or Go 6976 (B). The cells were fed on day 6 with media containing DMSO or the appropriate concentration of the PKC inhibitor. Cells were collected and counted on day 8. Values represent percentage of cells in control (DMSO only) dishes on day 8.

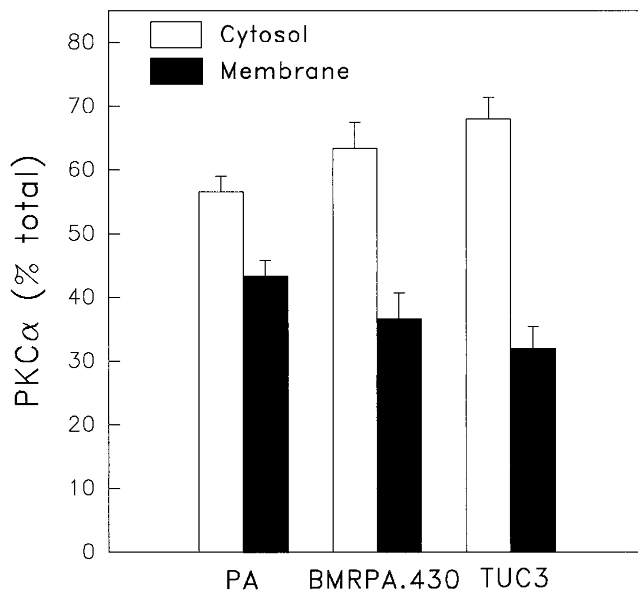


FIG. 5. Subcellular distribution of PKCα in dispersed pancreatic acini, BMRPA.430 and TUC3 cells. Subcellular fractions from BMRPA.430 and TUC3 cells were prepared and relative levels of PKCα in the membrane and cytosolic fractions were determined by immunoblotting and densitometry as described in Methods. Values represent the mean ± SEM of three experiments.

PKCθ, a novel isoform, was detected in dispersed acini, as well as BMRPA.430 and TUC3 cells, albeit at low levels. PKCθ is the major PKC isoform expressed in skeletal muscle and is also expressed in hematopoietic cells [24, 25]. In erythroleukemic cells, DMSO-induced differentiation is associated with downregulation of PKCθ, suggesting a role for this isoform in differentiation [25]. TUC3 cells are less differentiated than BMRPA.430 cells with respect to morphology and enzyme expression and, therefore, PKCθ levels in these cell lines may vary. However, low level expression of PKCθ in BMRPA.430 and TUC3 cells prevented us from comparing levels of this isoform in the two cell lines.

To determine whether PKC activity is involved in cell growth, we examined the effects of PKC inhibitors on the growth of the non-transformed and transformed acinar cell lines. The PKC inhibitor CGP 41 251 inhibits most PKC isoforms, although it has been demonstrated to preferentially inhibit conventional isoforms [18]. The biphasic effects of CGP 41 251 on BMRPA.430 cell growth may be

due to inhibition of PKC isoforms at low concentrations (<1 μM) and inhibition of other kinases at concentrations >1 μM. CGP 41 251 was more than 100-fold more potent in inhibiting TUC3 cell growth, indicating that PKC activation is an important event in TUC3 cell growth. Go 6976, a specific inhibitor of calcium-dependent PKC isoforms, was also more effective in inhibiting TUC3 cell growth versus BMRPA.430 cell growth. Taken together, these data indicate a role for calcium-dependent PKC isoforms in regulating acinar cell growth. The actions of Go 6976 are most likely due to the inhibition of PKCα, since this was the only calcium-dependent PKC isoform detected in the two acinar cell lines. Moreover, the IC_{50} values observed for BMRPA.430 and TUC3 cells with Go 6976 (8 and 2 nM, respectively) are similar to the IC_{50} value obtained for inhibiting PKCα activity *in vitro* (2.3 nM) [20].

The fact that these inhibitors are more effective in decreasing TUC3 cell growth suggests that the increased cell proliferation observed in ras-transformed acinar cells is mediated, at least in part, by an increase in PKCα activity. Translocation of PKCα to the membrane often occurs upon activation of this isoform. However, an increase in membrane-associated PKCα was not observed in either acinar cell line when compared with the subcellular localization of PKCα in dispersed pancreatic acini. Nevertheless, although the relative levels of cytosolic and membrane-associated PKCα were similar in dispersed acini and the two cell lines, it is possible that the levels of total cellular PKCα and/or PKCα activity may be upregulated in one or both cell lines. In addition, preliminary immunohistochemical studies in this laboratory indicate that PKCα is localized to the nuclei of TUC3 cells, whereas it is predominantly cytoplasmic in BMRPA.430 cells (not shown). Hence, ras-induced translocation of PKCα to the nucleus may be involved in increased acinar cell proliferation. We are presently exploring this possibility.

Consistent with our findings, elevated PKCα levels were observed in both primary and metastatic pancreatic tumors when compared with normal pancreatic tissue, and overexpression of PKCα in a human pancreatic carcinoma (HPAC) cell line resulted in a marked increase in cell proliferation, anchorage-independent growth and tumorigenicity in athymic mice [26]. Moreover, when pancreatic carcinoma cells were exposed to tumor necrosis factor-α or interferon-α, growth arrest and differentiation were associated with PKCα down-regulation [27, 28]. These findings, as well as the results from the present study, indicate a role for PKCα in acinar cell growth. Further experiments are required to determine how ras-induced transformation activates PKCα and the mechanisms by which this activation leads to increased cell proliferation. The transformed and non-transformed acinar cell lines used in the present study should serve as valuable tools for this purpose.

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