The PKCα-D294G Mutant Found in Pituitary and Thyroid Tumors Fails to Transduce Extracellular Signals

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Abstract

Protein kinase C (PKC) is a key regulator of cell proliferation, differentiation, and apoptosis and is one of the drug targets of anticancer therapy. Recently, a single point mutation (D294G) in PKCα has been found in pituitary and thyroid tumors with more invasive phenotype. Although the PKCα-D294G mutant is implicated in the progression of endocrine tumors, no apparent biochemical/cell biological abnormalities underlying tumorigenesis with this mutant have been found. We report here that the PKCα-D294G mutant is unable to bind to cellular membranes tightly despite the fact that it translocates to the membrane as efficiently as the wild-type PKCα upon treatment of phorbol ester. The impaired membrane binding is associated with this mutant's inability to transduce several antitumorigenic signals as it fails to mediate phorbol ester–stimulated translocation of myristoylated alanine–rich protein kinase C substrate (MARCKS), to activate mitogen-activated protein kinase and to augment melatonin-stimulated neurite outgrowth. Thus, the PKCα-D294G is a loss-of-function mutation. We propose that the wild-type PKCα may play important antitumorigenic roles in the progression of endocrine tumors. Therefore, developing selective activators instead of inhibitors of PKCα might provide effective pharmacological interventions for the treatment of certain endocrine tumors. (Cancer Res 2005; 65(11): 4520-4)

Introduction

Recent advances in cancer biology show that the tissue microenvironment in which tumor cells develop critically affect various steps of tumor progression (1). It is now appreciated that the neighboring stroma cells affect the rate of tumor growth, the extent of invasiveness, and the capacity of tumor dells to metastasize (1). Therefore, the microenvironment and macroenvironment surrounding the genetically aberrant tumor cells can either positively or negatively regulate the transition from early-stage tumors to invasive malignancies. The pituitary tumorigenesis is generally believed to be promoted by hormones and growth factors (2). However, pituitary cells are also under the regulation of inhibitory hormones, such as dopamine and glucocorticoids. The dysregulation of the intracellular signaling pathways and the evasion of the antitumorigenic surveillance from the tissue macroenvironment or microenvironment seem critical for the oncogenesis of endocrine tumors.

Protein kinase C (PKC) is a family of serine/threonine kinases that is involved in the control of neoplastic transformation, carcinogenesis, tumor cell invasion, and drug resistance (3). It is now appreciated that the role of PKC in tumorigenesis is complex and depends largely on the cell/tissue type and PKC isozyme involved (3). As for PKCα, the emerging consensus is that it generally plays a cytostatic role *in vivo* (3).

In 1993, a single point mutation (Asp^{294}-to-glycine) in PKCα was found in a subpopulation pituitary tumors with more invasive phenotype, suggesting a role in tumor progression (4). Subsequently, the same PKCα-D294G mutation was also found in thyroid (5) and breast cancer (6), indicating its pathogenic roles in endocrine and/or endocrine-related cancers. Although the Asp^{294}-to-glycine mutation of PKCα is implicated in endocrine tumorigenesis (2, 5), the molecular mechanisms underlying the mutation remain to be established. Here we report that the PKCα-D294G mutant is unable to bind to cellular membranes tightly and that it fails to transduce several antitumorigenic signals. This failure may contribute to the progression of endocrine tumors.

Materials and Methods

Expression constructs. Bovine PKCα in pcDNA3 was a gift from A. Toker (Harvard Medical School, Boston, MA), Myc-p42-ERK2 was from C.J. Marshall (Institute of Cancer Research, London, United Kingdom) and GFP-MARCKS was from N. Saito (Kobe University, Japan). We generated all other expression constructs using PCR-based site-directed mutagenesis, which were verified by DNA sequencing.

Cell culture and transfection. All cell lines used in this study were from American Type Culture Collection (Manassas, VA). Cell culture and transfection conditions were done as described (7).

Western analysis. The immunoblotting was done as described (7). Anti-Hsp70 was obtained from NeoMarkers (Freemont, CA), anti-flotillin-1 from BD Biosciences-PharMingen (San Diego, CA), anti-ERK1/2 from Promega (Madison, WI), anti-β-actin and anti-FLAG (clone M2) from Sigma-Aldrich (St. Louis, MO), anti-phospho-MARCKS (phosphoserine-152/3) from Abcam (Cambridge, MA), and anti-Myc (clone 9E10) from Roche Applied Science (Nutley, NJ).

Subcellular fractionation. For preparation of total cell membranes, cells, or tissues were lysed in isotonic buffer without detergent [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L EGTA, 20 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, 1 μmol/L okadaic acid, and a cocktail of protease inhibitor] by 30 strokes of a tight-fitting Dounce homogenizer. After 10 minutes centrifugation at 1,000 × *g* at 4°C, the resulting supernatant was further centrifuged at 300,000 × *g* for 1 hour at 4°C. The supernatant after the second centrifugation was designated as the cytosolic fraction, whereas the pellet was designated as total membranes.

Triton X-114 phase partitioning and other membrane protein extraction methods. Extraction of membrane proteins using precondensed Triton X-114 as well as alkali, high salt, and urea was done as described (7).

Immune-complex kinase assay. Total cell lysate or subcellular fractions were incubated with paramagnetic Dynabeads that had been coated with anti-FLAG or control antibodies. The immunoprecipitate

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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complexes were washed stringently as described (7). The PKC assay was carried out at 30°C for 10 minutes in 30 μL of kinase buffer [20 mmol/L MOPS (pH 7.4), 5 mmol/L $MgCl_2$, and a cocktail of protease inhibitors] containing 200 μmol/L [γ-^{32}P]ATP (specific activity, 2.8×10^{-4} μCi/pmol), with or without 300 μmol/L $CaCl_2$, 40 μg/mL phosphatidylserine, 108 μg/mL phosphatidylcholine, and 1.6 μg/mL diacylglycerol (prepared as multilamellar vesicles) using 250 μmol/L myristoylated alanine–rich protein kinase C substrate (MARCKS; 151-175) peptide or 6.6 μg/mL recombinant human Raf-1 protein (as a glutathione *S*-transferase fusion protein) as a substrate. All reactions proceeded linearly. The phosphorylation of peptide substrates was determined in a P81 filter assay, whereas that of the protein substrates was analyzed by SDS-PAGE followed by phosphorimaging. The correction of background and the derivation of specific kinase activity were done as described (7).

Myristoylated alanine–rich protein kinase C substrate translocation assay. CHO-K1 cells were cultured in 35-mm MatTek plates at 3×10^5 per well and transfected using PolyFectin (Qiagen, Chatsworth, CA). Forty hours post-transfection, the culture medium was changed to a HEPES buffer. Cells were exposed to 100 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and imaged using a Zeiss LSM 510 microscope. Fluorescence on the plasma membrane and cytoplasm was quantified using the "Line Scan" function in MetaMorph 5.0 (Universal Imaging, Downingtown, PA). For alternative quantification of GFP-MARCKS translocation, cells were grown in 60-mm plates. After TPA treatment for 1.5 minutes, cells were fractionated as described above. For each sample, 20% of cytosolic fraction and all of the total cellular membrane fraction were subjected to Western analysis.

p42 mitogen-activated protein kinase activation assay. COS-1 cells were cotransfected with Myc-p42 mitogen-activated protein kinase (MAPK) and PKCα expression constructs. Forty hours post-transfection, cells were treated with 400 nmol/L TPA for 20 minutes. Myc-p42 MAPK was immunoprecipitated and its activity was analyzed using myelin basic protein as a substrate as previously described (8).

Melatonin-stimulated neurite outgrowth assay. N1E-115 cells were plated at 0.25×10^5 per well in 24-well plate and transfected with PKCα expression plasmids. Six hours post-transfection, the culture medium was replaced by DMEM containing 1 nmol/L melatonin (Calbiochem, La Jolla, CA) and 15% FCS. Twenty-four hours later, cells were photographed. Cells containing at least one process greater than the soma diameter were classified as cells with neurites.

Results and Discussion

PKCα-D294G mutant retains its ability of membrane translocation but displays altered strength of membrane binding. We hypothesized that the Asp^{294}-to-glycine mutation would change the strength of interaction between the PKCα-D294G mutant and cellular membranes but not the membrane translocation itself. To test this hypothesis, we first confirmed that the PKCα-D294G mutant did translocate to membranes upon stimulation with TPA (Fig. 1*A*) as observed by others (4, 6). Although this mutant was found to have a slightly smaller membrane pool than that of the wild-type PKCα at the basal state, it reached the same membrane pool size as the wild-type PKCα upon stimulation with TPA. Next, we adopted the Triton X-114 phase partitioning procedure to remove loosely bound proteins from membranes by allowing tightly bound membrane proteins to partition into the detergent-rich phase, whereas loosely bound membrane proteins partition into the aqueous phase. Surprisingly, some wild-type PKCα but none of the PKCα-D294G mutant was found in the detergent-rich phase of Triton X-114 extraction (Fig. 1*B*, *top two*) by Western blotting in which flotillin-1, a marker for tightly membrane-bound proteins and heat shock protein 70 (Hsp70), a marker for cytosolic proteins were used as a positive and a negative control, respectively. We further established that the tight membrane binding of PKCα was not conferred by the negative charge of side chain of amino acid at

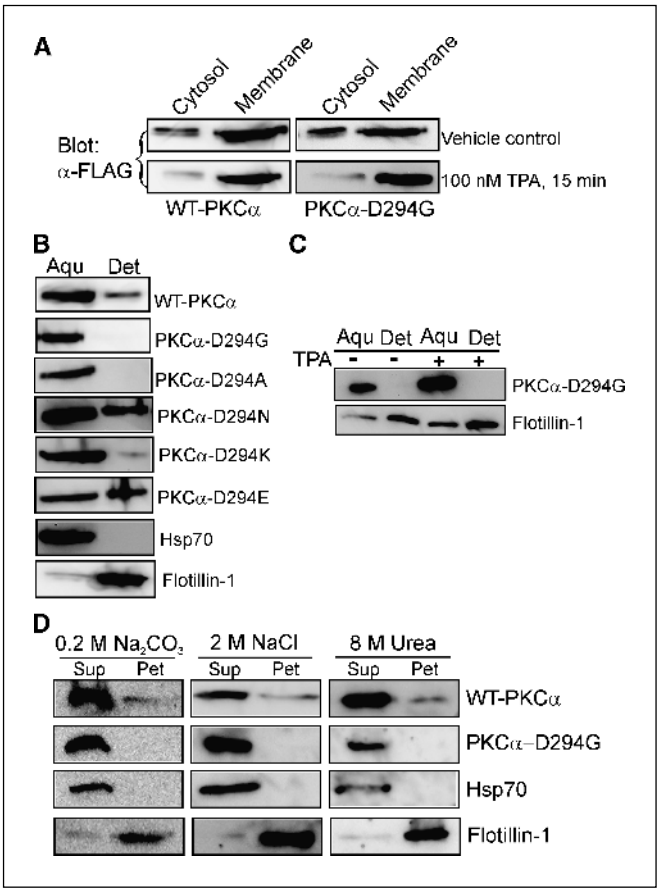

Figure 1. PKCα-D294G mutant translocates to the membrane upon treatment of TPA but displays altered membrane-binding strength. *A,* TPA-elicited translocation of FLAG-PKCα from cytosol to membrane fraction was determined by subcellular fractionation of transiently transfected COS-1 cells followed by Western analysis. Vehicle control: 0.01% (v/v) DMSO. *B,* PKCα-D294G does not bind to membranes tightly as the wild-type counterpart. COS-1 cells transiently transfected with indicated expression constructs of FLAG-PKCα were subject to Triton X-114 phase partitioning followed by Western analysis using antibodies to FLAG-tag or marker proteins. Approximately 17% of the aqueous phase and all of the detergent-rich phase were loaded for Western analysis. *C,* PKCα-D294G mutant does not bind to the membrane tightly even after the treatment of TPA. Experiments were performed as in (*B*) except cells were treated with 100 nmol/L TPA or vehicle as a control for 20 minutes before Triton X-114 phase partitioning. *D,* PKCα-D294G mutant does not bind to the membrane tightly as analyzed by extractions with alkali, high salt, and urea. All pellet and ~20% of the supernatant in each sample were used in Western analysis. FLAG-tagged PKCα was detected by anti-FLAG antibody. Data are typical of at least three to five independent experiments. *Aqu,* aqueous phase; *Det,* detergent-rich phase in Triton X-114 phase partitioning; *Sup,* supernatant; *Pet,* pellet.

position 294 as we observed that PKCα mutants (D294N and D294K) carrying a substituted residue that is either uncharged or positively charged were still found in the detergent-rich phase (Fig. 1*B*, *fourth* to *fifth*). As the PKCα-D294A mutant also lost its capacity to bind membrane tightly (Fig. 1*B*, *third*), it seems that the size and the polarity of the side chain of amino acid residue at position 294 are critical for the tight membrane interaction of PKCα. Treating cells with TPA did not restore the tight membrane binding of PKCα-D294G mutant as shown by its absence in the detergent-rich phase of Triton X-114 extraction (Fig. 1*C*). We verified the results from phase partitioning by employing additional three procedures that use distinct underlying biochemical principles to remove loosely bound membrane proteins from biological membranes. Extractions by 0.2 mol/L sodium carbonate (pH 11.5), 8 mol/L urea, or

2 mol/L NaCl removed all PKCα-D294G from membranes, but some wild-type PKCα remained in the pellet fraction (Fig. 1*D*). Thus, one of the major cell biological defects of the PKCα-D294G mutant is the loss of tight membrane binding even upon treatment of TPA, consistent with the proposition by others that this mutant may have reduced stability of membrane association (5, 9).

The PKCα-D294G mutant fails to elicit 12-*O*-tetradecanoylphorbol-13-acetate–induced phosphorylation and cytoplasmic translocation of myristoylated alanine–rich protein kinase C substrate. MARCKS, a widely expressed protein involved in the regulation of cell motility and membrane traffic, has been implicated in tumor suppression (10). Upon phosphorylation by PKC *in situ* at the plasma membrane, MARCKS translocates to the cytosol. To test the possibility that the PKCα-D294G cannot transduce extracellular signals, we used a system in which the PKC-dependent phosphorylation and displacement of MARCKS from the plasma membrane and its subsequent cytoplasmic translocation are studied using a GFP-MARCKS construct in CHO-K1 cells (11). We first examined the ability of the PKCα-D294G to phosphorylate a MARCKS peptide and found that this mutant was able to phosphorylate the MARCKS peptide as efficiently as the wild-type PKCα in the test tube (Fig. 2*A*). Hence, the Asp^{294}-to-glycine mutation does not affect the ability of PKCα-D294G to recognize or phosphorylate MARCKS. Next, we transiently cotransfected CHO-K1 cells with expression constructs for GFP-MARCKS (11) and wild-type PKCα or PKCα mutants. After treatment with 100 nmol/L TPA for 1.5 minutes, only wild-type PKC could initiate a rapid translocation of membrane GFP-MARCKS to the cytoplasm with similar kinetics as reported by others (11). However, there was minimal increase in the cytoplasmic translocation of GFP-MARCKS in cells cotransfected with PKCα-D294G mutant (a few percentage increase compared with ~150% increase in cells cotransfected with wild-type PKCα), as determined by fluorescence confocal microscopy (Supplementary Fig. S1). The results of morphologic analysis were confirmed by cell fractionation followed by Western blotting (Fig. 2*B-D*). We further assessed the phosphorylation status of MARCKS our Western analysis using a

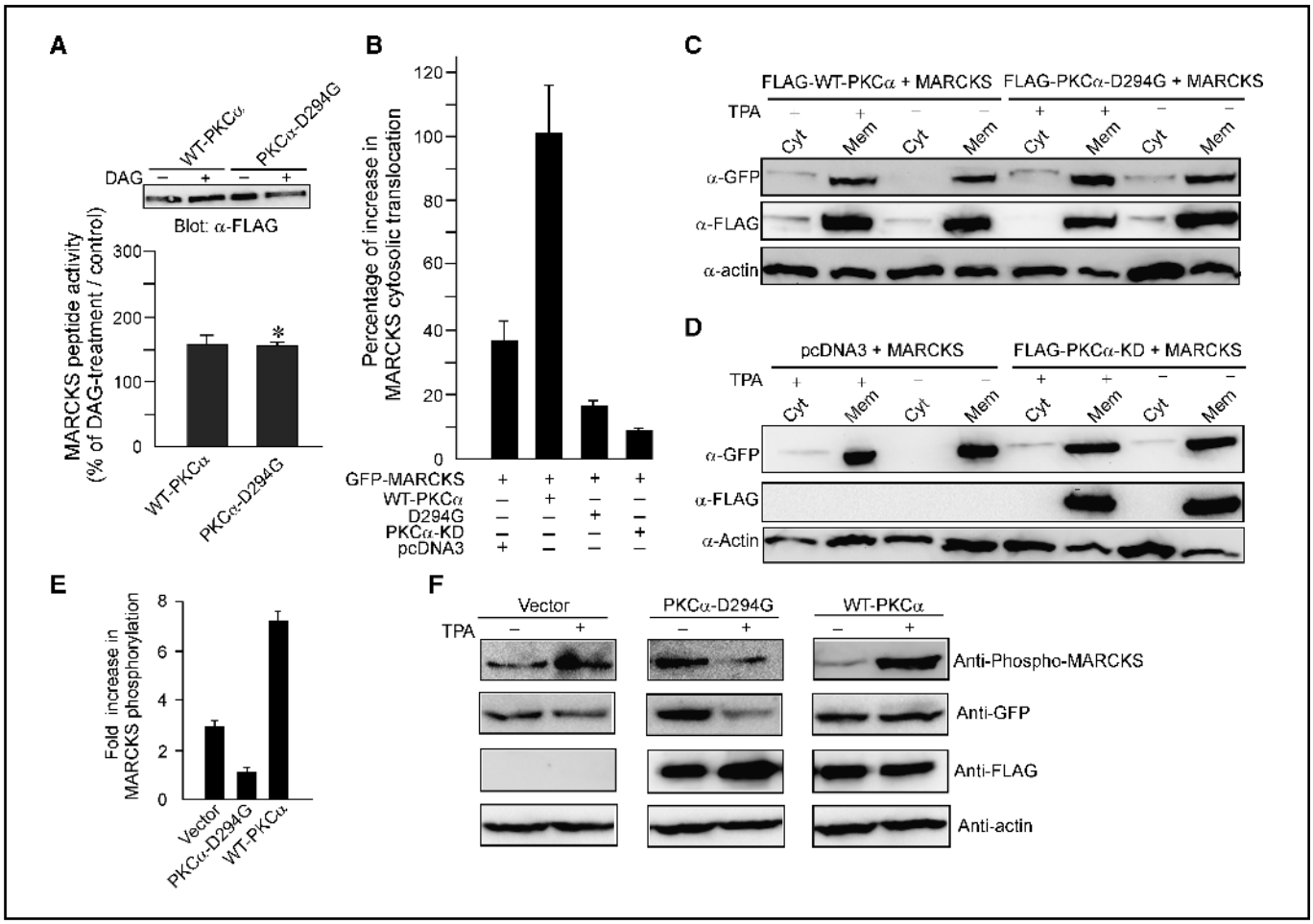

Figure 2. PKCα-D294G mutant fails to mediate TPA-elicited cytoplasmic translocation of MARCKS. *A,* catalytic activities of PKCα-D294G towards an MARCKS peptide are not compromised as determined in immunocomplex kinase assay without or with DAG and cofactors using a MARCKS peptide as a substrate. A representative Western blot used to determine the specific activity is shown. The PKC activity is expressed as a percentage of activation by DAG/calcium compared with that of the control. *B-D,* PKCα-D294G failed to initiate cytoplasmic translocation of MARCKS. GFP-MARCKS and the indicated FLAG-tagged PKCα expression constructs were cotransfected into CHO-K1 cells. After treatment with 100 nmol/L TPA for 1.5 minutes, cells were fractionated before Western analysis with antibodies to GFP (for MARCKS), FLAG-tag, and β-actin. Translocation of MARCKS to the cytosol (*B*) was quantified through Western analyses (*C-D*). *E-F,* no increase of phosphorylation of MARCKS cotransfected with PKCα-D294G in cells after TAP treatment. CHO-K1 cells were transfected and treated with TPA as in (*B-D*). The postnuclear cell lysate was concentrated using a chloroform/methanol precipitation method and subjected immunoblotting with a polyclonal antibody against MARCKS phosphorylated at Ser^{152}/Ser^{153}. *E,* quantification of increase in MARCKS phosphorylation after TPA treatment. *F,* a representative Western analysis used to derive (*E*) in which anti-GFP and anti-FLAG were used to determine the mass of GFP-MARCKS and FLAG-PKCα, respectively. Anti-actin was used as a loading control. *Cyt,* cytosolic fraction; *Mem,* total membrane fraction; *KD,* kinase dead. *Columns,* means of three independent experiments; *bars,* ±SE. *, $P > 0.05$ compared with WT-PKCα.

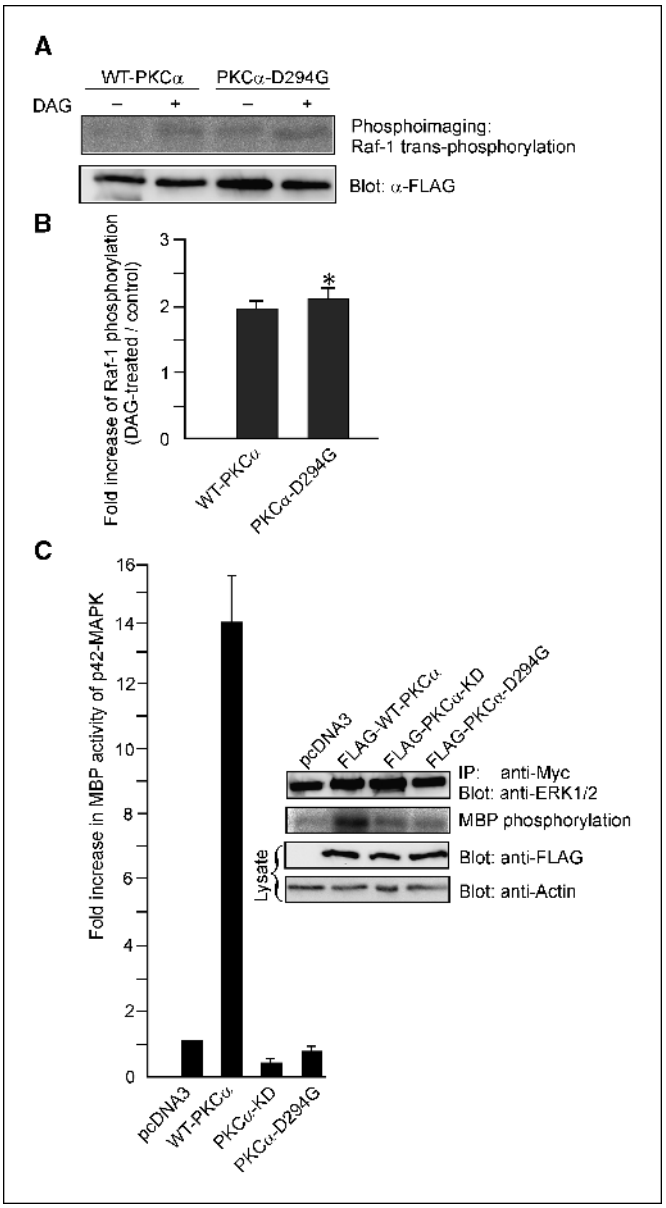

Figure 3. PKCα-D294G is unable to activate p42-MAPK in cells. *A-B,* PKCα-D294G is competent in phosphorylating Raf-1 as the wild-type counterpart *in vitro* as determined in an immunocomplex kinase assay with or without DAG and cofactors. *A,* a typical phosphorimaging graph for quantifying phosphorylation of Raf-1 protein that had been resolved by SDS-PAGE. *B,* the PKC activity is expressed as fold of activation in the presence of DAG compared with that of the control. *C,* PKCα-D294G is unable to activate p42-MAPK in cells as determined in an immunocomplex kinase assay with MBP as a substrate. A representative autoradiograph (via phosphorimaging) of phosphorylated MBP that had been resolved by SDS-PAGE (*second*), along with Western blots to derive the specific activity of immunoprecipitated p42-MAPK (*top*) and to control for PKCα expression and protein content (*bottom*). *MBP,* myelin basic protein. *Columns,* means of three independent experiments; *bars,* ±SE. *, $P > 0.05$ compared with WT-PKCα.

phosphospecific antibody against MARCKS. Notably, in cells cotransfected with wild-type PKCα, there was at least a 7-fold increase in MARCKS phosphorylation upon stimulation of TPA, whereas no increase of MARCKS phosphorylation in cells cotransfected with PKCα-D294G mutant was observed (Fig. 2*E* and *F*), confirming that PKCα-D294G could not phosphorylate MARCKS in cells upon TPA treatment. We conclude that despite the fact that there is no defect in PKCα-D294G's ability to phosphorylate MARCKS in the test tube, in cells, this mutant fails to induce PKC phosphorylation-dependent cytoplasmic translocation of MARCKS upon stimulation of extracellular signals. It is conceivable that this mutation in PKCα may disrupt the response of the cells harboring the mutant to extracellular tumor suppressive signals that activate cytostatic PKCα (1, 3, 10).

The PKCα-D294G mutant fails to activate mitogen-activated protein kinase upon treatment of 12-*O*-tetradecanoylphorbol-13-acetate in cells. Traditionally, the aberrant activation of extracellular-regulated kinase (ERK) was linked to cell proliferation and survival. However, it is now known that prolonged stimulation of ERK leads to differentiation and apoptosis (12). In prostate and

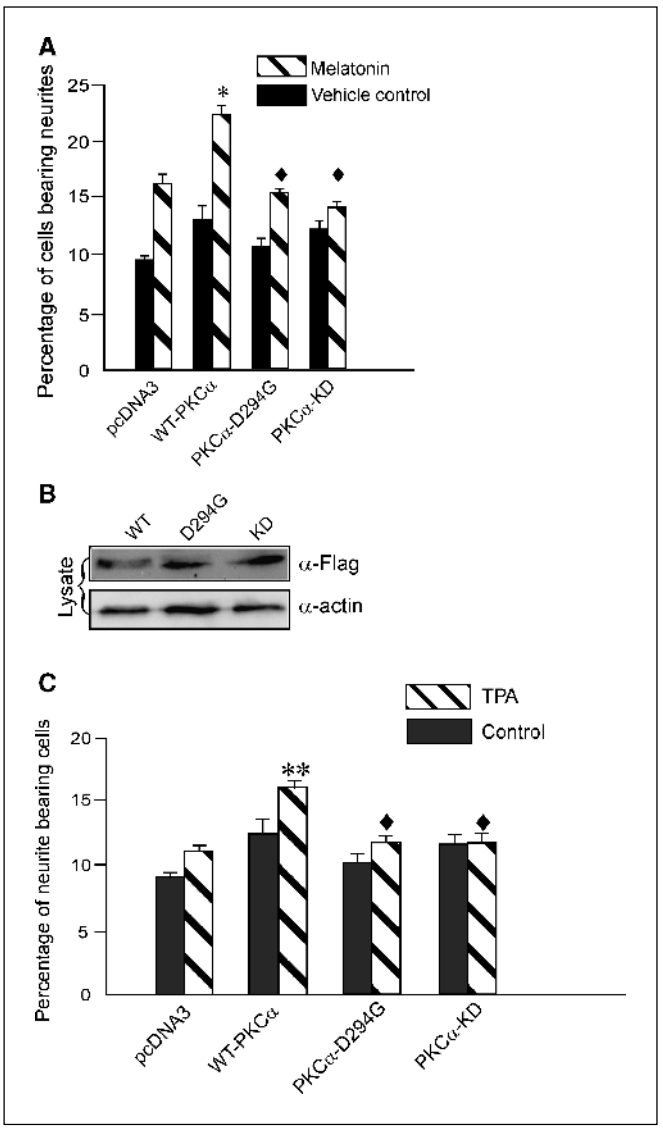

Figure 4. PKCα-D294G is unable able to augment melatonin-stimulated neurite outgrowth in N1E-115 cells. *A,* N1E-115 cells transfected with the indicated FLAG-PKCα constructs or empty vector control were treated with 1 nmol/L melatonin for 24 hours and photographed for analysis of cell morphology. *B,* a representative Western of total cell lysates for the verification of expression of exogenous FLAG-PKCα. *C,* PKCα-D294G fails to augment neurite outgrowth in N1E-115 neuronal cells after TPA treatment. Six hours post-transfection, the culture medium was changed to 15% FCS. Twenty-four hours later, cells were treated with 100 nmol/L TPA for 20 minutes and photographed. *Columns,* means of three to six independent experiments; *bars,* ±SE. *, $P < 0.001$ compared with vector control; **, $P < 0.05$ compared with the empty vector control; ♦, $P > 0.05$ compared with the control.

pancreatic cancer cells, the activation of MAPK via PKCα results in increased p21(waf/cip1) and subsequent growth arrest and induction of apoptosis (13, 14). In colorectal cancer, the activation of ERK via PKCα leads to decreased tumor cell proliferation as well as increased tumor cell differentiation and apoptosis (15). Therefore, under certain conditions, ERK1-2 can play antiproliferation and proapoptotic roles. By debilitating the growth inhibitory and proapoptotic signal transduction via the activation of ERK pathway, the pituitary and thyroid tumor cells with the PKCα-D294G mutation can continue to progress toward a more malignant phenotype. To examine the effect of Asp^{294}-to-glycine mutation on signal transduction, we studied the ability of PKCα-D294G in the activation of MAPK via Raf-1 in COS-1 cells (8). As in the case of MARCKS phosphorylation, the PKCα-D294G mutant was found to phosphorylate a recombinant human Raf-1 protein as well as the wild-type PKCα could in the test tube (Fig. 3*A* and *B*). Consistent with previous findings by others (8), we found that the wild-type PKCα activated cotransfected Myc-p42 MAPK after TPA treatment by ~12-fold in COS-1 cells. In contrast, PKCα-D294G behaved as the catalytically inactive PKCα mutant in its inability to activate MAPK under the same experimental conditions (Fig. 3*C*), presumably due to its altered strength of membrane interaction. In the context of pituitary tumors, the levels of phosphorylated ERKs are drastically reduced in pituitary tumors in mice lacking the dopamine D2 receptor (16). The activation of PKC by TPA induced a blockage of cell proliferation (16). Thus, the PKCα-D294G mutant could segregate the transduction of extracellular signals leading to the activation of MAP kinase that is pivotal in the negative regulation of cell proliferation and in promoting apoptosis when activated by the PKCα pathway (13).

PKCα-D294G mutant fails to augment melatonin-stimulated neurite outgrowth. At physiological concentrations, melatonin can inhibit cancer cell proliferation and reduce metastasic capacity, whereas at pharmacological concentrations, melatonin exhibits cytotoxic activities and induces apoptosis (17). In neuronal cells, melatonin activates PKCα via the ERK pathway (18) and elicits the translocation of PKCα to membranes as well as induction of differentiation in the form of neurite outgrowth (19). Therefore, we examined whether the Asp^{294}-to-glycine mutation in PKCα would result in communication breakdowns between cells with this mutation and extracellular antitumorigenic signals using a cellular model of melatonin-induced neurite outgrowth. Consistent with previous findings (19), we found that the neurite outgrowth in N1E-115 was augmented by the wild-type PKCα ($P < 0.001$ compared with empty vector; Fig. 4*A* and *B*). The PKCα-D294G mutant, on the other hand, lost its ability to mediate melatonin-stimulated neurite outgrowth and was comparable with the empty vector or the kinase-dead (K368R) PKCα mutant (Fig. 4*A*). To verify that the activation of PKC is responsible for neurite outgrowth, we treated N1E-115 cells transfected with PKCα constructs with 100 nmol/L TPA for 20 minutes and obtained comparable results (Fig. 4*C*). Because the deregulated cytoskeleton dynamics of cells often lead to tumor invasion and metastasis and because melatonin plays important roles in cancer inhibition, the PKCα-D294G mutant may confer invasive advantages to the host pituitary/thyroid tumors by secluding the tumor cells from extracellular oncostatic signals such as melatonin (17).

It is noteworthy that the PKCα-D294G mutant is found only in tumor cells with invasive behavior and elevated PKCα mutant protein levels but not found in the adjacent normal tissues (4, 5). From our findings, it seems that PKCα-D294G mutant can influence the progression of endocrine tumors by severing the transduction of extracellular signals that are pivotal in the suppression of tumor growth and in the enhancement of apoptosis of cancer cells. It can also be speculated that because PKC is known to have a variety of binding partners in cells (20), the overexpressed mutant PKCα-D294G protein can interfere with intracellular homeostatic mechanisms through sequestering other signaling proteins. Our study also implicates that the indiscriminate inhibition of PKC is not an ideal approach to the treatment of cancer. Isozyme-specific and cell type– and/or tissue-specific modulation (including the activation) of PKC may constitute a novel strategy in signal transduction therapy of cancers.

Acknowledgments

Received 12/20/2004; revised 3/10/2005; accepted 4/5/2005.

Grant support: National Medical Research Council (Ministry of Health, Singapore) and Biomedical Research Council, Singapore.

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We thank Dr. Y. Yuan for editing the article.

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Cancer Res 2005;65:4520-4524.