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GH3B6 Pituitary Tumor Cell Proliferation is Mediated by PKCα and PKCε via ERK 1/2-dependent Pathway

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Key Words
Pituitary • Cell proliferation • PKCα • PKCε • ERK 1/2

Abstract
Background: In this report, we explored the role of PKCα and PKCε as mediators of phorbol 12-myristate13-acetate (PMA)-induced proliferation in pituitary tumor GH3B6 cells, and determined if the ERK1/2 and Akt pathways were activated. Methods: The GH3B6 cell proliferation was estimated by BrdU incorporation and the cell cycle progression by flow cytometric cell cycle analysis. We determined the expression of PKCα and PKCε in membrane and cytosolic fractions by western blotting. The subcellular redistribution of both PKC isozymes was analyzed by confocal microscopy. Results: Incubation with PMA for 15 min stimulated PKCα and PKCε activation, which was correlated with the phosphorylation of ERK1/2 but not Akt. The activation of both these PKC isozymes was closely associated with the stimulation of proliferation and the cell cycle progression induced by PMA in GH3B6 cells, an effect that was blocked by the inhibitors of PKCα (Gö6976) and PKCε (εV1-2). In addition, the pretreatment with the inhibitor of ERK1/2 (PD98059) prevented the mitogenic activity induced by treatment with PMA for 15 min. Conclusion: We demonstrated that the activation of PKCα and PKCε by phorbol ester in tumor pituitary GH3B6 cells led to cell proliferation and cell cycle progression, effects that involved ERK1/2 activation.

Introduction

Pituitary adenomas are common benign monoclonal neoplasms accouting for approximately 15 % of intracranial tumors [1]. Although most are manageable or have clinically indolent courses, several subset of the tumor can cause significant morbidity and early mortality [2]. In recent years, the demonstration that pituitary adenomas are monoclonal in origin has provided further evidence that pituitary neoplasia arises from the replication of a single mutated cell, in which growth results from either inappropriate expression, the activation of proto-oncogenes, or the inactivation of tumor suppressor genes

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[3]. The common protooncogenes that have been analyzed in pituitary tumors include the proteins involved in proliferative signal transduction, such as Protein Kinase C (PKC) [4]. The PKC family is comprised of 10 known isozymes, which display ubiquitous, tissue or cell type specific expression, and play crucial roles in signal transductions related with cell proliferation [5], apoptosis [6], cell differentiation [7], and hormone release [8]. Moreover, in recent years it has become evident that PKCs can impact on the cell cycle in either a positive or negative way, depending on the cell type and isozyme specificity [9]. Indeed, PKC isozymes have been shown to regulate the progression of cells from G_1 to S phase as well as the transition from G_2 to M phase [10].

PKC isozymes have been grouped into three subclasses, according to their regulatory properties, which are defined by specific domains in the proteins. The conventional or classical PKCs include PKCα, βI, βII, and γ, with these isozymes being activated by Ca^{2+} and/or by diacylglycerol (DAG) and phorbol esters. The novel PKCs, δ, ε, θ, and η, which are Ca^{2+} independent, can also be activated by DAG and phorbol esters. Finally, the atypical PKCs, which include PKCζ and PKCι, are unresponsive to both Ca^{2+} and DAG/phorbol esters [11]. PKCs are the main mediators for the phorbol ester tumor promoters, such as phorbol 12-myristate 13-acetate (PMA), with individual PKC isozymes displaying differential sensitivity [12]. Depending on the cell type, various inactive cytoplasmic PKC isozymes may, upon phorbol ester activation, associate with different subcellular membranes [13, 14]. Recently we have demonstrated that short PMA stimulus was able to induce PKC isozymes translocation to different membrane compartments in normal and tumoral lactotroph cells [15, 16].

Depending on the cell type and the stimulus, the PKCs may mediate some of their cell effects through the activation of the extracellular-signal regulated kinase (ERK) 1/2 [17]. This MAPK is ubiquitously expressed in mammalian cells and is implicated in the regulation of cell proliferation as well as in cell death [18, 19]. Moreover, there is evidence that in various cell types, the ERK/MAPK pathway can be activated in either a PKC-dependent or PKC-independent manner in response to different stimuli [20, 21], with PKC also being reported to collaborate in the activation of the Akt pathway [5]. In addition, Akt was shown to act downstream of phosphoinositide 3-kinase (PI3-K) and play a critical role in cell survival and oncogenesis [22].

Although PKCs have a clear role in tumorigenesis, it has been a challenging task to determine the relative contribution of the individual isozymes, in order to define their specific roles in this process [23]. PKCε seems to be involved in tumor development, tumor cell invasion, and metastasis in several tissues [24]. Also, PKCα has been described to increase the cell proliferation in tumor cells, with this effect being mediated by ERK1/2 activation [25]. Therefore, a greater knowledge of the roles of the individual PKC isozymes in tumor development is necessary before being able to propose the use of PKC as a therapeutic target.

PKCα, β, δ, ε, ζ and θ have been shown to be expressed in the pituitary gland as well as in adenoma GH3 cells [12]. Also, in a previous report, we demonstrated that the activation of PKCε by phorbol ester was able to induce a proliferation of lactotroph cells from a primary pituitary culture through ERK1/2 activation [15]. In adenomatous pituitary cells, it has been observed that the enzyme activity and expression of PKCs were higher than in normal human or rat pituitaries. Furthermore, these levels were significantly higher in invasive tumors than in non-invasive tumors [26]. However, the role the specific isozymes of PKC and which signalling pathways are involved in tumorogenesis and cell proliferation of pituitary adenoma still need to be resolved.

The aim of the present work was to explore the role of PKCα and PKCε as mediators of PMA-induced proliferation in the pituitary tumor GH3B6 cell, and to determine if the ERK1/2 and Akt pathways are activated.

Here, we demonstrated that the activation of PKCα and PKCε by phorbol ester in pituitary tumor GH3B6 cells led to cell proliferation and cell cycle progression, effects that involved ERK1/2 activation.

Methods and Materials

Cell culture

The rat GH3B6 lacto-somatotroph pituitary tumor cell line, that secretes high levels of prolactin, was cultured in HAM-F12 medium supplemented with 5% fetal calf serum and 12% horse serum (Invitrogen; Carlsbad, USA), in an oven with a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. For cellular proliferation assays the cells were seeded on glass coverslips (13 mm) and placed at the bottom of 24-well culture plates (Corning, New York). For the other techniques, the cells were plated on 6-well culture plates (Corning, New York, USA), and the culture medium was filtered through 0.2 μ Nalgene membranes (Nalge Company; New York, USA). All cell culture grade reagents were obtained from the Sigma (St Louis, USA). After 3 days of culture and a confluence of 70%, the cells were submitted to different experimental protocols.

Cell treatments

Phorbol ester. Phorbol 12-myristate 13-acetate (PMA, 400 nM; Sigma; St Louis, USA) was solubilized in DMSO before being added to cell media for a short time: 15 min or for a long time: 3 h.

PKC Inhibitors. The pan- PKCs inhibitor Bisindolylmaleimide I (BIM, 4 μM; Sigma, St Louis, USA) [27]; the inhibitor of calcium-dependent PKC Gö6976, used to inhibit PKCα isozyme (5 μM; Calbiochem, San Diego, USA) [28, 29] or the PKCε translocation inhibitor peptide Myr-PKC εV1-2 (εV1-2, 50 and 100 μM; Biomol, Plymouth Meeting, USA) [30], were added separately for 30 min. Then, the cells were washed, and incubated with 400 nM of PMA for an additional 15 min in HAM-F12.

Specific inhibitor of ERK1/2

The inhibitor of mitogen-activated kinase effector kinase (MEK) PD98059 (Calbiochem, San Diego, USA) [31] was used to inhibit ERK1/2 activation. The cells were treated with PD98059 (50 and 100 μM) for 30 min, washed, and incubated with 400 nM of PMA for 15 min.

At the end of each experimental condition, the cells were processed for 5-bromo-2′-deoxyuridine (BrdU) detection, and subjected to western blotting and cell cycle analyses by flow cytometry.

Bromodeoxyuridine incorporation

The immunocytochemical detection of BrdU was used to label proliferating cells in the S-phase of the cell cycle, according to Oomizu and co-worker [32] with modifications [33]. After applying the different experimental protocols mentioned above, the culture medium was replaced and BrdU (3 mg/ml) was added for 3 h. The cells attached to the coverslips were fixed with 4 % formaldehyde in PBS for 30 min at room temperature (RT), washed in PBS, and permeabilised with 0.5 % Triton X-100 for 10 min. Non-specific immunoreactivity was blocked with 1 % PBS-BSA for 30 min at RT, and the cells were incubated overnight with a monoclonal antibody against BrdU (Amersham; Buckinghamshire, England) at 4 °C in a wet chamber. After washing in PBS, the cells were incubated with biotinylated anti-mouse IgG diluted 1:100 for 30 min at room temperature (GE Healthcare; Buenos Aires, Argentina). The coverslips were washed again with PBS, and incubated with the avidin-biotin-peroxidase complex (ABC; Vector; Burlingame, USA). The immunoreactivity for BrdU was visualized with 3,3 - diaminobenzidine tetrahydrochloride (DAB) as a chromogen, and the nuclei were stained brown. The coverslips were then mounted on glass slides with glycerol. Controls were performed by applying the same protocol, but omitting the BrdU antibody.

A total of 1 000 immunoreactive cells were examined by light microscopy in randomly chosen fields of each glass slide, in order to establish the percentage of immunoreactive pituitary cells for BrdU. Three slides were analyzed for each group, derived from the same cell preparations. Experiments were replicated at least three times with separate batches of cell preparations.

Subcellular fractionation

In order to obtain cytosolic and membrane fractions, the GH3B6 cells were washed in ice-cold PBS, and then harvested by scraping with hypotonic Hepes (HHB) buffer [10 mM HEPES (pH 7.4), 5 mM MgCl2, and 40 mM KCl] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cell homogenates were centrifuged at 100 000 g for 45 min at 4 °C with the supernatants collected corresponding to the cytosolic fraction. The pellet was resuspended in HHB supplemented with 1% (v/v) Triton X 100, followed by a brief sonication, and then designated as the membrane fraction.

Western Blot analysis

The total protein concentration was measured using a BioRad kit (Bio-Rad Protein Assay; Bio-Rad; Hercules, USA). Thirty micrograms (30 μg) from the total homogenate and subcellular fractions were run in 12 % polyacrylamide gel (Sigma; St Louis, USA). Then, to estimate the corresponding molecular weights, the Full Range Rainbow Molecular Weight Marker was used (Amersham; Buckinghamshire, England). Proteins were transferred to a nitrocellulose membrane, and nonspecific binding was blocked with PBS containing 5 % nonfat dried milk, 0.1 % Tween 20 (blocking buffer) at RT. The membranes were rinsed and incubated for 2 h with the following appropriate primary antibodies: PKCα rabbit polyclonal antibody (1:300), PKCε rabbit polyclonal antibody (1:300), total ERK1 rabbit polyclonal antibody (1:200) (Santa Cruz Biotechnology; Santa Cruz, USA), phosphorylated ERK 1/2 mouse monoclonal antibody (1:700) (Sigma; St Louis, USA), total Akt rabbit polyclonal antibody (1:300) (Santa Cruz Biotechnology; Santa Cruz, USA) and phosphorilated Akt rabbit polyclonal antibody (1:300) (Santa Cruz Biotechnology; Santa Cruz, USA). The blots were incubated with a peroxidase-conjugated (HRP) goat anti-rabbit secondary antibody (Jackson, West Grove, USA) and goat anti-mouse secondary antibody (Pierce; Rockford, USA), diluted in a blocking buffer (1:5000). The membranes were thoroughly rinsed in PBS/0.1 % Tween-20, and the HRP-coupled secondary antibody was revealed with ECL western blot detection reagents (Amersham; Buckinghamshire, England) following the manufacturer's instructions. Emitted light was captured on Hyperfilm (Amersham; Buckinghamshire, England), with signals being scanned and quantified with Scion Image software (V. beta 4.0.2, Scion Image Corp., Frederick, MD, USA) at three different exposure times. The β-actin expression (1:5000; mouse monoclonal antibody; Sigma; St Louis, USA) was used as an internal control to confirm the equivalent total protein loading.

Flow Cytometric Cell Cycle Analysis

GH3B6 cells were harvested, washed in PBS, fixed with 70% ethanol, washed again with citrate-phosphate buffer at pH-7.8, and incubated with RNAse (10 μg/μl) (Ribonuclease A, Sigma R5503; St Louis, USA). This was followed by DNA staining with propidium iodide (50 μg/ml) (Sigma P4170; St Louis, USA) in order to analyze the cellular DNA content. Cell cycle analysis was performed on a Coulter flow cytometer

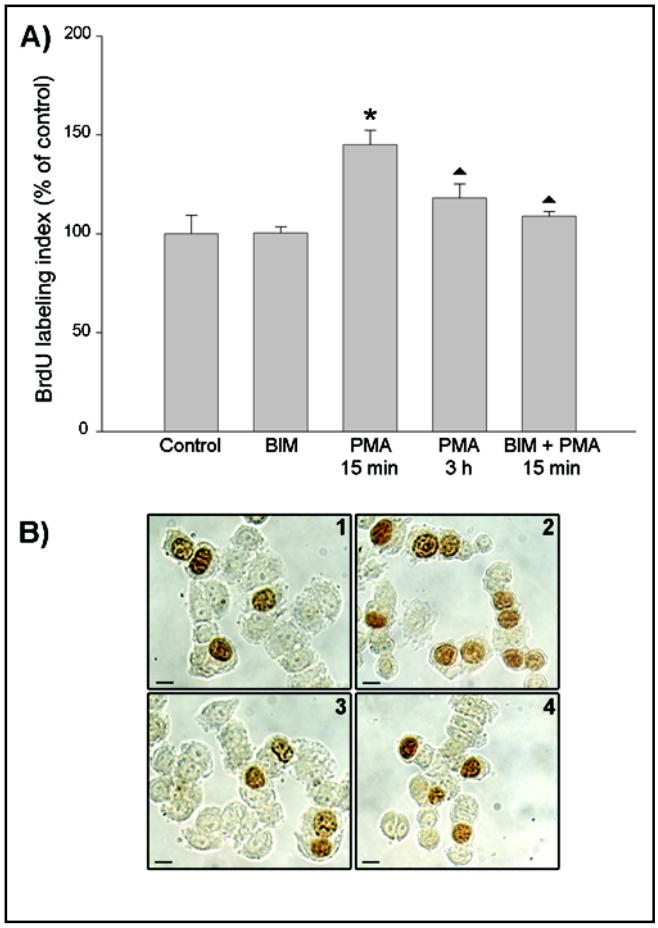

Fig. 1. Effect of PMA on GH3B6 cell proliferation. These cells were treated with PMA (400 nM) for 15 min or 3 h and the cell proliferation was quantified by incorporation of BrdU for 3 h. The PKCs inhibitor BIM (4 μM) was added 30 min before the incubation with the phorbol ester for 15 min. A) The BrdU-labelling index is relative to the control, which represents 24% of the cell that are proliferating (these control data were set to 100 %). The data are represented as means ± S.D. of three independent experiments. * p <0.001 vs control, ▲ p <0.001 vs PMA 15 min. B) Representative images of immunocytochemical detection of BrdU in control (1) and treated cells: PMA 15 min (2), PMA 3 h (3) and BIM + PMA (4). The BrdU-labelled cells with nuclei stained brown can be distinguished and accurately quantified. Bars = 10 μm.

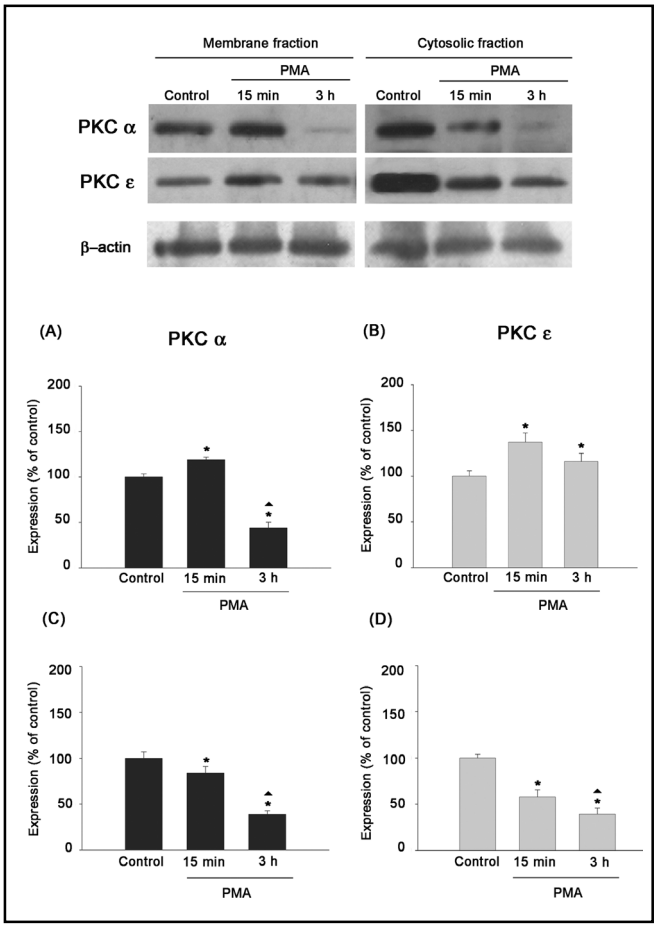

Fig. 2. Activation of PKCα and PKCε by PMA. Western blot analysis of PKCα and PKCε expression from membrane (A, B) and cytosol (C, D) fractions of GH3B6 cells stimulated with PMA (400 nM) for 15 min or 3 h. Equal amounts of protein from each sample were loaded on 12% SDS-PAGE and subjected to electrophoresis and immunobloting. The protein expression index is represented as a percentage relative to the untreated control (control data were set to 100 %). The data are expressed as means ± S.D. of three independent experiments. * p <0.01 vs control, ▲ p <0.01 vs 15 min.

(BD FACS Canto II). The percentage of cells in the S and G_2/M phase fractions was combined to serve as a proliferative index.

Confocal Laser Scanning Microscopy

Pituitary cells from different experimental groups were fixed in 4% formaldehyde, permeabilized in 0.25% Triton X-100 in PBS, blocked for 1 h in 3 % PBS-BSA and incubated with the following primary antibodies: PKCα rabbit polyclonal (1:300, Santa Cruz Biotechnology; Santa Cruz, USA) or PKCε rabbit polyclonal (1:300, Santa Cruz Biotechnology; Santa Cruz, USA) for 1 h. The coverslips were washed with PBS, and incubated with anti-BrdU (GE Healthcare; Buenos Aires, Argentina) for 1 h. Then, these cells were washed, and further incubated with an Alexa 488 anti-rabbit or Alexa 594 anti-mouse secondary antibody (1:1000; Invitrogen; Carlsbad, USA) for 1 h. Images were then obtained using an inverted confocal laser scanning microscope FluoView FV 1000 (Olympus;

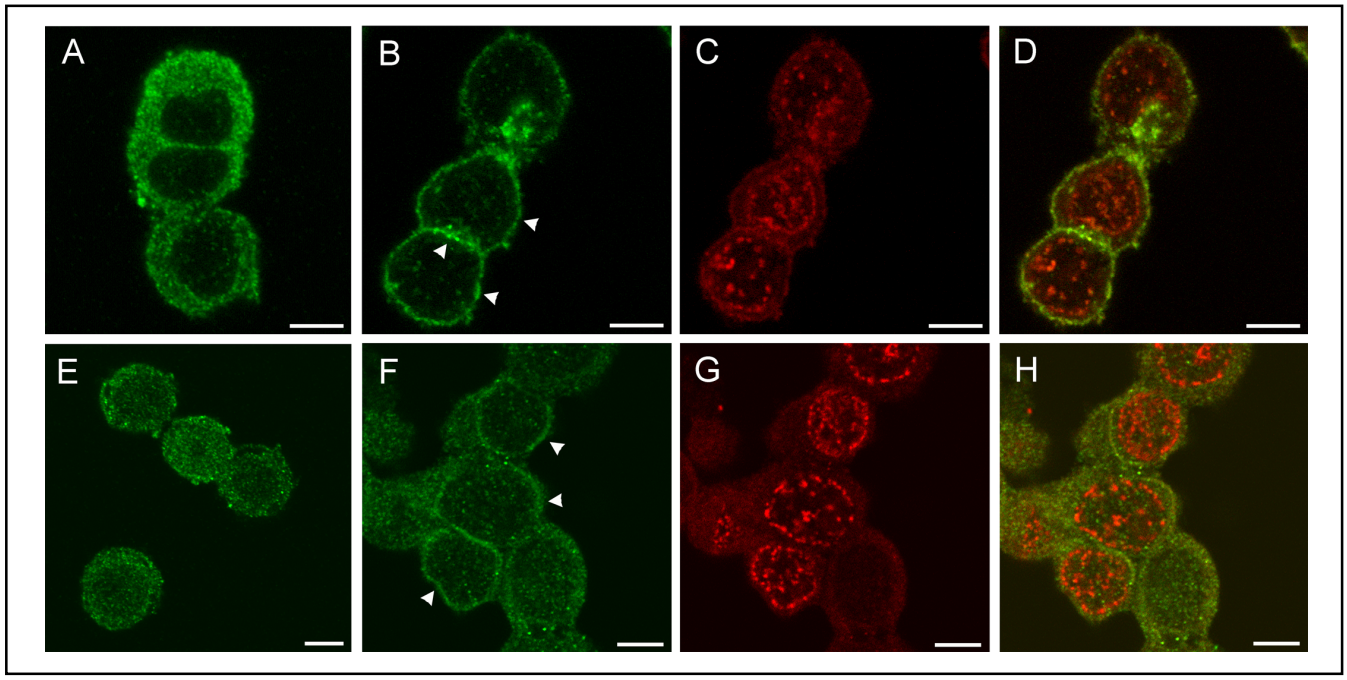

Fig. 3. Translocation of PKCα and PKCε in response to PMA-mitogenic stimulus. The intracellular localization of PKC isozymes in GH3B6 proliferating cells was analyzed by confocal laser scanning microscopy. A: Green fluorescence represents PKCα in untreated cells. B: The treatment with PMA induced a notable PKCα translocation from cytoplasm to plasma membrane (arrowhead). C: Red fluorescence represents BrdU in cells treated with PMA. D: PKCα (green) and BrdU (red) in PMA treated cells. E: PKCε in control cells. F: PKCε translocation to plasma membrane (arrowhead) in GH3B6 cells incubated with PMA. G: Red fluorescence represents BrdU in cells treated with PMA. H: PKCε subcellular localization in cells treated with PMA and that have incorporated BrdU. The photographs correspond to a representative experiment from a total of three which had similar results. Bar = 10 μm.

Tokyo, Japan). An Ar-ion 488 nm laser was used for excitation of green fluorescence and 543 nm for excitation of red fluorescence. Serial z-axis sections were collected with a 60 or 100X objective. Analysis of confocal microscopy images were performed using the software FV10-ASW 1.6 Viewer.

Statistical analysis

Experimental points represent the mean ± S.D of three replicates measured on three independent cell cultures. Statistical analysis was carried out using ANOVA, followed by the Fisher test using the InfoStat program. Significance levels were chosen as $p < 0.05$ for the Fisher test.

Results

PMA induces GH3B6 cell proliferation by PKCs activation

The immunocytochemical detection of BrdU was used to investigate whether the activation of PKCs by PMA stimulated pituitary adenoma cell proliferation. As shown in Fig. 1A-B, the PMA treatment (400 nM) for 15 min increased a 45% the number of GH3B6 cells that incorporated BrdU into DNA, consistent with an augmentation of pituitary cells that undergoing mitosis ($p < 0.001$).

It is known that the overexposure to phorbol esters leads the down-regulation of PKCs, which can be then exploited to study their function [34]. In order to ascertain whether the depletion of PKCs induced by long exposure to phorbol ester had any effect on cell proliferation, GH3B6 cells were incubated with PMA for 3 h. This treatment significantly decreased the cell proliferation, when compared to short PMA treatment ($p < 0.001$), indicating that PKC down-regulation could be responsible for the partial blockade of GH3B6 proliferation.

To confirm if activation of the PKCs was required for PMA-induced GH3B6 proliferation, the cells were pretreated with BIM, a PKCs inhibitor. The increase in the cell proliferation caused by stimulation with PMA for 15 min was blocked when GH3B6 cells were preincubated with BIM for 30 min ($p < 0.001$).

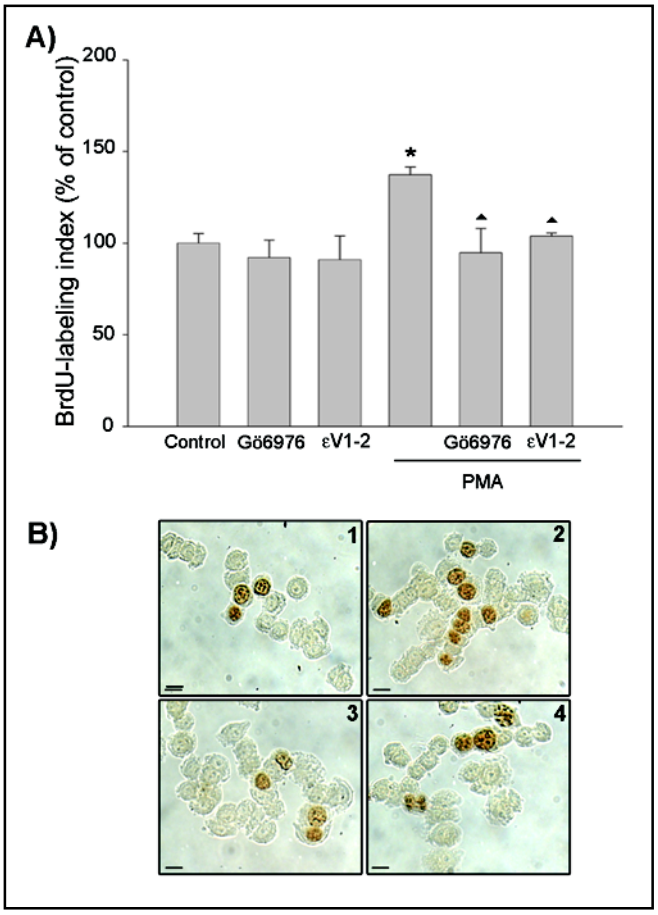

Fig. 4. PMA-induced GH3B6 proliferation through PKCα and PKCε. The cells were pretreated with the inhibitors of PKCα (Gö6976, 5 μM) or PKCε (εV1-2 peptide, 50 μM) for 30 min before PMA (400 nM) treatment for 15 min. After the treatments the cultures were incubated with BrdU for an additional 3 h. A) The BrdU-labelling index is expressed relative to the control cells (control data were set to 100 %). The data are represented as means ± S.D. of three independent experiments. * p <0.001 vs control, ▲ p <0.001 vs PMA. B) Example of BrdU-labelled GH3B6 cells with brown nuclear staining in control (1), and after PMA (2), Gö6976 + PMA (3) and εV1-2 + PMA (4) treatments. Bars = 10 μm.

PMA activates PKCα and PKCε

Western bloting. The translocation of PKCs from the cytosol to the membranes has been closely associated with their activation. To determine the effects of PMA on PKCα and PKCε activation, the expression of both isoforms was examined by western blotting in the membrane and the cytosolic fractions. Treatment with PMA for 15 min induced an increase in PKCα and PKCε expression at the membrane fraction (p <0.01) (Fig. 2A and B), with a concomitant decrease in the cytosol fraction (p <0.01) (Fig. 2C and D), indicating their translocation from the cytosol to the membrane fraction. However, phorbol ester administration for 3 h diminished the expression of PKCα and PKCε in the membrane and the cytosolic fractions compared with 15 min phorbol ester treatment, probably due to the down-regulation caused by long term exposure to PMA.

Confocal Microscopy. The subcellular redistribution of PKCs by PMA action is the key for dictating the access to isozyme-specific substrates and ultimately for conferring functional selectivity. In order to relate the intracellular translocation of PKCs with the proliferation of GH3B6 cells, we investigated the subcellular localization of PKCα and PKCε in cells that have incorporated BrdU after PMA treatment.

In untreated GH3B6 cells, the immunofluorescence of PKCα and PKCε was mainly cytoplasmic, which could be indicative of the inactive state (Fig. 3). After PMA mitogenic stimulus we visualized BrdU positive cells with a marked PKCα and PKCε translocation to plasma membrane.

Activation of PKCα and PKCε induces GH3B6 cell division

Taking into account the presented data, we suggest that there is a close correlation between the proliferation of GH3B6 induced by PMA and the activation of PKCα and PKCε. Thus, we hypothesized that PKCα and PKCε activation could be required for PMA-induced GH3B6 cell growth. To investigate this concept, we analyzed the proliferation and cell cycle progression by using inhibitors for PKCα (Gö6976) and for PKCε (εV1-2) in cell treated with PMA for 15 min. As shown in Fig. 4A-B, the mitogenic effect of the phorbol ester was completely blocked (p <0.001) when the GH3B6 cells were preincubated with Gö6976 and εV1-2.

The PMA treatment for 15 min also stimulated entry into the cell cycle, increasing the fraction of cells in the S and G_2/M phases (p <0.05) (Fig. 5A-B). A corresponding reduction in the number of cells in G_1 was observed (not shown). To investigate the involvement of PKCα and PKCε activation on the accumulation of cells in the proliferative fraction (S + G_2/M), we treated GH3B6 cells with inhibitors of both isozymes and the cell cycle progression was analyzed. Preincubation with Gö6976 and εV1-2 blocked the rise of cells in the S and G_2/M phases induced by PMA (p <0.05).

These experiments led us to propose that PKCα and PKCε could be key mediators of the mitogenic effect of PMA in GH3B6 cells.

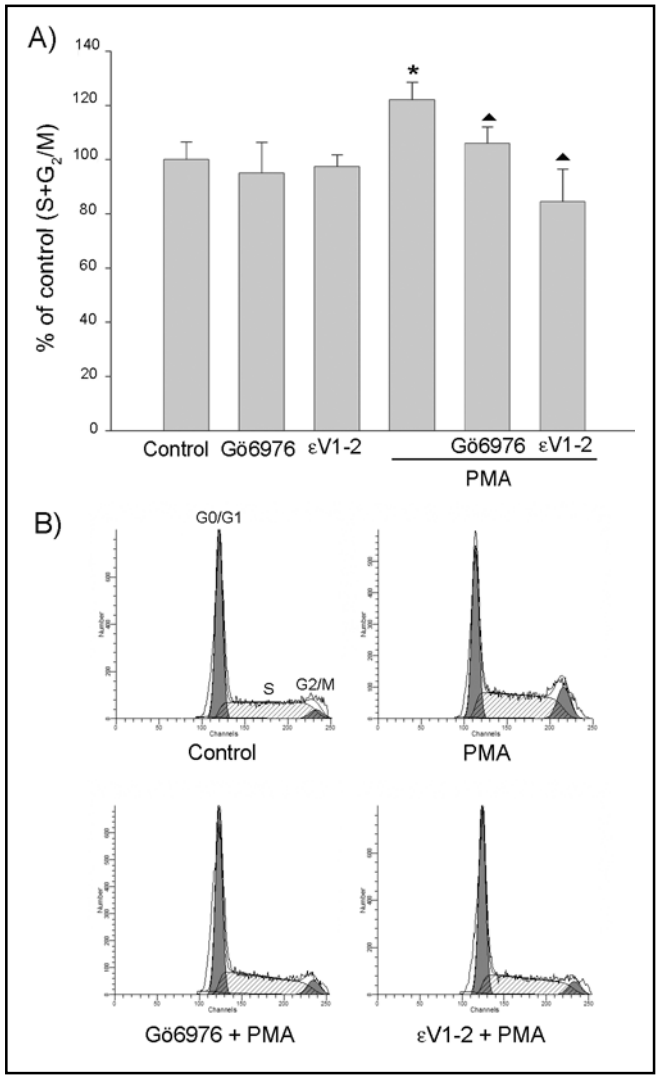

Fig. 5. PKCα and PKCε activation by PMA induced cell cycle progression. After pretreament with Gö6976 (5 μM) or εV1-2 peptide (50 μM) for 30 min, GH3B6 cells were treated with PMA (400 nM) for 15 min. Cells were collected 3 h after treatment, stained with propidium iodide and analyzed for cell cycle distribution using flow cytometry. A) The percent cells in the proliferative fraction ($S + G_2/M$) is expressed relative to the control cells (control data were set to 100 %). Similar results were observed in three independent experiments. The data are expressed as means ± S.D. * p <0.05 vs control, ▲ p <0.05 vs PMA. B) The image is representative of a cell cycle profile in control (1) and treated cells with PMA alone (2) or after the pretreatments with PKCα (3) and PKCε (4) inhibitors.

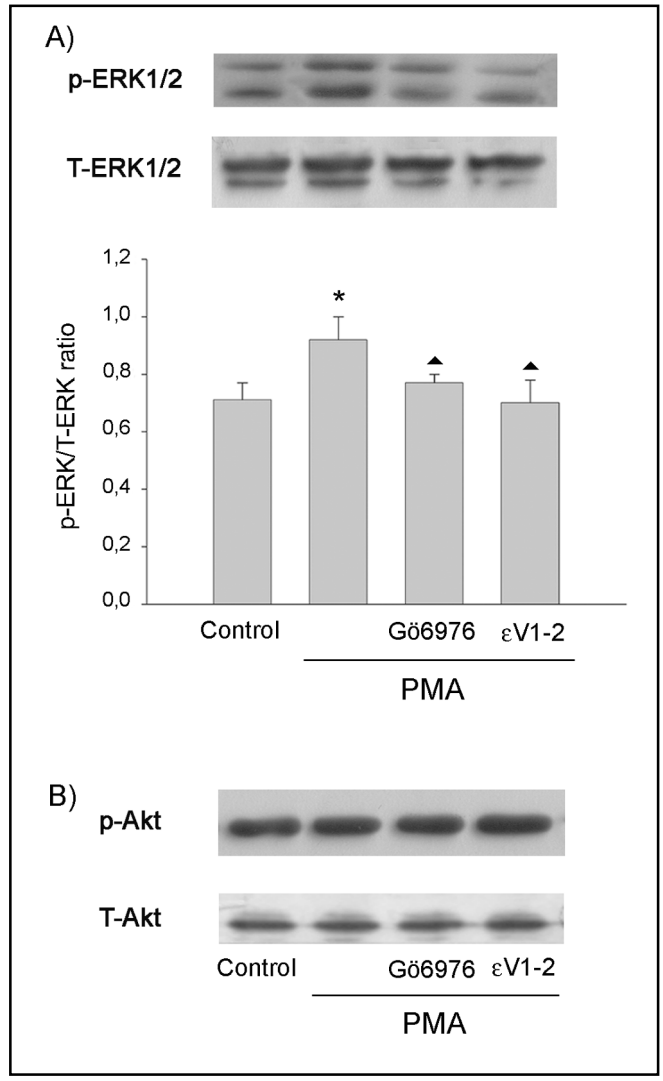

Fig. 6. PKCα and PKCε activation induces phosphorylation of ERK1/2. GH3B6 cells were pretreatment with Gö6976 (5 μM) or εV1-2 peptide (50 μM) for 30 min before PMA (400 nM) incubation for 15 min. A) The phosphorylated (p) and total (T) ERK1/2 expression levels from total extracts. The bands correspond to a representative experiment from a total of three with similar results. The graph represents the pERK/tERK ratio of the mean ± S.D. of three independent experiments. * p <0.001 vs control, ▲ p <0.001 vs PMA. B) Akt expression was determined by western blot in total extracts. A representative panel of three independent experiments is shown.

PKCα and PKCε activate ERK1/2 but not Akt

The proliferative signalling pathways modulated by PKCs in tumoral pituitary cells are not yet fully understood. To investigate whether the activation of PKCα and PKCε induced by PMA stimulated the ERK1/2 and Akt pathways, the expression of both phosphorilated kinases was determined in total cell extracts. Treatment with PMA for 15 min stimulated

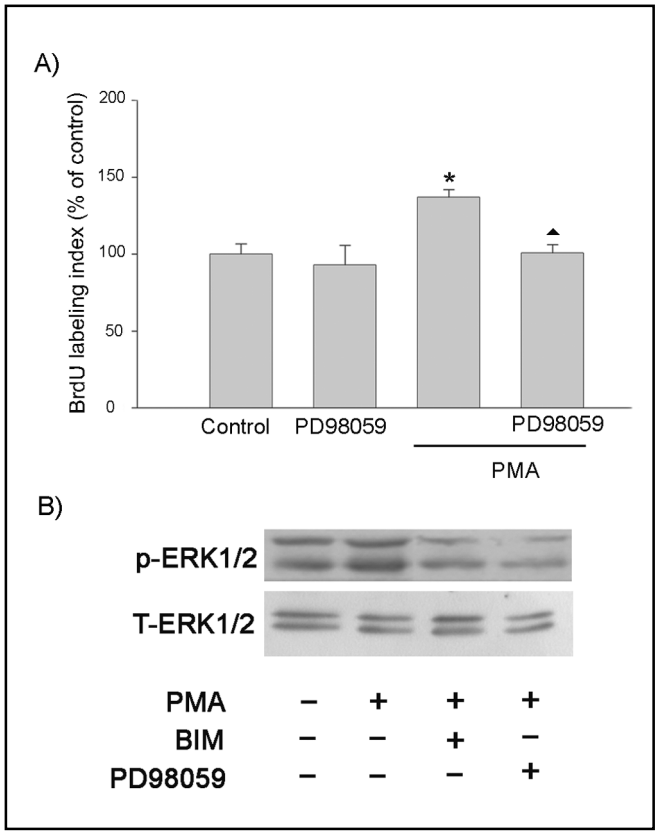

Fig. 7. ERK1/2 activation mediates the proliferative effect of PMA. A: Cell proliferation assay in GH3B6 cells treated with PMA (400 nM) for 15 min, either in the presence or absence of PD98059 (50 μM) added 30 min before the phorbol ester incubation. The BrdU-labelling index is expressed relative to the control cells (control data were set to 100 %). The data are represented as means ± S.D. of three independent experiments. * p <0.001 vs control, ▲ p <0.001 vs PMA. B: Western blot analysis in GH3B6 cells treated with PMA (400 nM) for 15 min, either in the presence or absence of BIM (4 μM) or PD98059 (50 μM) added 30 min before the phorbol ester incubation. A representative panel of three independent experiments is shown.

phosphorylation of ERK1/2 but not of Akt (Fig. 6A-B). The inhibitor of PKCα (Gö6976) and PKCε (εV1-2) blocked the activation of ERK1/2 stimulated by the PMA treatment. However, corresponding to the lack of response to Akt activation observed with PMA, the inhibitors of both PKC isozymes did not induce changes in the expression of phosphorylated Akt. These results suggest that the activation of PKCα and PKCε isozymes are required to stimulate the ERK1/2 pathway.

ERK1/2 mediates the proliferative effect induced by PKCs activation

To confirm the role of the ERK1/2 pathway on the GH3B6 proliferation induced by PKC activation, we used an inhibitor of MEKs (PD98059). Preincubation with PD98059 for 30 min significantly inhibited the cell proliferation stimulated by a 15 min treatment with PMA (p <0.001) (Fig. 7A).

The treatment with BIM and PD98059 inhibited the ERK1/2 phosphorylation induced by PMA, suggesting that the activation of PKCs mediated the proliferative effect of phorbol ester through the ERK 1/2 pathway (Fig. 7B).

Discussion

Pituitary tumors may originate from either functional mutations or overexpression of ubiquitously expressed proto-oncogenes that are components of common proliferative pathways. Abnormalities in the expression of membrane and nuclear receptors, growth factors, transcription factors, and their signalling proteins have been proposed to play a relevant role in cell transformation and/or clonal expansion [3]. However, knowledge about the molecular mechanisms responsible for pituitary tumor progression is still incomplete.

Studies on various normal and cancer cells have confirmed the involvement of PKC isozymes in mitogenesis, survival and malignant transformation. In addition, it is now appreciated that the role of PKCs in tumorogenesis is complex, and depends largely on the cell/tissue type and PKC isozyme involved [35]. In the present study, we first explored whether the activation of PKCs by PMA was required to induce pituitary adenoma proliferation by taking into account that PKCs can be either selectively activated or down-regulated by phorbol ester [36]. The activation of PKCs by PMA for 15 min stimulated GH3B6 proliferation whereas a prolonged activation for 3 h diminished this proliferative response. These results indicate that the activation of PKCs was required to induce cell proliferation, an effect that was partially reversed by the depletion of these kinases after PMA incubation for 3 h. Furthermore, the general PKC inhibitor, BIM, blocked the GH3B6 proliferation induced by PMA, demonstrating that the phorbol ester effect was mediated by PMA-responsive PKC isozymes and not by other endogenous phorbol ester mediators [34].

The molecular mechanism by which PKCs may upregulate cell proliferation in pituitary adenomas is not yet clear. However, the ERK1/2 pathway has emerged as a

central regulator of cell proliferation, which controls both cell growth and cell cycle progression [18]. The ERK1/2 pathway has been implicated in the PMA-induced growth stimulatory response [37]. In our study, the ERK1/2 pathway was stimulated by PMA in the GH3B6 tumor cells as indicated by its phosphorylation after phorbol ester treatment for 15 min and by its blockade by both the PKC inhibitor BIM and the MEK inhibitor PD98059. This MEK inhibitor also reversed the PMA-induced growth stimulatory response. Taken together, our data suggest that ERK1/2 are downstream targets of PKCs, which mediate the proliferative response of GH3B6 cells to PMA.

Despite the PKCs having a clear role in the proliferation of pituitary tumor cells, little is known about the contribution of different isozymes in this process. Related to this, the present work is the first study that demonstrates that both PKCα and PKCε mediate the proliferative effect of PMA in GH3B6 cells. The localization of PKCα and PKCε in plasma membrane after PMA stimulus in BrdU positive cells observed at confocal microscopy level was correlated with the increase of both PKC isozymes expression in membrane fraction obtained by western blot. These results suggest that there is a close relationship between the plasma membrane localization of both isozymes and the GH3B6 cell proliferation. PKC translocation to the plasma membrane has been related with the ERK1/2 activation [38], which in turn are involved in cell proliferation [39]. Particularly, it has been reported that PMA induces PKCα and PKCε translocation from cytosol to plasma membrane, ERK1/2 activation and proliferation of NIH/3T3 cells [40].

The participation of both PKC isozymes on the GH3B6 cell growth was confirmed by the use of the inhibitors which were able to block the mitogenic activity induced by PMA. In a previous investigation using normal pituitary cells, we observed that a short PMA treatment induced lactotroph proliferation by the specific translocation and activation of PKCε [15]. In the present study, the results indicated that the activation of PKCα and PKCε is involved in the proliferation of GH3B6 cells induced by PMA, and these kinases may provide putative therapeutic targets for the control of pituitary adenoma growth. Supporting this suggestion, it has been previously reported in the pituitary tumor HP75 cells that PMA increased the tumoral invasion, a process that was blocked by the inhibition of PKCα [27].

The PKCα and PKCε have been suggested to play important roles in tumorigenesis, invasion, and metastasis in different cell types. Although, the downstream events following PKC activation are poorly understood, the MEK/ERK pathway is thought to have an important role [41]. It has also been reported that PKCα and PKCε regulate the cell growth of different carcinomas through ERK1/2 phosphorylation [42, 43]. There is also evidence to support that the involvement of PKC in the PI3K/Akt pathway, which appears to be determined in a cell-type and stimulus-specific way [5]. Our results using inhibitors of PKCα and PKCε demonstrated that the activation of both isozymes by PMA stimulated the phosphorilation of ERK1/2 but not of Akt. This evidence strongly supports a proliferative role for PKCα and PKCε in adenoma pituitary cells, and indicates that this effect is dependent on ERK1/2 but independent of the Akt pathway.

Molecular analysis of human pituitary neoplasias has shown that cell cycle desregulation is significantly implicated in pituitary tumorigenesis, with an important number of genetic and epigenetic alterations occurring in pituitary tumors, involving specific regulators of the cell cycle [44]. Evidence has also accumulated in recent years supporting the idea that the effect of phorbol esters on cell proliferation is conferred by the ability of PKC isozymes to regulate the cell cycle machinery [9]. However, little is known about the implication of specific PKC isozymes on the cell cycle progression of pituitary tumors. In our investigation, we observed that the activation of PKCα and PKCε increased the percentage of GH3B6 cells in the proliferative fraction ($S + G_2/M$), an effect that was blocked by treatment with the inhibitors of both isozymes. These results suggest that PKCα and PKCε activation could contribute to the regulation of the GH3B6 cell cycle. Moreover, the activation of PKC by PMA promotes human colon carcinoma HCT116 cell growth, through downregulation of the cyclin-dependent kinase inhibitors [45]. In recent years, it has been demonstrated that the PKCε ?and PKCι inhibition blocked G_1/S transition by the accumulation of cells in the G_0/G_1 phase in non-small cell lung cancer cells and in prostate carcinoma DU-145 cells respectively [46, 47]. Concerning pituitary tumors, it has been proposed that more than 80% of these present alterations in at least in one of the cell cycle regulators during G_1/S transition [48].

Abnormalities in cell signalling pathways are frequently seen in pituitary adenomas [49]. Previous work has highlighted the importance of studying PKCs, because these kinases have a higher enzyme activity in human pituitary tumors compared to normal pituitary cells [26]. Moreover, a dose-dependent inhibition of cell growth has

been demonstrated in pituitary tumor cell cultures treated with hypericin, a PKC inhibitor [50].

We decided to evaluate PKCα and PKCε activation in response to PMA, because these enzymes are usually involved in tumorigenesis [23] and are the most expressed isozymes in human pituitary adenomas [51]. It has also been reported that PKCα is not only overexpressed in human pituitary tumors, but is also structurally altered in an invasive subpopulation of these tumors [52]. However, this PKCα mutant is unable to bind to cellular membranes tightly and subsequently fails to transduce many signals [53]. With regard to the involvement of MAPK pathways activated by PKCs in pituitary tumors, it has been shown that MEK1/2 as well as its down-stream regulator ERK1/2 is over-phosphorylated, and hence over-activated in all types of pituitary adenomas, compared to normal pituitary cells [49].

The PKC family is undoubtedly an attractive target for therapeutic intervention, given its role in tumorigenesis and its potential for enhancing the cytotoxicity of existing drugs [54]. Recently, it has been reported that enzastaurin (LY317615), a PKCβ-selective inhibitor, in combination with other drugs showed a good tolerance with preliminary evidence of anticancer activity, particularly in thyroid cancer [55]. The PKCs/ERK1/2 pathways in pituitary adenomas have attracted considerable attention because they usually act in more aggressive cancers, and might allow the implementation of new drugs, selective inhibitors of these kinases, in the treatment of pituitary disorders in the future.

Conclusions

Summing up, our study suggests that the activation of PKCα and PKCε by phorbol ester in tumor pituitary GH3B6 cells leads to cell proliferation and cell cycle progression, effects that involve ERK1/2 activation. A better understanding of the specific PKC isozymes participation in pituitary tumoral cell growth and the corresponding signalling downstream pathways activated, in response to different stimuli, will help to clarify the mechanisms involved in pituitary tumorogenesis.

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