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Activation of PKC epsilon induces lactotroph proliferation through ERK1/2 in response to phorbol ester

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ABSTRACT

The aim of this investigation was to contribute to current knowledge about intracellular mechanisms that are involved in lactotroph cell proliferation, by evaluating the role of PKCα, PKCε and extracellular-signal regulated kinase (ERK) 1/2 in response to phorbol 12-myristate13-acetate (PMA). In primary pituitary cultures, the activation of protein kinase C (PKC) by PMA for 15 min stimulated lactotroph proliferation; whereas a prolonged activation for 3–8 h diminished this proliferative effect. The use of PMA for 15 min-activated PKCε and ERK1/2, whereas incubation with PMA for 3 h induced PKCα activation and attenuated the PMA-triggered phosphorylation of ERK1/2. The following inhibitors: PKCs (bisindolylmaleimide I), PKCε (εV1 peptide) and ERK1/2 (PD98059) prevented the mitogenic activity induced by PMA for 15 min. Lactotroph cells stimulated with PMA for 15 min showed a translocation of PKCε to membrane compartment and nucleus. These results thus establish that PKCε plays an essential role in the lactotroph proliferation induced by PMA by triggering signals that involve ERK1/2 activation.

1. Introduction

Pituitary cells are physiologically regulated by hormones and growth factors that stimulate their hormone production and proliferation. The systems that mediate these responses are important for normal homeostasis, but dysregulation of these signalling pathways can promote tumor development (Asa and Ezzat, 2002). Related to this, the prolactinomas are the most prevalent neoplasms in the human pituitary, demonstrating that the lactotroph cells play a critical role in the pituitary growth disorders (Sarkar, 2006).

In previous reports, we demonstrated that transcription factors such as Pit-1 and c-Myc, as well as growth factors FGF-2 and VEGF, are involved in the complex process of prolactinoma development (Mukdsi et al., 2004, 2005, 2006). Although extracellular signals that regulate lactotroph proliferation and their interactions have already been studied, the current investigation is focused on the intracellular signal transduction pathways that lead to cell proliferation in the pituitary gland. Related to this, recent reports have described the involvement of the mitogen-activated protein kinase (MAPK) pathway in regulating the cell proliferation in normal pituitary lactotrophs induced by bFGF (Chaturvedi and Sarkar, 2005). In addition, existence of cross-talk between the estrogen receptor, protein kinase A, and MAPK signalling pathways in the regulation of PRL cell proliferation in primary cultures has been previously demonstrated (Yamakawa and Arita, 2004).

To date, numerous studies have demonstrated the relevance of the protein kinase C (PKC) family in mediating signals that regulate cellular proliferation (Basu and Sivaprasad, 2007). Furthermore, the PKC family is also involved in apoptosis (Yoshida, 2007), cell differentiation (Lin et al., 2007), and hormone release (Garrido-García et al., 2006).

Eleven distinct PKC isozymes have been identified in mammalian cells and 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 isoforms 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. Furthermore, the atypical PKCs, which include PKCζ and PKCλ/ι, are unresponsive to both Ca^{2+} and DAG/phorbol esters (Ohno and Nishizuka, 2002; Griner and Kazanietz, 2007). Finally, although it has been reported that many effects of phorbol esters attributed to PKCs may probably involve other targets, it has been long known that PKC is the main receptor for the phorbol ester tumor promoters (Kazanietz, 2002).

There has been particular interest in the opposing roles generally observed for PKCs with respect to proliferation, survival and the promotion of cancer. For example, PKCε has been shown to be

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up-regulated in various types of cancers, whereas PKCα is down-regulated (Knauf et al., 2002; Weichert et al., 2003; Pan et al., 2005). In some cancers there is a striking correlation between changes in the PKC expression of some isoforms and the progression of the disease, suggesting the potential use of these kinases as prognostic markers.

Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), stimulate a rapid association of PKC with cell membranes. This translocation reflects activation of cytosolic enzymes and leads later to down-regulation (Srivastava et al., 2002). The down-regulation of different PKC isotypes may therefore play an important role in cell proliferation. Immunofluorescence and subcellular fractionation studies have demonstrated that activation of PKCs by phorbol esters is accompanied by the movement or translocation of each PKC isozyme to a different cell compartment, such as a plasma membrane, Golgi apparatus, mitochondria or nuclear membrane (Csukai et al., 1997; Majumder et al., 2000; Quittau-Prévostel et al., 2004; Kenessey et al., 2006). This differential redistribution is the key for dictating the access to isozyme-specific substrates and ultimately confers functional selectivity.

The MAPK cascade, which involves Raf, mitogen-activated kinase effector kinase (MEK) and extracellular-signal regulated kinase (ERK) 1/2, is ubiquitously expressed in mammalian cells and is implicated in the regulation of cell proliferation as well as cell death (Leevers and Marshall, 1992; Caughlan et al., 2004). 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 stimulus (Qiu and Leslie, 1994; Lehoux and Lefebvre, 2007).

It has been demonstrated that PKCα, β, δ, ε, ζ and θ are expressed in the pituitary gland as well as in GH3 cells (MacEwan et al., 1999). Numerous endogenous extracellular stimuli activate PKCs in the normal pituitary gland such as neuropeptides, growth factors and hormones (Wang and Maurer, 1999; Lachowicz and Rebas, 2002; Gutiérrez et al., 2005, 2008), thereby regulating the hormone secretion (Garrido-Gracía et al., 2006), cell proliferation (Zhu et al., 2005) and cell death (Hamilton et al., 1996), depending on the type of stimulus applied to the particular cell as well as the specific PKC isoform translocation.

A higher expression of PKC baseline levels in adenomatous than in normal pituitary cells has been reported (Alvaro et al., 1992). Furthermore, PKC activation with PMA increased the invasion of a human pituitary tumor-derived cell line (HP75 cells), a process that was attenuated by using PKC inhibitors (Hussaini et al., 2007). However, little is known about the specific isoform of PKC and the ERK1/2 signal pathway contribution in the regulation of lactotroph cell proliferation.

The aim of this investigation was to study the participation of PKCα and ε in lactotroph cell proliferation stimulated by PMA, and also to determine if the ERK/MAPK pathway is activated by these kinases. In addition, we examined the subcellular translocation of the PKC isoform that is involved in the mitogenic lactotroph in response to phorbol ester.

2. Materials and methods

2.1. Animals

Three-month-old female rats of the Wistar strain were used. Large pools of animals were used for each culture at random cycle stages. The rats were raised in our laboratory under controlled temperature (21 ± 3 °C) and lighting conditions (14 h light/10 h dark), having free access to commercial lab chow and tap water.

Animal conditions were in compliance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare, and the local Institutional Animal Care Committee.

2.2. Dissociation of anterior pituitary cells

The protocol for the dissociation of pituitary cells was described previously (De Paul et al., 1997). Briefly, anterior pituitaries excised from female rats were placed in minimal essential medium for suspension culture (SMEM), then minced, digested with 0.4% trypsin and dispersed with Pasteur pipettes. The cell yield was $(1.5–2) \times 10^6$ per pituitary, and the cell viability, tested with Trypan Blue exclusion, was always better than 90%. The final suspension was adjusted to 1×10^6 cells/ml of medium. The dispersed cells were plated on glass coverslips (22 mm × 22 mm), and placed at the bottom of six-well culture plates (Corning, New York, USA). The cells were seeded at a density of 5×10^5 cells/well, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% fetal calf serum and 8% horse serum (Invitrogen, Carlsbad, USA), in an oven with a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 3 days.

All culture media were filtered through 0.2 μm Nalgene membranes (Nalge Company, New York, USA). The cell culture grade reagents were obtained from the Sigma (St. Louis, USA).

2.3. Cell treatments

After 3 days of culture, the medium was discarded and replaced with fresh DMEM, and the following treatments were applied.

2.3.1. Phorbol ester

Phorbol 12-myristate 13-acetate (400 nM; Sigma, St. Louis, USA) was solubilized in DMSO before being added to cell media for short time: 15 min and long times: 3, 5 or 8 h. Then, the cells were processed to evaluate lactotroph cell proliferation by bromodeoxyuridine (BrdU) detection, protein expression by western blotting and mRNA expression by PCR.

2.3.2. PKC inhibitors

The inhibitor of conventional and novel PKC, bisindolylmaleimide I (BIM, 4 μM; Sigma, St. Louis, USA) (Hussaini et al., 2007), or the specific PKCε translocation inhibitor peptide, Myr-PKCεV1-2, (εV1, 50 and 100 μM; Biomol, Plymouth Meeting, USA) (Eichholtz et al., 1993) was added for 30 min. Then, the cells were washed, incubated with 400 nM of PMA for an additional 15 min in DMEM and finally processed to evaluate lactotroph cell proliferation and protein expression.

2.3.3. PD98059 MEK inhibitor

The inhibitor of mitogen-activated kinase effector kinase (PD98059; Calbiochem, San Diego, USA) (Alessi et al., 1995) was used to inhibit ERK1/2 activation. The cells were treated with PD98059 (50 and 100 μM) for 30 min, washed, incubated with 400 nM of PMA for 15 min, and then processed to evaluate lactotroph cell proliferation.

2.4. Immunocytochemical detection of lactotroph proliferation

Cells at DNA-synthesizing stages and lactotrophs were individualized by dual-immunocytochemical detection of BrdU and PRL, according to Oomizu et al. (1998) with modifications (Gutiérrez et al., 2005). To conclude the experimental protocols, the culture medium was replaced and BrdU (3 mg/ml) was added for 24 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. 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 (Amersham, Buckinghamshire, England). 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 stained brown. Then, PRL immunocytochemistry for lactotroph cell detection was performed on the same coverslip. The cells were incubated with rabbit anti-rat PRL at 1:3000 dilution (NIHDDK, Bethesda, USA) in a wet chamber for 1 h at 37 °C followed by washing with PBS and incubation in biotinylated anti-rabbit IgG 1:150 for 30 min (Vector, Burlingame, USA). After a wash in PBS, the avidin–biotin–peroxidase complex was applied for 30 min at RT. The immunoreactivity for PRL was detected with chloronaphthol, which caused the immuno-stained lactotroph cells to acquire a purplish blue color. The coverslips were then mounted on glass slides with glycerol. Controls were performed, applying the same protocol, but omitting the BrdU or PRL antibodies.

A total of 1000 PRL-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 both PRL and BrdU. Three slides were analyzed for each group derived from the same cell preparations.

2.5. Western blot analysis

2.5.1. Preparation of total cell extract

After different experimental conditions, the pituitary cells were rinsed with PBS before being lysed on ice by addition of 200 μl of cold PBS containing 1.25% Igepal CA-630, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin. This was followed by scraping and transfer of the lysate to a centrifuge tube placed on ice. After 30 min, the lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C to pellet the Igepal CA-630-insoluble material, and the supernatants were then withdrawn and stored in aliquots frozen at −20 °C until required.

2.5.2. Subcellular fractionation

In order to obtain cytosolic and membrane fractions, the pituitary 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 $MgCl_2$, and 40 mM KCl] containing 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. After 30 min incubation on ice, cell homogenates were centrifuged at 100,000 × *g* for 45 min at 4 °C. The supernatants collected corresponded to the cytosolic fraction. The pellet was resuspended in HHB supplemented with 1% (v/v) Triton-X 100, followed by brief sonication, and designated as the membrane fraction.

The total protein concentration was measured using BioRad kit (Bio-Rad Protein Assay, Bio-Rad; Hercules, USA). Thirty micrograms (30 μg) from total homogenate and subcellular fractions were run in 12% acrylamide gel (Sigma, St. Louis, USA). To estimate the corresponding molecular weights, Full Range Rainbow Molecular Weight Marker was used (Amersham, Buckinghamshire, England). The proteins were transferred to a nitrocellulose membrane and non-specific binding was blocked with PBS containing 5% non-fat 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 mouse monoclonal antibody (1:300), PKCε rabbit polyclonal antibody (1:300), total ERK1 rabbit polyclonal antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, USA) and anti phosphorylated ERK1/2 mouse monoclonal antibody (1:700) (Sigma, St. Louis, 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 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). Signals were 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.

2.6. Immunogold electron microscopy

The subcellular localization of PKCε in lactotroph cells was examined by electron microscopy. Pituitary cells, stimulated with PMA for 15 min, were scraped, washed, centrifuged and fixed in a mixture of 4% formaldehyde, 1.5% glutaraldehyde and 0.1 M cacodylate buffer, pH 7.3, at room temperature for 5–6 h, with osmiun fixation being omitted. The pellet was dehydrated in a series of increasing concentrations of ethanol, and then embedded in a 1:1 solution of LR White (Sigma, St. Louis, USA) overnight. The following morning, samples were placed in fresh LR White, embedded in gelatin capsules, and polymerized at 50 °C for 48 h. Thin sections were cut in a JEOL ultramicrotome with a diamond knife. Grids were incubated with PKCε rabbit polyclonal antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4 °C. The sections were washed with distilled water, and incubated with secondary antibody goat anti-rabbit conjugated to 15 nm colloidal gold particles (Electron Microscopy Sciences, Hatfield, USA), diluted 1:15 in blocking buffer. To validate the specificity of the immunostaining, the following controls were performed: (1) replacement of primary antiserum with 1% BSA in 0.1 M phosphate buffer, pH 7.3, plus 0.15 M sodium chloride (PBS); (2) replacement of primary antiserum with diluted preimmune serum followed by the secondary antibody. Sections were examined in a Zeiss LEO 906-E electron microscope and photographed with a megaview III camera.

Lactotroph cells were confirmed by ultrastructural immunocytochemistry for PRL using a specific antibody (antisera raised against rat PRL diluted 1:3000; NIHDDK, Bethesda, USA).

2.7. Statistical analysis

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

3. Results

3.1. Lactotroph cell proliferation induced by PMA

With the aim of investigating whether the activation of PKCs by PMA stimulates lactotroph cell proliferation, BrdU-labelling index was determined in cultured pituitary cells subjected to double labelled immunocytochemistry. In the control group, the PRL positive cells that underwent mitosis reached 14%. As shown in Fig. 1, the activation of PKCs by PMA (400 nM) applied for 15 min promoted a significant increase in the lactotroph cell number (60%) compared to the control ($p < 0.001$). However the exposition of pituitary cells to PMA for 3 or 5 h decreased the similar manner the lactotroph cell proliferation with respect to the values attained after PMA for 15 min ($p < 0.001$), reaching values similar to the control. The treatment with phorbol ester for 8 h also significantly inhibited the lactotroph proliferation with respect to the 15 min, test producing lower values than that observed in the control. To confirm whether the activation of PKCs is required for PMA-induced lactotroph proliferation, the cells were pretreated with BIM, a PKCs inhibitor. This pretreatment with BIM for 30 min blocked the stimulation of the lactotroph proliferation induced by PMA for 15 min ($p < 0.001$) (Fig. 2).

3.2. Activation of PKCα and ε by PMA

It has been well established that translocation of PKCs from cytosol to membrane is closely associated with its activation. In order to determine the activation of PKCα and ε by PMA in pituitary cells, the expression of both isoforms was examined by western blotting of cytosolic and membrane fractions. A rapid translocation (78%) of PKCε from cytosolic to membrane fraction was detected after 15 min with PMA, which was followed by a down-regulation after 3 h of stimulus ($p < 0.05$) (Fig. 3A and B). In contrast, isoform α exhibited a different pattern of activation compared to those attained with PKCε, with a significant translocation of PKCα from cytosolic to membrane fraction (52%) being observed when pituitary cells were exposed to phorbol ester for 3 h ($p < 0.05$) (Fig. 3C and D).

3.3. Specific inhibition of PKCε

Taking together the results reported above, we suggest that PKCε was the PKC isozyme activated on the membrane fraction

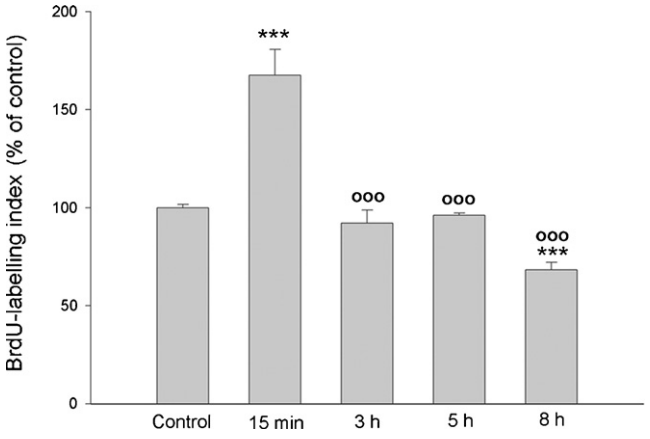

Fig. 1. Effect of PMA on the lactotroph cell proliferation. The cells were treated with 400 nM PMA for different times, and then incubated with BrdU for 24 h. The BrdU-labelling index is expressed relative to the control cells (control data were set to 100%). The data is represented as a mean ± S.E.M. of three independent experiments. ***$p < 0.001$ vs. control, ○○○$p < 0.001$ vs. 15 min.

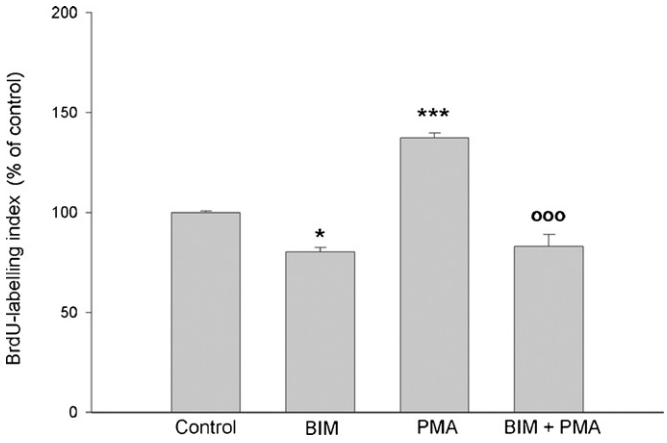

Fig. 2. Effect of PKC inhibitor on the lactotroph cell proliferation. The cells were pretreated with BIM (4 μM) for 30 min before PMA (400 nM) treatment for 15 min and then incubated with BrdU for 24 h. The BrdU-labelling index is expressed relative to the control cells (control data were set to 100%). The data is represented as a mean ± S.E.M. of three independent experiments. $^{*}p < 0.05$ vs. control, $^{***}p < 0.001$ vs. control, $^{ooo}p < 0.001$ vs. 15 min.

after short PMA treatment, which produced the highest lactotroph cell proliferation. To investigate whether the PKCε activation is required for PMA-induced lactotroph growth, the cells were preincubated with a specific peptide inhibitor of PKCε translocation (Myristolated-PKCεV1-2 peptide inhibitor) and then treated with PMA for 15 min. As shown in Fig. 4, the treatment with both doses of this inhibitor completely blocked ($p < 0.001$) the PKCε translocation to the membrane fraction (Fig. 4A) and the lactotroph cell proliferation (Fig. 4B) induced by PMA. The consequence of these results is that we have shown that PKCε activation by PMA is required in order to stimulate lactotroph proliferation.

3.4. Down-regulation of PKCε by prolonged stimulation with PMA

To ascertain whether the down-regulation of PKCε induced by long exposure to PMA is required to trigger the inhibition of lactotroph proliferation, the expression of PKCε was measured from whole cell extracts. Phorbol ester administration to pituitary cells for 3–8 h down-regulated the PKCε isoform expression with respect to that observed in the control cells ($p < 0.01$) (Fig. 5), indicating that PKCε down-regulation could be responsible for decrease in the lactotroph cell proliferation (Fig. 1).

3.5. Effect of PMA on ERK1/2 activation

To investigate the role of PKC in mediating ERK1/2 activation (MAP kinase), the ERK1/2 phosphorylated expression (ERK1/2-P) in total pituitary cell extracts treated with PMA was quantified. The treatment with phorbol ester for 15 min caused a significant increase in ERK1/2 activation ($p < 0.01$), whereas over-exposition for 3 or 5 h reduced markedly the phosphorylation of these kinases ($p < 0.01$) (Fig. 6).

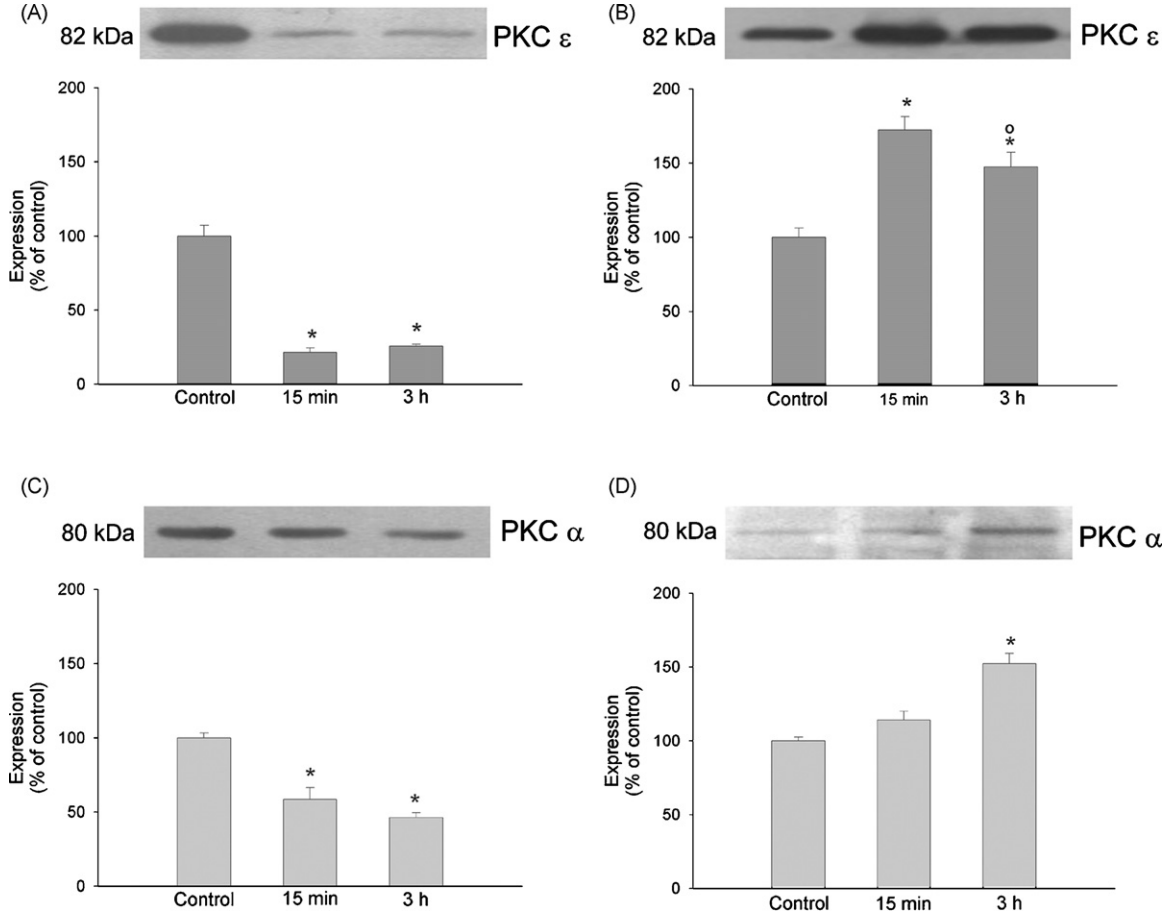

Fig. 3. Subcellular distribution of PKCα and ε in pituitary culture induced by PMA (400 nM) for 15 min and 3 h from cytosol (A, C) and membrane (B, D) fractions. Western blot results are representative of one out of three independent experiments. The protein expression index is represented as a percentage relative to the untreated control (control data were set to 100%). The data is expressed as a mean ± S.E.M. of three independent experiments. $^{*}p < 0.05$ vs. control, $^{o}p < 0.05$ vs. 15 min.

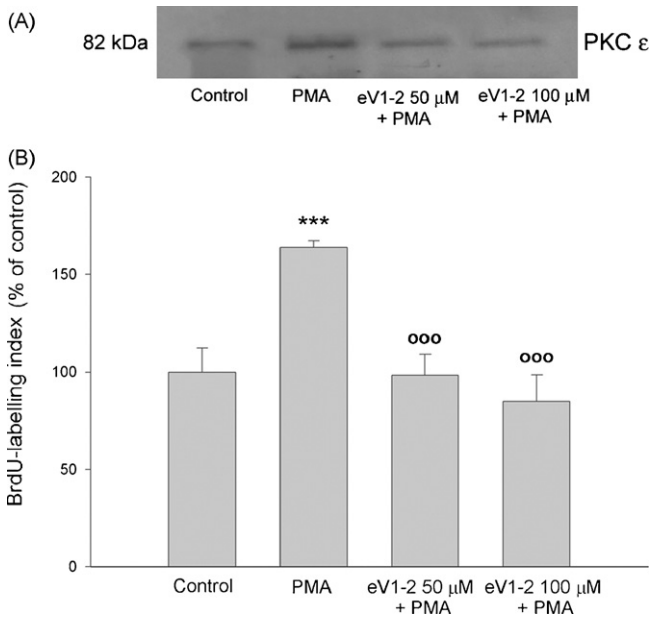

Fig. 4. Effect of PKCε inhibitor on the lactotroph cell. The cells were pretreated with εV1-2 peptide (50 and 100 μM) for 30 min before PMA (400 nM) treatment for 15 min. (A) Western blot analysis of PKCε expression in membrane fraction. (B) After the treatments the cultures were incubated with BrdU for an additional 24 h. The BrdU-labelling index is expressed relative to the control cells (control data were set to 100%). The data is represented as a mean ± S.E.M. of three independent experiments. $^{***}p < 0.001$ vs. control, $^{○○○}p < 0.001$ vs. 15 min.

To determine whether ERK1/2 participates in PMA-induced lactotroph cell proliferation, pituitary cells were pretreated with a MEK inhibitor (activator of the ERK1/2) (PD98059) and then exposed to PMA for 15 min. Preincubation with 50 and 100 μM PD98059 for 30 min significantly inhibited in a dose-dependent manner the lactotroph proliferation induced by PMA ($p < 0.001$) (Fig. 7).

3.6. Immunolocalization of PKCε by electron microscopy

Bearing in mind that the biological function of PKC depends on its spatial localization, we attempted to determine the fine localization of PKCε by means of electron microscopy immunogold labelling in lactotroph cells, mitogenically stimulated by PMA for 15 min. In untreated cells, the enzyme was identified in the cytoplasm, being scarcely dispersed in the cytoplasmic matrix, and also in the nucleus (Fig. 8A and B). After PMA stimuli for 15 min, the lactotroph cells showed a predominant PKCε immunogold labelling associated with membranes such as plasma membrane, rough endoplasmic reticulum and Golgi networks. In addition, PKCε was also translocated in the nucleus of lactotroph cells upon PMA stimulation, as observed by an intense immunogold labelling in this compartment (Fig. 8C–E).

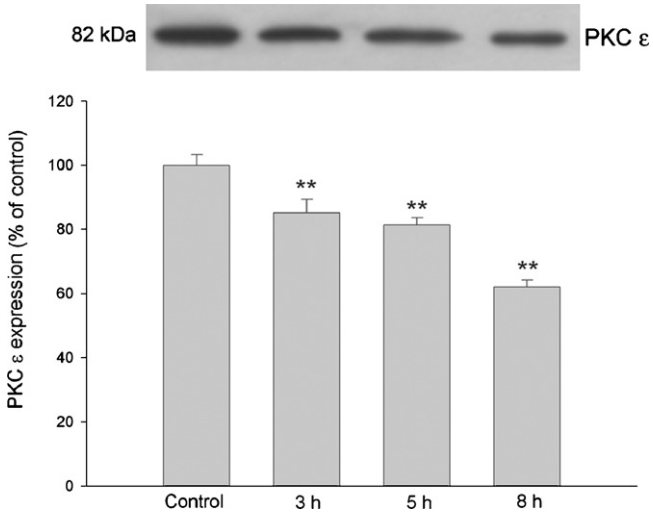

Fig. 5. PKCε expression in pituitary culture treated with PMA (400 nM) for 3, 5 or 8 h from total extracts. The protein expression index is represented as a percentage relative to the untreated control (control data were set to 100%). The data is expressed as a mean ± S.E.M. of three independent experiments. $^{**}p < 0.01$ vs. control.

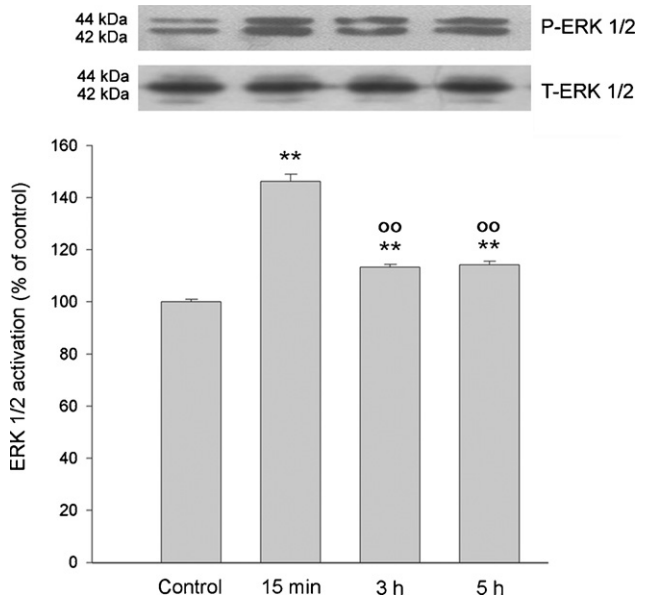

Fig. 6. Phosphorylated (P) and total (T) ERK1/2 expression induced by PMA from total extracts of pituitary cells. The cells were treated with PMA (400 nM) for 15 min, 3 or 5 h. The protein expression index is represented as a percentage relative to the untreated control (control data were set to 100%). The data is expressed as a mean ± S.E.M. of three independent experiments. $^{**}p < 0.01$ vs. control; $^{○○}p < 0.01$ vs. 15 min.

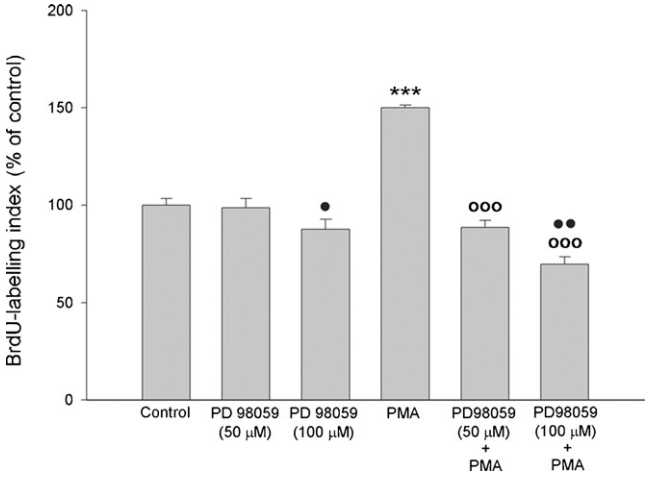

Fig. 7. Effect of MEK inhibitor on the lactotroph cell proliferation. The cells were pretreated with PD98059 (50 and 100 μM) for 30 min before PMA (400 nM) treatment for 15 min. Then, the cultures were incubated with BrdU for another 24 h. The BrdU-labelling index is expressed relative to the control cells (control data were set to 100%). The data is represented as a mean ± S.E.M. of three independent experiments. $^{***}p < 0.001$ vs. control, $^{○○○}p < 0.001$ vs. 15 min, $^{•}p < 0.05$ vs. PD98059 (50 μM), $^{••}p < 0.01$ vs. PD98059 (50 μM).

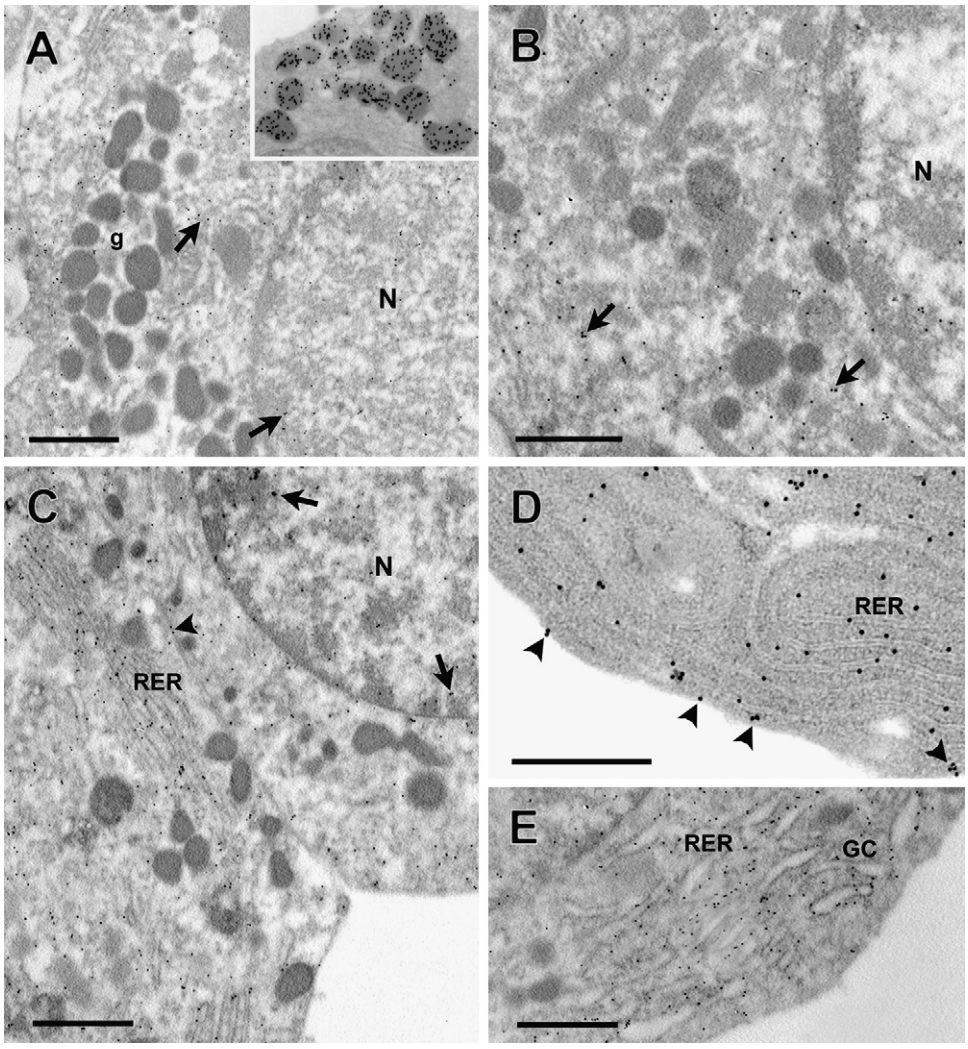

Fig. 8. Representative transmission electron micrographs of thin sections of control (A, B) and PMA treated for 15 min (C–E) lactotroph cells immunolabelled for PKCε. (A, B) Typical control lactotrophs show PKCε immunogold labelling sparse in cytoplasmic matrix and scarcely distributed in the nucleus (N) (arrows). The characteristic large and polymorphic mature secretory granules (g, about 500–900 nm in diameter) stored in the cytoplasm, are unlabelled for PKCε and serve as negative control. Inset: secretory granules from lactotroph cells specifically identified by immunocytochemistry for PRL. Bar 1 μm. (C–E) Lactotroph cells stimulated with PMA for 15 min exhibit a stronger immunoreactivity for PKCε associated with the plasma membrane (arrowhead), rough endoplasmic reticulum (RER), Golgi complex (GC), as well as that of the nucleus (N), than that observed in the control. The treated lactotroph cells display signs of enhanced metabolic activity characterized by a well developed RER and Golgi complex, both of which are immunolabelled for PKCε. In the nucleus the immunogold particles are attached to the chromatin and nuclear matrix (arrows). Bar 1 μm.

4. Discussion

In this report, we have investigated the mechanisms that participate in the regulation of the lactotroph proliferation, specifically the involvement of PKCα, PKCε and ERK1/2. The treatments with PMA employed in this study have enabled us to demonstrate the direct contribution of PKCα and ε on the regulation of lactotroph proliferation. Depending on the duration of the stimulus applied by phorbol esters, PKCs are either selectively activated or down-regulated (Ballester and Rosen, 1985). These effects of phorbol ester on lactotroph cell proliferation were determinate by the measurement of the BrdU-labelling index after PMA treatments. The activation of PKCs by PMA for 15 min stimulated the lactotroph proliferation whereas a prolonged activation for 3–8 h diminished this proliferative response. These results strongly indicate that the activation and down-regulation of PKCs are essential in the regulation of lactotroph proliferation. Furthermore, the PKC inhibitor, BIM, blocked the lactotroph proliferation induced by the shortest PMA exposure, demonstrating that PKCs are involved in this effect.

In general, the translocation of PKCs from the cytosol to the membranes is commonly associated with their activation. Our results have demonstrated that in pituitary cell cultures, PMA activated the PKCα and ε in different manners. The PKCε was rapidly translocated to the membrane fraction at 15 min, whereas the PKCα achieved this translocation only after 3 h of phorbol ester treatment. This difference in the time activation of PKC could expose a differential sensitivity in response to phorbol ester, as has been previously demonstrated (MacEwan et al., 1999). The stimulation of lactotroph proliferation observed after PMA treatment for 15 min was closely related to the highest PKCε translocation to the membrane fraction. The decrease in PKCε expression observed after long-time PMA activation could indicate that the down-regulation of this isoform may be responsible for inhibiting lactotroph proliferation. The participation of PKCε in lactotroph proliferation was confirmed by the specific inhibitor, epsilon V1 peptide, which was able to block the translocation of this isoform to the membrane fraction and consequently to prevent the mitogenic activity induced by PMA stimulation for 15 min. The present work is the first study that

demonstrates that the PKCε activation is closely correlated with the stimulation of lactotroph proliferation induced by PMA. The participation of PKCε on cell growth was also reported in other cell types (Basu and Sivaprasad, 2007). Moreover, it was shown that in prostatic epithelial cell lines and primary human non-small cell lung cancer, the over-expression of PKCε exerted an oncogenic activity (Wu et al., 2002; Bae et al., 2007).

The signal pathways by which PKCs participate in the lactotroph cell proliferation induced by PMA are not well understood. It has been previously described that the ERK/MAP kinase pathway, activated in a PKC-dependent or independent manner, is associated not only with cell proliferation but also with growth inhibition and cell differentiation (Qiu and Leslie, 1994; Greco et al., 2005; Wen-Sheng and Jun-Ming, 2005; Matsumoto et al., 2006). In our study, the ERK/MAP kinase pathway was activated by PKCs, as indicated by the phosphorylation of ERK1/2 which occurred after a short PMA treatment and its subsequent blockade by the down-regulation of PKCs induced by a long activation with phorbol ester. Therefore, our findings suggest that ERK1/2 are downstream targets of PKCs. The participation of ERK1/2 on the mitogenic activity of lactotroph cells was confirmed using the MEK inhibitor PD98059, which was able to eliminate the PMA-induced PRL cell proliferation. The stronger inhibitor effect induced by 100 μM of PD98059 (53%) compared to that of 50 μM (41%), may indicate that other signalling pathways are contributing to inhibit ERK1/2. Related to this, it has previously been reported that high concentrations (100–200 μM) of PD98059 can activate AMP kinase pathways, thereby inducing ERK1/2 inhibition (Dokladda et al., 2005).

In our present study, the highest activation of PKCε, obtained after 15 min of PMA, was directly related to the increase in the phosphorylation of ERK1/2 levels, indicating that this isoform is required for PMA-triggered ERK-MAPK signalling and cellular growth. The activation of PKCε by the PMA that induced an increase in the lactotroph cell number, and the subsequent reversal of this effect by the use of its specific inhibitor, implies that this novel PKC isoform is involved in lactotroph cell proliferation. Since the membrane targeting and activation of PKCs is mediated by phorbol ester, we decided to determine the specific subcellular localization of PKCε in lactotroph cells subject to a short stimulation with the PMA that induced the mitogenic effect on this cell type. This isoform was mainly translocated to membrane compartments which included plasma membrane, rough endoplasmic reticulum and Golgi complex and also to the nucleus of lactotroph cells upon short PMA treatment. This fine localization of PKCε in lactotroph cells using immunogold labelling has not been previously reported and constitutes ultrastructural evidence of PKC activation, a finding that was closely associated with the highest PKCε expression achieved in membrane fraction after short PMA stimulation. It is well known that the PKC function requires its translocation to a different cellular compartment. In GH3B6, TRH induced a specific translocation of PKCε from the cytoplasm to the plasma membrane (Collazos et al., 2006), while arachidonic acid translocated PKCε to the Golgi complex and nucleus (Shirai and Saito, 2002). Related to this, PKC isoform translocation to the plasma membrane was indispensable for ERK1/2 phosphorylation (Kuriyama et al., 2004), which is them involved in cell proliferation (Sharma et al., 2007). The PKCε translocation to the rough endoplasmic reticulum and Golgi complex may be interpreted in terms of membrane interaction with many partners (Poole et al., 2004), some of which are adaptor proteins and thus able to maintain each PKC isoform next to a subset of proteins and away from the other substrates (Schechtman and Mochly-Rosen, 2001). Several reports have indicated that PKCε interacts with the Golgi membrane coatomer protein β′-COP (RACK2) (Csukai et al., 1997), which is involved in vesicular trafficking (Dorn and Mochly-Rosen, 2002). The nuclear translocation of PKC is associated with nuclear envelope and interchromatin domains, suggesting a direct involvement of this kinase in the phosphorylation of the nuclear proteins that are responsible for the regulation of DNA replication (Zini et al., 1995).

Summing up, our observations demonstrate that PKCε plays an essential role in the lactotroph proliferation induced by short PMA stimuli, by triggering signals that involve ERK1/2 MAPK activation. These data highlight the importance of studying the key PKC isozyme responsible for transmitting the PMA trigged proliferative signals The understanding of how PKCε is modulated by different mitogenic stimuli will help unravel the mechanisms involved in the proliferative process of the pituitary gland and should provide insight into the pathogenesis of pituitary growth disorders.

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