Upregulation of PKC δ- and downregulation of PKC α-mRNA and protein by a phorbol ester in human T84 cells

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Abstract Protein kinase C (PKC) family members are downregulated by chronic activation at the protein level in most cell types. In T84 human epithelial cells 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) caused persistent translocation of PKC δ to the membrane compartment and a 400% increase of PKC δ-mRNA after 24 h. In contrast, PKC α protein was completely downregulated and its mRNA was decreased to 60% of control levels after 24 h. This is the first report of PKC δ-mRNA upregulation by TPA which was previously only shown for PKCβ. In view of the antimitogenic actions of PKC δ this pattern of regulation may serve to preserve growth control even in the presence of chronic cell activation.

Key words: Protein kinase C δ; Protein kinase C regulation; Protein kinase C mRNA regulation

1. Introduction

The protein kinase C (PKC) family of multifunctional serine/threonine protein kinases consists of at least 12 family members which are widely distributed in eukaryotes [1–3]. Among those PKC α [2], a 'classical' (cPKC) Ca^{2+}-dependent isoform, and PKC δ [3], a 'novel' (nPKC) Ca^{2+}-independent isoform are present in most central nervous and peripheral cell types [1,4]. Prolonged activation of PKC α by phorbol esters caused a rapid loss of PKC enzyme activity and protein while PKC α-mRNA was not affected in several studies [5,6]. Much less is known about the regulation of the more recently discovered PKC δ, particularly at the mRNA level. PKC δ was shown to exert antiproliferative actions upon overexpression in CHO [7] or NIH 3T3 cells [8,9] and to cause differentiation in myeloid 32D cells [10]. PKC δ is also a candidate for mediating the antiproliferative effects of phorbol esters observed in some pituitary and colon carcinoma cell lines [11,12]. Its mode of regulation may determine long-term responses of cells to prolonged activation of PKC in vivo, in particular with regard to the regulation of proliferation [13]. For example, we observed alterations of PKC δ protein and mRNA expression in human colonic and pituitary adenomas [14].

To learn more about its mode of regulation we examined the responses of PKC δ protein and mRNA to chronic activation by the phorbol ester TPA in a highly differentiated human colon carcinoma cell line T84. For comparison we also examined the regulation of PKC α which has not previously been investigated in this cell type.

2. Materials and methods

Chemicals were purchased from Merck, Darmstadt, Germany and restriction enzymes from Boehringer, Mannheim, Germany except when noted otherwise and were of the highest purity available.

2.1. Construction of PKC probes

Isotype specific human PKC cDNA fragments were subcloned into pSP72 or pSP73 plasmids (Serva, Heidelberg, Germany). A 194 bp *Sau*3A fragment of G3PDH derived from a G3PDH cDNA fragment (ITC, Heidelberg, Germany) was inserted into a *Bam*HI restricted pSP73 plasmid in 3′5′ orientation.

A PKC α fragment was generated by reverse transcription with MMLV reverse transcriptase (Gibco, Eggenstein, Germany) of 1 μg RNA of T84 cells and PCR according to Kawasaki [15]. PCR was performed with the upper primer ATTTAAGGAACCACAAGCA and lower primer AGACCCTGAACAGTTGATC for 40 cycles at 1 min 93°C, 1 min 49°C and 1 min 30 s 72°C. The 882 bp cDNA fragment was restricted with *Mae*II and *Stu*I and the 363 bp fragment was inserted into a pSP73 plasmid restricted with *Cla*I and *Pvu*II.

The PKC δ fragment was obtained from human blood cell buffy coat cDNA. A 498 bp cDNA fragment of PKC δ was detected using the upper primer CTGCTCTGGGGACTGGTGAA and lower primer GCAGATGAGGTGGGTGAGAAAG by PCR with reverse transcription. For this purpose 1 μg RNA were transcribed with MMLV reverse transcriptase (Gibco, Eggenstein, Germany) according to Kawasaki [15]. Amplification of cDNA was performed for 40 cycles at 1 min 15 s 92°C, 1 min 30 s 56°C and 1 min 72°C. The 498 bp PCR product was restricted with *Mae*II and *Hin*dIII and the 315 bp fragment was inserted into a *Cla*I and *Hin*dIII restricted pSP73 plasmid. All inserts were sequenced according to Sanger.

2.2. Transcription in vitro

Plasmids containing isotype specific gene probes for the PKC subtypes α, δ or G3PDH were linearized with *Bgl*I, *Hpa*I and *Xba*I, respectively. PKC α fragments were transcribed with T7 RNA polymerase and PKC δ and G3PDH with Sp6 RNA polymerase (Boehringer Mannheim, Germany). The transcripts had a specific activity of 2000 Ci/mmol except G3PDH which had a specific activity of 20 Ci/mmol. Complete transcription of the probes was controlled on denaturing polyacrylamide gels.

2.3. RNase protection analysis

In brief, 20 μg of total cellular RNA obtained using an acid phenol-chloroform extraction method [16] was hybridized with 300 000 cpm in 20 μl 40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA, 80% formamide. After submerse denaturation for 10 min at 85°C and cooling down slowly overnight to 54°C RNase digestion was performed with 1000 U/ml RNase T1 (Boehringer Mannheim, Germany), 5 mM EDTA, 0.3 M NaCl, 10 mM Tris-HCl pH 7.4 for 1 h at 30°C. RNase digestion was stopped with proteinase K and phenol chloroform extraction. After ethanol precipitation, washing and denaturing at 95°C for 3 min probes were run on a 6% denaturing polyacrylamide gel at 22 W for 2.5 h. Autoradiography was performed for 1–2 days using MP-hyperfilms (Amersham, Buchler, Germany). For quantification of PKC signals autoradiograms were scanned by laser densitometry (Molecular Dynamics, Sunnyvale, USA) relative to G3PDH using QuantImage software (Molecular Dynamics, Version 3.2 beta).

2.4. Cell culture

T84 cells were grown in DMEM/F12 (1:1) medium with penicillin and streptomycin (Boehringer Mannheim, Germany) containing 5%

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FCS at 37°C in a humid atmosphere containing 5% CO_2. Cells were subcultured with HBSS, 1 mM EDTA, 0.025% trypsin, pH 7.2 before reaching confluency and were grown on 10 cm ∅ petri dishes or 96 well plates for 2 days prior to experiments.

2.5. Immunoblotting

Denaturing gel electrophoresis was performed with 8% polyacrylamide gels according to Laemmli [17]. Proteins were blotted by alkaline semidry transfer [18,19] to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). Prestained molecular weight markers (Sigma, St. Louis, USA) were run in parallel to protein probes to control for completeness of transfer. Polyclonal antibodies were used generated against oligopeptides of rat PKC α (amino acids 313–326; Boehringer Mannheim, Germany) and human PKC δ (amino acids 658–676; Calbiochem, La Jolla, USA; cat. no. 539607, lot. no. B10897). Membranes were blocked in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20 (Sigma, Deisenhofen, Germany) with 2% non-fat dry milk for 1 h at room temperature. Antibodies to PKC α were incubated at 4 μg/ml and PKC δ at 10 μl/ml in TBS with 1% non-fat dry milk for 2 h at room temperature. Visualization of PKC signals was achieved with protein A conjugated to alkaline phosphatase (Calbiochem, La Jolla, USA) and BCIP/NBT (Sigma, Deisenhofen, Germany). For evaluation of the specificity of PKC signals we used single recombinant human PKC isotype proteins (Calbiochem, La Jolla, USA) generated in insect cells and competition with the peptides against which the antibodies were generated according to the manufacturers' protocols.

2.6. Quantification of immunoblots

PVDF membranes of PKC immunoblots were made transparent by soaking in 60% dimethyl sulfoxide and 40% absolute ethanol according to [20]. Membranes were sealed in plastic wraps and scanned with a laser densitometer (Molecular Dynamics). Relative intensities of PKC expression were determined by integrating the PKC signal subtracted from background signal using QuantImage software (Molecular Dynamics) which gave a linear signal between 5 and 40 μg protein loaded per lane with the amounts of PKC present in T84 cells.

2.7. Cytosolic and membrane homogenates and quantification of proteins

Cells grown to 80% confluency on 10 cm ∅ cell culture plates were washed in PBS and homogenized in cold 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl pH 7.5, 0.1% mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (added freshly), 0.1% (w/v) leupeptin, 0.1% aprotinin (Bayer AG, Wuppertal, Germany) by sonification (Braun, Melsungen, Germany) with 3 pulses of 10 s at 100 W on ice. All subsequent steps were performed at 4°C. Homogenates were centrifuged at 100 000 × *g* for 30 min. Supernatant was collected as the cytosolic fraction; the sediment was resuspended in homogenization buffer containing additionally 1% (v/v) Nonidet P-40. After incubation for 30 min on ice the homogenate was centrifuged at 14 000 × *g* for 30 min at 4°C. Supernatant was collected as the membrane fraction. Protein was determined by a modification [21] of the Bradford assay [22]. The remaining cytoskeletal proteins contained no detectable PKC protein.

3. Results

PKC δ protein was detectable by immunoblotting of T84 cell cytosolic and membrane protein and was completely eliminated by preincubation of the antibody with the immunogen (Fig. 1A). Treatment of T84 cells with TPA resulted in the rapid reduction of cytosolic PKC δ being apparent after 30 min (Fig. 2A, lanes 5,6) and led to almost complete loss after 24 h (Fig. 2A, lanes 1,2). The membrane-associated fraction increased in parallel within 30 min (Fig. 2A, lanes 5,6) and persisted for 24 h (Fig. 2A, lanes 1,2) indicating continued translocation of PKC δ from cytosol to membrane. PKC δ-mRNA was demonstrated by ribonuclease protection analysis (RPA) in 20 μg of unselected RNA. A G3PDH probe labeled to a low specific activity was hybridized together with the PKC δ probe and served as an internal marker for RNA loading. Treatment with 100 nM TPA caused an elevation of PKC δ-mRNA which was detectable after 4 h (Fig. 3A, lanes 5,6), persisting throughout the experiment and reaching 400% of basal values after 24 h (Fig. 3A, lanes 3,4).

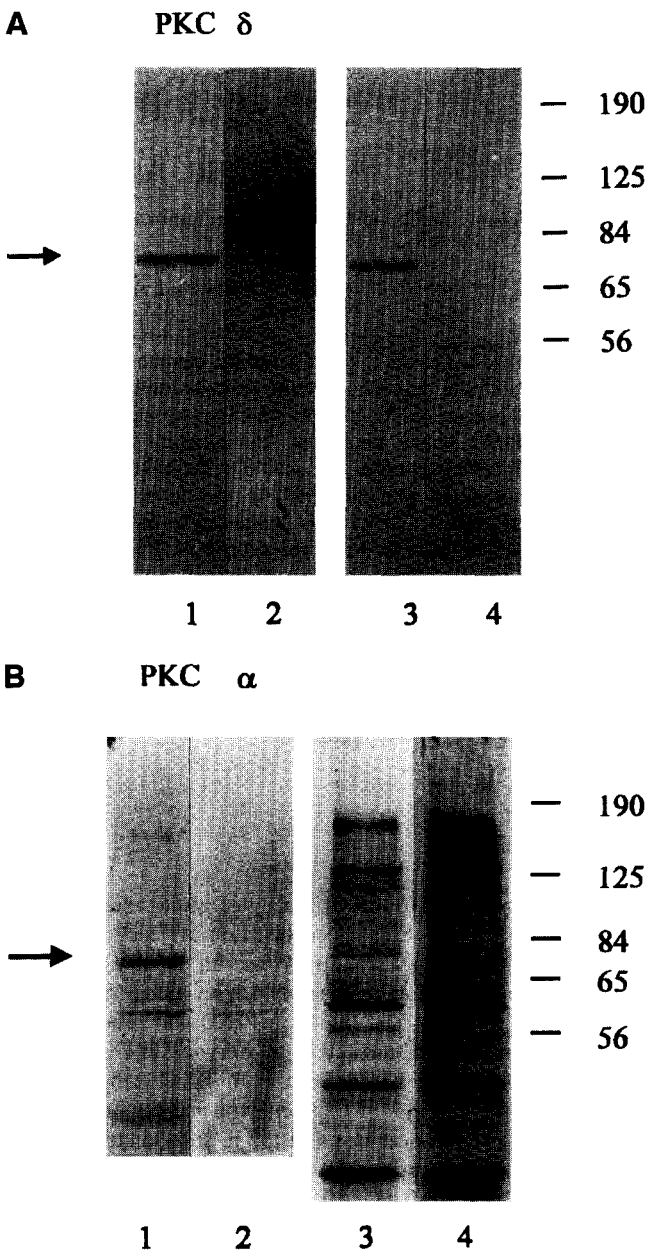

Fig. 1. Detection of PKC δ (A) and PKC α (B) expression by immunoblots. Cytosolic or membrane proteins were separated by SDS-PAGE using 20 μg per lane for PKCδ and 40 μg per lane for PKCα. For PKC δ and α lanes 1,3 show isotype specific detection in cytosol and membrane fractions, respectively; lanes 2,4 show the corresponding competition with the peptide against which the isotype specific antibodies were generated. Positions of molecular weight markers in kDa are shown to the right of each analysis.

In order to control for the possible regulation of G3PDH by TPA Northern blotting was performed which did not show a significant change of its expression.

PKC α was expressed at relatively low but detectable levels in the cytosolic compartment of T84 cells while the membrane signal was at the limit of detection (Fig. 1B). Cytosolic levels decreased upon TPA treatment and were almost undetectable

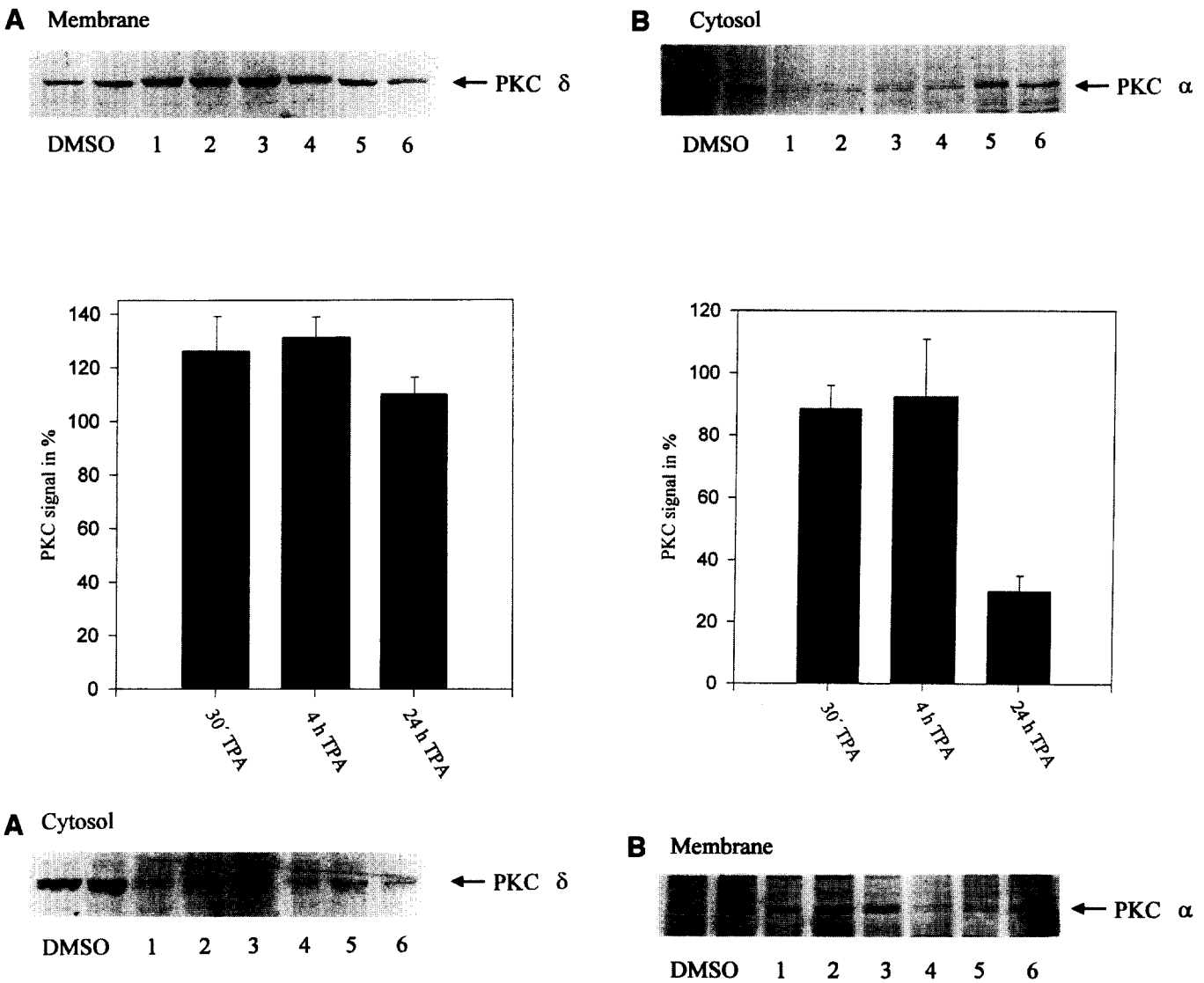

Fig. 2. Time-dependent expression of PKC δ (A) and α (B) protein during treatment of T84 cells with 100 nM TPA. 40 μg of cytosolic or membrane protein were analyzed per lane. Lanes 1,2 show signals of PKC δ or α of cells treated with TPA for 24 h, lanes 3,4 for 4 h and lanes 5,6 for 30 min. DMSO (24 h for controls) content was 0.1% (v/v) in all experiments. One representative blot is shown and the graphic analysis shows the results of 3 independent analyses relative to DMSO-treated cells as obtained by laser densitometry.

after 24 h (Fig. 2B, lanes 1,2). The membrane signal of PKC α showed no detectable change between 30 min and 24 h despite repeated attempts (Fig. 2B). PKC α-mRNA was readily detectable by RPA and suprisingly also decreased over 24 h of TPA treatment to 60% of initial levels (compare lane 1 = DMSO with lanes 2,3 = 24 h TPA; Fig. 3B). The time course of the decrease of PKC α-mRNA was relatively slow and a decrease was not yet apparent after 4 h of TPA treatment (Fig. 3B, lanes 4,5).

4. Discussion

Persistent activation of PKC by phorbol esters has been shown to lead to downregulation of both classical and novel PKCs in numerous cell types (for review see [4]). Regarding

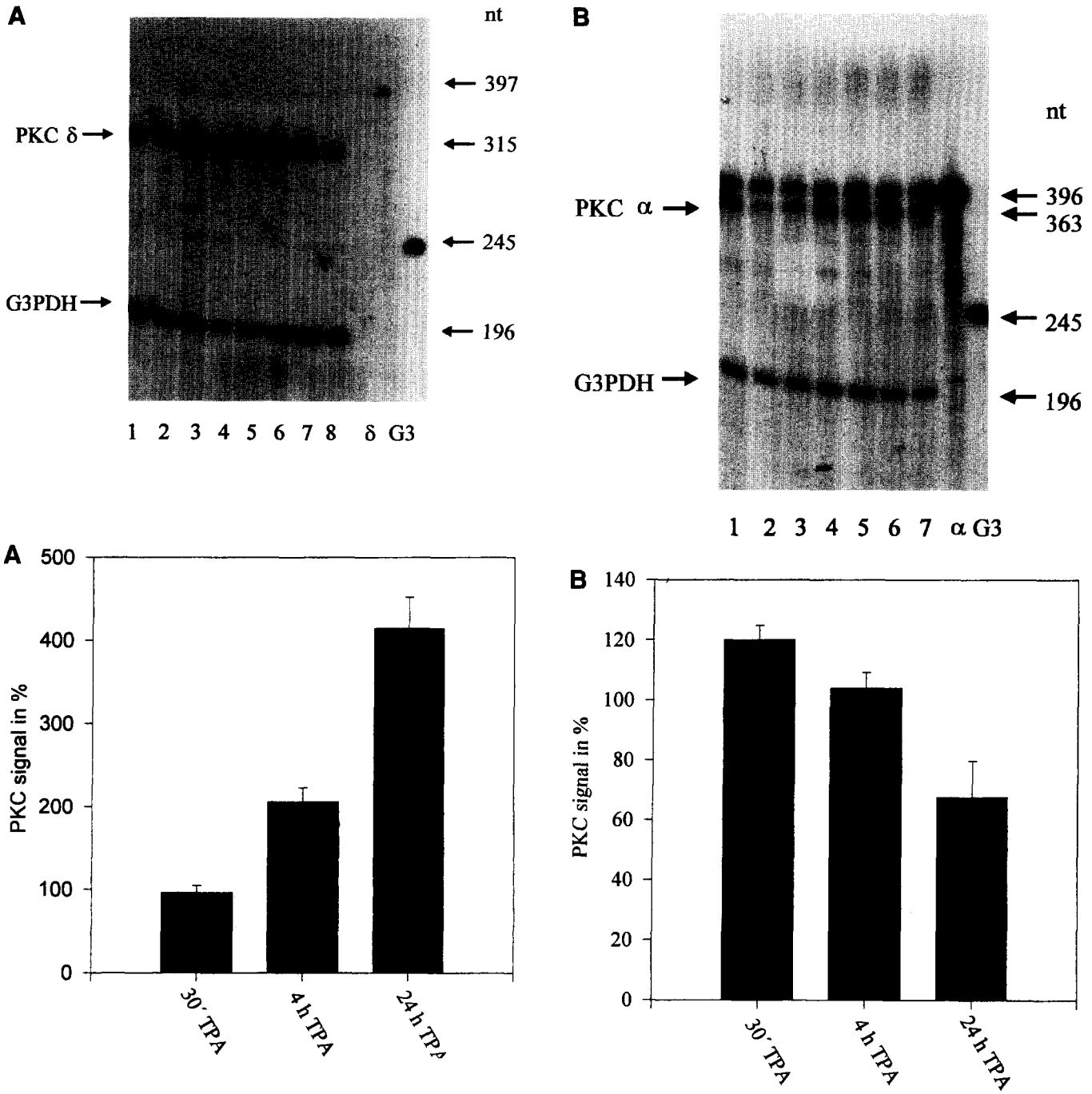

Fig. 3. Time course of PKC δ and α mRNA expression during treatment of T84 cells with 100 nM TPA. One representative RNase protection analysis of 20 μg total cellular RNA is shown in the upper panel. Each lane shows results from independent RNA extracts. Two 10 cm ∅ plates of T84 cells were treated with 100 nM TPA for 24 h (PKC δ: lanes 3,4; PKC α: lanes 2,3), 4 h (PKC δ: lanes 5,6; PKC α: lanes 4,5), 30 min (PKC δ: lanes 7,8; PKC α: lanes 6,7). 24 h DMSO (0.1% (v/v)) treated cells were analyzed in lanes 1,2 for PKC δ or in lane 1 for PKC α. PKC δ or α and G3PDH indicate the position of the protected fragments. The probes of transcription in vitro were used as molecular weight markers (PKC α = 396 nt, PKC δ = 397 nt, G3PDH = 245 nt). The analyses of three independent experiments are shown in the lower panel as obtained by laser densitometry in which RNA signals were corrected for G3PDH.

PKC α phorbol esters have been shown to accelerate degradation of the enzyme without affecting mRNA levels in NIH 3T3 or B82L cells [5,6]. In primary melanocytes a TPA-induced decrease of PKC α-mRNA similar to that described in this study has been reported [23]. This demonstrates that transcriptional or posttranscriptional downregulation of PKC α may occur in addition to posttranslational regulation.

In contrast, PKC δ was activated by TPA treatment as indicated by translocation and was persistently elevated in the membrane compartment. In parallel there was a persistent 400% increase of PKC δ-mRNA induced by TPA. This is reminiscent of the behaviour of PKC β which was shown to be upregulated by activation of PKC in lymphocytes [24], melanocytes [23], pituitary cells [25] and PC12 cells [26] at the protein and mRNA level. This upregulation is explained by the finding that the PKC β-promoter is positively regulated

by TPA [27,28]. The human PKC δ-promoter has not been reported to date but may be expected to contain similiar sequences. Alternatively, posttranscriptional regulation may occur.

The levels of PKC δ-protein were not elevated to the same degree as those of mRNA. However, it is important to stress that PKC δ was persistently elevated in the membrane compartment by TPA which caused downregulation of PKC α to undetectable levels. Although this was not investigated in the present study, levels of PKC protein are thought to be controlled by the rate of degradation upon translocation to the membrane by calpain or similar proteases. In the case of PKC α this process appears to be rapid and the loss of PKC protein is accentuated by a decrease in its mRNA levels. In the case of PKC δ the degradation of the protein appears to be counteracted by an elevated rate of resynthesis as suggested by the elevated levels of its mRNA.

PKC δ has been shown to exert antiproliferative effects in several cell types [7–9]. Its upregulation by chronic stimulation may thus present a protective mechanism against proliferative effects caused by cellular overactivation. Indeed, in T84 cells continous treatment with phorbol esters causes a persistent decrease of proliferation lasting for at least 18 days (Assert and Pfeiffer, unpublished). It will be of interest to study its regulation in other cell types.

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