Nuclear localization of protein kinase Cα and its association with nuclear components in Neuro-2a neuroblastoma cells

Klaus Buchner[a,*], Carsten Lindschau[b], Ferdinand Hucho[a]

[a]*Institute for Biochemistry, Free University of Berlin, Thielallee 63, 14195 Berlin, Germany*
[b]*Franz Vollhard Clinic, Max Delbrück Centre for Molecular Medicine, 13122 Berlin-Buch, Germany*

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Abstract Using activity measurements and Western blotting, we demonstrated that PKCα is constitutively present in nuclei of Neuro-2a neuroblastoma cells. Confocal microscopy revealed that PKCα is present in the nucleoplasm and that this localization does not change after stimulation with phorbol ester. However, as revealed by extraction experiments, phorbol ester leads to a firmer association of PKCα with nuclear components. Our findings suggest that PKCα not only associates with lipids but also with proteins inside the nucleus. The presence of active PKCα inside the nucleus allows the enzyme to phosphorylate not only proteins at the nuclear envelope but also proteins in the nucleoplasm.

Key words: Protein kinase C; Protein kinase Cα; Nucleus; Nucleoplasm; Confocal microscopy; Phorbol ester

1. Introduction

The members of the protein kinase C (PKC) family are involved in the transduction of a wide variety of cellular signals [1,2]. Besides its role in short-term events, such as the regulation of receptors or ion channels by phosphorylation, PKC isoforms play a pivotal role in the regulation of proliferation and differentiation of cells [3,4]. Since these processes are dependent on the control of nuclear events, it is obvious that to enable PKC growth-regulatory actions, some signal must reach the nucleus after PKC activation. PKC may bring about the transduction of signals toward and into the cell nucleus by acting in the cytoplasm, e.g., by triggering a nucleus-directed kinase cascade [5]. In addition, evidence has been accumulated over the past few years which shows that PKC can directly regulate nuclear processes. It can be translocated to the nucleus after stimulation or can be constitutively present in the nucleus and activated there (for review see [6]).

The presence of PKC isoforms in nuclei has been demonstrated for a variety of cell types [6]. As each cell type apparently expresses a defined set of PKC isoforms that are specifically located within the cell [7], no general rule can be established: in different cell types, different PKC isoforms are found at the nucleus and in subnuclear compartments [6]. The mechanism by which the different isoforms are directed to the nucleus and retained there is not clear. It is very likely that specific interactions with anchoring components of the respective nuclear compartment are necessary to achieve the observed distribution. Several reports exist on the interaction of PKC with cytoplasmic components like the cytoskeleton [8] and with PKC-binding proteins [9–11], but little is known about the association of PKC isoforms with nuclear components.

In this study we show that PKCα is present in the nuclei of Neuro-2a neuroblastoma cells and that stimulation of these cells leads to a more stable association of PKCα with structures inside the nucleus.

2. Materials and methods

2.1. Materials

DMEM, fetal calf serum and penicillin/streptomycin were from GIBCO-BRL. P81 ion-exchange-chromatography paper and DEAE-cellulose (DE 52) were from Whatman. [γ-^{32}P]ATP was from NEN. Phorbol-12 myristate, 13 acetate (PMA), dibutyryl-cAMP and other chemicals were from Sigma. Peptide GS, a specific substrate peptide for PKC, derived from glycogen synthase [12], was from Bachem, Heidelberg.

The monoclonal antibodies against PKCα were from UBI, the monoclonal antibody against neural cell adhesion molecule (N-CAM) was from Boehringer, Mannheim. The monoclonal antibody against a nuclear pore complex protein used in this study was raised in rat against a nuclear envelope preparation. It shows exactly the same staining pattern as a monoclonal antibody that is directed against a nuclear pore complex protein and will be described elsewhere in detail (manuscript in preparation).

2.2. Cell culture and stimulation

Mouse neuroblastoma cells (Neuro-2a) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5% CO_2.

PMA, dissolved in DMSO, was added to the medium to a final concentration of 160 nM. The final concentration of DMSO was 0.06%. Stimulation was carried out for 30 min unless stated otherwise.

2.3. Isolation of nuclei

Isolation of nuclei was performed essentially as described earlier [13]. The cells were washed twice with ice-cold PBS. The cells were then scraped off in PBS and spun down at 500×*g* for 5 min at 4°C. The cell pellet was resuspended in STM 0.25 (50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgSO_4$, 2 mM dithioerythritol (DTE), 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)).

Unless otherwise noted, the following manipulations were performed either with ice-cold reagents or at 4°C. A solution of 10% Nonidet P-40 was added to a final concentration of 0.025%. The pellets were homogenized with 30 strokes in a glass–glass Dounce-homogenizer (S-type). The homogenate was adjusted to 1.4 M sucrose by addition of an appropriate volume of STM 2.1 (50 mM Tris-HCl, pH 7.4, 2.1 M sucrose, 5 mM $MgSO_4$, 2 mM DTE, 10 μg/ml leupeptin, 1 mM PMSF). Ten milliliters of this suspension were transferred to each centrifuge tube and cushioned between 1 ml of STM 2.1 and 2 ml of STM 0.8 (50 mM Tris-HCl, pH 7.4, 0.8 M sucrose, 5 mM $MgSO_4$, 2 mM DTE, 10 μg/ml leupeptin, 1 mM PMSF). The tubes were filled to 14 ml with STM 0.25 and centrifuged at 100 000×*g* for 65 min in a swinging bucket rotor. The pellets containing the nuclei were resuspended in a small volume of STM 0.25.

2.4. Preparation of plasma membranes

A plasma membrane fraction was obtained from the interface be-

*Corresponding author. Fax: (49) 30-838-3753

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tween 1.4 M and 0.8 M sucrose of the gradient described above. The material was carefully removed from the phase border and diluted with 4 vol. of STM 0.25. The membranes were sedimented at 5000×*g* for 20 min and the pellets resuspended in STM 0.25.

2.5. Extraction of nuclei

For extraction with EDTA/EGTA, aliquots of stock solutions were added to suspensions of nuclei to final concentrations of 10 mM EGTA and 2 mM EDTA. After incubation on ice for 30 min with repeated mixing, nuclei were centrifuged for 15 min at 1000×*g* at 4°C. The pellets were resuspended in STM 0.25. NP-40 was added to a final concentration of 1%. After 30 min incubation on ice, nuclei were centrifuged as described above. Proteins in the EDTA/EGTA extracts and the NP-40 extracts were precipitated with 10% trichloroacetic acid (TCA) prior to solubilization in SDS-PAGE sample buffer. The pellets remaining after the NP-40 extraction were directly solubilized in sample buffer. To remove interfering DNA, these samples were mixed with 2 μl benzonase® (Merck, Darmstadt), incubated for 5 min at room temperature and then boiled for 5 min.

2.6. Western blotting

Subcellular fractions were subjected to SDS-PAGE (10% polyacrylamide gels) and transferred to nitrocellulose membranes. Proteins were stained with Ponceau red to control the efficiency of blotting and equal loading of the gel. The membranes were then blocked for 2 h with a solution of 10% (w/v) nonfat dry milk dissolved in TBS-T (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20). Incubation with the primary antibody was performed for 2 h at room temperature or at 4°C overnight, followed by 3 washes with TBS-T and incubation with the secondary antibody. Finally, blot membranes were washed 3 times with TBS-T and once with TBS. Colour development with alkaline phosphatase-coupled secondary antibody was performed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt). Horseradish peroxidase-coupled antibodies were detected with the ECL system (Amersham) according to the supplier's instructions.

All antibodies were diluted with TBS containing 5% (w/v) nonfat dry milk and (with the exception of horseradish peroxidase-coupled antibodies) 0.02% NaN_3.

2.7. Determination of PKC activity

Aliquots of nuclei were extracted with 1% NP-40 for 30 min on ice and then centrifuged for 15 min at 10 000×*g* at 4°C. The supernatants were applied to small DEAE anion exchange columns (bed vol. 0.5 ml) equilibrated with buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTE, 1 mM PMSF, 10 μg/ml leupeptin). After washing the columns with 2.5 ml of buffer A, PKC was eluted with 1.5 ml of 100 mM NaCl in buffer A. The eluates were concentrated in Centricon 30 microconcentrators (Amicon, Witten). Aliquots of the concentrated eluates were used to determination of PKC activity in a filter binding assay. The reaction mixture (total volume 50 μl) contained 20 mM triethanolamine, pH 7.4, 4 mM Mg-acetate, 0.1 mM $CaCl_2$, 400 μg/ml histone HIIIS or 50 μM peptide GS, 50 mM mercaptoethanol, 20 μM ATP supplemented with [γ-^{32}P]ATP to give an activity of 6–8×10^6 cpm/ml and, for assays under stimulating conditions, 50 μg/ml phosphatidylserine and 5 μg/ml diolein. After an incubation at 30°C for 10 min, 40 μl of the incubation mixture was applied to P81 ion exchange filters. The filters were washed 3 times with 0.5% phosphoric acid. Radioactivity was determined by measuring Cerenkov-radiation in water.

2.8. Immunofluorescence microscopy

Immunofluorescence microscopy was carried out as described in detail elsewhere [14]. In short, Neuro-2a neuroblastoma cells were cultured on multiwell microscope slides (Dunn). For stimulation, cells were treated with 160 nM PMA (phorbol-12-myristate-13-acetate) for 30 min. Cells were fixed with 3.7% paraformaldehyde for 10 min followed by permeabilization with 0.3% Triton X-100 in PBS for 10 min. After quenching with 100 mM glycine and blocking with 1% BSA in PBS, cells were incubated with the first and the second antibody (FITC- or Texas-Red-conjugated) for 60 min at room temperature in each case. Coverslips were mounted on slides with Fluoromount-G (Serva). For conventional fluorescence microscopy a Leitz DMIRB inverted microscope (Leica) was used. Optical sections of the immunostained cells were obtained with confocal laser scanning microscopy (MRC-600, Bio-Rad, coupled to an inverted Nikon Diaphot microscope or Leica TCS 4D, coupled to a Leica RBE microscope).

3. Results and discussion

3.1. Characterization of isolated nuclei

As shown before, nuclei prepared from Neuro-2a cells appeared to be intact and devoid of visible cytoplasmic structures attached to them [13]. Characterization of the nuclear preparation by measuring marker enzyme activities and by Western blot analysis of marker proteins further demonstrated a very small degree of contamination with proteins of other subcellular compartments. Lactate dehydrogenase-activity (marker for cytosol) and cytochrome *c* oxidase activity (marker for mitochondria) were not detectable with 100 μg of nuclear protein applied in the respective enzyme assays. The degree of contamination with plasma membrane proteins was checked using a monoclonal antibody against N-CAM, a protein specific for the plasma membrane. Whereas a strong signal was obtained for the plasma membrane fraction, faint signals were visible for the homogenate and no signals were detectable with the nuclear fraction (Fig. 1).

3.2. PKC-activity associated with isolated nuclei

PKC-activity associated with nuclei was measured after chromatography on small ion exchange columns. Nuclear extracts from cells stimulated with PMA contained more PKC activity than nuclear extracts from control cells (Fig. 2a). This could already be seen after 5 min stimulation, but the effect was more pronounced after 30 min.

Next, we addressed the question of which PKC isoforms were causing the measured activity. As shown by northern blot studies [15], Neuro-2a cells express the PKC isoforms α, ε, δ and ζ. Using Western blotting we found strong signals for PKCα and PKCε, whereas antibodies against PKCδ and PKCζ gave only faint signals (data not shown). Considering our assay conditions and the substrate used, our results

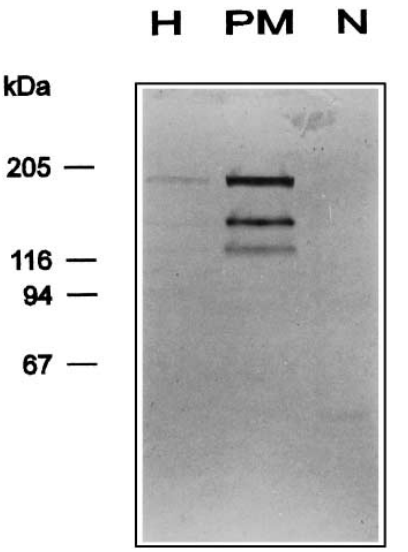

Fig. 1. Characterization of subcellular fractions. Western blot analysis of homogenate (H), plasma membrane (PM) and nuclear (N) fractions with a mAb against N-CAM shows strong enrichment of immunoreactivity in the plasma membrane fraction and virtually no signal with the nuclear fraction. Thirty micrograms of protein of each fraction was loaded on the gel.

strongly suggest a classical PKC isoform as basis for the nuclear PKC activity. Therefore, we concentrated our further experiments on PKCα.

Western blot analysis revealed that PKCα is indeed present in nuclei of Neuro-2a cells. Again, we found more PKCα in extracts from nuclei prepared from cells stimulated with PMA than in control extracts (Fig. 2b). Very similar results were obtained, when total nuclear protein (without extraction) was used (data not shown). Fig. 2b also shows that PKCα is completely extracted with 1% NP-40; after extraction no PKCα could be detected in the pellets.

Whereas the activity measurements showed a 2–3-fold increase, the Western blot analysis indicated a larger increase. This difference may be due to the rather long procedure (including ion-exchange chromatography) necessary for the activity measurements, which can easily lead to a partial inactivation of the enzyme

3.3. Immunocytochemistry

Immunocytochemical analysis using a monoclonal antibody against PKCα confirmed our result (obtained by Western blotting) that PKCα is present in Neuro-2a cell nuclei (Fig. 3a,c). To exactly demonstrate the location of the nuclei, the cells were also probed with a rat monoclonal antibody against a pore complex protein, giving the boundaries of the nucleus (Fig. 3b,d).

PKCα was present in the cytoplasm and in the nucleus, leaving a central region within the nucleus unstained (Fig. 3a). Since we used confocal laser scanning microscopy, we can be sure that the immunofluorescence signals detected in the nuclear regions truly reflect nuclear localization of PKCα, and is not caused by staining of PKC in areas above or below the nucleus. Furthermore, we could clearly localize PKCα inside the nucleus, whereas in earlier studies using conventional microscopy it was not possible to unequivocally distinguish between nucleoplasmic and nuclear envelope staining [16,17].

In contrast to what could be expected from activity measurements and Western blotting, we could not observe an increase in nuclear PKCα immunoreactivity after PMA stimulation (Fig. 3c). In preparations with high cell density, contact sites between cells especially showed a strong increase of PKCα after PMA stimulation, thus showing that PMA was active in these cells (data not shown).

3.4. Association of PKCα with nuclear components

At first sight, it seems that the results obtained with isolated nuclei are not in agreement with the immunocytochemistry data. In order to resolve this apparent contradiction, we carried out extraction experiments and analysed the association of PKC within nuclei in more detail. Extraction of nuclei from control cells with buffers containing EDTA and EGTA resulted in release of the major part of nuclear PKCα into the supernatant, whereas only a minor part remained associated with nuclear structures and could subsequently be extracted with NP-40 (Fig. 4). In contrast to unstimulated cells, only a small portion of PKCα could be extracted from nuclei of PMA-stimulated cells with EDTA/EGTA, whereas the major portion was found to be extractable with NP-40 (Fig. 4).

Taken together, these findings suggest that in control cells the majority of PKC is rather weakly associated with nuclei. Therefore, it could have been partially lost during the isolation procedure. PMA, on the other hand, led to a firmer association of PKCα with nuclei, therefore rendering the majority of nuclear PKC extractable only with detergent, and thus preventing a loss during the isolation of nuclei.

In contrast to immunocytochemical studies on other cell types [18,19] we found no indications for an association of

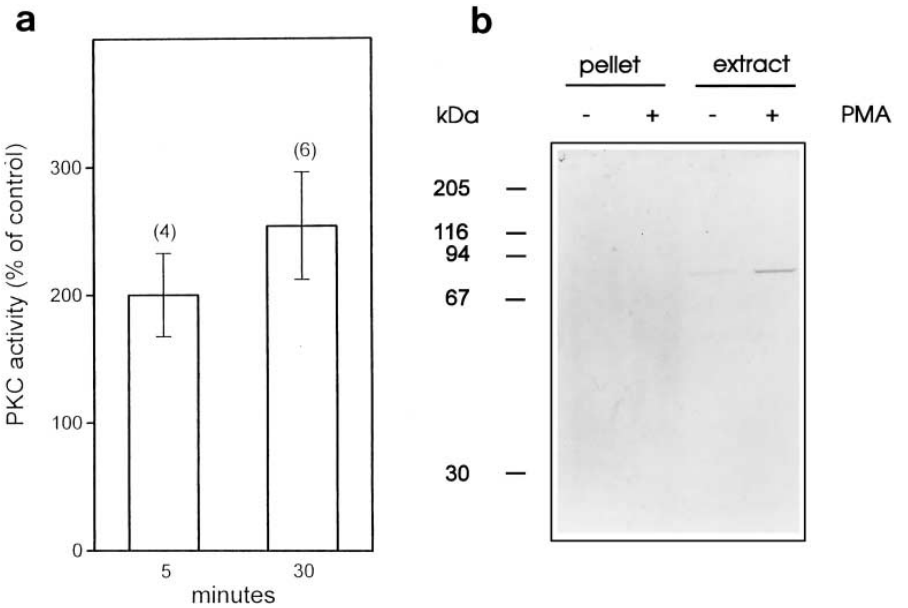

Fig. 2. PKCα in isolated nuclei from Neuro-2a cells. a: PKC activity in isolated nuclei after stimulation with 160 nM PMA for 5 and 30 min, expressed as percentage (±SEM) of activity found in control nuclei. Equal amounts of nuclear protein were used for extraction and activity determinations in each experiment. Numbers of experiments in parentheses. b: Western blot analysis with a mAb against PKCα applied to NP-40 extracts of isolated nuclei. Equal amounts of nuclear protein (100–200 μg) were used for extractions. In each experiment the entire pellets and extracts, respectively, were loaded on the gel. In the case of the extracts, proteins were precipitated with TCA. The absence of signals in the pellets after detergent extraction demonstrates a complete extraction. Extracts of nuclei from cells stimulated with 160 nM PMA for 30 min contain more PKCα than extracts from nuclei of control cells.

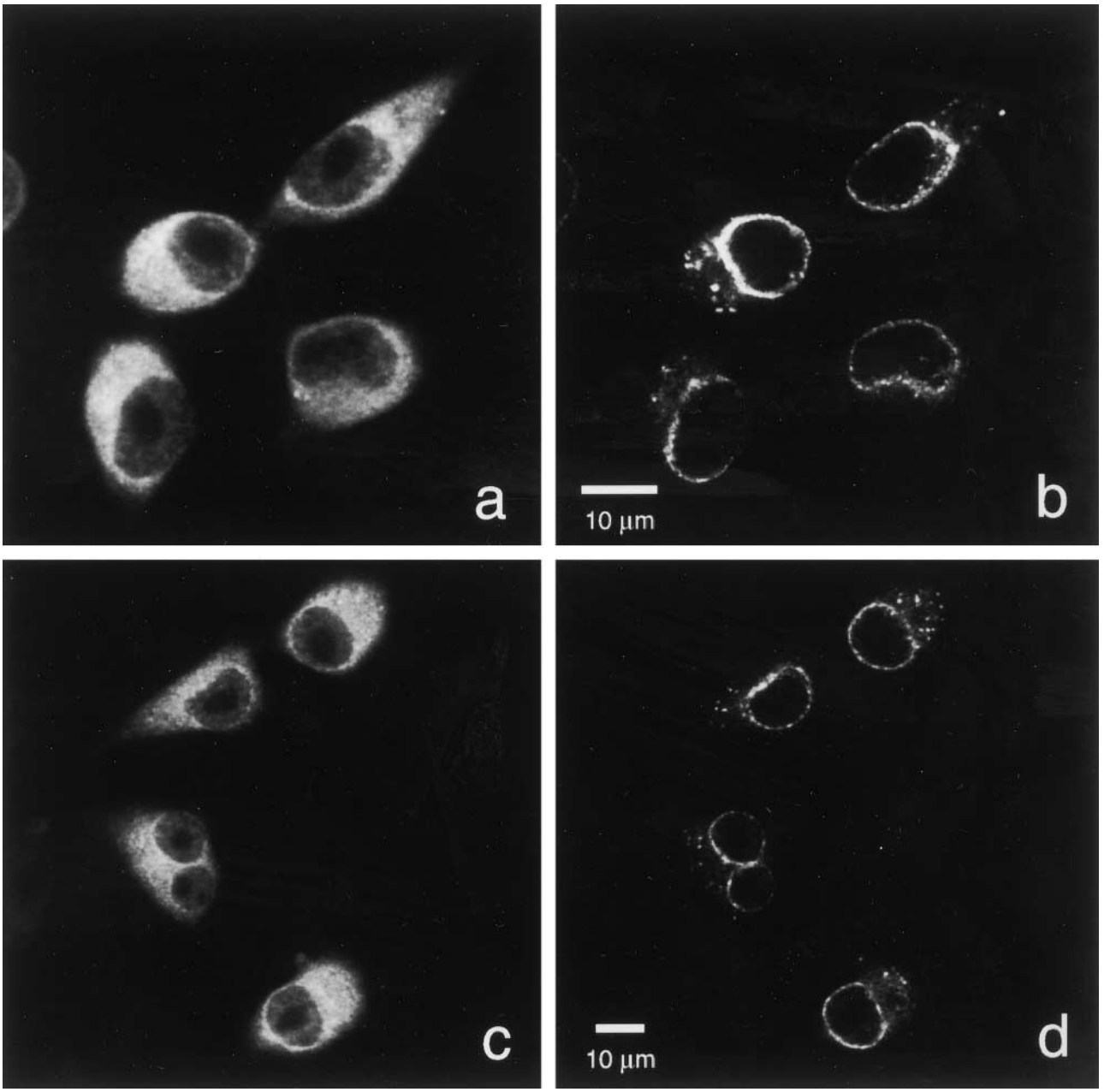

Fig. 3. Subcellular distribution of PKCα in Neuro-2a cells. Double-labeling experiments with a mAb against PKCα (a,c) and a mAb against a nuclear pore complex protein (b,d). The confocal images show that PKCα is localized in the cytoplasm and in the nucleus in control (a) and in PMA-stimulated (c) cells; PMA (160 nM for 30 min) apparently induces no change of PKCα localization in these cells. Staining with an antibody against a nuclear pore complex protein (b,c) delineates the boundaries of the nuclear envelope.

PKCα with ER-structures in control and PMA-stimulated cells. This makes it very unlikely that the results of our extraction experiments on isolated nuclei may be due to contamination of the nuclear fractions with ER membranes.

Since we could detect PKC inside the nucleus by immunocytochemistry, the association of PKCα with nuclear components most likely does not only occur with the lipids of the nuclear envelope but also with intranuclear, proteinaceous components. In recent experiments involving overlay assays, we were indeed able to detect nuclear proteins which bind activated PKC (Rosenberger et al., manuscript in preparation).

Since even conventional PKC isoforms under certain circumstances can show lipid-independent activity [20–22], it appears to be very likely that, after stimulation with PMA, PKC which is associated with intranuclear components, is in an active state.

The presence of active PKC in the interior of the nuclei would not only allow phosphorylation of nucleoplasmic proteins that are located near the membrane, but also of proteins further inside the nucleus, for example, proteins bound to chromatin. A couple of DNA-regulatory proteins (e.g. DNA-polymerases and DNA topoisomerases) and transcription factors (e.g. Fos, Jun and CREB) have been shown to be substrates of PKC (for review see [6]).

However, most of them have only been shown to be in vitro-substrates. Therefore, the function of nuclear PKC is far from clarified. To further elucidate the function of PKC

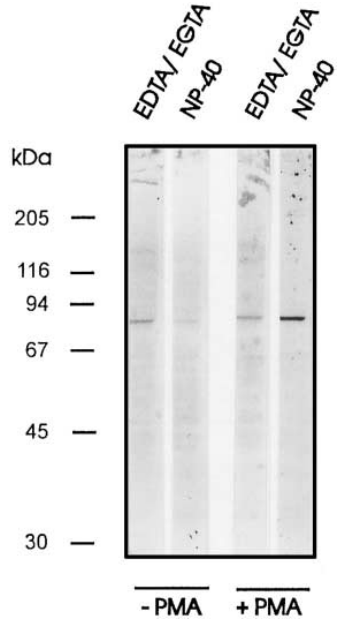

Fig. 4. Differential extraction of PKCα from Neuro-2a nuclei. Western blot analysis with a mAb against PKCα. Whereas the major portion of the enzyme could be extracted from control nuclei (−PMA) with EDTA/EGTA, the major portion of PKCα from PMA-stimulated cells (+PMA) could only be recovered in a subsequent extraction with NP-40. Extractions were performed with equal amounts of nuclei (100–300 μg of protein). The extracted proteins were precipitated with 10% TCA prior to loading on the gel.

localized inside the nucleus, nuclear PKC substrates must be identified in intact cells and assigned a role in the context of a defined biological process.

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