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TPA induces translocation but not down-regulation of new PKC isoform η in macrophages, MDCK cells and astrocytes

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Abstract New type protein kinase C (PKC) η was found to be expressed in RAW 264.7 and J774A.1 macrophages, Madin-Darby canine kidney (MDCK) cells and astrocytes by Western blot analysis. Both cytosol and membrane in macrophages and astrocytes express this isoform, however, the expression in the membrane is more abundant than that in the cytosol. On the other hand, only membrane PKCη was detected in MDCK cells. Exposure of the cells to 1 μM TPA for 10 min resulted in the translocation of PKCη from the cytosolic to the membrane fraction. This translocation maintained at a constant level after 1.5, 3, 6 and 24 h TPA treatment. However, another new type PKCδ which expressed in the macrophages and astrocytes was down-regulated after long-term (6 and 24 h) TPA treatment. The immunoreactive band of PKCη in J774A.1 macrophages was blocked by the control PKCη antigenic peptide. Incubation of RAW 264.7 macrophages with UTP (1, 10 and 100 μM) resulted in the accumulation of inositol phosphates, indicating the presence of P_2 receptor-coupled PLC pathway in these cells. This natural activator UTP also induced translocation of PKCη from cytosol to the membrane in RAW 264.7 macrophages after 1, 5 or 10 min treatment. Immunofluorescence microscopy revealed that in RAW 264.7 cells, PKCη is located in the cytoplasm organelle, plasma membrane and nuclear envelope. Stimulation of the cells with TPA resulted in translocation to the plasma membrane. This translocation of PKCη was still apparent after 24 h treatment with TPA.

Key words: Protein kinase C η; TPA; UTP; Macrophage; MDCK cell; Astrocyte

1. Introduction

Protein kinase C (PKC) comprises a family of isozymes that are pivotal in intracellular signal transduction [1,2]. It is activated by increased amount of diacylglycerol (DAG) in membranes, which results from hydrolysis of inositol phospholipids by phospholipase C (PLC) or from phosphatidylcholine (PC) by PC-PLC or by phospholipase D (PLD) [3]. This phospholipid-dependent serine/threonine kinase comprises at least 12 isoforms with different pattern of tissue expression and is divided into conventional (α, β_I, β_{II} and γ), new (δ, ε, η and θ) and atypical (ζ, λ, ι and μ) isoforms [1]. Activation of PKC requires association with the membrane and is induced by a number of activators and cofactors, these requirement differ for each isoform [4]. With the exception of the so called atypical PKC isoforms, the activity of membrane-associated PKC is activated by the second messenger DAG.

In addition to the natural activator DAG, PKC is activated with high specificity by the tumor promoting phorbol esters, such as TPA [5]. For this reason, phorbol esters are often used in the study of the mechanism of PKC activation. From a response elicited by phorbol ester, it is common to infer PKC involvement in the regulation of cellular processes [5]. When cells are activated by physiological stimulants or by phorbol esters that act via PKC, the conventional and new but not atypical isoform ζ in many different cell types become tightly associated with membrane fractions [6–9]. After long-term treatment with phorbol esters, down-regulation of both conventional and new [8] but not atypical isoform ζ was also observed [7,8,10,11]. Here we demonstrated that RAW 264.7 and J774A.1 macrophages, Madin Darby canine kidney (MDCK) cells and astrocytes expressed new PKC isoform η and this isoform was translocated but not down-regulated after treatment of these cells with TPA. This result was further confirmed by immunofluorescence staining. UTP, which acts through P_2 receptor coupled PLC pathway in RAW 264.7 macrophages to mediate phosphatidylinositol hydrolysis, also translocated PKCη from the cytosol to the membrane.

2. Materials and methods

2.1. Materials

Affinity purified rabbit polyclonal antibody against peptide sequence unique to PKCη and control peptide were purchased from Santa Cruz Biotechnology (California). Rabbit affinity-purified antibody against PKCδ-specific peptide sequence, Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin and streptomycin were obtained from Gibco BRL (Gaithersberg, MD). Fluorescein isothiocyanate (FITC) conjugates of goat anti-rabbit IgG was from Cappel (Aurora, Ohio). TPA was from LC Services (Woburn, MA). UTP was from Sigma Chemical Co. (St. Louis, MO). Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. Myo-[^{3}H]inositol (23.5 Ci/mmol) was from Dupont-New England Nuclear. The horseradish peroxidase-labeled donkey anti-rabbit second antibody and the ECL detecting reagent were purchased from Amersham International.

2.2. Cell cultures

RAW 264.7 and J774A.1 macrophages and MDCK cells, purchased from ATCC (Rockville, MD), were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary culture of astrocytes was prepared from cerebellum of 8-day old Wistar rats as described previously [12]. All the cells were plated in 145 mm Petri dishes for PKCη and δ assay and for nuclear protein separation; plated in 6-well plates for phosphatidylinositol hydrolysis assay and plated on 24-mm glass coverslip in 35 mm dishes for immunofluorescence staining. Cells were grown in an atmosphere of 5% CO_2/95% O_2 humidified air at 37°C.

2.3. Preparation of cell extracts and immunoblot analysis of PKCη and PKCδ in macrophages, MDCK cells and astrocytes

Cells in 145 mm petri dishes were treated with 1 μM TPA in the growth medium for periods of 10 min, 1.5, 3, 6 or 24 h and RAW 264.7 cells were treated with UTP for 1, 5 and 10 min before harvesting. The cells were then rapidly washed with ice-cold phosphate buffer saline (PBS), scraped and collected by centrifugation at 1000×*g* for 10 min. The preparation of cell extracts and immunoblot analyses of

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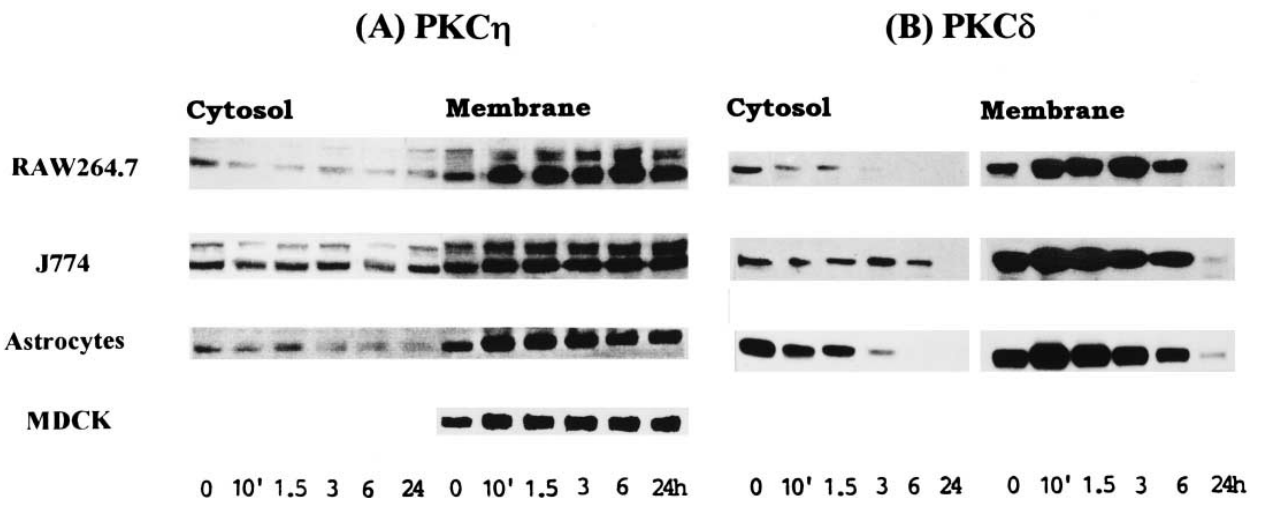

Fig. 1. Translocation and down-regulation of PKCη (A) and PKCδ (B) in macrophages, astrocytes and MDCK cells in response to TPA. Cells were treated with 0.1% DMSO (24 h) or 1 μM TPA for different times (10 min and 1.5, 3, 6 and 24 h) and then fractionated into cytosolic and membrane fractions, the proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose paper, and immunodetected with PKCη- and δ-specific antibodies. The autoradiography was obtained from ECL detection with three similar results.

PKCη and δ detected by ECL reagents were performed as described previously [8,12]. The peptide block of PKCη was performed by adding antigenic PKCη peptide in the PKCη antibody solution before incubation with the nitrocellulose membrane.

2.4. Measurement of phosphatidylinositol hydrolysis in RAW 264.7 macrophages

Phosphatidylinositol hydrolysis in RAW 264.7 macrophages was assessed by measuring the accumulation of [^{3}H]inositol phosphates in cells labeled with myo-[^{3}H]inositol (2.5 μCi/ml) for 24 h as previously described [12]. Cells were then washed three times with physiological saline solution (PSS) to remove free myo-[^{3}H]inositol. After 20 min preincubation at 37°C in PSS containing 10 mM LiCl, UTP at 1, 10 or 100 μM was added and incubation was performed for another 15 min.

2.5. Preparation of nuclear extracts

RAW 264.7 cells grown in 145 cm dishes were treated with 1 μM TPA in the growth medium for 10 min or 24 h before harvesting. The cells were then rapidly washed with ice-cold PBS, scraped. The collected cells were resuspended in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 mM NaF and 1 mM Na_3VO_4) and incubated 15 min on ice. Cells were lysed by the addition of 0.5% NP-40 and mixed by inversion, and the nuclei were pelleted. The nuclear pellet is resuspended in extraction buffer B (added 1 mM EDTA and replaced 10 mM KCl with 450 mM NaCl in buffer A) and incubated for 15 min at 4°C on rocking platform and then the nuclear extracts were centrifuged.

2.6. Immunofluorescence staining

Raw 264.7 cells grown in coverslips were treated with 1 μM TPA in the growth medium for 10 min or 24 h. The cells were then rapidly washed with PBS and fixed at room temperature for 15 min with 3% paraformaldehyde. After washing with PBS, cells were blocked and permeabilized with 1% BSA in Tris-buffer saline containing 0.1% Triton X-100 for 20 min at room temperature. The cells were then incubated with anti-PKCη antibody (1 : 50) for 30 min, washed extensively, and then stained for 30 min with anti-rabbit IgG-fluorescein (1 : 2000). After additional washes, the coverslips were rinsed in distilled water and mounted on glass slides using mounting medium (2% *n*-propyl gallate in 60% glycerol and 0.1 M PBS, pH 8.0). Optical sections of the immunostained cells were visualized and photographed with an Zeiss Axiovert inverted microscope equipped with epifluorescence. In negative controls, the primary antibody was omitted and no background staining was seen.

3. Results

3.1. Expression of PKCη and δ in macrophages, astrocytes and MDCK cells after short-term and long-term TPA treatment

Using PKCη-specific antibody, the abundant expression of this isoform in both cytosol and membrane of RAW264.7 and J774A.1 macrophages was found (Fig. 1A). It appeared in the immunoblot as a doublet with a major immunoreactive band

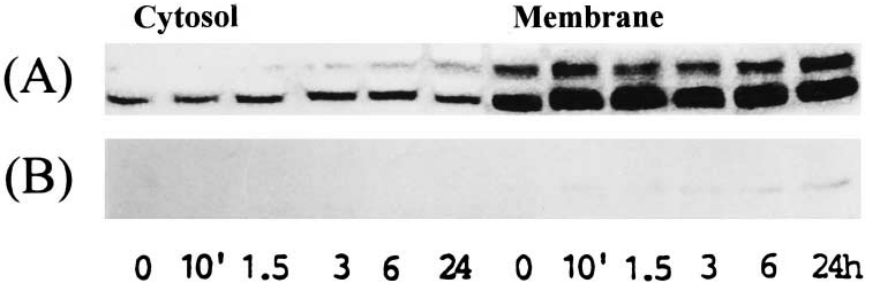

Fig. 2. Protein immunoblots showing translocation of PKCη in J774A.1 macrophages after treatment with TPA. Transferred nitrocullose paper was blotted with PKCη antibody in the absence (A) or presence (B) of PKCη peptide antigen. Cells were treated with 1 μM TPA for different times (10 min and 1.5, 3, 6 and 24 h) and separated into cytosolic and membrane fractions that were subjected to SDS-PAGE. Transferred nitrocellulose papers were blotted with PKCη antibody (A) and PKCη antibody plus PKCη peptide antigen (B), respectivley. The autoradiography was obtained from ECL detection with three similar results.

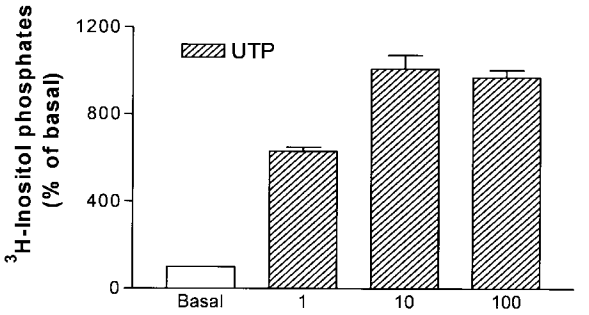

Fig. 3. Stimulation of [^{3}H]inositol phosphates formation induced by UTP in RAW 264.7 macrophages. Prelabeled cells were washed with physiological salt solution and lithium-dependent accumulation of [^{3}H]inositol phosphates was measured in the presence of UTP at indicated concentrations at 37°C for 15 min. Results are expressed as the mean ± S.E.M. of three experiments. Basal [^{3}H]inositol phosphates accumulation was 400 ± 21 cpm/well.

at 82 kDa and one minor band above 82 kDa. Inclusion of the control antigenic PKCη peptide, which was used for raising this antibody, in the immunoblot analysis prevented completely the detection of these two immunoreactive bands of PKCη (Fig. 2). The expression of PKCη in astrocytes is in both cytosol and membrane as well, however, that in MDCK cells is only in the membrane (Fig. 1A). After treatment of cells with 1 μM TPA for 10 min, 1.5, 3, 6 and 24 h, translocation of PKCη was seen after 10 min treatment. This translocation was maintained even after long-term 24 h treatment (Fig. 1A). On the other hand, another new type PKCδ, which also abundantly expressed in the macrophages and astrocytes, was translocated after 10 min TPA treatment and gradually down-regulated after 1.5, 3 and 6 h treatment. A complete down-regulation of PKCδ was seen after 24 h TPA treatment (Fig. 1B).

3.2. *Effect of UTP on phosphatidylinositol hydrolysis and on PKCη translocation in RAW 264.7 macrophages*

UTP (1 μM) induced a 6-fold increase of inositol phosphates formation in RAW 264.7 macrophages. Both 10 μM and 100 μM UTP induced a maximal 10-fold increase of inositol phosphates formation, indicating the presence of P_2 receptor coupled-PLC pathway to mediate phosphatidylinositol turnover in these cells (Fig. 3). A 1, 5 and 10 min exposure of RAW 264.7 macrophages to 100 μM UTP, the natural activator, also induced translocation of PKCη from cytosol to the membrane (Fig. 4).

3.3. *PKCη is located in the cytosol, plasma membrane and nuclear envelope in RAW 264.7 cells*

Immunofluorescence staining showed the expression of PKCη in certain cytoplasm organelle, plasma membrane and at the nuclear envelope in RAW 264.7 cells (Fig. 5A). Western blot of nuclear extracts did show the expression as well (Fig. 5E). Stimulation of cells with TPA leads to translocation of PKCη to the plasma membrane (Fig. 5C). This event is lasted after 24 h treatment with TPA (Fig. 5D), confirming the translocation but not down-regulation of PKCη to plasma membrane in Western blot analysis (Fig. 1A)

4. Discussion

The new type PKCη has a unique tissue distribution [13,14]. Unlike other members of the PKC family, nPKCη is expressed predominantly in epithelial tissues, including skin, tongue, esophagus, stomach, intestine, trachea and bronchus. In situ hybridization and immunohistochemical staining indicated that nPKCη is localized in differentiated or differentiating epithelial cells rather than proliferating basal cells [14]. Particularly in the skin, it is localized exclusively in the uppermost granule layers [15], which consists of the terminally differentiating keratinocytes with keratohyalin granules. In addition to epithelial cells, we found nPKCη also expressed in macrophages RAW 264.7 and J774A.1, astrocytes and MDCK cells in the present study. There is a doublet with molecular weight around 82 kDa in the immunoblot of macrophages but not astrocytes and MDCK cells (Fig. 1A). Osada et al. [13] reported a 82 kDa PKCη expressed in transfected COS cells, while Livneh and coworkers [16,17] reported a 86 kDa PKCη expressed in transfected COS cells and in several skin-derived cell lines. The doublet in the present macrophages might be equivalent to 82 and 86 kDa PKCη, respectively, since they were completely blocked by PKCη antigenic peptide (Fig. 2). Translocation of PKCη from cytosol to the particulate fraction was seen in response to 10 min treatment with TPA in macrophages, MDCK cells and astrocytes [18] (Fig. 1A) and in response to 1, 5 and 10 min treatment with UTP in RAW 264.7 macrophages (Fig. 4). The translocation of PKCη was still apparent after 24 h treatment with TPA in condition complete down-regulation of another new type PKCδ in macrophages and astrocytes [12] was seen (Fig. 1B). Therefore, nPKCη was translocated but not down-regulated by TPA in these three types of cells. Previous report [16] suggested that PKCη is the nuclear receptor for phorbol esters in skin-derived cell lines. Greif et al. [17] using immunofluorescence staining indicated the distribution of PKCη throughout the nucleus but not cytosol of human epidermal carcinoma A431 cells, skin keratinocyte cell line HaCaT and skin squamous carcinoma cell line SCL-1. PKCη was involved in the transcriptional activation of the human transglutaminase 1 gene which is expressed during the terminal differentiation of keratinized squamous epithelium to form cornified cell envelop in differentiated keratinocytes [19]. However, by chemical and morphological analyses, Chida et al. [20] reported the localization of PKCη exclusively on the rough endoplasmic reticulum and outer nuclear membrane in keratocytes. Our results from RAW 264.7 cells are somewhat similar to this finding. Western blot showed the expression of PKCη in nuclear extracts (Fig. 5E) in addition to cytosol and membrane and immunofluorescence staining confirmed this result (Fig. 5A). Short-term treatment (10 min) with TPA resulted in the translocation of this isoform to the plasma membrane, and

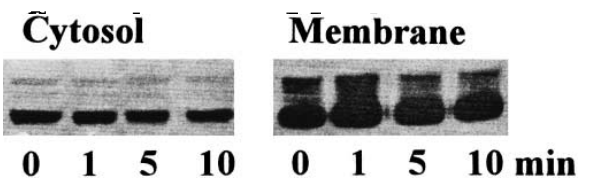

Fig. 4. Immunoblot detection of translocation of PKCη in RAW 264.7 macrophages by UTP. Cells were treated with 100 μM UTP for 1, 5 and 10 min, the cytosolic and membrane fractions were prepared as described in Section 2.3. Samples (100 μg of protein) were separated by SDS-PAGE, transferred to nitrocellulase paper and immunoreacted with PKCη antibody (1 : 300 dilution). The autoradiography was a representative of three similar results.

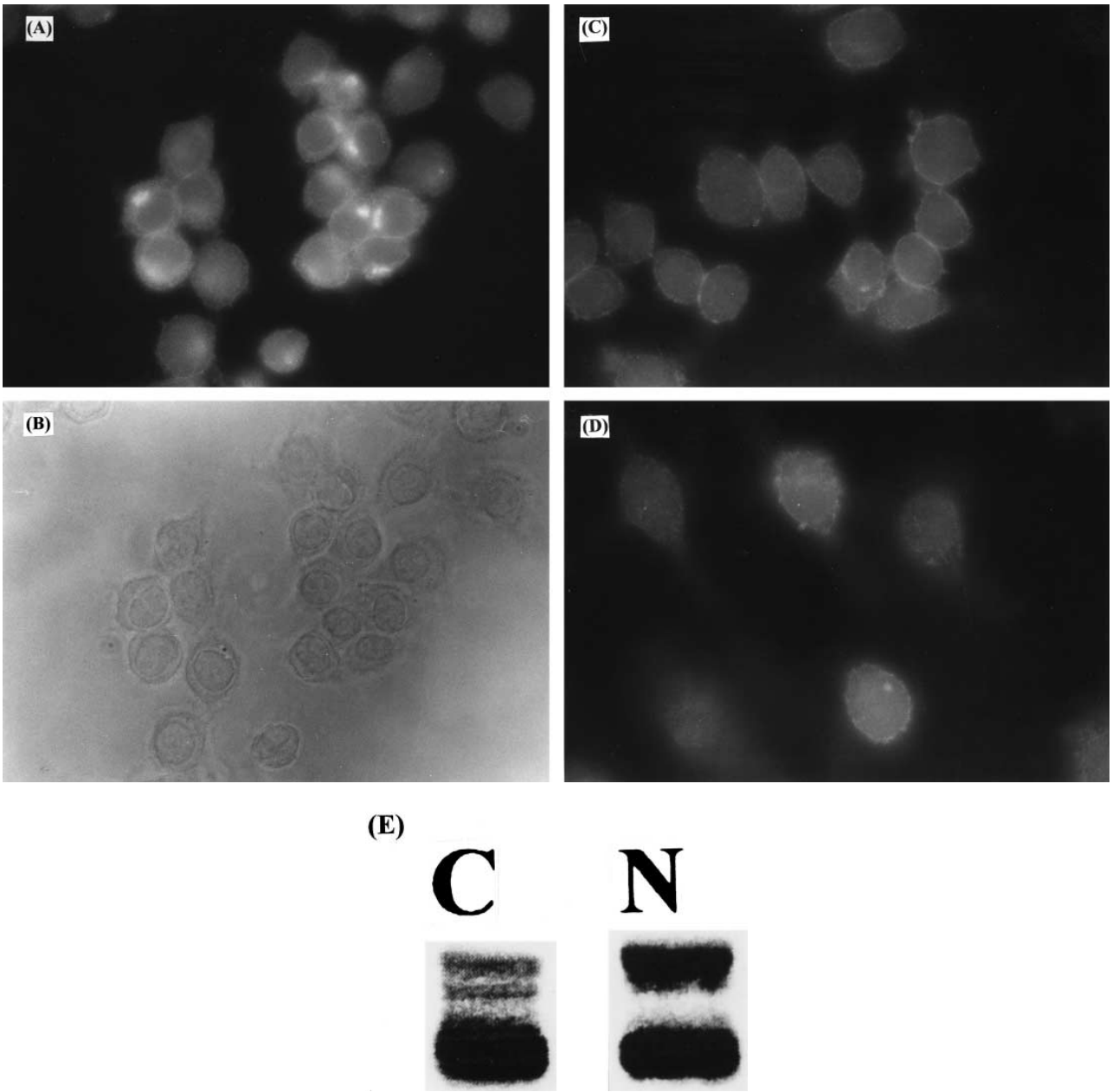

Fig. 5. PKCη is located in the cytoplasm, plasma membrane and nuclear envelope. Immunofluorescent staining of RAW 264.7 macrophages with affinity-purified PKCη antibody (1 : 50). Cells were fixed and stained as described in Section 2. Control (A) and image of phase contrast (B), after TPA 10 min stimulation (C), after TPA 24 h treatment (D) and Western blot of PKCη in (N) nuclear extracts and (C) cytosol (E).

this phenomenon is still apparent after 24 h TPA treatment (Fig. 1, Fig. 5C and 5D). PKCη in astrocytes and MDCK cells was not related to the regulation of ATP-induced phosphatidylinositol hydrolysis [18] and arachidonic acid release [21], respectively. The functional role of PKCη in nonepithelial cells, especially in macrophages, remains to be investigated.

The translocation of PKCη and resistance to down-regulation by TPA would suggest that in TPA-down-regulated cells, often described as PKC-depleted cells, the cellular pathways previously identified as PKC independent may still be regulated by PKCη. Hence, interpretation of experiments involving phorbol ester treatment, at least in the cells which expressed PKCη, should take into account the activation of this isoform. The nuclear PKCη is also resistant to down-regulation by TPA in A431, HaCa T and SCL-1 cells [17]. In contrast, complete down-regulation of PKCη in murine epidermis by 18 h TPA or bryostatin treatment was reported by Gschwendt et al. [11].

In summary, PKCη expressing cells could be activated by TPA and natural activator, while this isoform is not down-regulated by TPA. PKCη may be responsible for some of the actions induced by phorbol esters or natural activator in the cell membrane or nucleus, at least in macrophages. The exact molecular mechanisms involved remain to be elucidated.

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