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Protein kinase C α, δ, ε and ζ in C_6 glioma cells

TPA induces translocation and down-regulation of conventional and new PKC isoforms but not atypical PKC ζ

Ching-Chow Chen*

Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei 10018, Taiwan, ROC

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Both the cytosol and membrane in C_6 glioma cells express abundance of PKCα, δ, ζ and trace amount of PKCε by Western blot analysis with isozyme-specific antibodies. These characteristics make this cell line a good model to study the properties of different classes of PKC isoforms in one cell type. Exposure of the cells to 100 nM TPA for 10 min resulted in the translocation of conventional PKCα (cPKCα) and new PKCδ (nPKCδ) and -ε from the cytosolic to the membrane fraction, while left atypical PKCζ (aPKCζ) unaffected. The extent of translocation of cPKCα induced by TPA was more prominent than that of nPKCδ and nPKCε. α-TPA, the inactive phorbol ester, did not induce translocation of these isozymes. After treatment of the cells with 1 μM TPA for 17 h, cPKCα, nPKCδ and nPKCε were almost completely down-regulated, whereas aPKCζ was still unaffected. The natural activators of this cell line, endothelin-1 and ATP also translocated cPKCα and nPKCδ. However, the extent of translocation induced by these two agonists was much less than that of TPA.

Protein kinase C; Isozyme, conventional, new and atypical; TPA; Natural activator

1. INTRODUCTION

Protein kinase C (PKC) is one of the major mediators of signals generated upon external stimulation of cells by hormones, neurotransmitters and growth factors. Molecular cloning analysis has shown that PKC is a family of at least ten isozymes, all having closely related structure but differing in their individual properties [1,2]. They are divided into three classes; one contains the putative Ca^{2+}-binding region C-2 and is Ca^{2+}-responsive (conventional PKCα, -β, -γ), another lacks this region and is Ca^{2+}-unresponsive (new PKCδ, -ε, -η, -Θ), the other also lacks this region and has only one cysteine-rich zinc finger-like motif in the region C-1 (atypical PKCζ, -λ) [2]. The presence of these PKC isozymes and their cell type-specific expression [3] strongly suggest that different isozymes have distinct functions within a given cell. As the phorbol ester TPA is known to activate PKC, mimicking the physiological activator diacylglycerol, PKC is thought to mediate TPA-induced biological effects [1]. The translocation and down-regulation of conventional PKC isozymes were studied by either enzyme activity assay or Western blot analysis [4–9], while those of new and atypical PKC isozymes could only be investigated by Western blot analysis with isozyme-specific antibodies [9–15]. The endothelin- and ATP-induced phosphatidylinositol (PI) breakdown in C_6 glioma cells was negatively regulated by phorbol ester [16–18] and abundance of conventional PKCα (cPKCα), new PKCδ (nPKCδ) and atypical PKCζ (aPKCζ) and trace amount of nPKCε was found in this cell line [19]. Therefore, this cell is a good model to study the properties of the three classes of PKC isoforms and further define which PKC isoform is involved in the negative regulation of receptor mediated PI hydrolysis [20,21]. Short term (10 min) and long term (17 h) treatment of the cells with TPA was studied in the present investigation. Here we demonstrated that the conventional PKC and new PKC isozymes were translocated and down-regulated by TPA, while atypical PKCζ was resistant to TPA with respect to translocation and down-regulation. The natural activators of C_6 glioma cells, endothelin-1 (ET-1) and ATP also translocated conventional and new PKC isozymes but not atypical PKCζ.

*Corresponding author. Fax: (886) (2) 391-5297.

2. MATERIALS AND METHODS

2.1. *Materials*

Rabbit polyclonal antibodies against peptide sequence unique to PKCα, -ε, -δ, -ζ, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin and streptomycin were purchased from Gibco BRL (Gaithersburg, MD). TPA and α-TPA were from L.C. Services Corp (Woburn, MA). ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF) was from Sigma (St. Louis, MO). Leupeptin was from Boehringer Mannheim (Mannheim, Germany). Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. [^{125}I]Protein A were from DuPont-New England Nuclear.

Stock solutions of TPA were made in dimethylsulfoxide (DMSO)

and diluted just prior to use. DMSO up to a concentration of 0.1% had no effect on cells.

2.2. *Cell culture and cell treatment with TPA and agonists*

C_6 glioma cells obtained from American Type Culture Collection (Rockville, MD) were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. All the cells were grown in 145 mm Petri dishes in an atmosphere of 5% CO_2/95% humidified air at 37°C. When the cells reached confluence, TPA, α-TPA, DMSO, ET-1 or ATP was added to the growth medium for 5 min, 10 min or 17 h prior to the harvest of cells, then the cells were rapidly washed with ice-cold phosphate buffer saline (PBS) and scraped, and were collected by centrifuging for 10 min at 1000 × *g*.

2.3. *Preparation of cell extracts*

The collected cells were lysed in ice-cold homogenizing buffer containing 20 mM Tris-Cl, pH 7.5, 1 mM dithiothreitol (DTT), 5 mM EGTA, 2 mM EDTA, 10% glycerol, 0.5 mM PMSF and 5 μg/ml leupeptin by a sonicator with four 10 s burst. The homogenates were centrifuged at 45,000 × *g* for 1 h at 4°C to yield the supernatants and pellets. The resulting pellets were resonicated in homogenizing buffer and centrifuged again at 45,000 × *g* for 1 h. These two supernatants were combined to get the crude cytosolic extract and the pellets were membrane fractions.

2.4. *Immunoblot analysis*

The cytosolic extracts and membrane fractions (100 μg of protein) were denatured by heating in Laemmli stop solution [22] and subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane for 1 h at 1 A current as described by Towbin et al. [23]. The membrane was incubated successively with 1% bovine serum albumin (BSA) in Tris buffer saline containing Tween-20 (TTBS) (50 mM Tris-Cl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween-20) at room temperature for 1 h, with rabbit antibodies to PKCα, PKCδ, PKCε and PKCζ diluted 1:250 in TTBS containing 1% BSA for 3 h, and with [^{125}I]protein A (0.4 μg, 4–6 μCi/20 ml) for 1 h. Following each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands were visualized and quantitated by Phosphor Imager-Image Quant (Molecular Dynamics, Sunnyvale, CA).

3. RESULTS

Using isozyme-specific antibodies, we detected the abundant expression of cPKCα, nPKCδ, aPKCζ in both cytosolic and membrane fraction and trace amount of nPKCε in membrane fraction of C_6 glioma cells with molecular mass of 80, 80, 80 and 90 kDa respectively by Western blot analysis (Fig. 1). Immunoreactivity against these bands was blocked by the presence of specific antigen peptides (data not shown). The expression of cPKCα is preferentially in the cytosolic fraction, while nPKCδ and trace amount of nPKCε are predominantly present in the membrane fraction and nPKCε in the cytosolic fraction is undetectable (Fig. 1). The aPKCζ has almost equal expression in the cytosolic and membrane fractions.

A 10 min exposure of cells to 100 nM TPA induced translocation of cPKCα, nPKCδ from the cytosolic to the membrane fraction. Although only trace amount of nPKCε was in the membrane fraction, the increased expression after 10 min treatment with TPA was also clearly seen from Western blot analysis (Fig. 1). However, aPKCζ was not translocated by TPA. The inactive

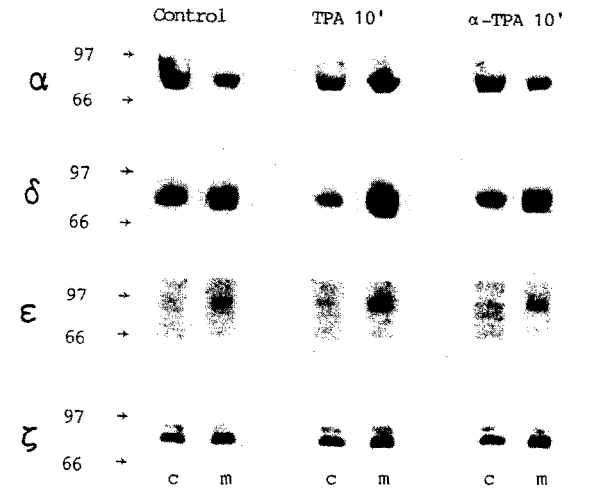

Fig. 1. Immunoblot detection of translocation of PKCα, -δ, -ε and -ζ in C_6 glioma cells induced by TPA. Cells were treated with 0.1% DMSO (control) or 100 nM TPA or 100 nM α-TPA for 10 min, and the cytosolic (c) and membrane (m) fractions were prepared as described in methods. Samples (100 μg of protein) were separated by SDS-PAGE, transferred to nitrocellulose paper and immunodetected with antibodies of PKCα, -δ, -ε and -ζ (1:250 dilution) as described under section 2.4. The autoradiography was obtained from Phosphor Imager-Image Quant.

phorbol ester, α-TPA, did not induce translocation of all these isozymes (Fig. 1). Fig. 2 shows the quantitative data of Western blot analysis from Phosphor Imager-Image Quant integration. The extent of translocation of cPKCα to the membrane was greater than 4-fold (Fig. 2A) and much higher than that of nPKCδ and nPKCε which were about 2-fold of translocation to the membrane (Fig. 2B and C). After a 17 h exposure to 1 μM TPA, both cytosolic and membrane cPKCα, nPKCδ were almost down-regulated (Fig. 3). The nPKCε which was only detected in the membrane fraction was also completely down-regulated by TPA. In contrast, treatment of TPA for 17 h did not induce down-regulation of aPKCζ (Fig. 3). A 5 min exposure of cells to 30 nM ET-1 or 100 μM ATP, the natural activators of C_6 glioma cells, also induced translocation of cPKCα and nPKCδ but not aPKCζ (Fig. 4). However, the extent of translocation of cPKCα induced by ET-1 and ATP was 157% and 147%, respectively, that of nPKCδ was 145% and 125%, respectively. Therefore, the translocation induced by these two agonists was much smaller than that of TPA.

4. DISCUSSION

C_6 glioma cells are a useful model to study the signal transduction of endothelin and ATP-induced PI turnover. When these two agonists bind to cell surface receptors, they activate a phospholipase C which hydrolyses PI with the formation of inositol 1,4,5-trisphosphate [16–18]. The abundance of cPKCα, nPKCδ and aPKCζ in both cytosol and membrane of this cell line makes it

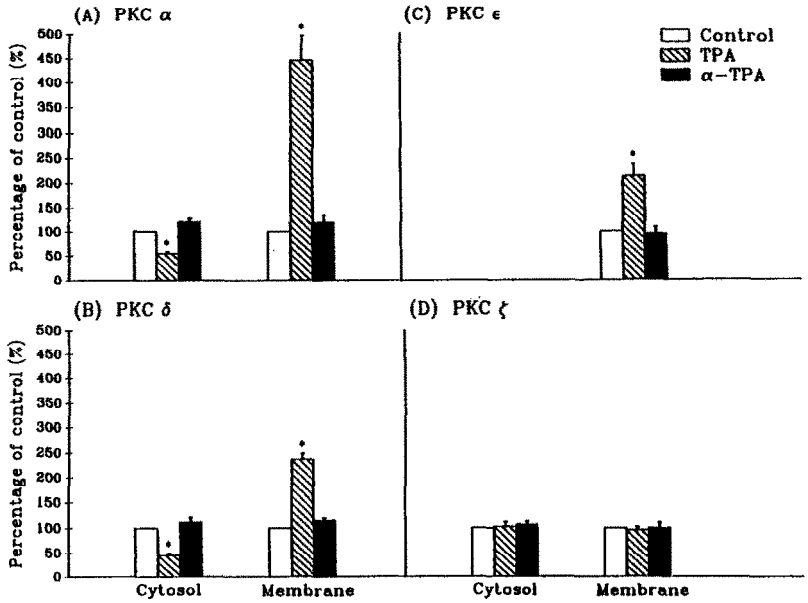

Fig. 2. Quantitative data of translocation of PKC isoforms in C_6 glioma cells from Western blot by Phosphor Imager-Image Quant analysis. Each PKC isoform in the cytosol and membrane after TPA treatment was evaluated. Data are presented as mean±S.E.M. for at least three experiments. *$P < 0.05$ as compared with the control. Since PKCε in the cytosol is undetectable, data were only obtained from membrane.

a useful model to study the properties of the three classes of PKC isozymes in one cell type. Translocation of PKC from cytosol to the particulate fraction has been domonstrated in response to phorbol esters and bradykinin stimulating PI turnover [10,24,25]. This particulate association has been considered to be a direct activation of PKC. Exposure of C_6 glioma cells to TPA for 10 min resulted in a translocation of cPKCα, nPKCδ and nPKCε to the membrane. Although similar results have been observed in rat fibroblasts, GH_4C_1 and Swiss 3T3 cells [9,10,13], neither cPKCα nor nPKCδ was expressed in the membrane fraction of these cell lines. Therefore, the differential extent of translocation between conventional and new PKC isoforms could only be quantitatively compared in the C_6 glioma cells. The translocation of cPKCα was more prominent than that of nPKC isozymes in this study. Similar observation was also found in the NG108–15 neuroblastoma cells (manuscript submitted). These results might imply when both conventional and new PKC isoforms coexist in a cell, the conventional isoforms might play a major role in the regulation of cell function. On the other hand, TPA did not induce translocation of aPKCζ. This result was in concert with aPKCζ in U937, HL-60, COS cells [11] and murine epidermis [12] but contrary to that in rat fibroblast [13] and human platelets [14]. The inactive phorbol ester, α-TPA, did not induce translocation of all these isozymes. The natural activators, ET-1 and ATP also translocated cPKCα and nPKCδ. This translocation was probable due to diacylglycerol formation from PI breakdown by these two agonists. ET-1 and ATP did not translocate atypical PKCζ either, indicating that TPA could mimic the action of natural agonists in C_6 glioma cells.

Another factor which regulates the PKC isozymes is their susceptibility to inactivation by proteolysis (down-regulation). In intact cells, differential down-regulation of conventional PKC isozymes by phorbol esters has been reported in different cell lines [5–8], and the down-

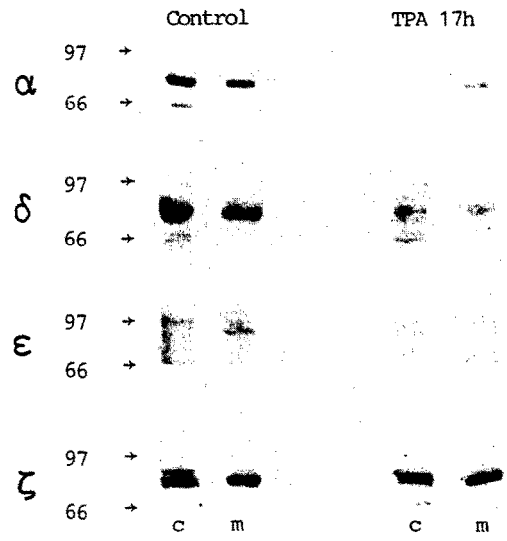

Fig. 3. Immunoblot detection of down-regulation of PKCα, -δ, -ε and -ζ in C_6 glioma cells induced by TPA. Cells were incubated with 0.1% DMSO (control) or 1 μM TPA for 17 h, then fractionated into cytosolic (c) and membrane (m) fractions. Samples (100 μg of protein) were separated by SDS-PAGE, transferred to nitrocellulose paper and immunodetected with antibodies of PKCα, -δ, -ε and -ζ (1:250 dilution) as described under section 2.4. Each autoradiography was obtained from Phosphor Imager-Image Quant after exposing nitrocellulose paper to phosphor screen.

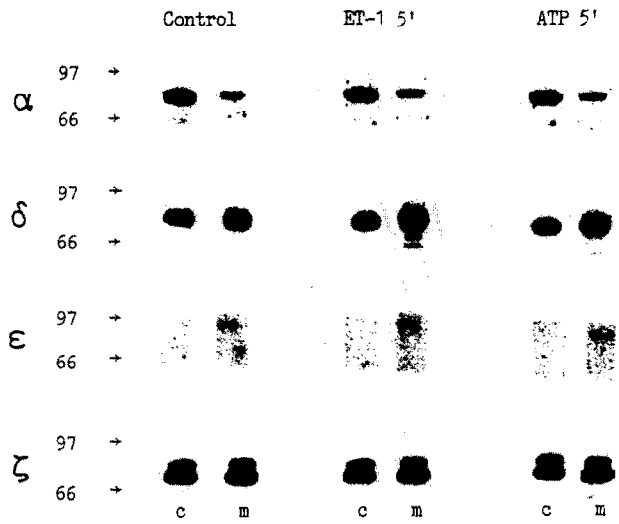

Fig. 4. Immunoblot detection of translocation of PKCα, -δ, -ε and -ζ in C_6 glioma cells by endothelin (ET-1) and ATP. Cells were treated with 30 nM ET-1 or 100 μM ATP for 5 min, and the cytosolic (c) and membrane (m) fractions were prepared as described in section 2. Samples (100 μg of protein) were separated by SDS-PAGE, transferred to nitrocellulose paper and immunodetected with antibodies of PKCα, -δ, -ε and -ζ (1:250 dilution) as described under section 2.4. The autoradiography was obtained from Phosphor Imager-Image Quant.

regulation of both new and atypical PKC isoforms by phorbol esters had also been reported in rat fibroblast [13]. In this study, conventional and new PKC isoforms were almost completely down-regulated by 17 h treatment with TPA, however, aPKCζ was not down-regulated. This latter observation of aPKCζ was also found in Swiss 3T3 cells [9], murine epidermis [12], rat mesangial cells [15], U937 cells [11] and NG 108–15 neuroblastoma cells (manuscript submitted) but opposite to that in rat fibroblasts [13]. However, only C_6 glioma cells, NG 108–15 neuroblastoma cells and U937 cells have the abundant expression of aPKCζ in both cytosolic and membrane fractions and the resistant down-regulation by TPA could be analyzed in these two fractions not just total cell lysate was done in Swiss 3T3 cells [9].

The aPKCζ partially purified from COS-7 cells transfected with cDNA or purified from bovine kidney is unable to bind phorbol ester and displays a protein kinase activity that is independent of diacylglycerol or Ca^{2+} [26,27]. The inability of TPA to induce both translocation and down-regulation of aPKCζ in C_6 glioma cells as well as NG 108–15 cells, Swiss 3T3 cells, murine epidermis and U937 cells may imply that in intact cells, aPKCζ behaves in the way predicted by its properties. Therefore, phorbol esters are not able to activate, consequently translocate and finally down-regulate this isozyme. The opposite result of translocation and down-regulation of aPKCζ in the rat fibroblasts by TPA [13] is unknown. The unique properties of aPKCζ indicate that this isoform may serve different functions in signal transduction from other isoforms. The mechanism by which aPKCζ is activated in intact cells remains unknown since it is insensitive to Ca^{2+} and diacylglycerol/phorbol ester. Phosphatidic acid, the product of phospholipase D on phosphatidylcholine, can increase the activity of this isozyme [27], but it is unclear that this effect would be significant in the presence of phosphatidylserine and PI in the intact plasma membrane. In addition, prolonged treatment of cells with TPA can no longer be used as an index for discriminating PKC-dependent and -independent cellular processes. The cellular responses that are not altered after long-term treatment with phorbol ester are not necessarily PKC-independent, but may be mediated by PKC isozymes that are resistant to down-regulation.

In conclusion, the properties of abundant expression of cPKCα, nPKCδ and aPKCζ in both cytosol and membrane of C_6 glioma cells was found and makes it a useful model which is superior to other cell lines to study the differential effect of activators or cofactors on the conventional, new and atypical PKC isoforms in intact cells. After the detailed properties of these three classes of PKC isoforms was analyzed, which PKC isoform is involved in the negative regulation of receptor-mediated PI hydrolysis will be performed and now in progress.

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