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Effects of Ca^{2+} on the activation of conventional and new PKC isozymes and on TPA and endothelin-1 induced translocations of these isozymes in intact cells

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Abstract

The effects of Ca^{2+} on the translocation of conventional and new protein kinase C isozymes in intact cells were studied by using C_6 glioma cells as a model system. Two conditions which monitor intracellular Ca^{2+} were performed: one is extracellular Ca^{2+}-depletion by treating the cells with physiological saline solution (PSS) without Ca^{2+} but containing 0.5 mM EGTA, the other is treating the cells with 1 μM ionomycin to induce Ca^{2+}-influx. In addition, the TPA and endothelin-1 induced translocations of conventional and new PKC isozymes under these two conditions were also comparatively studied. When the intact cells were treated with Ca^{2+}-free, EGTA containing PSS, the membrane-bound conventional PKCα (cPKCα) was greatly reduced and cytosolic cPKCα was slightly increased. However, neither membrane bound nor cytosolic new PKCδ (nPKCδ) was affected by extracellular Ca^{2+}-depletion. On the other hand, when the cells were treated with 1 μM ionomycin, the translocation of cPKCα itself was observed while nPKCδ was not affected. In extracellular Ca^{2+}-depletion, the translocation of cPKCα induced by 100 nM TPA still occurred although the extent of translocation was smaller than that induced by TPA under normal Ca^{2+} conditions; however, that induced by 30 nM ET-1 was blocked. After the cells were treated with 1 μM ionomycin, the translocation of cPKCα induced by 30 nM TPA was further increased compared to 1 μM ionomycin or 30 nM TPA alone, while that induced by ET-1 was only slightly further increased. All these results suggested that in intact cells, the activation of cPKCα was operated by both the intracellular Ca^{2+} level and diacylglycerol and that of nPKCδ was operated by diacylglycerol alone as predicted by their properties from purified enzyme or cDNA. In addition, the translocation of cPKCα induced by the natural activator ET-1 seemed to be more dependent on Ca^{2+} than TPA in intact cells.

Key words: Protein kinase C; Isozyme, conventional and new; Extracellular Ca^{2+}-depletion; Ionomycin; TPA, ET-1

1. Introduction

Protein kinase C (PKC) plays a major role in the signal transduction pathway of a variety of ligands, such as hormones, growth factors and neurotransmitters [1,2]. Most isozymes of PKC are activated by 1,2-diacylglycerol (DAG) and undergo translocation from the cytosol to the plasma membrane [3–7]. This translocaiton of PKC can also be mimicked by phorbol esters such as 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) [8]. Molecular cloning analysis has shown that PKC is a family of at least ten isozymes and divides into three groups: 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 third also lacks this region and has only one cysteine-rich zinc finger-like motif in the region (C-1) (atypical PKCζ,λ). Therefore, Ca^{2+} rather than DAG might be involved in the differential activation of conventional and new PKC isoforms. A role for Ca^{2+} in the translocation of conventional PKC induced by TPA was indicated by early studies of the binding of the kinase to plasma membrane in a cell-free system [9,10], and the presence of Ca^{2+} during the disruption of unstimulated cells resulted in an increase in PKC activity and/or immunoreactivity in the particulate fraction [11,12]. In intact cells, using enzyme activity assays, only conventional PKC has been reported to be rapidly translocated by Ca^{2+} influx induced by depolarization or ionophore [13,14]. However, it was not known which conventional isoform was translocated. In addition, whether the activation of new and atypical PKC isoforms was regulated by Ca^{2+} in intact cells was not addressed either. C_6 glioma cells have been reported from this laboratory to contain an abundance of conventional PKCα (cPKCα), new PKCδ (nPKCδ) and atypical PKCζ. TPA could mimic the natural activators, ATP and endothelin-1 (ET-1), to activate conventional and new but not atypical PKC isoforms [15]. Therefore, we used this cell line to study the effect of Ca^{2+} on the activation of conventional and new PKC isoforms. Extracellular Ca^{2+}-depletion was performed by treating the cells with physiological saline solution (PSS) without Ca^{2+} but containing 0.5 mM EGTA and Ca^{2+}-influx was induced by treating the cells with 1 μM ionomycin. Furthermore, the TPA and ET-1 induced translocations of cPKCα and nPKCδ under these two conditions were also comparatively studied.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies against peptide sequence unique to PKCα, -δ, Dulbecco's modified Eagle's medium (DMEM), fetal calf

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serum (FCS), penicillin and streptomycin were purchased from Gibco BRL (Gaithersburg, MD). TPA was from L.C. Services Corp. (Woburn, MA). ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF) and ionomycin were 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 was from DuPont-New England Nuclear.

Stock solutions of ionomycin and 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

C_6 glioma cells from American Type Culture Collection (Rockville, MD) kindly supplied by D.M. Chuang (Molecular Neurobiology, NIMH, NIH), were grown in DMEM supplemented with 10% FCS, 100 U/ml pencillin and 100 μg/ml streptomycin. All the cells were grown in 145 mm Petri dishes in an atomosphere of 5% CO_2/95% humidified air at 37°C. In the experiments for studying the effects of extracellular Ca^{2+}-depletion on PKC, confluent cells were first washed three times with PSS (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 20 mM HEPES, pH 7.4), then Ca^{2+}-free PSS ($CaCl_2$ was omitted and 0.5 mM EGTA was added) was added and incubated for 20 min at 37°C. Then 100 nM TPA or DMSO was added and incubated for another 10 min. In the experiments for studying the effect of Ca^{2+} influx on PKC, the confluent cells were added with DMSO, 1 μM ionomycin, 30 nM TPA or 1 μM ionomycin plus 30 nM TPA, and incubated for 10 min at 37°C. For the experiment of natural activator, ET-1, the concentration of 30 nM was used and incubated for 5 min. After the incubation, the cells were rapidly washed with ice-cold phosphate buffered saline 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 the membrane fractions.

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [15]. The cytosolic extracts and membrane fractions (100 μg of protein) were denatured by heating in Laemmli stop solution and subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and 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 and PKCδ, diluted 1 : 250 in TTBS containing 1% BSA for 3 h, and with [^{125}I]protein A (0.4 μg, 4–6 μCi/20ml) 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).

2.5. Statistical analysis

Data were presented as mean ± S.E.M. Statistical analyses were done with Student's *t*-test.

3. Results

3.1. Effects of extracellular Ca^{2+}-depletion on the translocation of cPKCα and nPKCδ and on TPA and ET-1 induced translocations of these two isozymes

When the intact cells were treated with Ca^{2+}-free PSS containing 0.5 mM EGTA, cPKCα in the membrane was decreased dramatically and that in the cytosol was slightly increased (Figs. 1 and 2A). However, neither cytosolic nor membrane nPKCδ was affected by extracellular Ca^{2+}-depletion (Figs. 1 and 2B). Therefore, extracellular Ca^{2+}-depletion changed the redistribution of cPKCα itself, especially decreased membrane bound cPKCα. In this condition, the translocation of nPKCδ induced by 100 nM TPA was similar to that in normal Ca^{2+} condition (Figs. 1 and 2B). As for cPKCα, 100 nM TPA still induced translocation of this conventional isozyme; however, the extent (209%) was less than that induced by TPA in normal PSS (445%) (Figs. 1 and 2A). On the other hand, when comparing to membrane cPKCα in Ca^{2+}-free, EGTA containing PSS as control in which the activity of this isoform was already decreased, the extent of translocation (450%) induced by 100 nM TPA was still as prominent as that in normal PSS (445%) (Figs. 1 and 2A).

When the cells were treated with 30 nM ET-1 for 5 min, translocation of both cPKCα and nPKCδ was shown. In extracellular Ca^{2+} depletion condition, the translocation of cPKCα induced by ET-1 was blocked as compared with the control in normal Ca^{2+}, although it still induced about 120% translocation as compared with

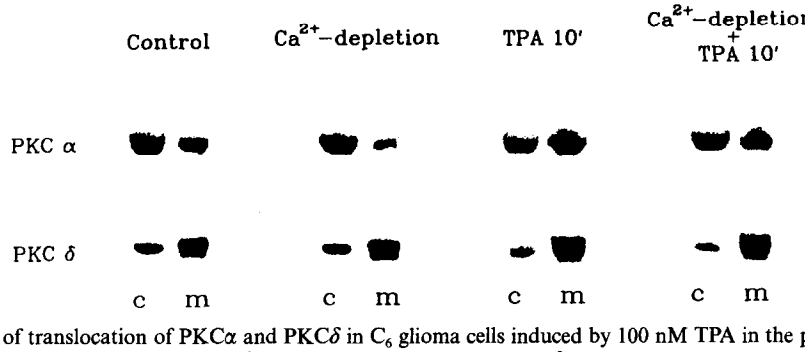

Fig. 1. Immunoblot detection of translocation of PKCα and PKCδ in C_6 glioma cells induced by 100 nM TPA in the presence and absence of Ca^{2+} in PSS. Cells were equilibrated in normal (Control) or Ca^{2+}-free, EGTA containing PSS (Ca^{2+}-depletion) for 20 min, then 0.1% DMSO (Control and Ca^{2+}-depletion) or 100 nM TPA (TPA 10 min and Ca^{2+}-depletion + TPA 10 min) was added and incubated for another 10 min. 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 and PKCδ (1 : 250 dilution) as described under section 2.4. The autoradiography was obtained from Phosphor Imager-Image Quant.

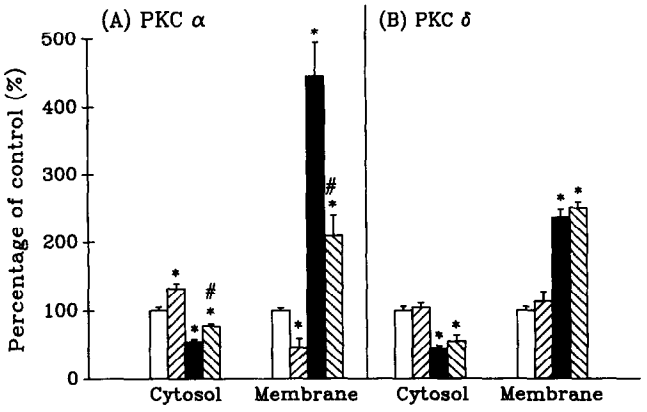

Fig. 2. Quantitative data of translocation of PKCα (A) and PKCδ (B) in C_6 glioma cells induced by 100 nM TPA in the presence and absence of Ca^{2+} in PSS. Western blots were analyzed by Phosphor Imager-Image Quant analysis. PKCα and PKCδ in cytosol and membrane after various treatment were evaluated. □ = Control; ▨ = Ca^{2+}-depletion; ■ ; TPA, 10 min; ▩ = Ca^{2+}-depletion + TPA 10 min. Data are presented as mean ± S.E.M. for at least four experiments. $^*P<0.05$ as compared with the control in normal Ca^{2+}-PSS. $^\#P<0.05$ as compared with Ca^{2+}-depletion.

the membrane cPKCα in Ca^{2+}-depletion. On the other hand, ET-1 induced translocation of nPKCδ was not affected (Fig. 3).

3.2. *Effects of ionomycin on the translocation of cPKCα and nPKCδ and on TPA and ET-1 induced translocations of these two isozymes*

When the cells were treated with 1 μM ionomycin, translocation of cPKCα itself was observed (Figs. 4 and 5A), while nPKCδ was not affected (Figs. 4 and 5B). Therefore, 1 μM ionomycin only increased the membrane bound cPKCα but not nPKCδ. In the presence of ionomycin, the translocation of nPKCδ induced by 30 nM TPA, was the same as that in the absence of ionomycin (Figs. 4 and 5B); however, that of cPKCα was further increased and the extent was 885% as compared with the control (Figs. 4 and 5A). On the other hand, when comparing to membrane cPKCα in ionomycin as control in which the activity of this isozyme was already increased, the extent of translocation induced by 30 nM TPA (299%) was almost the same as that in normal control (311%) (Figs. 4 and 5A). The translocation of nPKCδ induced by 30 nM ET-1 was not affected by ionomycin treatment, either (Fig. 6). However, further increase of the ET-1 induced translocation of cPKCα in the presence of ionomycin was only slight. When comparing to membrane cPKCα in ionomycin as control, the extent of translocation induced by ET-1 (125%) was much smaller than that in normal control (166%) (Fig. 6).

4. Discussion

The conventional PKCα, -βI, -βII and -γ contain the putative Ca^{2+}-binding region C_2 in the regulatory domain and are Ca^{2+}-responsive and dependent on Ca^{2+} for activity [16–18]. The other new PKCδ, -ε and -η and atypical ζ lack this region and are Ca^{2+}-unresponsive and not dependent on Ca^{2+} for activity [19–24]. All these results were from in vitro studies carried out on the pure recombinant isoforms or on the pure naturally expressed proteins from brain or kidney. In this study, we treated intact cells with Ca^{2+} free, EGTA-containing PSS to reduce the intracellular Ca^{2+} level [25] or with ionomycin to increase the intracellular Ca^{2+} level [26]. The results showed that membrane-bound cPKCα itself was dramatically reduced in extracellular Ca^{2+}-depletion but increased after ionomycin treatment. On the other hand, membrane bound nPKCδ was not affected by these two different treatments. The intracellular Ca^{2+} level was reduced from about 150 nM to 50 nM by extracellular Ca^{2+} chelation with EGTA [25] and increased from 200 nM to 750 nM after 1 μM ionomycin treatment [26] in C_6 glioma cells. The results from present study imply that in intact cells, both Ca^{2+}-dependent conventional and Ca^{2+}-independent new PKC isoforms behave in the way predicted by their properties and any input signal which affects intracellular Ca^{2+} levels may alter the activation of conventional PKC isoform itself while leaves new

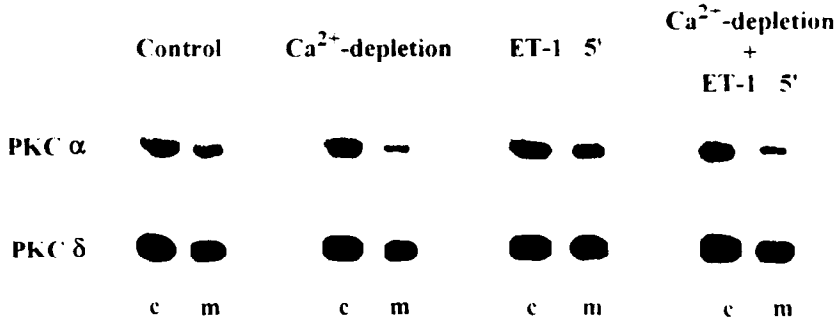

Fig. 3. Immunoblot detection of translocation of PKCα and PKCδ in C_6 glioma cells induced by 30 nM ET-1 in the presence and absence of Ca^{2+} in PSS. Cells were equilibrated in normal (Control) or Ca^{2+}-free, EGTA containing PSS (Ca^{2+}-depletion) for 20 min, then 30 nM ET-1 (ET-1 5 min and Ca^{2+}-depletion + ET-1 5 min) was added and incubated for another 5 min. The cytosolic (c) and membrane (m) fractions were prepared as described in section 2.

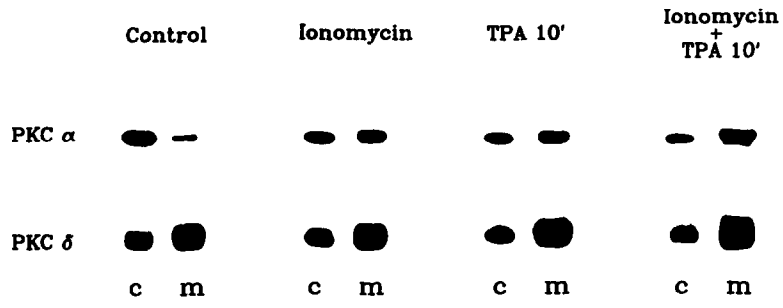

Fig. 4. Immunoblot detection of translocation of PKCα and PKCδ in C_6 glioma cells induced by 30 nM TPA in the absence and presence of 1 μM ionomycin. Confluent cells were treated with 0.1% DMSO (Control), 1 μM ionomycin (Ionomycin), 30 nM TPA (TPA, 10 min) or 1 μM ionomycin first then 30 nM TPA (Ionomycin+TPA 10 min) for 10 min at 37°C. The cytosolic (c) and membrane (m) fractions were prepared as described in section 2. Samples (100 μg of protein) are separated by SDS-PAGE, transferred to nitrocellulose paper and immunodetected with antibodies of PKCα and PKCδ (1:250 dilution) as described in section 2.4. The autoradiography was obtained from Phosphor Imager-Image Quant.

PKC isoform unaffected in physiological condition. From conventional PKC activity assay by using histone as the exogenous substrate, the membrane associated enzyme activity in pinealocytes was also decreased by treating intact cells with 3 mM EGTA and increased by high K^+ treatment [14]. However, Borner et al. [12] used either EGTA or Ca^{2+} containing extraction buffers to lyse rat fibroblasts and claimed that 80% of cPKCα resided in the cytosol and 20% was membrane-bound when extraction was performed in the presence of EGTA. On the other hand, subcellular fractionation in the presence of Ca^{2+} revealed that only 30% of cPKCα was located in the cytosol and 70% in the membrane fraction. In fact, at least two types of membrane-associated PKC are distinguishable; one that can be extracted by metal ion chelators and the other that is stable to chelators but can be dissociated with detergents [27]. Sonication of membranes in the presence of chelating agents (e.g. 1–10 mM EDTA, or EGTA) is valuable in extracting peripheral membrane proteins [28]. Therefore, the peripheral membrane bound PKC was extracted to the cytosolic fraction when sonicated the cells in the presence of EGTA. Results obtained from Borner et al. [12], Kiley et al. [29] and Akita et al. [30] were not surprising and couldn't be explained by the dependence of this conventional isoform on Ca^{2+}. The results from intact cells in the present experiment reflect more physiological significance.

Using inside-out erythrocyte vesicles and measuring [^{3}H]PDBu binding, synergism between Ca^{2+} and TPA for intracellular translocation of PKC was reported [9]. In addition, using cell homogenates and measuring PKC activity, addition of TPA to the homogenates in the presence of Ca^{2+} resulted in a plasma membrane binding of PKC which subsequently remained bound to the membrane independent of Ca^{2+} [10]. In the present study, in intact cells, the Ca^{2+} on the TPA induced translocation of cPKCα and nPKCδ was examined. In extracellular Ca^{2+}-depletion condition, TPA still induced translocation of cPKCα although the extent was less (209%) than that in normal Ca^{2+} condition (445%). However, comparing to membrane cPKCα in extracellular Ca^{2+} depletion as control in which this isoform was already decreased, the extent of translocation induced by TPA (450%) was still as prominent as that in normal condition (Fig. 2A). On the other hand, the translocation of cPKCα induced by natural activator, ET-1, was blocked in extracellular Ca^{2+}-depletion condition, although ET-1 still induced 120% translocation as compared with membrane cPKCα in extracellular Ca^{2+}-depletion as control. After ionomycin treatment, the translocation of cPKCα itself was shown and TPA induced translocation in this condition was much greater than that induced by ionomycin or TPA alone. If comparing to the membrane cPKCα after ionomycin treatment as control in which this isoform was already increased, the extent of translocation induced by TPA in this condition (299%) was almost the same as that in normal condition (311%) (Fig. 5A). On the other hand, the translocation of cPKCα

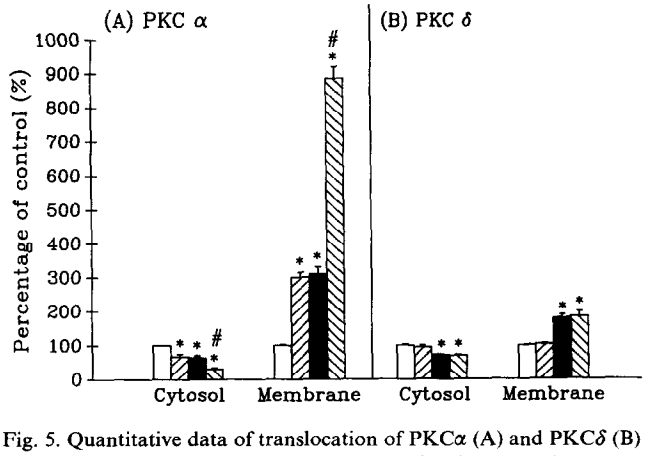

Fig. 5. Quantitative data of translocation of PKCα (A) and PKCδ (B) in C_6 glioma cells induced by 30 nM TPA in the absence and presence of 1 μM ionomycin. Western blots were analyzed by Phosphor Imager-Image Quant analysis. PKCα and PKCδ in cytosol and membrane after various treatment were evaluated. □ = control; ▨ = Ionomycin; ■ = TPA, 10 min; ▧ = Ionomycin + TPA 10 min. Data are presented as mean ± S.E.M. for 3 experiments. $^*P < 0.05$ as compared with the control. $^\#P < 0.05$ as compared with ionomycin alone.

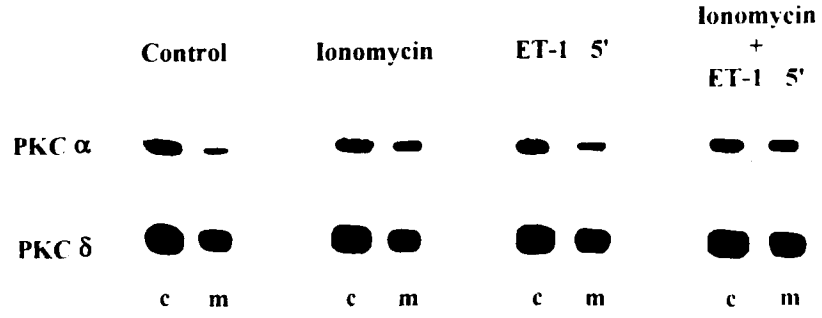

Fig. 6. Immunoblot detection of translocation of PKCα and PKCδ in C_6 glioma cells induced by 30 nM ET-1 in the absence and presence of 1 μM ionomycin. Confluent cells were treated with 0.1% DMSO (Control), 1 μM ionomycin (Ionomycin), 30 nM ET-1 (ET-1 5 min) or 1 μM ionomycin first then 30 nM ET-1 (Ionomycin + ET-1 5 min) for 5 min at 37°C. The cytosolic (c) and membrane (m) fractions were prepared as described in section 2.

induced by ET-1 in this condition was only slightly greater than that induced by ionomycin or ET-1 alone. When comparing to the membrane cPKCα after ionomycin treatment as control, the extent of translocation induced by ET-1 was much smaller (125%) than that in normal condition (166%). As for nPKCδ, either TPA or ET-1 induced translocation was not affected by extracellular Ca^{2+}-depletion or ionomycin treatment. Therefore, both translocations of cPKCα and nPKCδ induced by TPA seemed to be not dependent on Ca^{2+}. However, ET-1 induced translocation of cPKCα seemed to be more dependent on Ca^{2+} although that of nPKCδ was not dependent on Ca^{2+} either. These results also imply that any input signal which could increase both intracellular Ca^{2+} level and DAG may produce more activation of conventional PKCα. The conclusion of independent effect of TPA on Ca^{2+} obtained from intact cells was in accordance with Gschwendt et al. [31] analysis that TPA together with membrane phospholipids but not Ca^{2+} appeared to be sufficient for activation of all PKC isozymes. The different Ca^{2+}-dependence of cPKCα translocation induced by ET-1 and TPA might be due to endogenous DAG was formed after ET-1 treatment, because DAG participated in forming an active PKC which was reversibly bound to the membrane, while TPA generated an irreversible PKC–membrane complex which was chelator-resistant [32,33].

In summary, the predicted properties of cPKCα and nPKCδ from cDNA and purified enzyme was really performed in intact cells, indicating that in physiological condition, any input signal which increases both intracellular Ca^{2+} and DAG may induce the activation of conventional PKC isoforms and that of new PKC isoforms are only activated by DAG. In addition, the translocation of cPKCα induced by natural activator, ET-1, seemed to be more dependent on Ca^{2+} than TPA in intact cells.

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