

Immunochemical identification and translocation of protein kinase C zeta in human neutrophils

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Abstract

Western blots of human polymorphonuclear leukocyte (PMN) extracts were immunostained with antibodies specific for various protein kinase C (PKC) isoforms. Two bands corresponding to PKC type ζ with apparent molecular masses of 81 kDa and 76 kDa were identified in the cytosolic fraction of resting cells, in addition to PKC types α and β . PKC ζ was apparently abundant, like PKC β , whereas PKC δ , $-\epsilon$, and $-\gamma$ were not detectable. Following short stimulation (5 min) of PMN with phorbol-12-myristate-13-acetate (1 μ g/ml), physical translocation of PKC ζ from the cytosol to the plasma membrane fraction occurred, although this isoform does not bind phorbol esters. These data show that, in addition to the two calcium-dependent isoenzymes α and β , human PMN express a calcium-independent isoenzyme ζ which translocates in stimulated cells, suggesting a role in the regulation of antibacterial activities.

Key words: Protein kinase C; Isotype; Neutrophil; Phorbol ester

1. Introduction

Protein kinase C, a family of phospholipid-dependent serine/threonine kinases, plays a pivotal role in agonist-stimulated cell functions [1–3]. To date, twelve PKC isoenzymes have been identified in mammalian tissues [4]. They have been classified into three subgroups [5]. The conventional PKCs (cPKC) PKC α , $-\beta$ I, $-\beta$ II, and $-\gamma$ require PS and are activated by calcium and DAG or phorbol esters. The novel PKCs (nPKCs) PKC δ , $-\epsilon$, $-\eta$ and $-\theta$ do not require calcium and exhibit enzyme activities in the presence of PS and DAG. Atypical (aPKC) PKC ζ and $-\lambda$ are dependent on PS but not DAG, phorbol esters or calcium for activation. Recently, two other isoforms, PKC ι and $-\mu$, have been identified; they may belong to the aPKC and nPKC subgroups, respectively [6,7]. PKC isoforms show subtly different enzymatic properties, different tissue expression, and specific intracellular locations, suggesting a different role for each isoform in cell functions [3–5].

In a number of cell models, activation of PKC by phorbol esters is generally associated with irreversible association and conversion of soluble PKC to a particulate or 'inserted' form [8–10]. This translocation of PKC has been used as an index of enzyme activation.

Polymorphonuclear leukocytes (PMN) play an impor-

tant role in host defences against microbial infections. A role of PKC has been proposed in the regulation of antibacterial PMN activities such as superoxide anion generation (respiratory burst) and release of granule contents induced by phorbol esters or chemoattractants [11–14]. To date, three PKC isoforms have been characterized in the human PMN, including two conventional calcium-dependent PKC isoforms, PKC α and $-\beta$, and a calcium-independent form which requires both PS and DAG for optimal activity [15–18].

In this study, we show that human PMN also express a calcium-independent isoform, PKC ζ , which is mainly present in the cytosol of resting cells. In addition, stimulation of PMN with PMA results in translocation of PKC ζ to membrane fractions, suggesting a potential role of PKC ζ in PMN antimicrobial activities.

2. Materials and methods

2.1. Materials

Antibodies against PKC α , $-\beta$, $-\delta$, $-\epsilon$, $-\gamma$, and $-\zeta$ were all from Gibco BRL (Cergy-Pontoise, France). They were raised in rabbits against amino acid sequences of V3 regions (α and β isoforms) and the amino acid sequences of V5 regions (δ , ϵ , and ζ isoforms). Phorbol-12-myristate-13-acetate (PMA), all protease inhibitors and materials for polyacrylamide gel electrophoresis were purchased from Sigma (St. Louis, MO). X-Ray films for autoradiography were obtained from Amersham International (UK).

2.2. Cell preparation

PMN from heparinized venous blood of healthy volunteers were isolated as previously described [29] except that cells were washed with phosphate-buffered saline containing 5 mM EDTA to minimize platelet contamination [20]. Red cells were removed by hypotonic lysis, and the PMN were washed and resuspended in calcium-free Hanks balanced salt solution (HBSS) at pH 7.4. Cell preparations usually contained 98% PMN and fewer than 0.5 platelets per PMN. Platelets were prepared from human blood as described elsewhere [21].

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Abbreviations: DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

2.3. Stimulation and fractionation of PMN

Suspensions of 5×10^7 PMN/ml were warmed to 37°C and stimulated for 5 min with 1 μ g/ml PMA or 0.1% dimethyl sulfoxide (vehicle) with gentle agitation. Cells were then diluted in four volumes of ice-cold buffer and spun down at $400 \times g$ for 10 min at 4°C. Pellets were resuspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 0.25 M saccharose, 50 mM 2-mercaptoethanol, 2 mM EDTA, 10 mM EGTA, 2 mM PMSF and anti-protease mixture consisting of 0.1% leupeptin, 0.35 mM antipain, 0.24 mg/ml chymostatin, 0.35 mM pepstatin and 10 μ g/ml aprotinin. PMN suspensions were cavitated (450 psi, 20 min) at 4°C and the homogenates were centrifuged ($400 \times g$, 10 min) to remove unbroken cells. The postnuclear supernatant was loaded on a discontinuous sucrose gradient (50% sucrose, 30% sucrose) and centrifuged for 1 h at $200,000 \times g$. The membrane fraction was collected in the 30% layer and washed once with lysis buffer.

2.4. Western blotting

Following SDS-PAGE on 10–12% acrylamide gels [22], protein was transferred to nitrocellulose filters (Hybond-C extra, Amersham). Rat brain protein extracts were run in parallel and served as positive controls for immunodetection of PKC isoforms. Filters were incubated for 2 h at room temperature in 50 mM Tris, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 10% (w/v) fat-free dried milk. Nitrocellulose membranes were washed with TBST and incubated overnight with specific antibodies against PKC α , - β , - δ , - ϵ , - γ and - ζ , all at a dilution of 1/1000. In some cases, PKC isozyme specific peptides were added to the primary antibody solution to block specific isozyme bands. After washing ($5 \times$) with TBST, a 1/30 000 dilution of horseradish peroxidase-labelled anti-rabbit IgG was added for 1 h at room temperature. The blots were developed using ECL western-blotting reagents (Amersham UK). Results shown are representative of three separate experiments.

3. Results

Human PMN have been shown to express cPKC α and - β isoforms [15–18]. To examine the possibility that human PMN express other PKC isoforms, Western blots using antipeptide antibodies specific to PKC α , - β , - γ , - δ , - ϵ , and - ζ isoforms were performed on cytosolic and plasma membrane fractions of resting PMN. All six isotypes tested were present in the rat brain cytosolic extract, used here as positive control (Fig. 1). We confirmed the presence of immunoreactive PKC α and - β , and demonstrated the presence of PKC ζ , which was detected as two bands (Fig. 1). None of these isoforms were observed in the plasma membrane fraction of resting cells (Fig. 1) or in the granular fraction (results not shown).

The reactivity of PKC ζ was intense, suggesting that it may be expressed at high levels, like PKC β . This latter was present as three components with apparent molecular masses of 80, 72, and 65 kDa (p80, p72, p65). P72 and p65 predominated. Immunodetection of all three was specifically blocked by PKC β peptide. Thus, p80 may represent intact PKC β , and p72 and p65 modified forms generated by proteolytic cleavage. A doublet of 81 kDa

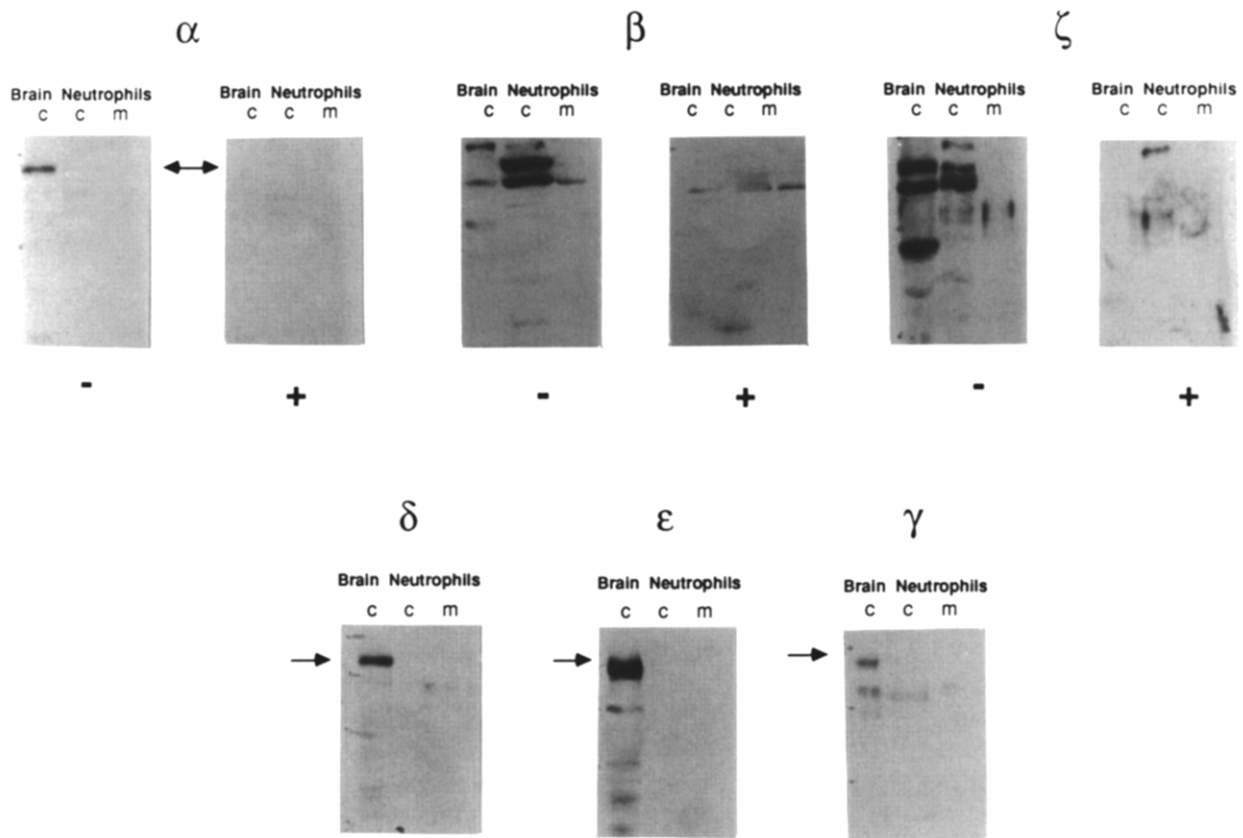


Fig. 1. Protein kinase C isoform immunoreactivity in cytosol and membrane fractions from human PMN by Western blot analysis. Cytosolic (c) and membrane (m) fractions of PMN were prepared as described in section 2, subjected to SDS-PAGE (50 μ g of protein/lane for cytosolic fraction and 80 μ g of protein/lane for membrane fraction). Proteins were transferred to nitrocellulose and immunoblotted with antipeptide antibodies specific to PKC α , - β , - δ , - γ , - ϵ , and - ζ . To confirm the specificity of staining, antisera were preincubated with (+) and without (-) the appropriate peptide antigen. Each left lane corresponds to a rat brain cytosolic fraction as a positive control.

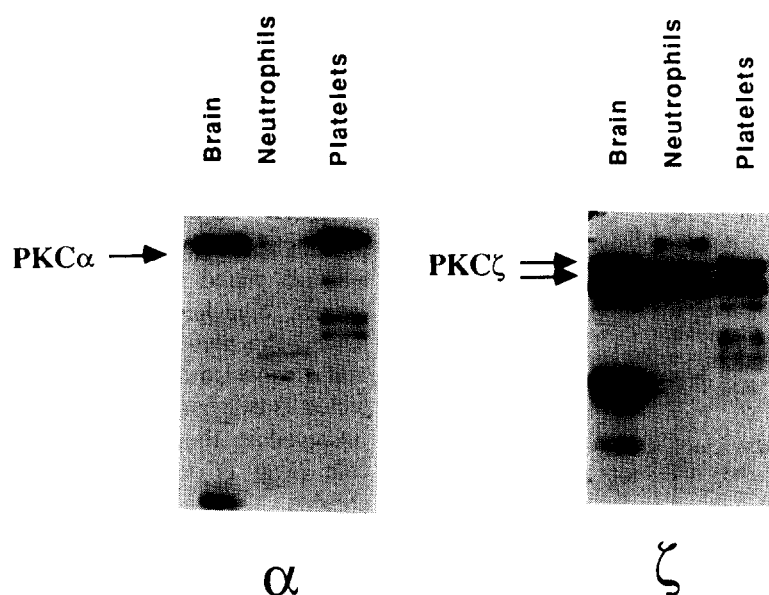


Fig. 2. Comparison of PKC isoforms in cytosol from human PMN and human platelets by Western blot analysis. Cytosolic fractions were prepared as described in section 2, subjected to SDS-PAGE ($50 \mu\text{g}$ of protein/lane), transferred to nitrocellulose, and immunoblotted with antibodies specific to PKC α and PKC ζ . The amount of soluble protein correspond to a cell equivalent of 5×10^6 PMN and 75×10^6 platelets. Each left lane contains a rat brain cytosolic fraction as a positive control.

and 76 kDa was recognized by the antibody against PKC ζ and was eliminated when the primary antibody was incubated with the peptide antigen used to generate the antibody. Both bands could correspond to PKC ζ since the existence of additional PKC ζ forms is predicted by genomic Southern blot analysis [23]. In the rat brain cytosolic extract, a lower molecular weight band (51 kDa) displaced by the PKC ζ peptide was also recognized by the PKC ζ antibody. This band could correspond to PKM form of PKC ζ . The PKC α antibody revealed a faint immunoreactive band of 80 kDa. No band was observed when Western blots were performed with antibodies specific for PKC δ , $-\epsilon$ and $-\gamma$.

Because the PMN preparation used here contained up to 0.5 platelets per PMN, we determined whether the PKC ζ signal was due to platelet contamination. Western blots were performed on platelet cytosol using antipeptide antibodies specific to PKC α and $-\zeta$. As shown in Fig. 2, the PKC ζ signal was intense in both PMN and platelets, while the PKC α signal was weak in PMN and strong in platelets, as reported previously [24]. These data indicate that the PKC ζ signal observed with the antibody against PKC ζ derives predominantly from PMN, not contaminating platelets.

PMA is a potent stimulant of PMN functions such as respiratory burst and exocytosis [11–14] and has been shown to induce PKC translocation from the cytosol to membrane fraction, a phenomenon which has been correlated with the respiratory burst [11]. To determine the possible involvement of PKC ζ in PMN functions, we examined the distribution of PKC ζ in PMA-stimulated

PMN. As shown in Fig. 3, short stimulation (5 min, 37°C) of PMN with a PMA concentration that causes superoxide production [11] induced physical translocation of PKC ζ from the cytosol to the plasma membrane, although this isoform does not bind phorbol esters [25].

4. Discussion

Our data confirm the presence of the two conventional calcium-dependent cPKC isoforms, α and β , in human PMN [15–17]. We also provide the first evidence that human PMN express an atypical calcium-independent PKC isoform, ζ , which was detected as two major components in the cytosolic fraction of resting PMN. The other PKC isoforms studied here, PKC δ , $-\epsilon$, and $-\gamma$ were undetectable, in agreement with other work [18]. It should be noted that detection of PKC isozymes is dependent on the characteristics of the antibodies used and the isoenzyme concentration. Other newly described calcium-independent PKC isoforms which were not studied here, such as η , θ , λ , ι and μ , may also have been present, as suggested by the work of Majumdar et al. [18], who characterized a calcium-independent kinase, termed nPKC, which requires both PS and DG for optimal activity. This nPKC was not recognized by an antibody directed against PKC ζ , although the authors mentioned the presence of ζ . PKC ζ and $-\beta$ but not PKC α were also identified in bovine PMN [26].

The relative expression of the different protein isoforms is difficult to determine by the Western blot

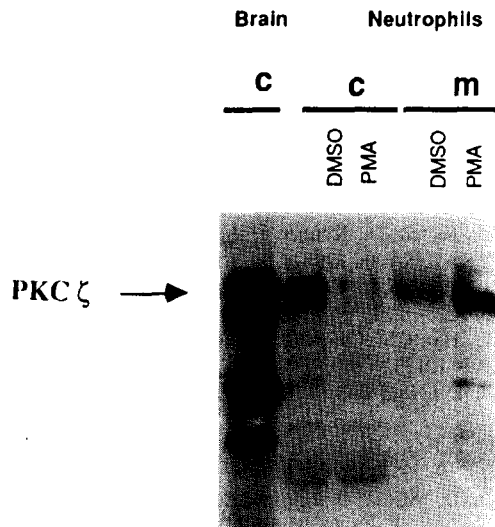


Fig. 3. PMA-induced translocation of PKC ζ from PMN cytosol to plasma membrane. Cells (50×10^6 PMN/ml) were exposed to $1 \mu\text{g/ml}$ PMA or 0.1% DMSO (control) for 5 min at 37°C . Cytosol (c) and membrane (m) proteins was subjected to SDS-PAGE ($50 \mu\text{g}$ of protein/lane for cytosolic fraction and $80 \mu\text{g}$ of protein/lane for membrane fraction) and immunoblotted with an antibody against PKC ζ .

technique, on account of differences in the titer and hybridization efficiencies of the respective antibodies. However, densitometric analysis of autoradiograms from immunoblots incubated with the same dilution of antibodies and developed after the same exposure, indicated that aPKC ζ may be expressed as abundantly as the β isoform, suggesting that it may play a role in PMN biological responses. This is further supported by the observation that stimulation of PMN with PMA induced a rapid (30 s, not shown) displacement of PKC ζ to the plasma membrane (Fig. 3). The PMA concentration used ($1 \mu\text{g/ml}$) also induces superoxide anion production and degranulation of primary granules, which supports the role of PKC ζ in these processes [11]. PKC α and PKC β were also shown to translocate after cell stimulation with PMA [16,17,27] and may play an important role in the regulation of PMN functions. Whether the isoforms act sequentially or synergistically remains to be determined.

The finding that aPKC ζ translocates from the cytosol to the plasma membrane in PMA-stimulated PMN is interesting, given that this isoform neither binds nor is activated by phorbol esters [25]. Translocation of this PKC isoform has also been reported in rat R6 embryo fibroblasts [28], human platelets [24,29] and smooth muscle cells [31]. The translocated PKC ζ was extracted by Triton X-100, indicating that it may share the characteristics of membrane 'inserted' form of PKC [8–10]. Possible mechanisms of PKC ζ redistribution include interaction of the PKC-binding domain with membrane-associated PKC-binding proteins [31–33] which may serve to anchor and compartmentalize PKC isoenzymes. PKC ζ may be associated with the membrane through

these proteins. It has been also proposed that aPKC ζ may have preserved a low-affinity binding site for phorbol esters in its single cysteine-rich domain and that high doses of PMA would trigger its redistribution. This hypothesis is supported by the binding of phorbol esters to a PKC γ mutant containing one set of cysteine-rich motifs with a structure similar to that of PKC ζ [34,35]. However, in PKC ζ , this motif differs in a single residue from the consensus (proline in position 11 of the motif). Restoration of this proline by site-directed mutagenesis of PKC ζ does not restore phorbol ester binding, suggesting that other amino acid residues besides the postulated consensus may be necessary for binding [36].

The physiological role of PKC ζ , as well the mechanisms by which the enzyme is activated in intact cells, remains unclear. However, it has recently been shown that phosphatidylinositol 3,4,5-trisphosphate (PIP3) can activate PKC ζ in vitro [37]. PIP3 might be a physiological activator of PKC ζ in PMN, since stimulation of these cells by formylated peptides triggers the activation of PI3-kinase, which phosphorylates phosphatidyl inositol 4,5-biphosphate (PIP2) to form PIP3 [38]. A role of PIP3 in PMN biological functions is further suggested by the observation that wortmanin, an inhibitor of PI3-kinase [39], depresses the PMN respiratory burst mediated by formyl peptides [40].

In conclusion, our data provide evidence that human PMN express substantial amounts of PKC ζ , mainly in soluble form. Stimulation of PMN by phorbol esters triggers redistribution of PKC ζ raising the possibility that PKC ζ , plays a role in the regulation of PMN anti-bacterial activities. The mechanism leading to PKC ζ activation, as well as its physiological significance, requires further studies.

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