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. PKCζ was apparently abundant, like PKCβ, whereas PKCδ, -ε, and -γ were not detectable. Following short stimulation (5 min) of PMN with phorbol-12-myristate-13-acetate (1 μg/ml), physical translocation of PKCζ 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) PKCα, -β, -δ, and -γ require PS and are activated by calcium and DAG or phorbol esters. The novel PKCs (nPKCs) PKCζ, -ε, -η, and -θ do not require calcium and exhibit enzyme activities in the presence of PS and DAG. Atypical (aPKC) PKCζ and -λ are dependent on PS but not DAG, phorbol esters or calcium for activation. Recently, two other isoforms, PKCι and -μ, 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 important 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, PKCζ and -β, 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, PKCζ, which is mainly present in the cytosol of resting cells. In addition, stimulation of PMN with PMA results in translocation of PKCζ to membrane fractions, suggesting a potential role of PKCζ in PMN antimicrobial activities.

2. Materials and methods

2.1. Materials

Antibodies against PKCζ, -β, -δ, -ε, -η, and -ζ 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 sequence 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].
2.3. Stimulation and fractionation of PMN

Suspensions of $5 \times 10^7$ PMN/ml were warmed to 37°C and stimulated for 5 min with 1 µg/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 x g for 10 min at 4°C. Pellets were resuspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 50 mM 2-mercaptoethanol, 2 mM EDTA, 10 mM EGTA, 2 mM PMSE and anti-protease mixture consisting of 0.1% leupeptin, 0.35 mM antipain, 0.24 mg/ml chymostatin, 0.35 mM pepstatin and 10 µg/ml aprotinin. PMN suspensions were cavitated (450 psi, 20 min) at 4°C and the homogenates were centrifuged (400 x 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 x 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 PKCa, -/B, -$\delta$, -$\gamma$, -$\epsilon$, and -$\zeta$, 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 x) 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 cPKCa and -$\beta$ isoforms [15-18]. To examine the possibility that human PMN express other PKC isoforms, Western blots using antipeptide antibodies specific to PKCa, -$\beta$, -$\gamma$, -$\delta$, -$\epsilon$, and -$\zeta$ isoforms were performed on cytosolic and plasma membrane fractions of resting PMN. All six isoforms tested were present in the rat brain cytosolic extract, used here as positive control (Fig. 1). We confirmed the presence of immunoreactive PKCa and -$\beta$, and demonstrated the presence of PKC$\zeta$, 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$\zeta$ was intense, suggesting that it may be expressed at high levels, like PKC$\beta$. 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$\beta$ peptide. Thus, p80 may represent intact PKC$\beta$, 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 µg of protein/lane for cytosolic fraction and 80 µg of protein/lane for membrane fraction). Proteins were transferred to nitrocellulose and immunoblotted with antipeptide antibodies specific to PKCa, -$\beta$, -$\delta$, -$\gamma$, -$\epsilon$, and -$\zeta$. 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.](image-url)
Fig. 2. Comparison of PKC isofoms 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 µg of protein/lane), transferred to nitrocellulose, and immunoblotted with antibodies specific to PKCa and PKCζ. The amount of soluble protein correspond to a cell equivalent of 5 x 10⁶ PMN and 75 x 10⁶ platelets. Each left lane contains a rat brain cytosolic fraction as a positive control.

and 76 kDa was recognized by the antibody against PKCζ and was eliminated when the primary antibody was incubated with the peptide antigen used to generate the antibody. Both bands could correspond to PKCζ since the existence of additional PKCζ 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 PKCζ peptide was also recognized by the PKCζ antibody. This band could correspond to PKM form of PKCζ. The PKCa antibody revealed a faint immunoreactive band of 80 kDa. No band was observed when Western blots were performed with antibodies specific for PKCδ, -ε and -γ.

Because the PMN preparation used here contained up to 0.5 platelets per PMN, we determined whether the PKCζ signal was due to platelet contamination. Western blots were performed on platelet cytosol using antipeptide antibodies specific to PKCa and -ζ. As shown in Fig. 2, the PKCζ signal was intense in both PMN and platelets, while the PKCa signal was weak in PMN and strong in platelets, as reported previously [24]. These data indicate that the PKCζ signal observed with the antibody against PKCζ 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 PKCζ in PMN functions, we examined the distribution of PKCζ 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 PKCζ 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, PKCδ, -ε, and -γ 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, τ 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 PKCζ, although the authors mentioned the presence of ζ. PKCζ and -β but not PKCa were also identified in bovine PMN [26].

The relative expression of the different protein isoforms is difficult to determine by the Western blot
PKCζ 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 PKCy mutant containing one set of cysteine-rich motifs with a structure similar to that of PKCζ [34,35]. However, in PKCζ, 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 PKCζ 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 PKCζ, as well as the mechanism 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 PKCζ in vitro [37]. PIP3 might be a physiological activator of PKCζ in PMN, since stimulation of these cells by formylated peptides triggers the activation of PI3 kinase, which phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to form PIP3 [38]. A role of PIP3 in PMN biological functions is further suggested by the observation that wortmannin, 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 PKCζ, mainly in soluble form. Stimulation of PMN by phorbol esters triggers redistribution of PKCζ, raising the possibility that PKCζ plays a role in the regulation of PMN antibacterial activities. The mechanism leading to PKCζ activation, as well as its physiological significance, requires further studies.

References
