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ROLE OF PROTEIN KINASE C ISOZYMES IN ACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 IN CHRONICALLY INFECTED PROMONOCYTIC CELLS: EVIDENCE AGAINST A ROLE OF PKCβI

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Protein kinase C (PKC) plays an important role in activation of human immunodeficiency virus type 1 (HIV-1). Because of a molecular and biochemical heterogeneity of PKC, we have studied the effects of PKC isozymes in HIV-1 activation in a latently infected promonocytic cell line, U1, using various PKC isozyme agonists. 12-Deoxyphrbol 13-phenylacetate (dPP), an agonist of both Ca^{++}-dependent and Ca^{++}-independent isozymes, and thymeleatoxin (TT), an agonist of Ca^{++}-dependent PKC isozymes, induced HIV-1 production at 10 nM with increase in a concentration dependent manner, whereas 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA), an PKCβI isozyme agonist,did not induce viral production at 100 nM. We verified that dPPA induced translocation of PKCβ isozyme with the isozyme-specific monoclonal antibody using flow cytometry. This study demonstrates that activation of PKC isozymes leads to an induction of latent HIV-1 in U1 cells whereas PKCβI isozyme may not be important.

Protein kinase C plays an important role in signal transduction, gene expression, cell growth and differentiation (1). PKC is found both in cytosol and bound to plasma membrane. Activation of PKC is accompanied by translocation of the enzyme to plasma or nuclear membrane. Recent molecular and biochemical characterizations have revealed the presence of at least ten isozymes of PKC in mammalian tissues (2). These isozymes are the products of distinct genes except PKCß (ß1 and ß2) derived from alternative splicing of a single gene (2,3). PKC isozymes are distinct with regard to tissue distribution, dependence on Ca^{++}, enzymological properties, and kinetics of activation and inactivation, and substrate specificity (1,4,5). They are classified into three groups: [A] the conventional Ca^{++}-dependent PKC (cPKC; α, β-I, β-II, and γ), [B] novel Ca^{++}-independent PKC (nPKC; δ, ϵ, η and γ), and [C] atypical PKC (aPKC; ζ and λ). The heterogeneity of PKC isozymes suggests that different isozymes may have different physiological roles.

Previous studies have indicated that PKC has an important role in retroviral replication (6,7). Jakobovits *et al* have reported that depletion of PKC significantly reduced *tat*-mediated HIV-1 transactivation (6). Selective inhibitors of PKC have been reported to inhibit virion production by HIV-1 infected cells. Lavie *et al* have reported that hypericin, a broad PKC

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inhibitor, may interfere with assembly and processing of viral precursor proteins (7). H-7, a non-selective inhibitor of protein kinases including PKC and cAMP-dependent protein kinase (PKA) has been reported to block HIV infectivity by inhibiting phosphorylation of $CD4^+$ receptor (8). Substrates of PKC include the *gag* product (9) and the key nuclear factors essential for HIV replication, such as the *rev* product (10), a cellular TAR-binding factor (11) and IκB (12), an inhibitory subunit of NF-κB complex. Divergent mode of antiviral activity by PKC inhibitors suggests that different isozymes may play roles in HIV replication.

In this study we examined the role of PKC isozymes including PKCβI on HIV-1 replication using isozyme agonists. Activation of PKC isozymes leads to an induction of latently infected HIV-1 in promonocytic cells. Meanwhile, activation of PKCβI isozyme by a specific agonist failed to induce latent HIV-1.

MATERIALS AND METHODS

Cell Line: U1, a latently HIV-1 infected promonocytic cell line was obtained from the AIDS Reagent and Reference Program, NIH, Division of AIDS, Rockville, MD and maintained in RPMI 1640 medium containing 10% fetal bovine serum. The cells carry HIV-1 provirus but do not produce viral particles (13).

Reagents: Monoclonal anti-PKCβ (both βI and βII isozymes) antibody with IgG_{2a} isotype was purchased from Seikagaku America, Rockville, MD. FITC-conjugated goat anti-mouse IgG_{2a} antibody was purchased from ICN Immunochemicals, Irvine, CA. 12-Deoxyphrbol 13-phenylacetate (dPP), deoxyphorbol 13-phenylacetate 20-acetate (dPPA), and thymeleatoxin (TT) were purchased from LC Services Corp., Woburn, MA. Stock solutions were made at 10 mg/ml in dimethyl sulfoxide and kept at -70°C until use. Phorbol myristate acetate (PMA) and propidium iodide were purchased from Sigma (St. Louis, MO).

Cell Viability: Cells were incubated in culture medium with various concentrations of PKC agonists at 37°C in 5% CO_2 atmosphere for 2 days. Cells were incubated with 50 μg of propidium iodide for 30 min at 4°C. Cell viability was analyzed with a FACScan flow cytometer (Beckton Dickinson, San Jose, CA).

HIV-1 Production: Cells were cultured for 2 days at 37°C in the presence or absence of PKC agonists. Viral particles released to culture medium by U1 cells were analyzed by reverse transcriptase assay and ELISA for HIV-1 p24 antigen.

Reverse Transcriptase Assay: A standard method was used as described (14) with a minor modification. In brief, 2 ml of culture medium was centrifuged at 15,000xg for 10 min at 4°C, and viral particles in the supernatant were precipitated overnight at 0°C with 10% polyethylene glycol. The viral pellet was recovered by centrifugation at 800xg for 45 min at 0°C, resuspended in 25 mM Tris-HCl buffer (pH 7.8) and denatured by 0.6% Triton X-100. The assay was done in triplicate using 96 well microtiter plate in 50 μl RT buffer containing 10 μCi ^{3}H-dTTP (ICN Immunochemicals, Irvine, CA) and poly dA oligo $(dT)_{12-18}$ or poly rA oligo $(dT)_{12-18}$ as template primer. ^{3}H labelled cDNA was precipitated in 10% ice cold trichloroacetic acid and collected on glass fiber filters using a cell harvester from Skatron Inc., Sterling, VA. Radioactivity was measured in a liquid scintillation counter.

ELISA for HIV-1 p24 Antigen: HIV-1 p24 antigen was measured by antigen capturing ELISA using a commercially available kit from Cellular Products, Inc., Buffalo, New York.

Immunofluorescence Analysis: Translocation of PKCβ to plasma membrane was assessed by an indirect immunofluorescence method as described previously (15). Briefly, one million U1 cells were incubated with or without 100 nM dPPA for 30 min at 37°C, washed 2x with ice cold PBS and permealized with ice cold 70% methanol for 5 min at -20°C. Then, cells were washed 2x with ice cold PBS and incubated with 2 μg of anti-PKCβ monoclonal antibody (IgG_{2a}) for 1 h on ice. Cells were washed 2x with ice cold PBS and counter stained with FITC-conjugated goat anti-mouse IgG_{2a} for 1 h on ice. Cells stained with FITC-conjugated goat anti-mouse IgG_{2a} were used as background control. Stained cells were analyzed with a FACScan flow cytometer (Beckton Dickinson, San Jose, CA).

RESULTS

Effects of PKC Agonists on HIV-1 Production: HIV-1 production by U1 cells in the presence and absence of PKC agonists was measured by RT assay (Fig.1) and ELISA for p24 antigen (Fig.2). Data shown are representatives of three experiments. Three different PKC isozyme agonists were used in this study: dPP, a broad isozyme agonist of both Ca^{++}-dependent and Ca^{++}-independent isozymes, dPPA, a specific agonist to PKCβI isozyme and TT, an agonist specific to Ca^{++}-dependent isozymes (16,17). RT activity and HIV-1 p24 antigen production were observed at 10 nM of dPP or TT, and incresed in a concentration dependent manner. DPP appeared to be more potent than TT. Viral production peaked at 100 nM of dPP, whereas it did not reach the same level at 200 nM of TT. On the other hand, dPPA failed to induce RT activity and p24 production up to 100 nM. PMA was used as a control since it has been known as a PKC activator and a inducer of latent HIV-1 (13).

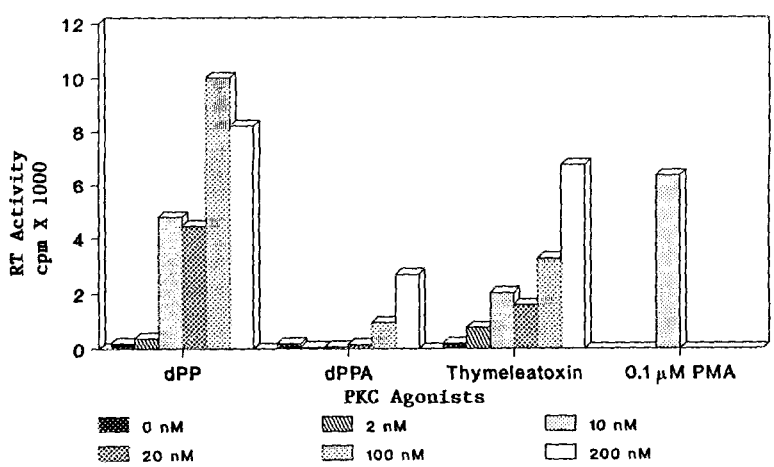

Fig. 1. Effect of PKC isozyme agonists on U1 cells for HIV-1 production. U1 cells were incubated for 2 days at 37°C with various concentrations of the agonists and supernatants were tested for RT activity.

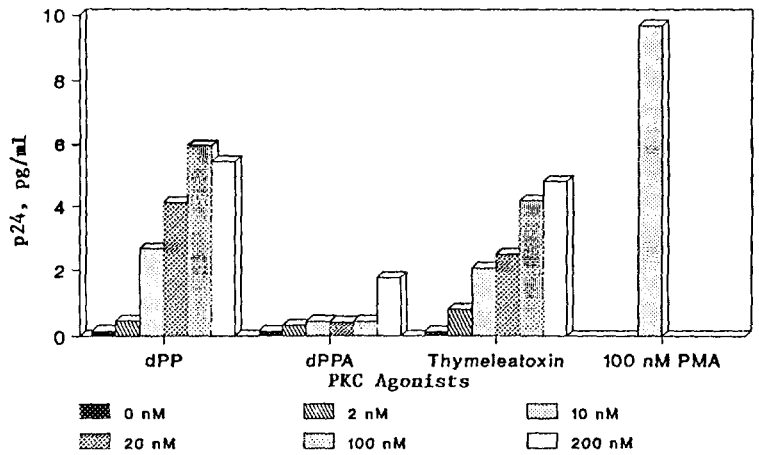

Fig. 2. Effects of PKC isozyme agonists on U1 cells for HIV-1 p24 production. U1 cells were incubated with various concentrations of the agonists for 2 days, and p24 in the culture supernatant was measured by ELISA for HIV-1 p24 antigen.

Effect of PKC Agonists on Cell Viability: PKC agonists were tested for their cytotoxic effects on U1 cells. No significant cytotoxicity was observed with any of the agonists (data not shown).

Immunofluorescence Analysis for PKCβ Activation: Since PKCβI agonist did not induce viral production, we tested if PKCβI was actually activated by dPPA. Cells treated with dPPA were analyzed with anti-PKCβ (both βI and βII) antibody using FACScan. PKC activation is accompanied by translocation of the enzyme from cytoplasm to plasma membrane, resulting in increase of fluorescence intensity as measured by flow cytometry (18). Cells treated with 100 nM of dPPA for 30 min showed increased fluorescence compared to untreated cells as shown Fig.3, indicating that PKCβ was activated by dPPA.

DISCUSSION

Protein kinase C plays an important role in intracellular signal transduction and in the regulation of cellular events such as, gene expression, cell proliferation and differentiation, and promotion of tumor development (1,4,5). Previous studies have shown that PKC plays a role in HIV-1 replication. The evidence includes 1) depletion of PKC reduces transactivation of HIV-1 long terminal repeat (LTR) by *tat* protein (6), 2) PKC phosphorylates the key nuclear proteins in expression of HIV-1 LTR (10-12), 3) HIV-1 proteins including *rev* and *gag* proteins are substrates of PKC (9,10) although phosphorylation of *rev* protein may not be important in HIV-1 replication, and 4) hypericin, a PKC inhibitor, interferes with assembly of viral particles (7). Recent biochemical and enzymological studies have characterized different isozymes of PKC and

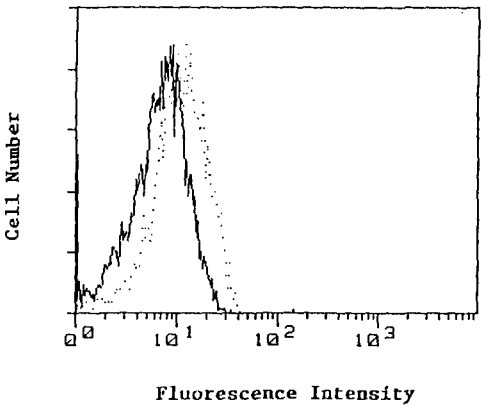

Fig. 3. Immunofluorescence of U1 cells treated with dPPA. U1 cells were incubated with 100 nM of dPPA for 30 min at 37°C. Cells were immunostained with anti-PKCβ monoclonal antibody (.....) and counterstained with FITC-labeled goat anti-mouse IgG_{2a}. Cells stained with FITC-labeled goat anti-mouse IgG2a (——) served as control. Stained cells were analyzed with flow cytometry.

the isozyme cDNAs have been cloned (1,4). The heterogeneity of PKC isozymes suggests that different isozymes may have different role in HIV-1 replication.

In the present study, We have used a latently HIV-1 infected promonocytic cell line, U1, which contains HIV-1 provirus in the chromosome but do not produce HIV particles unless it is stimulated by PMA, TNFα, or IL-6 (13). This cell line, therefore, provided a good model system to study the role of PKC isozymes in mediating signals required for activation of the host cells to increased HIV-1 production. we have demonstrated that activation of PKC isozymes lead to induction of viral expression and activation of PKCβI isozyme may not be sufficient in activation of latent HIV-1 provirus. Activation of Ca^{++}-dependent isozymes by TT leads to strong induction of viral production although not as strong as activation of both Ca^{++}-dependent and Ca^{++}-independent isozymes by dPP. This suggests that both groups of isozymes are important in HIV-1 induction. Activation of PKCβI isozyme by dPPA failed to induce viral production suggesting selective activation of PKCβI may not be sufficient for induction of HIV-1 expression.

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