

# Protein Kinase C $\theta$ Is a Specific Target for Inhibition of the HIV Type 1 Replication in CD4<sup>+</sup> T Lymphocytes<sup>\*[5]</sup>

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Integration of HIV-1 genome in CD4<sup>+</sup> T cells produces latent reservoirs with long half-life that impedes the eradication of the infection. Control of viral replication is essential to reduce the size of latent reservoirs, mainly during primary infection when HIV-1 infects CD4<sup>+</sup> T cells massively. The addition of immunosuppressive agents to highly active antiretroviral therapy during primary infection would suppress HIV-1 replication by limiting T cell activation, but these agents show potential risk for causing lymphoproliferative disorders. Selective inhibition of PKC $\theta$ , crucial for T cell function, would limit T cell activation and HIV-1 replication without causing general immunosuppression due to PKC $\theta$  being mostly expressed in T cells. Accordingly, the effect of rottlerin, a dose-dependent PKC $\theta$  inhibitor, on HIV-1 replication was analyzed in T cells. Rottlerin was able to reduce HIV-1 replication more than 20-fold in MT-2 (IC<sub>50</sub> = 5.2  $\mu$ M) and Jurkat (IC<sub>50</sub> = 2.2  $\mu$ M) cells and more than 4-fold in peripheral blood lymphocytes (IC<sub>50</sub> = 4.4  $\mu$ M). Selective inhibition of PKC $\theta$ , but not PKC $\delta$  or  $\zeta$ , was observed at <6.0  $\mu$ M, decreasing the phosphorylation at residue Thr<sup>538</sup> on the kinase catalytic domain activation loop and avoiding PKC $\theta$  translocation to the lipid rafts. Consequently, the main effector at the end of PKC $\theta$  pathway, NF- $\kappa$ B, was repressed. Rottlerin also caused a significant inhibition of HIV-1 integration. Recently, several specific PKC $\theta$  inhibitors have been designed for the treatment of autoimmune diseases. Using these inhibitors in combination with highly active antiretroviral therapy during primary infection could be helpful to avoid massive viral infection and replication from infected CD4<sup>+</sup> T cells, reducing the reservoir size at early stages of the infection.

The novel protein serine/threonine kinase C  $\theta$  (PKC $\theta$ ) isoform is selectively expressed in T lymphocytes but not B lymphocytes, erythrocytes, polymorphonuclear neutrophils, monocytes, or macrophages (1). Other PKC isotypes ( $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ ,

$\zeta$ , and  $\eta$ ) are also expressed in T cells, but PKC $\theta$  is essential for T cell receptor/CD3-mediated activation events (2, 3). Among novel PKCs, PKC $\theta$  displays the highest amino acid similarity with PKC $\delta$ , not only in the catalytic or kinase core and the diacylglycerol binding regulatory domain C1 but mainly in the NH<sub>2</sub>-terminal V1 domain (2). However, V3 domain of PKC $\theta$  is unique and does not show significant similarity with any other PKC isoforms, including PKC $\delta$ . As a result, PKC $\theta$  provides a molecular basis for isotype selectivity and the non-redundant activity of distinct PKC isoenzymes in T cells (4).

PKC $\theta$  kinase activity is regulated by phosphorylation of the activation-loop residue Thr<sup>538</sup> on the catalytic domain during T cell activation (5). Residue Thr<sup>538</sup> has been described as critical for PKC $\theta$  catalytic activity for regulating phosphorylation of the hydrophobic motif and for enabling CD3-mediated nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation (6). PKC $\theta$  also shows the unique property of being translocated to the plasma membrane lipid rafts during the immunological synapse between T cells and antigen-presenting cells (7–9). Recruitment of PKC $\theta$  to the immunological synapse induces the activation of intracellular signaling pathways as the mitogen-activated protein kinase and the NF- $\kappa$ B pathway (10), essential for the regulation of T cell growth-promoting genes as *IL-2* (interleukin-2) (3, 11). NF- $\kappa$ B is also critical for the replication of the human immunodeficiency virus type 1 (HIV-1) in human blood CD4<sup>+</sup> T cells (12). The main NF- $\kappa$ B inhibitor, I $\kappa$ B $\alpha$ , binds to the NF- $\kappa$ B nuclear localization signal to keep it inactive in the cytoplasm in the absence of activation. Upon T cell activation, I $\kappa$ B $\alpha$  is phosphorylated by the I $\kappa$ B kinase complex and degraded in the proteasome (13), releasing the nuclear localization signal and allowing NF- $\kappa$ B translocation to the nucleus, where binds to cognate sequences in inducible gene promoters (14), as the HIV-1 long terminal promoter (LTR).

The main target for HIV-1 infection is the CD4<sup>+</sup> T cell population, in particular memory CD4<sup>+</sup> T cells that are generated by antigen recognition (15). The viral genome can be permanently integrated in the chromosomes of these cells, producing latent reservoirs with long half-life. HIV-1-infected memory T cells remain undetectable by the immune system and the highly active antiretroviral therapy (HAART)<sup>4</sup> when they are in a resting state, but they are able to release new batches of virions after

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<sup>4</sup> The abbreviations used are: HAART, highly active antiretroviral therapy; CXCR4, chemokine (CXC motif) receptor 4; LUC, luciferase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PHA, phytohemagglutinin; PMA, 5-phorbol 12-myristate 13-acetate; RLUs, relative luciferase/Renilla units; VSV, vesicular stomatitis virus; PBL, peripheral blood lymphocytes.

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transitory activation during antigen recognition or inflammatory processes (16–18). As a consequence, HIV-1-integrated proviruses are the major cause for the impossibility of eradicating the infection despite HAART (19). In an attempt to eliminate these viral reservoirs, PKCs have been appointed as specific targets for anti-latency drugs to reactivate and destroy viral reservoirs (20). PKC activators as prostratin (21, 22), non-tumorigenic phorbol ester derivatives (23), and the jatrophone diterpene SJ23B (24) induce potent reactivation of viral reservoirs through the activation of NF- $\kappa$ B and Sp1, but their suitability as adjuvant of HIV-1 treatment remains to be proved in clinical trials. On the other hand, the opposite strategy may also be considered to reduce the size of latent reservoirs from the beginning of the infection. The use of PKC inhibitors has been proposed to induce immunosuppression in transplantation and autoimmune diseases (3). Because HIV-1 causes a massive infection of activated CD4<sup>+</sup> T cells and contributes to lymphocyte activation during primary infection (25–27), the use of PKC inhibitors as adjuvant for HAART would decrease the pool of activated CD4<sup>+</sup> T cells, lessening the virus production and diminishing the size of latent reservoirs from the beginning of the infection. Because PKC $\theta$  is selectively expressed in T cells and is essential for T cell activation and function, specifically targeting PKC $\theta$  will limit the immunosuppressive effect to the major targets for HIV-1 infection.

To test the hypothesis that specific inhibition of PKC $\theta$  will be useful for reducing HIV-1 replication in T cells, we analyzed the antiviral effect of rottlerin, a cell-permeable inhibitor of PKCs that is highly specific of PKC $\theta$  when used at low concentration (<6.0  $\mu$ M). Evidences that the selective inhibition of PKC $\theta$  activation in T cells could be a useful target for designing pharmacological or genetic strategies for preventing HIV-1 replication and spread are provided.

### EXPERIMENTAL PROCEDURES

**Cells**—Jurkat and MT2 cell lines were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (PAN Biotech GmbH, Aidenbach, Germany), 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, and 100 units/ml penicillin (Lonza, Basel, Switzerland) at 37 °C. Peripheral blood lymphocytes (PBLs) were isolated from blood of healthy donors by centrifugation through a Ficoll-Hypaque gradient (Lymphocyte separation medium, Lonza). Cells were collected in supplemented RPMI 1640 medium and maintained at 37 °C,  $2 \times 10^6$  cells/ml. Phytohemagglutinin (PHA)-treated T lymphocytes were obtained from PBLs cultured for 3 days in the presence of 5  $\mu$ g/ml PHA (Sigma) and 300 units/ml IL-2 (Chiron, Emeryville, CA). Resting PBLs were maintained in culture at  $2 \times 10^6$  cells/ml in supplemented RPMI in the absence of stimulus. Jurkat and MT2 cells stably transfected with pGeneClip-iPKC $\theta$ -C1 or pGeneClip-iPKC $\theta$ -1 and pGeneClip-iPKC $\theta$ -3 were cultured at 37 °C in supplemented RPMI 1640 medium with 2  $\mu$ g/ml puromycin (Invitrogen).

**Vectors**—Vector pNL4.3 wild-type (wt) that contained the HIV-1 complete genome and induced an infectious progeny after transfection was kindly provided by Dr M.A. Martin (28). Vector pNL4.3-Renilla was obtained by replacing the gene *nef* of the HIV-1 proviral clone pNL4.3 with the Renilla luciferase

gene, as previously described (29). LTR-LUC vector containing the luciferase (LUC) reporter gene under the control of HIV-1 LTR U3+R region (LAI strain) was described previously (30). 3 $\kappa$ B-LUC vector that contains a luciferase gene under the control of three  $\kappa$ B consensus motifs of the immunoglobulin  $\kappa$ -chain promoter was described previously (31). Plasmid pCMV-tat101 was previously described (31). DNA for vesicular stomatitis virus (VSV) G glycoprotein was cloned in the pcDNA3.1 plasmid (pcDNA-VSV) (32). GeneClip U1 Hairpin Cloning System kit (Promega Biotech Iberica, Madrid, Spain), containing the pGeneClip vector, was used to generate the small hairpin RNA (shRNA) plasmids (pGeneClip-iPKC $\theta$ -1 and pGeneClip-iPKC $\theta$ -3) containing two different small interference RNA (siRNA) sequences directed against mRNA encoding for PKC $\theta$ . Sequences used to generate pGeneClip-iPKC $\theta$ -1 vector were iPKCt1-s (5'-TCTCGATTGGCTTGTCCAACCTTAAAGTTCTCTAAAGTTGGACAAGCCAACTCT-3') and iPKCt1-as (5'-CTGCAGGATTGGCTTGTCCAACTTTAGAGAACTTAAAGTTGGACAAGCCAAATC-3'). Sequences used to generate pGeneClip-iPKC $\theta$ -3 vector were iPKCt3-s (5'-TCTCGTAGAGAAGAGAGTTCTTTAAGTTCTCTAAAGAACTCTTCTCTACCT-3') and iPKCt3-as (5'-CTGCAGGTAGAGAAGAGAGTTCTTTAGAGAACTTAAAGAACTCTTCTCTAC-3'). Scrambled sequences used to generate pGeneClip-iPKC $\theta$ -C1 vector were iPKCtC1-s (3'-TCTCGGTTGCTTCCTGTATTAACAAGTTCTCTGTAAATACAGGAAGCAACCCT-5') and iPKCtC1-as (3'-CTGCAGGGTTGCTTCCTGTATTAACAGAGAACTTGTAAATACAGGAAGCAACC-5'). Each pair of primers was annealed and cloned in linearized pGeneClip vector according to the manufacturers' instructions. pRL-TK Renilla or pSV- $\beta$ -galactosidase vector, used as controls of transfection efficiency, were purchased from Promega. Plasmids were purified using Qiagen Plasmid Maxi kit (Qiagen Iberia, Madrid, Spain) following the manufacturer's instructions.

**Reagents and Antibodies**—Rottlerin or mallotoxin is a cell-permeable inhibitor of PKC $\theta$  ( $IC_{50} \geq 1.25 \mu$ M) (33) and PKC $\delta$  ( $IC_{50} \geq 6.0 \mu$ M) that was purchased from Calbiochem. Rottlerin also inhibits PKC $\alpha$ , - $\beta$ , - $\gamma$ , - $\epsilon$ , - $\eta$ , and - $\xi$  isoenzymes at high concentration (>30  $\mu$ M). Bisindolylmaleimide I (Gö6850) is a highly selective cell-permeable inhibitor of PKC $\alpha$ , - $\beta$ I, - $\beta$ II, - $\gamma$ , - $\delta$ , and - $\epsilon$  isoenzymes ( $K_i = 10$  nM), structurally similar to staurosporine, supplied by Merck Chemicals (34). Bisindolylmaleimide was used at 1.0  $\mu$ M to ensure efficient inhibition of PKC $\delta$  without inhibition of protein kinase A ( $K_i = 2.0 \mu$ M) (35). PHA was used at 5  $\mu$ g/ml, and 5-phorbol 12-myristate 13-acetate (PMA) (Sigma) was used at 25 ng/ml. Purified anti-human CD3 (clone OKT3) and CD28 (clone CD28.2) were used for T cell activation (eBiosciences, San Diego, CA). Polyclonal antibody against human PKC $\theta$  (clone C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against phospho-PKC $\theta$  (Thr<sup>538</sup>), phospho-PKC $\delta$  (Thr<sup>505</sup>), and phospho-PKC $\zeta/\lambda$  (Thr<sup>410/403</sup>) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against p65/RelA (clone C-20), I $\kappa$ B $\alpha$  (clone C-21), phospho-I $\kappa$ B $\alpha$  (Ser<sup>32</sup>) (clone 14D4), and full-length native HIV-1 p24 antigen (clone 491) were supplied by Santa Cruz Biotechnology. Specific antibody against the  $\beta$ -isoform of actin was obtained

from Sigma. Secondary antibodies conjugated with horseradish peroxidase were purchased from GE Healthcare. Antibodies against CD4 and the chemokine (CXC motif) receptor 4 (CXCR4) conjugated with phycoerythrin were purchased from BD Biosciences.

**PKC Kinase Assays**—PKC $\theta$  and PKC $\delta$  enzymatic activity was assayed using an immunoprecipitation kinase assay described previously (36) with minor modifications. Briefly, Jurkat cells were incubated with rottlerin or bisindolylmaleimide for 18 h and then collected and washed once with  $1 \times$  PBS. The pellet was resuspended in PKC lysis buffer (25 mM HEPES, pH 7.5, 20 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM NaF, and 4  $\mu$ g/ml each of aprotinin and leupeptin). The lysates were incubated on ice for 30 min and then centrifuged at  $13,000 \times g$  for 5 min. The supernatants were collected as cytosolic fractions, and protein concentration was determined by the method of Bradford (37). Cytosolic extracts (250–500  $\mu$ g) were immunoprecipitated for 18 h at 4 °C using 2  $\mu$ g of anti-PKC $\theta$  or anti-PKC $\delta$  antibodies. The immunoprecipitates were then incubated with goat anti-rabbit IgG (whole molecule) antibody conjugated with agarose (Sigma) for 1 h at 4 °C while gently rotating. The agarose-bound antigen-antibody complexes were washed three times with  $2 \times$  kinase buffer (40 mM Tris, pH 7.4, 20 mM MgCl<sub>2</sub>, 20  $\mu$ M ATP, and 2.5 mM CaCl<sub>2</sub>) and resuspended in 20  $\mu$ l of  $2 \times$  kinase buffer. The enzymatic reaction was started by adding 20  $\mu$ l of reaction buffer containing 3  $\mu$ g of PKC $\epsilon$  peptide substrate ERMRPRKRQGS-VRRRV, 50  $\mu$ g/ml phosphatidylserine, 4.1  $\mu$ M diacylglycerol, and 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP at 3000 Ci/mM to the immunoprecipitated samples, which were subsequently incubated for 10 min at 30 °C. The enzymatic reaction was stopped by spotting 20  $\mu$ l of the reaction mixture onto an individually pre-cut strip of phosphocellulose P81 paper (Millipore Iberica, Madrid, Spain). After air drying, the P81 strips were sequentially washed 3 times for 10 min each in 1% phosphoric acid solution (Sigma) with constant gentle stirring. A radioactive control was set up to measure the total [ $\gamma$ -<sup>32</sup>P]ATP counts introduced into the reaction by spotting 5  $\mu$ l of the [ $\gamma$ -<sup>32</sup>P]ATP reaction buffer on a P81 strip. The control was dried for 2 min, and counts were read without previous washing. As a negative control, one reaction with the goat anti-rabbit IgG antibody conjugated with agarose (Sigma) was performed without adding the primary antibody to the cytosolic extracts. A complete reaction mixture lacking of PKC $\epsilon$  peptide substrate was used as the blank solution. Radioactivity on each air-dried P81 paper was counted in a Beckman LS 6000L scintillation counter (Beckman Instruments, Fullerton, CA). The corrected cpm was determined by subtracting the blank control value.

**Transfection Assays**—Stable transfection of Jurkat and MT2 cells with pGeneClip-iPKC $\theta$ -C1 or with pGeneClip-iPKC $\theta$ -1 and pGeneClip-iPKC $\theta$ -3 vectors was performed by electroporation with an Easyjet Plus Electroporator (Equibio, Middlesex, UK). In brief,  $5 \times 10^6$  cells were collected in 250  $\mu$ l of RPMI without supplement and mixed with 5  $\mu$ g of plasmid DNA in an electroporation cuvette with a 2-mm electrode gap (Equibio). Cells were transfected by two pulses at 280 V, 150 microfarads, and 330 ohms. After transfection, cells were incubated in sup-

plemental RPMI at 37 °C for 18 h, and then puromycin was added to the culture medium at 0.5  $\mu$ g/ml/day until selection. For transient transfections,  $5$ – $10 \times 10^6$  cells were resuspended in 350  $\mu$ l of RPMI without supplements and mixed with 5–10  $\mu$ g of plasmid DNA in an electroporation cuvette with a 4-mm electrode gap (Equibio). Jurkat cells were transfected by one pulse at 280 V and 1500 microfarads, and resting PBLs were transfected by 1 pulse at 320 V and 1500 microfarads. After transfection, cells were incubated in supplemental RPMI for 18 h at 37 °C. Luciferase and Renilla activities were assayed with the Luciferase Assay System (Promega), and  $\beta$ -galactosidase activity was measured with the  $\beta$ -Galactosidase Enzyme Assay System (Promega) according to manufacturer's instructions. Relative luciferase and Renilla units (RLUs) were measured in supernatants with a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN) after the addition of the appropriate substrate;  $\beta$ -galactosidase activity was measured at 420 nm in a microplate reader Sunrise (Tecan Group Ltd., Männedorf, Switzerland). RLUs were normalized by measuring both  $\beta$ -galactosidase activity and total protein concentration by the method of Bradford (37) using a bovine serum albumin standard curve.

**HIV-1 Infection**—Infectious supernatants were obtained from calcium phosphate transfection of 293T cells with plasmids pNL4.3-wt or pNL4.3-Renilla. VSV-pseudotyped  $\Delta$ Env-NL4.3-Luc virus was obtained by co-transfection of full-length HIV-1 DNA not expressing the HIV envelope (pNL4.3-Luc-R\_E\_) (AIDS Research and Reference Reagent program, NIAID, National Institutes of Health) and pcDNA-VSV, which expresses G protein of vesicular stomatitis virus (38, 39). Classic antiviral assays were performed by pretreating PBLs activated with anti-CD3/CD28 or PHA/IL-2 for 3 days or MT-2 cells with different concentrations of rottlerin for 30 min and infecting for 2 additional hours with HIV-1 NL4.3-wt strain or recombinant HIV-1 NL4.3-Renilla with gentle rotation at room temperature. Cells were then centrifuged at  $600 \times g$  for 30 min at 25 °C. After extensive washing with  $1 \times$  PBS, rottlerin was added again and left in culture for 2–7 days. Supernatants were collected, and HIV-1 p24 antigen was measured by immunoblotting or using an enzyme-like immunoassay (Innotest<sup>TM</sup> HIV Ag mAb; Innogenetics, Barcelona, Spain). Cells were lysed for quantifying Renilla or luciferase activity as described above.

Inhibitory concentrations 50 (IC<sub>50</sub>) were determined by incubating PBLs activated with anti-CD3/CD28 or PHA/IL-2 for 3 days or MT-2 cells in a 96-well microtiter plate in the presence of 0.5 ng of p24/well of recombinant HIV-1 NL4.3-Renilla as well as increasing concentrations of rottlerin. Production of Renilla (RLUs), corresponding to HIV-1 replication, was measured 48 h post-infection. IC<sub>50</sub> was calculated using GraphPad Prism Software (sigmoidal dose-response formula). Cell viability was evaluated in cells treated in parallel as described below. Half-maximal cytotoxic concentrations 50 (CC<sub>50</sub>) were calculated using GraphPad Prism Software (sigmoidal dose-response formula).

**Cell Viability and Proliferation**—Cell viability was determined with the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability assay (Promega) following the manufacturer's instructions. Briefly,  $1 \times 10^5$  cells were harvested by centrifugation, washed

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twice with  $1 \times$  PBS, and resuspended in lysis buffer. After incubation for 10 min at room temperature to stabilize luminescent signal, cell lysates were deposited in an opaque-walled multiwell plate and analyzed in an Orion Microplate Luminometer with Simplicity software (Berthold Detection Systems, Oak Ridge, TN). Cell viability was also determined by observing the cells in bright-field with Leica DMI 4000B Inverted Microscope (Leica Microsystems, Barcelona, Spain) or by staining with propidium iodide and analysis in a FACScalibur Flow Cytometer (BD Biosciences) using CellQuest software.

Cell proliferation was measured with the CellTiter-96 Aqueous Non-radioactive Cell Proliferation assay (Promega) according to the manufacturer's instructions. Briefly,  $2 \times 10^4$  cells per well were cultured in a 96-well microtiter plate in the absence of any stimulus or with  $5 \mu\text{g/ml}$  PHA for 72 h. Compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium was mixed with phenazine methosulfate solution and then added to the culture medium. Absorbance was measured at 490 nm after incubation for 3 h. The proliferation rate for each cell type in response to PHA was compared with their proliferation rate in the absence of stimulus.

**Immunoblotting Assays**—Cytosolic, nuclear, and membrane protein fractions were obtained as described previously (40, 41). Protein concentration was determined by the Bradford method (37). Eighty micrograms of protein extracts from each fraction were fractionated by SDS-PAGE and transferred onto Hybond-ECL nitrocellulose paper (GE Healthcare). After blocking and incubation with primary and secondary antibodies, proteins were detected with SuperSignal West Pico/Femto Chemiluminescent Substrate (Pierce). Images were acquired in a Bio-Rad Geldoc 2000.

**DNA Affinity Immunoblotting Assay**—The DNA affinity immunoblotting assay was performed as described previously (6) with minor modifications (42). Briefly,  $100 \mu\text{g}$  of nuclear protein extracts from Jurkat cells were incubated at  $4^\circ\text{C}$  for 30 min with  $25 \text{ nM}$  concentrations of a  $5'$ -labeled biotin probe that contains the double  $\kappa\text{B}$  consensus motif located in the HIV-1 LTR promoter ( $5'$ -AGCTTACAAGGGACTTTCCGCTGGG-GACTTTCCAGGGA- $3'$ ) or with a probe that contains the estrogen receptor DNA element, a non-related NF- $\kappa\text{B}$  gene (43). Biotin-probe/protein complexes were captured by incubating with streptavidin-agarose resins (Pierce) at  $4^\circ\text{C}$  for 30 min and then collected by centrifugation at  $3000 \times g$  for 10 min. Protein complexes were fractionated by SDS-PAGE, and the presence of p65/RelA protein bound to DNA probes was detected by immunoblotting with a specific antibody. As internal control,  $10 \mu\text{g}$  of protein were separated before adding each probe and resolved by immunoblot using an antibody against  $\beta$ -actin. Densitometry analysis was performed in a Gel Doc 2000 System (Bio-Rad) by using Quantity One software (Bio-Rad). Gel bands were quantified, and background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated regarding to the internal control for each lane.

**Analysis of Reverse Transcriptase Products**—Reverse transcriptase products were detected by semiquantitative PCR as described previously (44) with modifications. Briefly, MT-2

cells were infected with HIV-1 NL4.3-wt clone (10 ng of p24 antigen/million of cells) for 18 h by spinoculation, as indicated above, and total DNA was extracted with QIAamp DNA Mini kit (Qiagen). The reaction mixture contained  $2.5 \mu\text{l}$  of buffer  $10\times$  with  $\text{MgCl}_2$ ,  $100 \mu\text{M}$  dNTPs,  $0.4 \mu\text{M}$  concentrations of each primer pair, and 2.5 units of TaqDNA polymerase (Roche Pharma, Madrid, Spain) in a final volume of  $25 \mu\text{l}$ . Five micrograms of DNA were added and amplified using a C1000 thermal cycler (Bio-Rad) with the following conditions: an initial 10-min denaturation step at  $95^\circ\text{C}$  and then 50 cycles consisting of  $94^\circ\text{C}$  for 30 s,  $55^\circ\text{C}$  for 2 min,  $72^\circ\text{C}$  for 1 min, and a final extension cycle of  $72^\circ\text{C}$  for 10 min. The following primers were used to amplify a short retrotranscription product, yielding a fragment of 138 bp: R/U5 (sense)  $5'$ -GGCTAACTAGGGAAAC-CCACTGCTT- $3'$ ; R/U5 (antisense)  $5'$ -GCTAGAGATTTTC-CACACTGACTAA- $3'$ . To amplify a long retrotranscription product, primers R/U5 sense and LTR/gag (antisense) ( $5'$ -CTCGCACCCATCTCTCTCTCTCTA- $3'$ ) were used, yielding a fragment of 306 bp. Amplification of a gene encoding for the ribosomal protein S18 was used as an internal control for data normalization by using the primers hRibS18 (sense,  $5'$ -GCCATGTCTCTAGTGATCCCTGAA- $3'$ ) and hRibS18 (antisense,  $5'$ -TTCTTCTTGGACACCCACGGT- $3'$ ) yielding a fragment of 458 bp. All amplified products were analyzed by electrophoresis on 3% Seakem-agarose gel containing 5 mg/ml ethidium bromide in  $0.5\times$  Tris acetate buffer. The Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) was used for sequencing the amplified DNA fragments according to manufacturer's instructions by using an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Densitometry analysis was performed as described above.

**Analysis of Proviral DNA Integration**—A semiquantitative nested-Alu PCR assay was performed for analyzing HIV-1 proviral integration as previously described (44) with modifications. Briefly, MT-2 cells were infected, and total DNA was extracted as described above. The reaction mixtures contained  $5 \mu\text{l}$  of buffer  $10\times$  with 2 mM  $\text{MgCl}_2$ ,  $300 \mu\text{M}$  dNTPs,  $0.4 \mu\text{M}$  concentrations of each primer pair, and 1.25 units of Taq DNA polymerase (Roche Applied Science) in a final volume of  $50 \mu\text{l}$ . Five micrograms of DNA were added and amplified using a C1000 thermal cycler (Bio-Rad) with the following conditions; first PCR cycling conditions were 50 cycles of  $94^\circ\text{C}$  for 45 s,  $50^\circ\text{C}$  for 1.5 min,  $68^\circ\text{C}$  for 4 min, and a final incubation of  $68^\circ\text{C}$  for 10 min. An aliquot of  $6 \mu\text{l}$  of the first PCR was added to the second PCR tube, with  $50 \mu\text{l}$  as the final volume. Second PCR cycling conditions were 50 cycles of  $94^\circ\text{C}$  for 45 s,  $53^\circ\text{C}$  for 1.5 min,  $72^\circ\text{C}$  for 4 min, and a final incubation of  $72^\circ\text{C}$  for 10 min. The following primers were used for the first Alu-PCR: LA1 (sense),  $5'$ -TGTGTGCCCGTCTGTTGTGT- $3'$ ; LA2 (Alu sequence) (antisense),  $5'$ -TGCTGGGATTACAGGCGTGAG- $3'$ . The following primers were used for the second nested PCR, yielding a fragment of 142 bp: NL1 (sense),  $5'$ -GTGCCCGTCTGTTGTGTGACT- $3'$ ; NL2 (antisense),  $5'$ -CCGAGTCCTG-CGTCGAGAGA- $3'$ . Five micrograms of DNA extracted from 8E5 cells, which contain a single integrated copy of HIV-1 (45), were used as control. Amplification of gene encoding for the ribosomal protein S18 was used as the internal control for data normalization. All amplified products were analyzed by elec-

trophoresis and sequenced as described above. Densitometry analysis was performed as described above.

**Semiquantitative RT-PCR Assay**—Total RNA from Jurkat and MT2 cells was isolated with RNeasy Mini kit (Qiagen) according to manufacturer's instructions. For semiquantitative RT-PCR assay, specific primers for amplifying PKC $\theta$  gene were PKCt-7s (5'-GCAATTTTCGACAAAGAATT-3') and PKCt-6as, (5'-AAATGTATGGTTGGAATTC-3'), yielding a fragment of 1.103 bp. Primers for amplifying human  $\beta$ -actin gene were  $\beta$ -actin-s (5'-TCACCCACACTGTGCCCATCTA-3') and  $\beta$ -actin-as (5'-AGTTGAAGGTAGTTTCGTGGAT-3'), yielding a fragment of 360 bp. RT-PCR assay was performed by using Qiagen OneStep RT-PCR kit (Qiagen). Briefly, the reaction mixture contained 10  $\mu$ l of buffer 5 $\times$ , 2 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.4  $\mu$ M concentrations of each primer pair, and 1  $\mu$ l of Qiagen OneStep RT-PCR enzyme mix in a final volume of 50  $\mu$ l. A total of 5  $\mu$ g of RNA was added, and thermal cycling conditions were as follows: an initial cycle of 48 °C for 45 min and 95 °C for 10 min; 25 cycles of 95 °C for 30 s, 49 °C for 1.5 min, 68 °C for 2 min; a final extension cycle of 68 °C for 10 min. Amplified products were analyzed and sequenced by electrophoresis as described above. Densitometry analysis was performed as described.

## RESULTS

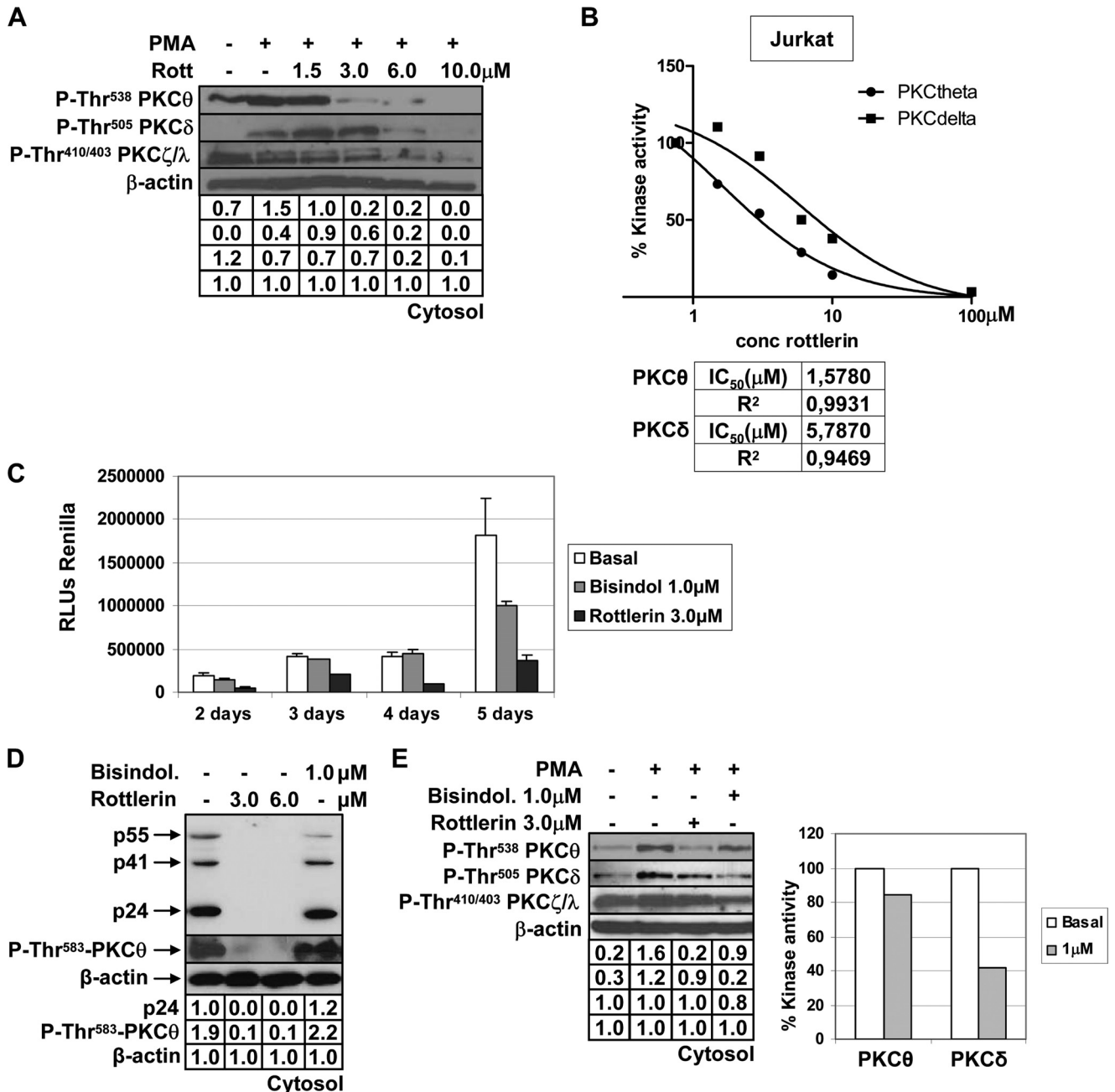
**Specific Inhibition of PKC $\theta$  by Rottlerin in T Cells**—Rottlerin is a dose-dependent, cell-permeable PKC $\theta$  inhibitor that has been described as a potent immunosuppressive agent for the development of novel therapies in T cell-mediated immune disorders (33). As rottlerin specificity may be controlled by the concentration in the culture medium, this compound has been used as an inhibitor of either PKC $\delta$  (46) or PKC $\theta$  (33). Then, first we determined the concentration of rottlerin that was able to inhibit selectively PKC $\theta$  but not PKC $\delta$  in T cells in long term culture. For this purpose, Jurkat cells were used as a model of T cells after analyzing that both PKC $\theta$  phosphorylation at Thr<sup>538</sup> and translocation to the plasma membrane lipid rafts were PMA-inducible events in these cells (see supplemental Fig. 1). To further evaluate the specificity of rottlerin for the inhibition of PKC $\theta$  but not PKC $\delta$ , the phosphorylation of PKC $\delta$  at Thr<sup>505</sup> was also analyzed. PKC $\delta$  phosphorylation at Thr<sup>505</sup> increases in association with PMA-dependent PKC $\delta$  activation along with the PMA-dependent translocation of PKC $\delta$  to the plasma membrane (47). Accordingly, Thr<sup>505</sup> phosphorylation has been described as a regulated event that enhances PKC $\delta$  activity (48).

Once we determined the suitability of Jurkat as a valid model for the analysis of PKC $\theta$  activity, these cells were incubated with PMA and rottlerin at increasing concentrations for 18 h. Rottlerin inhibited the phosphorylation of PKC $\theta$  (Thr<sup>538</sup>) at 3.0  $\mu$ M but not the phosphorylation of PKC $\delta$  (Thr<sup>505</sup>) or PKC $\zeta/\lambda$  (Thr<sup>410/403</sup>). However, significant inhibition of both PKC $\delta$  and PKC $\zeta/\lambda$  phosphorylation was observed with rottlerin  $\geq$ 6.0  $\mu$ M (Fig. 1A). Correlation between changes in the phosphorylation at Thr<sup>538</sup> in PKC $\theta$  and Thr<sup>505</sup> in PKC $\delta$  and the kinase activity due to the presence of rottlerin was determined by using a radioactive enzymatic assay in the same conditions of culture (Fig. 1B). The effect of rottlerin for preventing HIV-1 replication in long term culture as a specific PKC $\theta$  inhibitor was eval-

uated in PBLs in comparison with the generic PKC inhibitor bisindolylmaleimide (Gö6850) (49). PBLs from healthy donors activated with anti-CD3/CD28 for 3 days were infected with NL4.3-Renilla clone by spinoculation and then cultured for 5 days. Upon integration into host chromosomes, this recombinant virus expresses the firefly luciferase Renilla, and consequently, Renilla activity in infected cells correlates with the rate of viral replication (29). 3.0  $\mu$ M rottlerin or 1.0  $\mu$ M bisindolylmaleimide were added to the culture medium only at the beginning of the experiment. Measurement of Renilla in HIV-1-infected T cells showed that the infection was less than 2-fold inhibited by 1.0  $\mu$ M bisindolylmaleimide, whereas rottlerin thwarted HIV-1 replication more than 4-fold (Fig. 1C). Analysis by immunoblotting of the cytosolic proteins from HIV-1-infected PBLs 5 days post-infection showed that there was no significant production of p24 antigen in the presence of rottlerin, whereas p24 was detected in cells that had been incubated with 1.0  $\mu$ M bisindolylmaleimide (Fig. 1D). The absence of PKC $\theta$  phosphorylation at Thr<sup>538</sup> induced by rottlerin correlated with the absence of p24 antigen in these cells. Efficacy of 1.0  $\mu$ M bisindolylmaleimide for inhibiting phosphorylation of PKC $\delta$  at Thr<sup>505</sup> but not PKC $\theta$  at Thr<sup>538</sup> was determined by immunoblotting of cytosolic protein extracts from Jurkat treated with PMA for 18 h (Fig. 1E). Correlation between changes in the phosphorylation at Thr<sup>538</sup> in PKC $\theta$  and Thr<sup>505</sup> in PKC $\delta$  and the kinase activity due to the presence of bisindolylmaleimide was determined by using a radioactive enzymatic assay in the same conditions of culture. Phosphorylation of PKC $\zeta/\lambda$  was not significantly modified in these conditions.

**PKC $\theta$  Inhibitor Rottlerin Thwarted HIV-1 Replication in T Cells**—Selective inhibition of PKC $\theta$  occurred with rottlerin at  $<$ 6.0  $\mu$ M. Next, we analyzed the inhibitory activity of rottlerin over HIV-1 replication with lower concentrations, and the IC<sub>50</sub> was calculated with a range of concentrations. MT-2 cells were pretreated with rottlerin for 30 min, then infected with NL4.3-Renilla clone by spinoculation and cultured for 5 days. High Renilla activity levels were detected after 5 days, but pretreatment with increasing doses of rottlerin resulted in dose-dependent inhibition of the Renilla activity, being as rottlerin at the highest concentration tested (3.0  $\mu$ M) was the most effective for the inhibition of HIV-1 replication (Fig. 2A). Lower concentrations than 3.0  $\mu$ M were able to delay HIV-1 replication but did not completely inhibit it. Then, we assayed the anti-HIV-1 activity of rottlerin in human PBLs from healthy donors that were activated for 3 days with anti-CD3/CD28 before being infected with the NL4.3-Renilla clone by spinoculation, proving that pretreatment with rottlerin also caused a dose-dependent inhibition of the Renilla activity associated to NL4.3-Renilla clone replication (Fig. 2B). Correspondence of Renilla production with p24 antigen synthesis was determined by quantification of p24 in the infected cell supernatants 5 days post-infection. Rottlerin IC<sub>50</sub> values for the inhibition of HIV-1 replication were calculated by infecting MT-2 cells or activated PBLs in a 96-well microtiter plate with the recombinant NL4.3-Renilla. After an incubation of 48 h, IC<sub>50</sub> was determined as 5.2  $\mu$ M for MT-2 cells and 4.4  $\mu$ M for PBLs by using GraphPad Prism Software (Fig. 2C). Similar results were obtained in cells that were previously infected and treated with rottlerin for 2 h

## Role of PKC $\theta$ in HIV-1 Replication

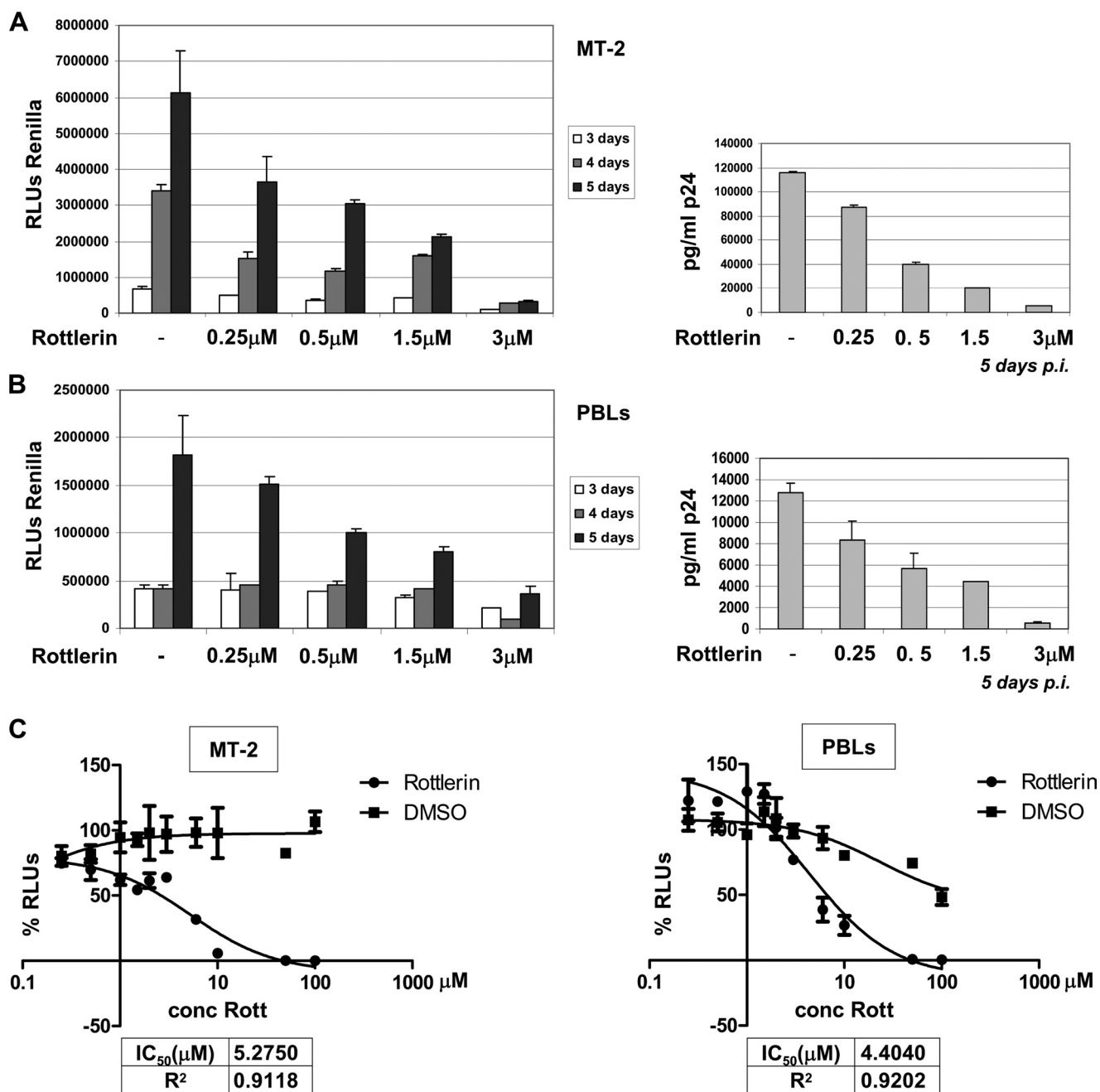


**FIGURE 1. Rottlerin at low concentration induced a selective inhibition of PKC $\theta$  phosphorylation but not PKC $\delta$  or PKC $\zeta$ .** *A*, Jurkat cells were incubated with PMA and rottlerin (Rott) at increasing concentrations for 18 h. Cytosolic protein extracts were analyzed by immunoblotting using antibodies against PKC $\theta$  phosphorylated at Thr<sup>538</sup>, PKC $\delta$  phosphorylated at Thr<sup>505</sup>, and PKC $\zeta/\lambda$  phosphorylated at Thr<sup>410/403</sup>.  $\beta$ -Actin was used as the internal loading control. *B*, PKC $\theta$  and PKC $\delta$  kinase activities were quantified using an immunoprecipitation kinase assay in cytosolic protein extracts from Jurkat cells treated with different concentration of rottlerin for 18 h. Inhibition of enzymatic activity of each PKC was analyzed, and IC<sub>50</sub> was calculated using GraphPad Prism Software (sigmoidal dose-response formula). *C*, PBLs from healthy donors activated with anti-CD3/CD28 for 3 days were infected with NL4.3-Renilla clone by spinoculation and then incubated for 5 days in the presence of rottlerin 3.0 or 1.0  $\mu$ M bisindolylmaleimide. Production of Renilla (RLUs), corresponding to HIV-1 replication, was measured at 2–5 days. The mean of RLUs corresponding to three independent experiments is represented as a bar diagram. Lines on the top of the bars correspond to the S.D. *D*, cytosolic protein extracts from activated PBLs infected for 5 days were analyzed by immunoblotting using antibodies against full-length native HIV-1 p24 antigen, PKC $\theta$  phosphorylated at Thr<sup>538</sup>, and  $\beta$ -actin as internal loading control. *E*, Jurkat cells were treated with 3.0  $\mu$ M rottlerin or 1.0  $\mu$ M bisindolylmaleimide (Bisindol.) for 30 min and then with PMA for an additional 30 min. Cytosolic protein extracts were analyzed by immunoblotting using antibodies against PKC $\theta$  phosphorylated at Thr<sup>538</sup>, PKC $\delta$  phosphorylated at Thr<sup>505</sup>, and PKC $\zeta/\lambda$  phosphorylated at Thr<sup>410/403</sup> and  $\beta$ -actin as internal loading control. Gel bands from all the immunoblotting assays were quantified by densitometry. PKC $\theta$  and PKC $\delta$  kinase activities in the presence of bisindolylmaleimide were quantified using an immunoprecipitation kinase assay in cytosolic protein extracts from Jurkat cells treated with 1.0  $\mu$ M bisindolylmaleimide for 18 h.

after infection, proving that pretreatment with rottlerin was not necessary to inhibit viral replication (data not shown).

**Effects of Rottlerin on Cell Viability and Proliferation**—The effect of rottlerin on cellular toxicity was evaluated in resting

PBLs, Jurkat, and MT-2 cells previously incubated with increasing doses of rottlerin for 72 h, 100  $\mu$ M being the highest concentration tested. Cell viability was measured by quantifying the levels of ATP present in metabolically active cells. Rottlerin

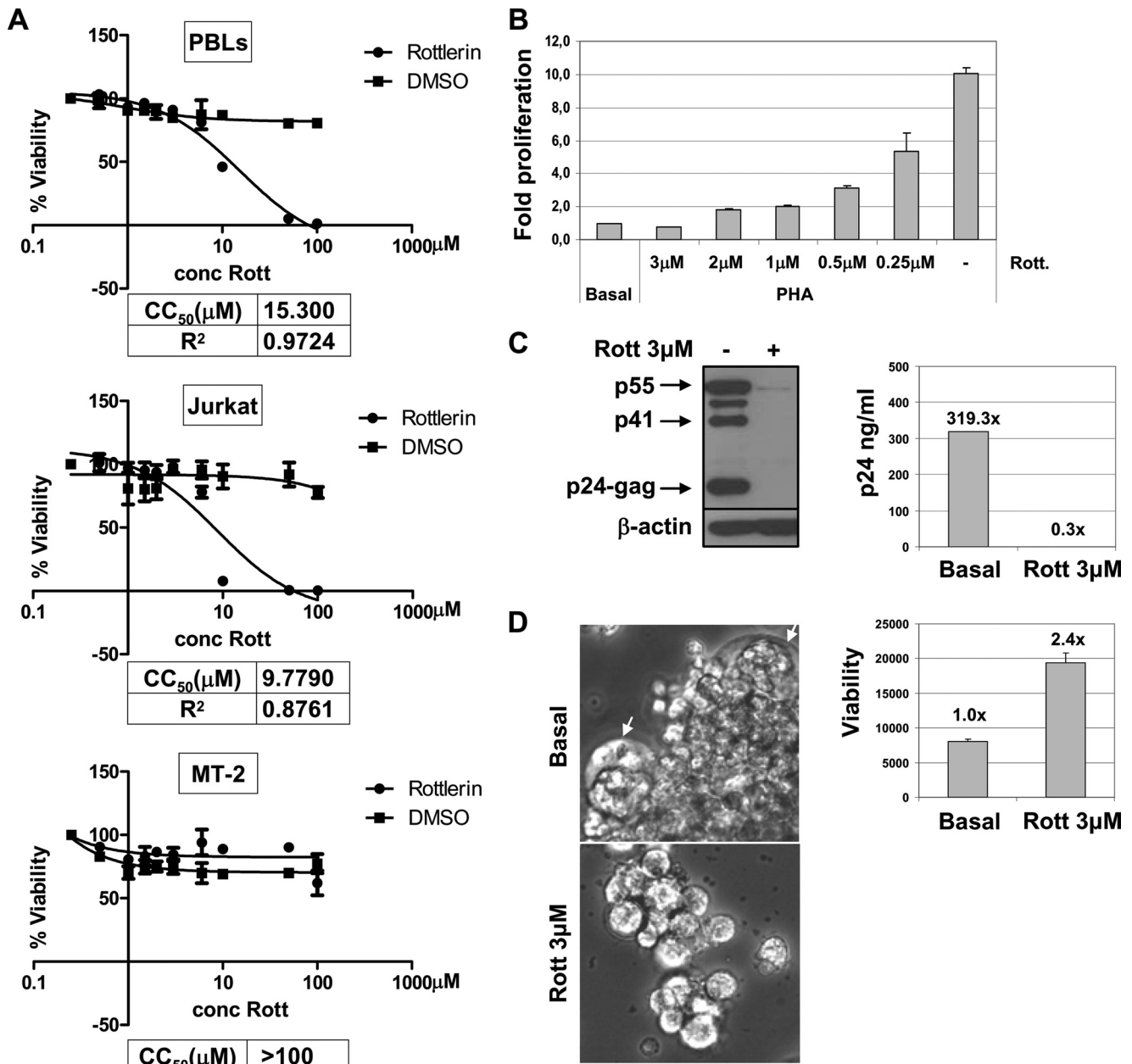


**FIGURE 2. Inhibition of HIV-1 replication in T cells by rottlerin.** A, MT-2 cells were infected with NL4.3-Renilla clone by spinoculation and then incubated for 5 days in the presence of increasing concentrations of rottlerin. Production of Renilla (RLUs), corresponding to HIV-1 replication, was measured at 3–5 days. B, PBLs obtained from healthy donors were activated for 3 days with anti-CD3/CD28 and then infected with NL4.3-Renilla clone by spinoculation. Renilla was measured at 3–5 days post-infection after incubation with increasing concentrations of rottlerin. Renilla production correlated with p24 antigen synthesis in the infected cell supernatants 5 days post-infection. In all cases the mean of RLUs corresponding to three independent experiments is represented as *bar diagrams*. Lines on the top of the bars correspond to S.D. C, PBLs obtained from healthy donors and activated for 3 days with anti-CD3/CD28 and MT-2 cells were infected with NL4.3-Renilla clone in 96-well microtiter plates in the presence of increasing concentrations of rottlerin or DMSO as negative control. Production of Renilla (RLUs), corresponding to HIV-1 replication, was measured 48 h post-infection. All measurements were done in triplicate, and the mean is represented with S.D. Inhibition of viral replication was analyzed, and IC<sub>50</sub> was calculated using GraphPad Prism Software (sigmoidal dose-response formula).

induced cytotoxicity at  $\geq 15 \mu\text{M}$  in PBLs and  $\geq 10 \mu\text{M}$  in Jurkat cells, but no significant cytotoxicity was observed in MT-2 cells even at the highest concentration tested (Fig. 3A). T cell proliferation capacity in the presence of rottlerin was also evaluated in PBLs incubated with PHA and rottlerin at different concentrations for 72 h. PHA-induced proliferation diminished as the concentration of rottlerin increased in the culture medium, keeping the proliferation of PHA-stimulated PBLs in the pres-

ence of the highest concentration of rottlerin assayed ( $3.0 \mu\text{M}$ ) at the same level of the resting cells (Fig. 3B). Similar results were obtained with Jurkat cells (data not shown). Low cytotoxicity during long term treatment with  $3.0 \mu\text{M}$  rottlerin was also observed in NL4.3wt-infected MT-2 cells after 7 days of culture. Analysis of p24 antigen synthesis by immunoblotting and ELISA showed that the production of this viral protein was severely limited in MT-2 cells treated with rottlerin (Fig. 3C).

## Role of PKC $\theta$ in HIV-1 Replication



**FIGURE 3. Analysis of cell viability and proliferation in the presence of rottlerin.** *A*, resting PBLs and Jurkat and MT-2 cells were incubated with increasing doses of rottlerin (*Rott*) for 72 h or DMSO as the negative control. Cell viability was measured by quantifying the levels of ATP present in metabolically active cells, and half-maximal cytotoxic concentration 50 ( $CC_{50}$ ) was calculated using GraphPad Prism Software (sigmoidal dose-response formula). All measurements were done in triplicate, and the mean is represented with S.D. *B*, PBLs were treated with PHA or/and rottlerin at different concentrations for 72 h, and cell proliferation capacity was assessed. The mean of data corresponding to three independent experiments is represented as a bar diagram. Lines on the top of the bars correspond to S.D. *C*, MT-2 cells were infected with NL4.3wt clone and then incubated for 7 days with or without 3.0  $\mu$ M rottlerin. Synthesis of p24 antigen was analyzed in cytosolic protein extracts by immunoblotting using  $\beta$ -actin as the internal loading control and in cell culture supernatants by ELISA. *D*, cell viability was analyzed by microscopy in bright field to observe the formation of syncytia (white arrows) and measured by quantifying the levels of ATP in living cells.

There was no significant cytopathic effect in these cells 7 days post-infection. Furthermore, cell viability was more than 2-fold increased in HIV-infected MT-2 cells treated with rottlerin, likely because the infection did not progress in these cells and they were protected by rottlerin from HIV-induced cytopathicity as syncytia (Fig. 3*D*).

**Mechanism of Inhibition of HIV-1 Replication by Rottlerin**—We observed that rottlerin inhibited HIV-1 replication from both NL4.3-Renilla or NL4.3wt clones in T cells. Progression of viral infection with these clones requires the fusion of the viral

membrane with CD4 and CXCR4 receptors, reverse transcription of the viral genome, integration, and transcription steps to induce Renilla activity. To determine whether rottlerin could inhibit HIV-1 replication by hampering the viral entry, the VSV-pseudotyped  $\Delta$ Env-NL4.3-Luc clone was used. The VSV protein mediates cell entry through a receptor-independent endocytic pathway (50). PBLs from healthy donors activated with anti-CD3/CD28, Jurkat, and MT-2 cells were preincubated for 30 min with rottlerin at different concentrations and further infected with the VSV-pseudotyped  $\Delta$ Env-NL4.3-Luc

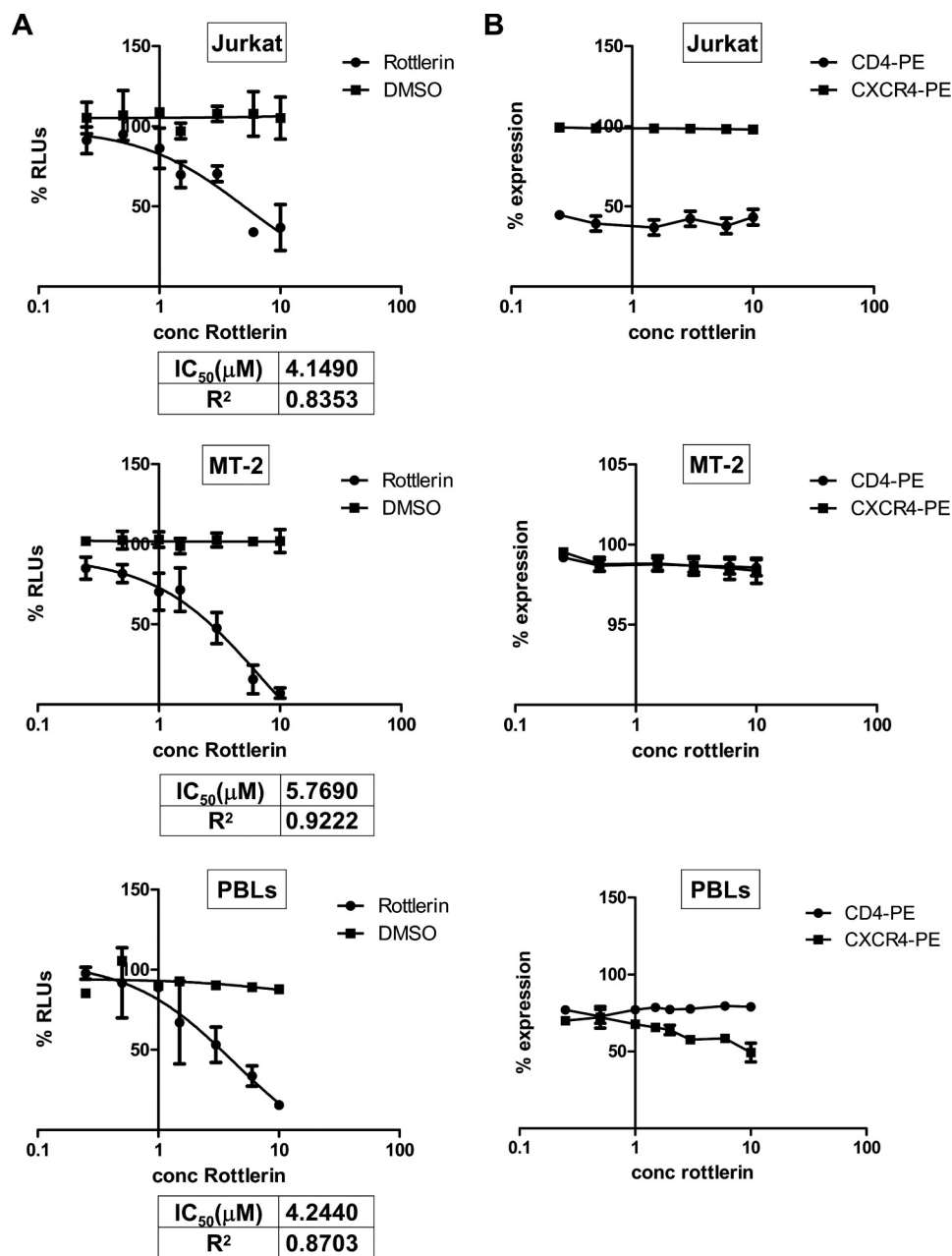
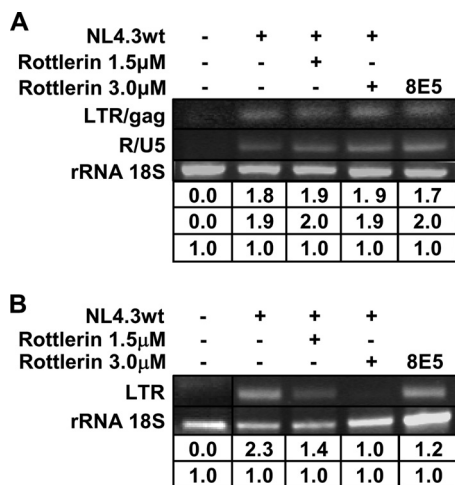


FIGURE 4. **Mechanism of inhibition of HIV-1 infection by rottlerin in T cells is independent of the viral entry.** *A*, PBLs from healthy donors activated with anti-CD3/CD28 for 3 days, Jurkat, and MT-2 cells were infected in 96-well microtiter plates with the VSV-pseudotyped  $\Delta$ Env-NL4.3-Luc clone for 48 h in the presence of rottlerin at different concentrations. Inhibition of viral replication was quantified, and IC<sub>50</sub> was estimated using GraphPad Prism Software (sigmoidal dose-response formula). *B*, Jurkat, MT-2, and resting PBLs were treated with several concentrations of rottlerin for 48 h, and changes in the expression on the cell surface of CD4 and CXCR4 were analyzed by flow cytometry. All measurements were done in triplicate, and the mean is represented with S.D. PE, phycoerythrin.

clone for 48 h in a 96-well microtiter plate. As shown Fig. 4*A*, rottlerin inhibited the luciferase activity driven by this recombinant virus that enters the cell independently of CD4 and CXCR4. There was no difference in the rottlerin-mediated inhibition of viral replication whether the cells were pretreated with this compound before being infected or after the infection (data not shown). We also determined whether rottlerin was able to induce endocytosis of CD4 and CXCR4. Jurkat, MT-2, and PBLs were treated with several concentrations of rottlerin for 48 h, and expression of CD4 and CXCR4 was analyzed by flow cytometry on the cell surface. There was no modification

of the expression of these receptors on the cell surface of Jurkat and MT-2 in response to increasing concentrations of rottlerin, although a decrease of 10% of CXCR4 expression was observed in PBLs with rottlerin at the highest concentration tested (10  $\mu$ M) (Fig. 4*B*). Overall, these results indicated that the inhibition of HIV-1 replication by rottlerin was not due to interference in viral entry. Consequently, rottlerin was also able to inhibit the replication of the viral clones JR-Renilla (24) and BX08-Renilla (51), both using CCR5 as co-receptor for entering the target cell (data not shown). Rottlerin did not modify CCR5 expression on the cell surface either.

## Role of PKC $\theta$ in HIV-1 Replication



**FIGURE 5. Effect of rottlerin on viral reverse transcription and proviral integration.** *A* and *B*, MT-2 cells infected with NL4.3wt by spinoculation were incubated with or without rottlerin for 18 h. Total RNA and genomic DNA were extracted. A semiquantitative RT-PCR assay was performed to amplify HIV-1 strong stop (R/U5) and full-length (LTR/gag) reverse transcriptase transcripts (*A*). Proviral integration was analyzed by a first-round Alu-PCR followed by nested PCR with internal primers targeted at the LTR (*B*). Ribosomal RNA 18 S was used as the housekeeping gene. Total RNA and genomic DNA from 8E5 cells, which contain a single integrated copy of HIV-1, were used as the positive control. Gel bands from immunoblotting assays were quantified by densitometry.

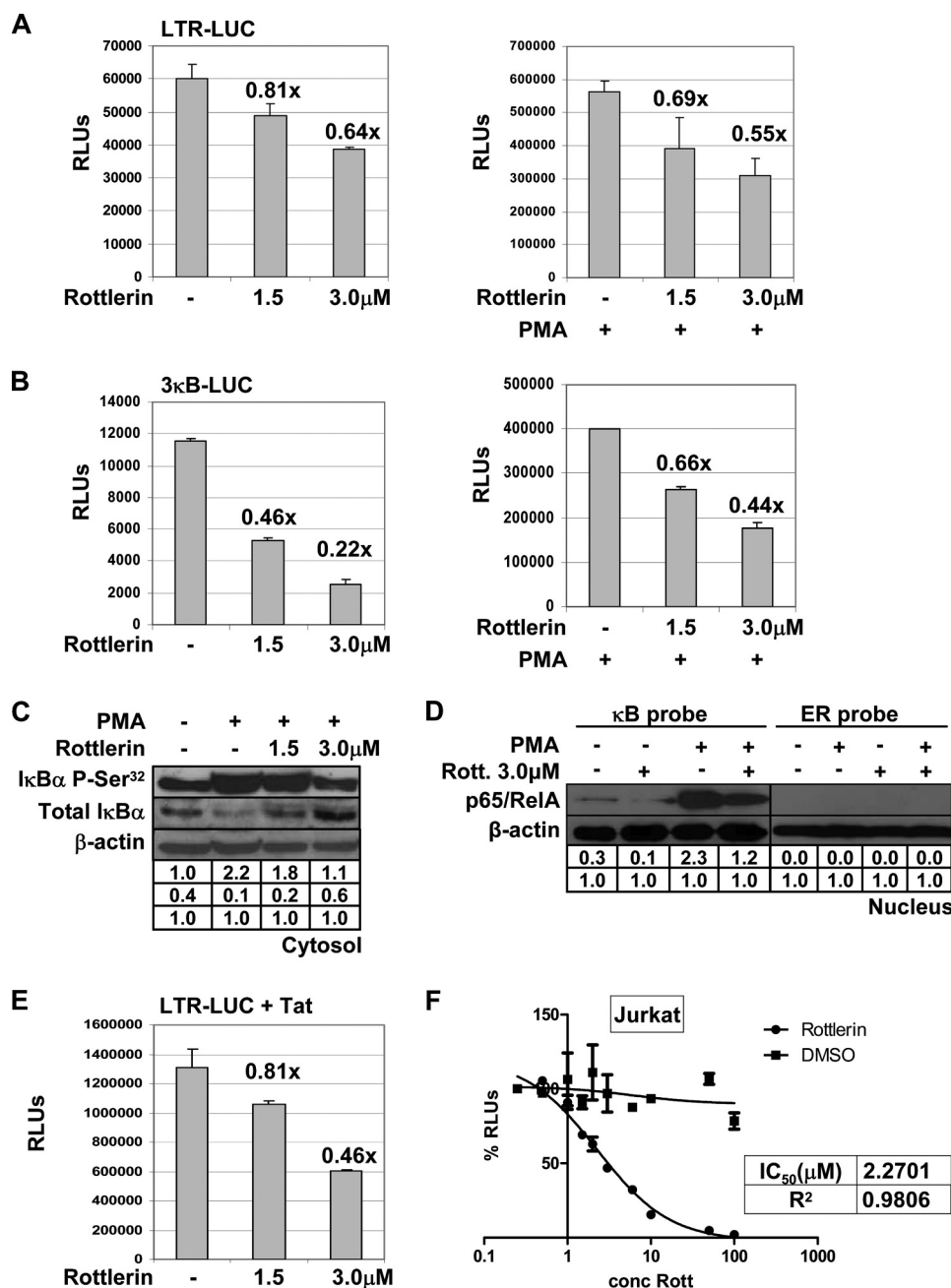
To analyze the effect of rottlerin on the viral reverse transcription, a semiquantitative RT-PCR assay was performed to amplify HIV-1 strong stop (R/U5) and full-length (LTR/gag) reverse transcriptase transcripts, which constitute early and late transcripts, respectively. Rottlerin at the highest concentration assayed (3.0  $\mu$ M) did not modified the amount of R/U5 and LTR/gag products obtained from MT-2 infected by spinoculation with NL4.3wt clone for 18 h, suggesting that rottlerin did not interfere with reverse transcription (Fig. 5*A*). HIV-1 integration was analyzed in the presence of rottlerin through quantification of proviral load in genomic DNA extracted from MT-2 cells infected by spinoculation with NL4.3wt clone for 18 h. A first-round Alu-PCR followed by a nested PCR with internal primers targeted at the LTR was performed. Ribosomal RNA 18 S was used to normalize the amount of integrated provirus. As a positive control, genomic DNA from 8E5 cells, which contain a single integrated copy of HIV-1, was used (45). As shown in Fig. 5*B*, HIV-1 proviral integration was significantly thwarted by rottlerin at the highest concentration tested (3.0  $\mu$ M).

**Effect of Rottlerin on LTR-dependent Transcriptional Activity—**The inhibitory effect of rottlerin on HIV-1 replication was analyzed at transcriptional levels in Jurkat cells by transfecting the plasmid LTR-LUC containing the complete HIV-1 LTR sequence upstream of the luciferase reporter gene. Transfected cells were treated with rottlerin for 30 min immediately after transfection and then activated or not with PMA for 18 h. Rottlerin induced a dose-dependent inhibition of HIV-1 LTR transactivation in basal conditions and upon activation with PMA (Fig. 6*A*). The upstream LTR promoter contains two binding sites for NF- $\kappa$ B, which is a major effector at the end of PKC $\theta$  pathway that can be activated with PMA. To further analyze whether the inhibition of HIV-1 replication and LTR-de-

pendent transactivation mediated by rottlerin was related to the inhibition of PKC $\theta$ -dependent NF- $\kappa$ B activity, Jurkat cells were transiently transfected with 3 $\kappa$ B-LUC expression vector and pretreated with rottlerin for 30 min immediately after transfection, and then PMA was added to the culture medium. Cells were collected after 18 h, and luciferase activity was measured. NF- $\kappa$ B-dependent luciferase expression diminished as rottlerin concentration increased, and this effect was observed with and without PMA (Fig. 6*B*). The inhibition of NF- $\kappa$ B activity mediated by rottlerin was further characterized, and we observed that both phosphorylation of I $\kappa$ B $\alpha$  at Ser<sup>32</sup> and I $\kappa$ B $\alpha$  degradation were repressed by rottlerin (Fig. 6*C*). This compound also induced 2-fold inhibition of both basal and PMA-dependent NF- $\kappa$ B nuclear binding activity (Fig. 6*D*). The LTR promoter also contains binding sites for the transcriptional factors NF-AT and Sp1, but rottlerin did not significantly decrease the activity of these factors (data not shown). The effect of rottlerin on Tat-induced LTR transactivation was also analyzed by co-transfection of LTR-LUC vector and the HIV-1 Tat expression vector CMV-Tat. Measurement of luciferase activity in cell lysates showed that rottlerin strongly inhibited Tat-mediated HIV-1 transactivation (Fig. 6*E*).

To further support the role of rottlerin in the inhibition of viral replication at the transcriptional level, Jurkat cells were transfected with the pNL4.3-Renilla vector that only requires the transcription step to express Renilla. Treatment of Jurkat with rottlerin immediately after transfection produced a strong dose-dependent inhibition of Renilla activity (Fig. 6*F*), with the lowest IC<sub>50</sub> observed for inhibiting HIV-1 replication (2.2  $\mu$ M).

**Generation of Jurkat and MT-2 Cells with Stable Interference of mRNA for PKC $\theta$ —**To prove that the inhibition of PKC $\theta$  was the major factor involved in rottlerin-dependent repression of HIV-1 replication, Jurkat and MT-2 cells with stable interference for PKC $\theta$  were generated. For this purpose, Jurkat and MT2 cells were co-transfected with two shRNA plasmids (pGeneClip-iPKC $\theta$ -1 and pGeneClip-iPKC $\theta$ -3) containing two different siRNA sequences directed against mRNA encoding for PKC $\theta$ . Vector pGeneClip-iPKC $\theta$ -C1 containing a scrambled siRNA sequence was used as negative control. PKC $\theta$  mRNA interference was analyzed by semiquantitative RT-PCR assay using  $\beta$ -actin as housekeeping gene (Fig. 7*A*), and more than 60–70% of stable RNA interference was observed in both Jurkat and MT-2. Specificity of the amplification reaction was determined by sequencing the amplified fragments. Synthesis of PKC $\theta$  protein was down-regulated in both Jurkat-iPKC $\theta$ -1,3 and MT2-iPKC $\theta$ -1,3 but not in the control cell lines Jurkat-iPKC $\theta$ -C1 and MT2-iPKC $\theta$ -C1 (Fig. 7*B*). Cell viability of Jurkat-iPKC $\theta$ -1,3 and MT2-iPKC $\theta$ -1,3 was not significantly reduced regarding to control cells C1 (Fig. 7*C*), but more than 2-fold decrease in NF- $\kappa$ B activity was observed in these cells (Fig. 7*D*). PHA-induced proliferation of Jurkat-iPKC $\theta$ -1,3 was not notably different from Jurkat-iPKC $\theta$ -C1 (data not shown). The ability of HIV-1 to infect and replicate in MT2-iPKC $\theta$ -1,3 and MT2-iPKC $\theta$ -C1 cells was analyzed by infection with NL4.3-Renilla clone. The effect of stable PKC $\theta$  interference on HIV-1 transcription was analyzed in Jurkat-iPKC $\theta$ -1,3 and Jurkat-iPKC $\theta$ -C1 cells by direct transfection of pNL4.3-Renilla vector. Cells were incubated for 3–4 days, and synthesis of



**FIGURE 6. Analysis of rottlerin-mediated modifications in LTR-dependent transcriptional activity.** *A* and *B*, Jurkat cells were transfected with LTR-LUC (*A*) or 3κB-LUC (*B*) expression vectors and treated with rottlerin or/and PMA immediately after transfection. After 18 h of incubation, Renilla activity was measured. RLUs were normalized according to protein concentration in each lysate and to β-galactosidase activity from pSV-β-galactosidase vector used as control of transfection efficiency. -fold mean of RLUs corresponding to three independent experiments is represented as a bar diagram. Lines on the top of the bars correspond to S.D. Numbers on the top of the bars correspond to -fold reduction regarding to the basal line. *C* and *D*, cytosolic protein extracts from Jurkat cells treated with rottlerin and/or PMA for 4 h were analyzed by immunoblotting using antibodies against total IκBα and IκBα phosphorylated at Ser<sup>32</sup> (*C*). Nuclear protein extracts from the same cells were analyzed by DNA affinity immunoblotting assay using a specific probe containing two κB consensus sites labeled with biotin. Protein-DNA complexes were captured with streptavidin-agarose resins and analyzed by immunoblotting with an antibody against p65/RelA. Non-related estrogen receptor (*ER*) DNA element probe was used as control of specificity (*D*). β-Actin was used as internal loading control. Gel bands were quantified by densitometry. *ER*, estrogen receptor. *E*, Jurkat cells were co-transfected with LTR-LUC and CMV-Tat expression vectors (2:1) and incubated 18 h with or without rottlerin. Renilla activity was measured, and RLUs were normalized according to protein concentration and β-galactosidase activity. *F*, Jurkat cells were transfected with pNL4.3-Renilla vector and incubated for 5 days with increasing concentrations of rottlerin. IC<sub>50</sub> was calculated using GraphPad Prism Software (sigmoidal dose-response formula). All measurements were done in triplicate, and the mean is represented with S.D.

Renilla was measured in cell lysates. HIV-1 replication was 4-fold reduced in MT2-iPKC $\theta$ -1,3 and more than 3-fold reduced in Jurkat-iPKC $\theta$ -1,3 in comparison with their control cells C1 (Fig. 7*E*). Measurement of Renilla correlated to p24 antigen production in both cases (Fig. 7*F*).

*Impairment of HIV-1 Infection in Human PBLs by Transient Interference of mRNA Encoding for PKC $\theta$* —Jurkat and resting PBLs were transiently co-transfected with pNL4.3-Renilla and both plasmids pGeneClip-iPKC $\theta$ -1 and pGeneClip-iPKC $\theta$ -3 or with empty plasmid pGeneClip or pGeneClip-iPKC $\theta$ -C1 as

## Role of PKC $\theta$ in HIV-1 Replication

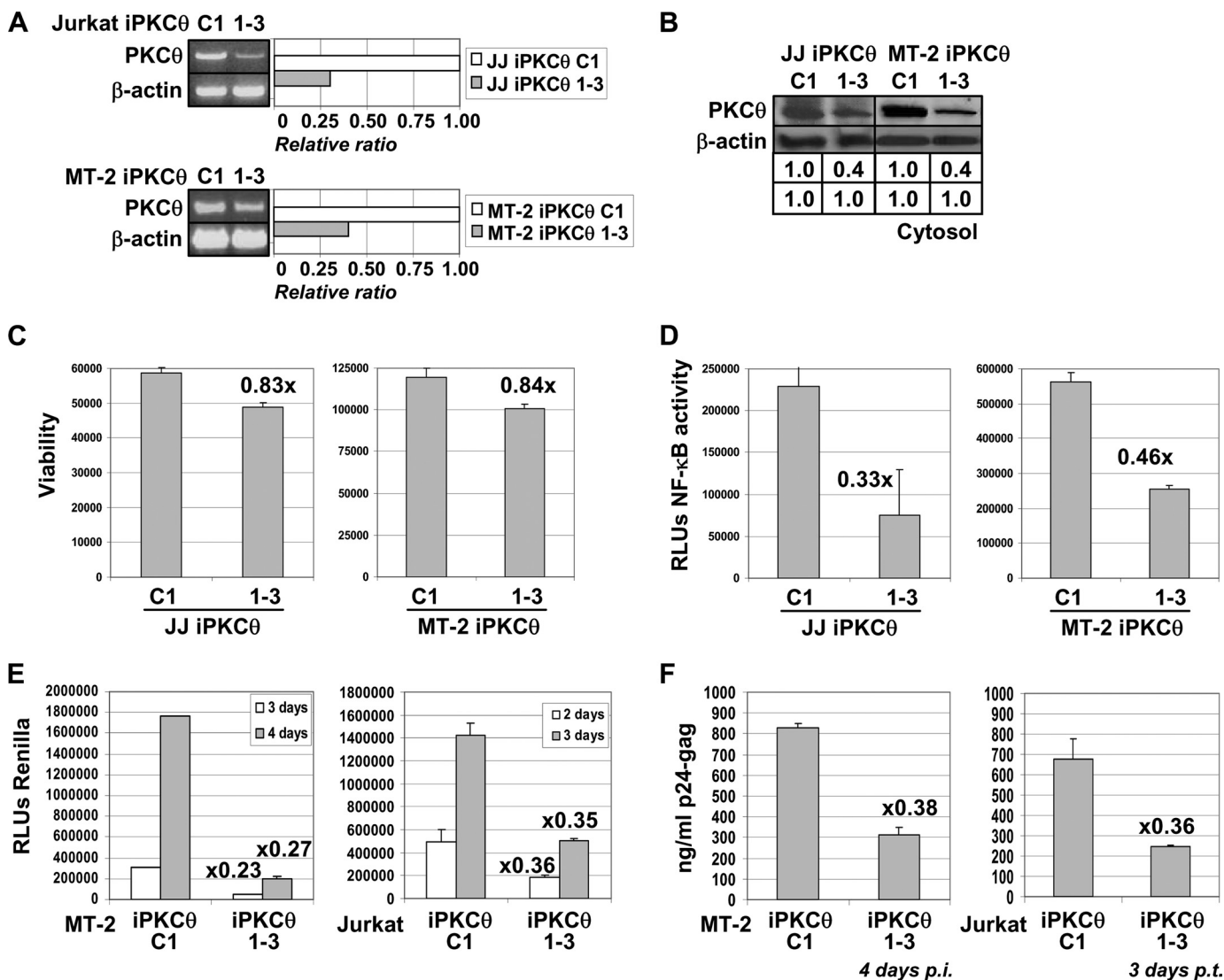


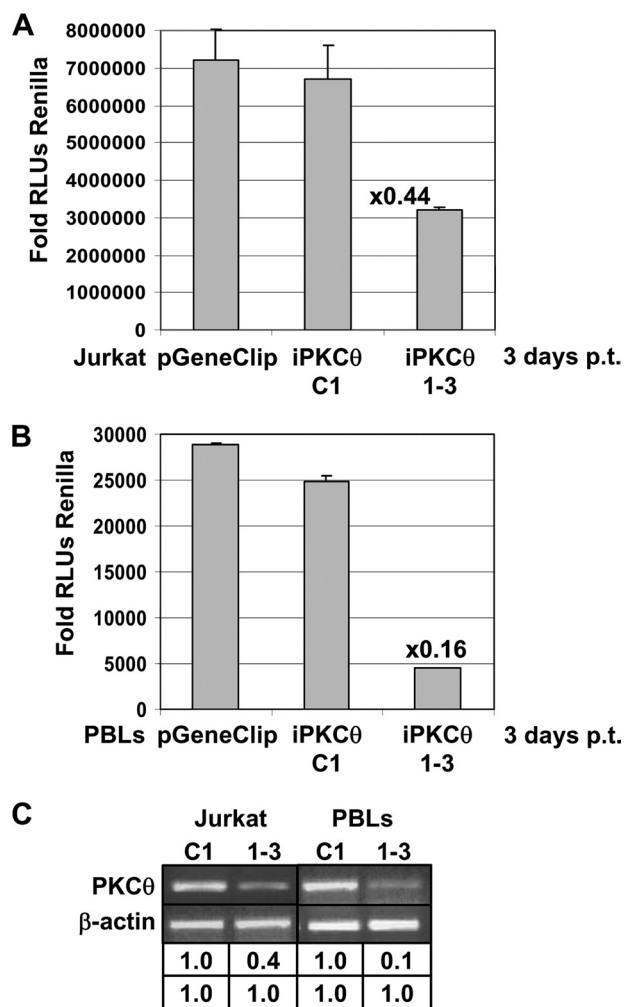
FIGURE 7. HIV-1 replication is hindered in Jurkat and MT-2 cells with stable interference of mRNA encoding for PKC $\theta$ . *A*, shown is an analysis of stable interference of mRNA for PKC $\theta$  in Jurkat-iPKC $\theta$ -1,3 and MT-2-iPKC $\theta$ -1,3 cell lines by semiquantitative RT-PCR assay using  $\beta$ -actin as the housekeeping gene in comparison with control cell lines, Jurkat-iPKC $\theta$ -C1, and MT-2-iPKC $\theta$ -C1. Gel bands were quantified by densitometry. *B*, cytosolic expression of PKC $\theta$  protein was analyzed in Jurkat-iPKC $\theta$ -1,3 and MT-2-iPKC $\theta$ -1,3 cell lines by immunoblotting using an antibody against total PKC $\theta$ .  $\beta$ -Actin was used as protein loading control. *C*, cell viability in Jurkat-iPKC $\theta$ -1,3 and MT-2-iPKC $\theta$ -1,3 cell lines was analyzed by quantifying the levels of ATP in living cells. *D*, NF- $\kappa$ B activity in Jurkat-iPKC $\theta$ -1,3 and MT-2-iPKC $\theta$ -1,3 cell lines was measured by transient transfection of 3 $\kappa$ B-LUC expression vector in the absence of stimulation. *E*, MT-2-iPKC $\theta$ -1,3 and MT-2-iPKC $\theta$ -C1 cell lines were infected with NL4.3-Renilla clone, and Jurkat-iPKC $\theta$ -1,3 and Jurkat-iPKC $\theta$ -C1 cell lines were transfected with pNL4.3-Renilla proviral clone. Renilla activity was measured 2–4 days post-infection (*p.i.*). *F*, production of p24 antigen in culture supernatants from the former experiment was measured by ELISA to determine the correlation with the synthesis of Renilla. In all cases, data corresponding to three independent experiments are represented as bar diagrams. Lines on the top of the bars correspond to S.D., and numbers on the top of the bars correspond to the reduction-fold relative to the basal line. *p.t.*, post transfection.

negative controls. Transfected Jurkat cells were incubated in the absence of stimulus. Transfected PBLs were activated with PHA and maintained for 3 days in culture. Measurement of Renilla showed that HIV-1 replication was more than 2-fold reduced in Jurkat cells (Fig. 8*A*) and more than 6-fold reduced in PBLs (Fig. 8*B*) due to PKC $\theta$  interference. Efficiency of PKC $\theta$  mRNA interference was assessed by semiquantitative RT-PCR assay with RNA obtained 3 days after transfection (Fig. 8*C*).  $\beta$ -Actin was used as a housekeeping gene.

### DISCUSSION

HIV-1 infection is characterized by a continuous viral replication that persists even in patients on HAART due to the exist-

ence of latent viral reservoirs. Accordingly, current therapeutic regimens are not fully suppressive, and viral escape occurs at a cellular and anatomical level. New strategies should be developed, intended not only for the elimination of transcriptionally silent reservoirs but also for the complete annulment of viral replication to avoid ongoing replication and continuous feeding of reservoirs. During primary stages of HIV-1 infection, there is a massive infection of activated memory CD4<sup>+</sup> T lymphocytes in the gut-associated lymphoid tissue (GALT) that leads to a sustained HIV-1 replication (26). Accordingly, the lower the pool of activated CD4<sup>+</sup> T cells, the lower the levels of virus production. Because HIV-1 infects both resting and activated CD4<sup>+</sup> T lymphocytes but only replicates in activated cells



**FIGURE 8. HIV-1 replication is impaired in human PBLs with transient interference of mRNA encoding for PKC $\theta$ .** *A* and *B*, Jurkat cells (*A*) and resting PBLs from healthy donors (*B*) were transiently co-transfected with pGeneClip-iPKC $\theta$ -1, pGeneClip-iPKC $\theta$ -3, and pNL4.3-Renilla vectors (2:2:1). pGeneClip-iPKC $\theta$ -C1 and pGeneClip vectors were used as negative controls. Renilla was measured in cell lysates 3 days after transfection. RLU<sub>s</sub> were normalized according to protein concentration in each lysate and to  $\beta$ -galactosidase activity from pSV- $\beta$ -galactosidase vector used as control of transfection efficiency. Data corresponding to three independent experiments are represented as bar diagrams. Lines on the top of the bars correspond to S.D., and numbers on the top of the bars correspond to the reduction fold regarding to the basal line. *C*, efficiency of transient mRNA interference for PKC $\theta$  was assessed in RNA extracted from Jurkat and PBLs from the former experiment 3 days after transfection by semiquantitative RT-PCR assay.  $\beta$ -Actin was used as housekeeping gene. Gel bands from immunoblotting assays were quantified by densitometry. *p.t.*, post-transfection.

(20), two potential strategies could be envisaged. First, the clinical use of PKC activators as adjuvant for HAART, such as prostratin (21, 22) and the jatrophone diterpene SJ23B (24), has been proposed for reducing the size of latent reservoirs. Second, the use of PKC inhibitors could be considered as an alternative adjuvant mechanism to decrease T cell activation and, therefore, to control viral replication. We observed that rottlerin, a dose-dependent PKC $\theta$  inhibitor, was able to inhibit HIV-1 replication in activated T cells. Rottlerin-mediated inhibition of HIV-1 replication was mostly dependent on PKC $\theta$  inhibition but not on the closely related PKC $\delta$  or PKC $\zeta$ . Interestingly, PKC $\theta$  is selectively expressed in the main viral producers, CD4<sup>+</sup> T lymphocytes (5, 41), where this PKC is phosphorylated

and translocated from the cytoplasm to the immunologic synapse during T cell activation to initiate signaling pathways that end in the main effector NF- $\kappa$ B (52). Rottlerin was able to reduce the phosphorylation of PKC $\theta$  at Thr<sup>538</sup> and its translocation to the lipid rafts on the plasma membrane, which are events essential for the kinase activity. We proved that rottlerin-mediated inhibition of HIV-1 replication was highly related to the inhibition of PKC $\theta$  activity, as the stable interference of mRNA for PKC $\theta$  in human T cells produced that these cells were highly refractory to HIV-1 infection. Although there are murine cells from PKC $\theta$ <sup>-/-</sup> mice previously described that could be used for these experiments (53), we decided to generate human T cell lines with stable interference for PKC $\theta$  mRNA to get a better model for HIV-1 infection.

One major consequence of PKC $\theta$  inhibition by rottlerin was the decrease of NF- $\kappa$ B activity, necessary for HIV-1 replication in T cells (12). However, NF- $\kappa$ B activity was only partially reduced and, although both NF-AT and Sp1 were also moderately diminished, an alternative mechanism should trigger the rottlerin-mediated inhibition of HIV-1 replication. Several experiments proved that rottlerin was not an inhibitor of viral entry, and consequently, this compound was able to inhibit the infection of both CXCR4- and CCR5-dependent viral clones. However, rottlerin caused a significant decrease in Tat-mediated LTR transactivation and proviral integration, which would explain the delay in viral replication. This last effect could be related to the fact that this compound is a cytostatic agent that induces a G<sub>1</sub>-S phase arrest of cell cycle (33), causing accumulation of cells in G<sub>0</sub>-G<sub>1</sub> and delaying DNA replication and cell growth (54). Consequently, PKC $\theta$ -dependent T cell proliferation was also thwarted in PBLs treated with rottlerin. In fact, PKC $\theta$ <sup>-/-</sup> murine T cells show a defective IL-2 production that is associated with a stable state of anergy (53). This unresponsive state could be reversed by exogenous IL-2 (55), but intriguingly, rottlerin-dependent inhibition of T cell proliferation could not be overcome by the addition of IL-2 (33), proving that rottlerin should modify other signaling pathways involved in T cell growth. This anergy-like state would be responsible for the inhibition of proviral integration and replication, as occur in human resting T cells that may be infected by HIV-1, but viral replication does not occur until T cell activation is produced (25, 56).

As most cytostatic agents are also able to induce massive apoptosis, one major concern was whether HIV-1 replication was restrained in rottlerin-treated T cells because it reduced cell viability. In fact, PKC $\theta$  displays opposing effects in T cell apoptotic mechanisms, and their balance determines the final fate of the cell. When rottlerin is used at  $\geq 10$   $\mu$ M, it causes unspecific blockade of several PKCs as well as the inhibition of CD3/CD28 signaling pathway, which culminates in Fas-mediated cell death (10, 57–60). However, doses  $< 6.0$   $\mu$ M proved to be highly specific for PKC $\theta$  inhibition and kept the viability of PBLs and Jurkat mostly at basal levels. Furthermore, MT-2 cells were surprisingly resilient to rottlerin-induced apoptosis ( $> 100$   $\mu$ M), probably due to the concomitant infection with HTLV-1, which appears to suppress apoptosis as a mechanism involved in the immortalization of the infected lymphocytes (61, 62). In fact, although MT-2 cells are usually destroyed by

## Role of PKC $\theta$ in HIV-1 Replication

cytopathic effect as HIV-1 infection progresses, rottlerin was able to increase MT-2 viability because HIV-induced cytopathic effect was not produced.

The use of cytostatic agents, such as cyclosporin A, hydroxyurea, mycophenolic acid, and its morpholinoethyl ester derivate mycophenolate mophetil, has been proposed in HAART regimens during HIV-1 primary infection and structured treatment interruption to further suppress HIV-1 replication by limiting T cell activation through the inhibition of proliferation and differentiation (63). In this context, the use of specific PKC $\theta$  inhibitors would be useful to avoid massive viral replication from activated CD4<sup>+</sup> T cells and to reduce the size of reservoirs from the beginning of the infection. Because PKC $\theta$  is selectively expressed in T cells, targeting specifically PKC $\theta$  will limit the immunosuppressive effect to the main targets for HIV-1 infection. Among the benefits of initiating HAART during HIV-1 primary infection would be the successful suppression of virus replication and spreading, restoration of CD4<sup>+</sup> T cell counts, and long term containment of plasma viral load (21, 64–71).

Rottlerin is a powerful, selective PKC $\theta$  inhibitor in which activity is very constant in long term culture, as its inhibitory effect is sustained from the beginning of the infection. This compound is only intended for research studies and not for clinical assays, as it has a narrow therapeutic range. Accordingly, with this study we did not try to evaluate the ability of rottlerin to inhibit HIV-1 replication as a compound that could be used as an effective inhibitor of this viral infection. Instead, this compound was used to determine the importance of PKC $\theta$  in HIV-1 infection and replication and to evaluate the use of this essential kinase as a potential target to inhibit HIV-1 replication in T cells. In this context, data obtained with rottlerin were reinforced by using specific interference of mRNA for PKC $\theta$ , obtaining similar results as those achieved with rottlerin. Besides, the inhibitory activity of rottlerin on HIV-1 infection was also analyzed in PKC $\theta$  non-expressing cells. For this purpose, the absence of expression of mRNA encoding for PKC $\theta$  in the human astrocytoma U87 cell line was assessed by RTPCR. U87 cells that stably express the CD4 receptor (U87.CD4) and the chemokine receptor CXCR4 (U87.CD4.CXCR4) were then infected with the recombinant NL4.3-Renilla. It was observed that rottlerin was not able to inhibit HIV-1 infection in these cells at a concentration within the range of low cytotoxicity (see Fig. 2 in [supplemental data](#)).

Several laboratories have developed molecules targeted as selective inhibitors of PKC $\theta$  (72, 73). These PKC $\theta$  inhibitors are designed for the treatment of inflammatory diseases as arthritis, asthma, multiple sclerosis, and colitis (74). No specific PKC $\theta$  inhibitor has entered the clinic yet, although some 4-indolylamino-5-phenyl-3-pyridinecarbonitrile analogs met the necessary criteria for advancement into *in vivo* efficacy studies (75). Most of these compounds were selective for PKC $\theta$  over PKC $\delta$ . There are also generic PKC inhibitors in clinical assays as sotrastaurin/AEB071 (Novartis), an inhibitor of classical and novel PKCs that is currently in phase II trials for preventing renal transplant rejection and for the treatment of psoriasis (76–78). It would be interesting to analyze the efficacy of these

new selective PKC $\theta$  inhibitors for preventing HIV-1 replication in human T cells, comparing its effectivity with rottlerin.

In conclusion, the use of cytostatic drugs in combination with HAART would maintain T lymphocytes quiescent and, therefore, refractory to productive HIV-1 infection. This strategy could be applied selectively in patients during HIV-1 primary infection that started HAART with high levels of CD4<sup>+</sup> T cells and plasma viral load to avoid high peaks of viral replication that would spread the infection and establish new reservoirs. Because of the potential risk of lymphoproliferative disorders, such agents could not be likely used in long term treatment. However, the restricted expression and essential function of PKC $\theta$  in T cell activation suggest that cytostatic agents able to selectively block PKC $\theta$  in T cells may be therapeutically useful to thwart integration and both Tat- and NF- $\kappa$ B-dependent replication of HIV-1 without causing a general immunosuppression.

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