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Differential effects of phorbol-13-monoesters on human immunodeficiency virus reactivation

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ARTICLE INFO

Article history:
Received 13 November 2007
Accepted 12 December 2007

Keywords:
HIV-1
Latency
Phorbol ester
Prostratin
PKC
NF-κB

ABSTRACT

The persistence of latent reservoirs of HIV-1 represents a major barrier to virus eradication in patients treated with antiretrovirals. Prostratin is a non-tumor promoting 12-deoxyphorbol monoester capable of up-regulating viral expression from latent provirus and therefore is potentially useful for HIV adjuvant therapy and similar properties might be elicited by related non-tumor promoting phorboids. We have therefore investigated a series of phorbol 13-monoesters for their capacity to reactivate HIV latency. Using a Jurkat T cell line containing latent HIV proviruses, we found that prostratin and phorbol-13-stearate effectively activate HIV-1 gene expression in these latently infected cells, with phorbol-13-stearate being at least 10-fold more potent than prostratin, and its activity rapidly decreasing with a shortening of the acyl side chain. We further demonstrated that phorbol-13-stearate and prostratin stimulate IKK-dependent phosphorylation and degradation of IκBα, leading to activation of NF-κB. Moreover, prostratin, phorbol-13-hexanoate and phorbol-13-stearate also activate the JNK and ERK pathways. Studies with isoform-specific PKC inhibitors suggest that the classical PKCs play a prominent role in the responses elicited by phorbol-13-stearate. Nevertheless, this compound induces a translocation pattern of the PKC isotypes α and δ to cellular compartments distinctly different from that elicited by prostratin and PMA.

1. Introduction

HIV infects several cell types during the course of infection and progression to acquired immune deficiency syndrome (AIDS). The persistence of latent HIV-infected cellular reservoirs represents the major hurdle to virus eradication with highly active anti-retroviral therapy (HAART), since latently infected cells remain a permanent source of viral reactivation [1]. As a result, a sudden rebound of the virus load after interruption of HAART is generally observed [2–4]. The HIV-1

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Abbreviations: AP-1, activator protein-1; ERK, extracellular regulated kinase; HIV-LTR, HIV long terminal repeats; IκB, κB inhibitor; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; NF-κB, nuclear factor *kappa* B; PMA, phorbol 12-myristate 13-acetate; P-13S, phorbol-13-stearate; TNFα, tumor necrosis factor-α.

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doi:10.1016/j.bcp.2007.12.004

establishes a persistent infection in $CD4^+$ T lymphocytes creating a persistent reservoir consisting mainly of latently infected resting memory $CD4^+$ T cells carrying an integrated provirus that is transcriptionally silent [5,6]. The extremely long half-life of these cells, combined with a tight control of HIV-1 expression, has been reported to make this reservoir ideally suited to maintain hidden copies of the virus [6].

The current therapies directed against viral proteins (HAART) have been problematic because of long-term toxicity, inhibitor resistance, and the inability to target persistent reservoirs. Therefore, it has been suggested that reactivation of the latent reservoirs could allow effective targeting and possible eradication of the virus [7]. Immunoactivation therapy to reduce the latent pool of HIV by treatment with the anti-CD3 antibody OKT-3 alone or in combination with interleukin-2, substantially failed to significantly decrease the viral reservoir [8].

Nevertheless, a host of small molecules including phorbol esters [9], ingenols [10] and 1,2-diacylglycerol analogs [11], has been suggested as agents to reactivate HIV and eradicate the pool of latently HIV-infected $CD4^+$ T cells. More recently, non-tumor-promoting phorbol deoxyphorbol esters such as prostratin and 12-deoxyphorbol 13-phenylacetate have been directly evaluated for their ability to reactivate latent virus both in latently infected cell lines and in primary memory T cells from HIV infected patients [9,12]. Prostratin was isolated for the first time from the poisonous New Zealand plant *Pimela prostrata* [13] and was later identified as the anti-viral constituent of the Samoan plant *Homalanthus nutans* [14]. Prostratin not only reactivates HIV-1 latency in ''vitro'' by protein kinase C (PKC)-dependent NF-κB activation [14,15], but also down-regulates the expression of the HIV-1 receptor CD4 and the co-receptor CXCR4, thus avoiding the new infection of $CD4^+$ cells [9,16].

The PKC family of serine/threonine kinases plays a central role in mediating the signal transduction of extracellular stimuli, which result in the production of the second messenger 1,2-diacyl-*sn*-glycerol (DAG). PKC is also the primary target of the phorbol ester tumor promoters and consists of a family of 12 members that are classified into three major subfamilies. The classical PKCs (α, β_I, β_{II} and γ) are Ca^{2+}- and DAG-dependent, whereas the novel PKCs (δ, ε, η and θ) are Ca^{2+}-independent but DAG-responsive. The atypical PKCs (ζ and λ/ι) lack the responses to both Ca^{2+} and DAG [17]. A highly conserved cysteine-rich motif (C1 domain) in the regulatory region of the PKCs acts as the specific receptor for DAG and phorbol esters [18,19]. The C1 domain displays a hydrophobic surface interrupted by a hydrophilic cleft. By sneaking into the hydrophilic cleft, phorbol esters and DAG provide a hydrophobic cap on the hydrophilic cleft, masking its polarity and facilitating the association of the C1 domain with the lipid bilayer and other hydrophobic surfaces [20]. However, phorbol derivatives with different lypophilicities exhibit different biological activities and potencies [21].

The translocation of PKCs from cytoplasm to plasma membrane and other subcellular localizations is the hallmark for PKC activation and several studies have shown that the subcellular translocation of PKC is isoform-, cell type-, and activator-specific, and, for phorbol 12, 13-diesters, is tightly regulated by lipophilicity [21,22]. Therefore, distinct patterns of localization of PKC, and therefore of access to substrates, might underlie distinct patterns of biological response to a given PKC ligand.

We report here the synthesis and biological evaluation of a homologous series of phorbol 13-monoesters, a type of compounds that have been so far largely overlooked in terms of bioactivity. The length of the fatty acid side chain of these compounds is critical to induce HIV-1 reactivation in a cellular model of HIV-1 latency.

2. Materials and methods

2.1. Cell lines and reagents

Jurkat T leukemia cells were grown at 37 °C and 5% CO_2 in supplemented RPMI 1640 medium (Cambrex Co., Barcelona, Spain), containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The CHO-K1 cells (CCL 61) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in complete DMEM. The anti-IκBα mAb 10B was a gift from Hay (St. Andrews, Scotland), the mAb anti-tubulin and the anti-ERK 1+2 antibody were purchased from Sigma Co. (St. Louis, MO, USA). The anti phospho-ERK 1 + 2 (sc-7383) and the anti-IKKγ (FL-419) were from Santa Cruz Biotechnology (CA, USA). The antibody anti-phospho-JNK (9255S) was from New England Biolabs (Hitchin, UK). The anti-p50 and anti-p65 antisera were a gift from Dr. Alain Israël (Institute Pasteur). The antibodies anti-PKCδ and anti phospho- PKCδ (Thr505) were from Cell Signaling Technology (Danvers, MA). The mAb anti-CD25 antibody (clone ACT-1, PE-labeled) was from Dako (Glostrap, Denmark). The mAbs anti-CD69 (clone FN50, FITC-labeled) and anti-CXCR4 (clone 12G5, PE-labeled) were from BD Biosciences Pharmigen (San Diego, CA, USA). The mAb anti-CD4 (clone 6D10, FITC-labeled) was from ImmunoTools (Friesoythe, DE). Dual-Color Reagent Mouse IgG1/FITC + Mouse IgG1/PE from DAKO (clone DAK-GO1 directed towards *Aspergillus niger* glucose oxidase) was used as negative control. The inhibitors Rottlerin and PD 98059 were obtained from Alexis Co. (Lausen, Switzerland) and the inhibitors Gö6983, Gö6976 and Gö6850 were from Calbiochem (EMD Biosciences, Inc. Darmstadt, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) was from MP Biomedicals (Irvine, CA, USA). Prostratin was obtained from Alexis Co. and all other reagents were from Sigma.

2.2. Plasmids

The plasmid pEV731 (pHR′-tat2x.flag-IRES-eGFP) was obtained from Dr. E. Verdin (Gladstone Institute of Virology, UCSF, CA, USA) [23]. The pEGFP-N1 derived plasmids PKCα-GFP and PKCδ-GFP were obtained from Dr. P.M. Blumberg (NCI, MD, USA). The GST-IκB$\alpha_{(1–54)}$ plasmid has been described elsewhere [24].

2.3. Synthesis of 12-hydroxy-13 phorbol monoesters

The synthesis of 1b–h is outlined in Fig. 2. Aliphatic phorbol 13-esters were prepared by treatment of phorbol (2) with an excess (5–10 molar equivalents) of acylating agent (Anhy-

drides for the synthesis of 1b, RCOOH/DCC/DMAP for the synthesis of 1c–1g) in a suitable solvent (pyridine for Ac_2O, CH_2Cl_2 for all other cases). The resulting 13,20-diesters were purified by gravity column chromatography on neutral alumina to remove unreacted acids and next transesterified by treatment with methanolic perchloric acid (pH 3). After neutralization (NaOAc), filtration and evaporation, the crude reaction mixture was purified by gravity column chromatography on silica gel (*ca.* 15 g/g of mixture) using ethyl acetate–petroleum ether mixtures, to afford the 13-monoesters in overall yield of 24–68% from phorbol. The 13-benzoate 1h could not be prepared this way, since transesterification of the allylic ester group was very sluggish, presumably because of the decrease reactivity of the ester carbonyl toward nucleophilic addition. Compound 1h could be directly prepared from phorbol by esterification with benzoic acid in the presence of DCC (2 molar equivalents each). Without DMAP, acylation occurred chemoselectively at the C-13 hydroxyl. The crude product was purified from unreacted phorbol and benzoic acid by gravity column chromatography on silica gel (petroleum ether–ethyl acetate 1:1 as eluant) to afford 1h in 25% yield. All final compounds were fully characterized spectroscopically (^1H and ^{13}C NMR, MS), and showed purity >95% by HPLC.

2.4. Generation of Jurkat-LAT-GFP cells

For the production of viral particles containing an HIV-derived vector, 5×10^5 293T cells were transfected with plasmids pEV731 (10 μg), pCMV-R8.91 (6.5 μg) and $pcDNA_3$-VSV (3.5 μg) in 10 cm dishes. After 16 h, medium was replaced, supernatants containing viral particles were harvested 24 h later and viral particles containing 150 ng of p24 were used to infect 10^6 Jurkat cells. After 96 h, the efficiency of the infection process was monitored by FACS analysis and the negative population was sorted (FACSCvantage SE, BD Bioscience) and cultured again in completed medium. Then the sorted cells were stimulated with TNFα for 24 h and then the GFP^+ population was analysed (Cell Quest-Pro software), sorted and cloned by limit dilution in 96 well plates. After 3 weeks the clones were stimulated with PMA (50 ng/ml) to induce the expression of the integrated LTR-GFP vector for 24 h and 4 out of 72 clones were selected for characterization. The percentage of GFP^+ cells was analysed by flow cytometry in an EPIC XL flow cytometer (Beckman-Coulter Inc. CA, USA). Ten thousand gated events were collected per sample. Finally, clon 8 was selected for further experiments and renamed Jurkat-LAT-GFP cells.

2.5. IKK kinase assay

Cells were lysed in NP-40 lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 mM NaF, 0.5 mM sodium vanadate, leupeptine (10 μg/ml), aprotinin (10 μg/ml), 1% (v/v) NP-40 and 10% (v/v) glycerol) for 15 min at 4 °C, and after centrifugation for 10 min at 13000 rpm, the supernatant was incubated with non-specific IgG and 25 μl protein A/G sepharose (preclearing) and incubated overnight on a spinning wheel. After centrifugation, the supernatants were incubated with 2 μg of anti-IKK-γ antibody and 25 μl protein A/G sepharose and incubated for 2–4 h on a spinning wheel at 4 °C. The precipitate was washed three times in cold lysis buffer and three times in cold kinase buffer (20 mM Hepes/KOH pH 7.4, 25 mM ß-glycerophosphate, 2 mM DTT, 20 mM $MgCl_2$). The kinase assay was performed in a final volume of 20 μl kinase buffer containing 40 μM ATP and 2 μg of the purified substrate protein GST-IκBα$_{(1–54)}$. After incubation for 20 min at 30 °C, the reaction was stopped by the addition of 5× SDS loading buffer. After separation by SDS-PAGE the proteins were transferred to nitrocellulose membranes and IκBα phosphorylation was detected by Western blot using the antibody anti-phospho-IκBα 5A5 (Cell Signaling Technologies).

2.6. Western blots

Jurkat-LAT-GFP cells (10^6 cells/ml) were stimulated with the indicated compounds. Cells were then washed with PBS and proteins extracted in 50 μl of lysis buffer (20 mM Hepes pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaF, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA) and 30 μg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare).

2.7. Isolation of nuclear extracts and mobility shift assays

Jurkat-LAT-GFP cells (10^6/ml) were stimulated with the agonists in complete medium as indicated. Cells were then washed twice with cold PBS and proteins from nuclear extracts isolated as previously described [24]. Protein concentration was determined by the Bradford method (Bio-Rad). For the electrophoretic mobility shift assay (EMSA), the consensus oligonucleotide probes NF-κB, 5′-AGTTGAGGGGACTTTCCCAGG-3′ and the commercial AP-1 site (Promega Biotech Ibérica, ES) were end-labelled with [γ-^{32}P]ATP. The binding reaction mixture contained 3 μg of nuclear extract, 0.5 μg poly(dI-dC) (GE Healthcare), 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μg/ml BSA, 4% Ficoll, and 100,000 cpm of end-labelled DNA fragments in a total volume of 20 μl. When indicated, 0.5 μl of rabbit anti-p50, anti-p65 or preimmune serum was added to the standard reaction before the addition of the radiolabelled probe. For cold competition, a 100-fold excess of the double stranded oligonucleotide competitor was added to the binding reaction. After 30 min incubation at 4 °C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris–borate, 89 mM boric acid and 1 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to X-ray film at −80 °C.

2.8. Expression of PKCs-GFP in cultured cells and visualization by confocal microscopy

CHO-K1 cells were grown on 12 mm round coverslips to 50–75% confluence. Transient transfection was conducted using

Jet Pei reagent (Polyplus transfection, France), following the manufacturer's instructions. The fluorescence became detectable 24 h after transfection, and all experiments were performed 3 days after transfection. Prior to observation, transiently transfected CHO-K1 cells were washed twice with PBS containing 1% FCS prewarmed to 37 °C. All PKC activators were diluted to specified concentrations in the same medium, and the final concentration of solvent (DMSO) was always less than 1%. After 10 min of treatment the cells were fixed and analysed with a confocal microscopy (Espectral TCS-SP2-AOBS, Leica).

2.9. *Isolation of peripheral human T cells and cytofluorimetric analyses of cell surface antigen*

Human PBMC, from healthy adult volunteer donors, were isolated by centrifugation of venous blood on Ficoll-hypaque® density gradients (GE Healthcare). Macrophages were removed by incubating the PBMC on 100 mm plastic Petri plates at 37 °C for 60 min, and the remaining cells were passed twice through a nylon wool column to deplete residual B cells and macrophages [25]. Nylon Wool T cells were usually 95% $CD3^+$ and less than 5% $CD25^+$. Cell surface expression of CD4, CXCR4, CD25 and CD69 antigens was measured by direct fluorescence using specific mAbs and analyzed by flow cytometry in an EPIC XL flow cytometer (Beckman-Coulter Inc. CA, USA).

3. Results

3.1. *Generation of latent HIV-1 infected Jurkat cells*

To establish cell lines with latent integrated HIV-1, an HIV-based retroviral vector containing the Tat and GFP genes under the control of the HIV-1 LTR promoter was used [23]. Jurkat cells were infected with viral particles containing this vector, and 96 h later the GFP^- cells were selected by fluorescence-activated cell sorting (FACS). This population seemingly contained both uninfected cells and cells with transcriptionally inactive proviruses. To activate HIV-1 expression, this population was stimulated with TNFα (10 ng/ml) for 24 h and the GFP^+ cells were sorted again and cloned by limiting dilution. Four out of 72 clones were selected and characterized for their responsiveness to OKT3, PMA and TNFα (Fig. 1A). Clone 8 (Jurkat-LAT-GFP cells) was selected for further studies, and, after treatment with PMA, OKT3 or TNFα, GFP expression was induced to a different extent, with PMA being the stronger activator (Fig. 1B). Re-analysis 14 and 28 days after Jurkat-LAT-GFP cells selection showed that more than 95% of the cells had no GFP expression, indicating transcriptional silencing in the absence of stimuli. As expected, the HIV-1 Tat protein was detectable only in stimulated Jurkat-LAT-GFP cells (data not shown).

3.2. *Effects of phorbol-13-monoesters in HIV-1 reactivation*

12-Deoxyphorbol-13-acetate (prostratin, **1a**) (Fig. 2), has been shown to reactivate HIV-1 latency in a cellular model similar to the Jurkat-LAT-GFP cells [26]. To study the effect of hydroxylation at C-12, phorbol 13-acetate (**1b**) was synthesized from phorbol (**2**) (Fig. 2), and Jurkat-LAT-GFP cells were stimulated with increasing concentrations of both **1b** and prostratin (**1a**) for 6 h. As expected, prostratin (**1a**) activated HIV-1 latency with an EC_{50} of 0.41 μM, while **1b** was totally inactive for HIV-1 reactivation (Fig. 3). Thus, the presence of a 12β-hydroxyl is detrimental for the activity of prostratin (**1a**). Having established that the substitution pattern of C-12 is critical for

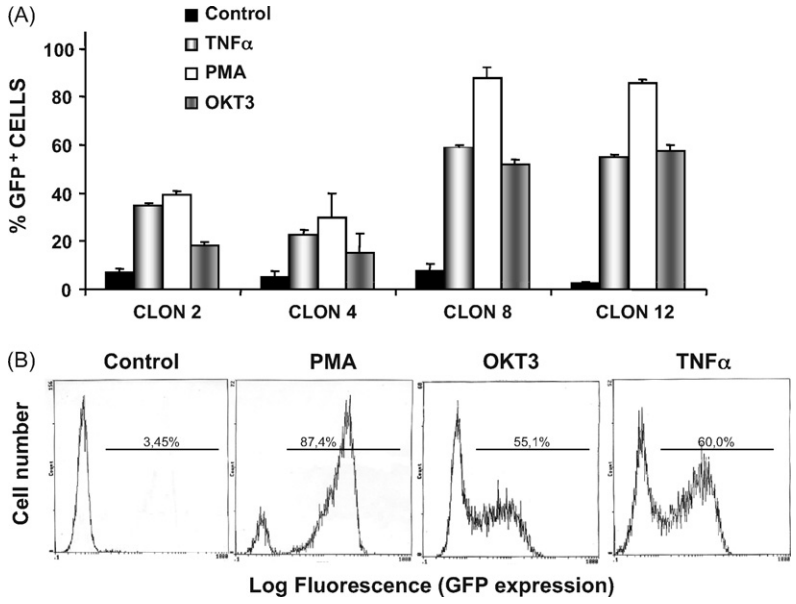
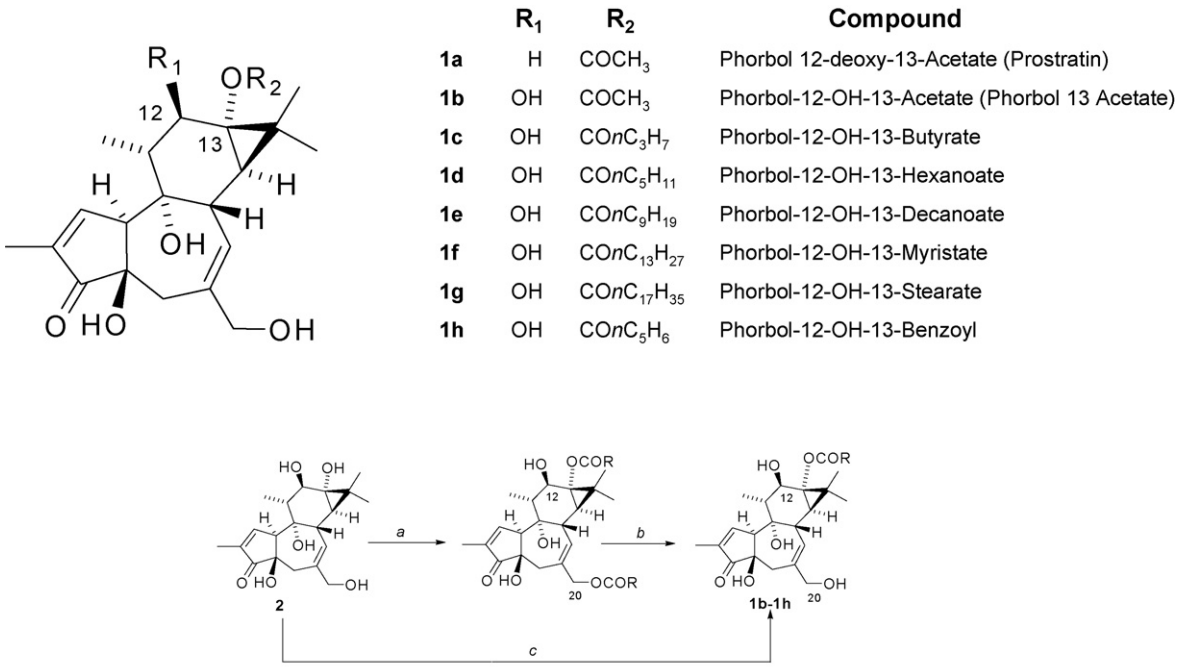


Fig. 1 – Characterization of the Jurkat-LAT-GPF cell line. (A) Cellular clones from latently infected Jurkat cells were stimulated with the agonists PMA (50 ng/ml), TNFα (10 ng/ml) and plastic coated OKT3 (2 μg/ml) for 24 h, and analyzed for GFP expression by flow cytometry. Results are represented as the percentage of GFP positive cells ± S.D. of three different experiments. (B) Clone 8 (Jurkat LAT-GFP) was selected for the further experiments, and the histograms represent the typical response of Jurkat-LAT-GFP cells to different stimuli. One out of three independent experiments is shown.



	R_1	R_2	Compound
1a	H	$COCH_3$	Phorbol 12-deoxy-13-Acetate (Prostratin)
1b	OH	$COCH_3$	Phorbol-12-OH-13-Acetate (Phorbol 13 Acetate)
1c	OH	$CO nC_3H_7$	Phorbol-12-OH-13-Butyrate
1d	OH	$CO nC_5H_{11}$	Phorbol-12-OH-13-Hexanoate
1e	OH	$CO nC_9H_{19}$	Phorbol-12-OH-13-Decanoate
1f	OH	$CO nC_{13}H_{27}$	Phorbol-12-OH-13-Myristate
1g	OH	$CO nC_{17}H_{35}$	Phorbol-12-OH-13-Stearate
1h	OH	$CO nC_5H_6$	Phorbol-12-OH-13-Benzoyl

Fig. 2 – Chemical structures of prostratin (1a), 12-hydroxyprostratin (1b) and the other 12-hydroxyphorbol-13 monoesters used in the study (1c–h) and synthesis of 1b–h.

activity, a series of analogues having a longer acyl residue at C-13 (C4, C6, C10, C14 and C18) was prepared (**1c–h**) (Fig. 2). Remarkably, recovery of activity was observed starting from the C6 ester, with the C18 analogue even outperforming prostratin by an order or potency (EC_{50} around 30 nM) for the capacity to induce HIV-1 reactivation from latency (Fig. 3).

3.3. *Effects of prostratin and 12-hydroxyphorbol-13-monoesters on NF-κB, JNK and ERK signaling pathways*

Several groups have independently demonstrated that prostratin reactivates HIV-1 latency by activating a PKC-dependent NF-κB pathway [15,26]. In order to study the role of prostratin (**1a**) and phorbol-13 monoesters in the activation of PKC-inducible transcription factors, Jurkat-LAT-GFP cells were stimulated with compounds **1b–1h** (10 μM) for 60 min, and nuclear extracts were obtained and analysed by electrophoretic mobility shift assay (EMSA) using NF-κB and AP-1-specific ^{32}P-labelled oligonucleotides. As shown in Fig. 4A, PMA stimulation strongly increased two NF-κB bands (Fig. 4A, compare lane 2 to lane 1) and one AP-1 band. The specificity of NF-κB bands was confirmed by cold oligonucleotide competition: wildtype κB oligonucleotides competed away-induced bands (Fig. 4B, lane 6), whereas AP-1 oligonucleotides did not diminish the intensity of the induced bands (Fig. 4B, lane 7). The addition of p65 (Fig. 4B, lane 4) and p50 (Fig. 4B, lane 3)

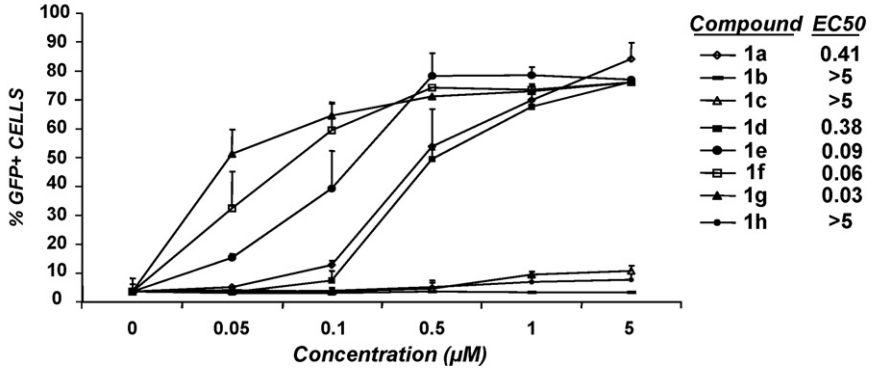


Fig. 3 – 12-Hydroxyphorbol-13 monoesters and prostratin antagonize HIV-1 latency. Jurkat LAT-GFP cells were stimulated with the 12-hydroxyphorbol-13 monoesters and prostratin at the indicated concentrations for 6 h, and next analyzed by flow cytometry. Results are represented as the percentage of GFP positive cells ± S.D. of four different experiments.

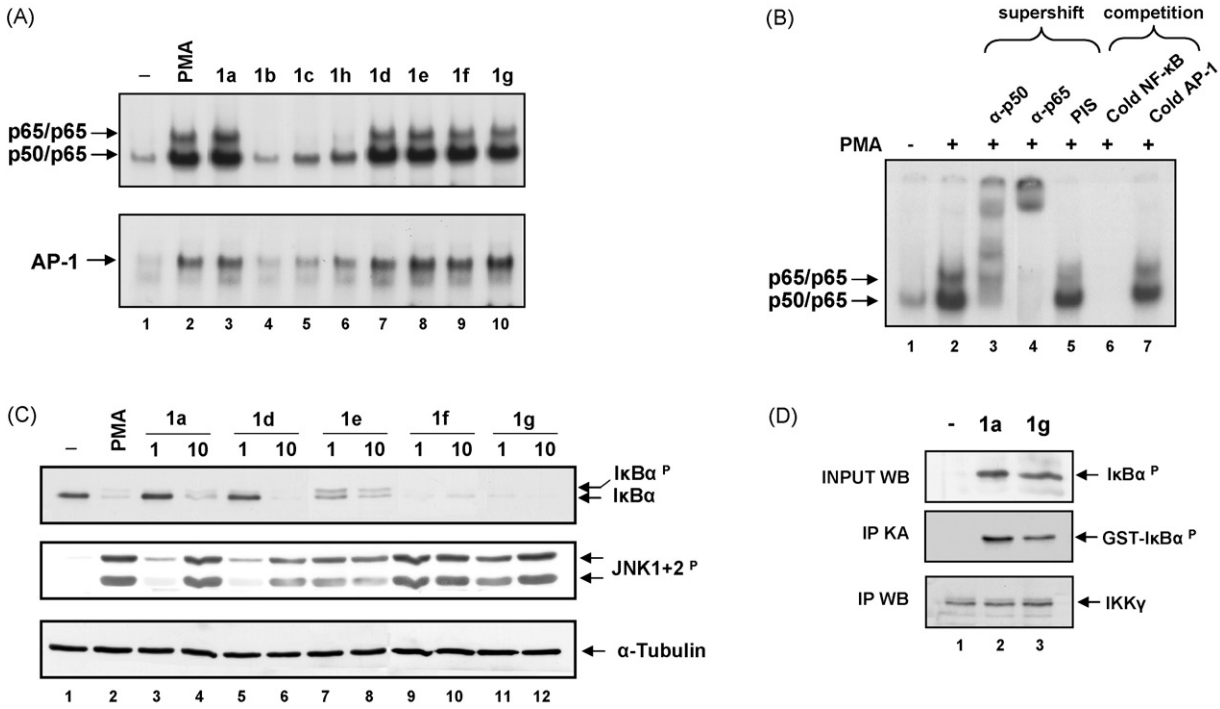


Fig. 4 – Prostratin and phorbol-13 stearate (P-13S) activate the NF-κB and the MAPK pathways. (A) Effect of phorbol 13 monoesters and prostratin on NF-κB and AP-1-DNA binding activity. Jurkat LAT-GFP cells were incubated with compounds 1a–1h (10 μM) and PMA (50 ng/ml) for 60 min. Nuclear cell extracts were then assayed by EMSA using γ-^{32}P-labelled NF-κB and AP-1 oligonucleotides (left panel). (B) The specificity of the NF-κB binding to DNA was demonstrated by supershift and cold competition assays. Supershift analysis with the indicated antibodies is shown in lanes 3, 4 and control with pre-immune serum (PIS) in lane 5. (C) Effect of phorbols 1a, 1d, 1e, 1f and 1g on IκBα degradation and JNK activation. Jurkat LAT-GFP cells were incubated with the selected phorbols (1 and 10 μM) during 15 min and IκBα degradation, JNK 1 + 2 activation, and the steady state levels of α-tubulin were analyzed using specific antibodies by Western blots. (D) Prostratin (1a) and phorbol-13-stearate (P-13S, 1g) activated IKK kinase activity. Cells were treated with the indicated compounds (10 μM) for 5 min, and half of the cell lysates were subjected to Western blot for the detection of endogenous IκBα phosphorylation (input). The other half of the lysates was used for IKKγ immunoprecipitation and the kinase activity was monitored using recombinant GST-IκBα (1–54) as substrate (IP KA). IKKγ was also detected by immunoblots as a control of the immunoprecipitated fraction.

antibodies indicated that the upper and lower bands contained p65 homodimers and p65-p50 heterodimers, respectively. The addition of pre-immune serum (PIS, lane 5) did not modify NF-κB EMSA complexes. PMA, prostratin (**1a**), phorbol 13-hexanoate (**1d**), phorbol 13-decanoate (**1e**), phorbol 13-miristate (**1f**) and phorbol 13-stearate (**1g**) (P-13S) induced a strong NF-κB and AP-1 binding to DNA (Fig. 4A, compare lanes 2, 3, 7–10 to lane 1). In contrast, phorbol 13-acetate (**1b**), phorbol 13-butyrate (**1c**) and phorbol 13-benzoate (**1h**) did not induce DNA binding to these transcription factors (Fig. 4A, compare lanes 4–6 to lane 1). Therefore, a close correlation between the NF-κB and AP-1 binding activity and HIV-1 latency reactivation was observed for phorbol derivatives in Jurkat-LAT-GFP cells.

Next, we investigated the biochemical pathways involved in NF-κB and AP-1 activation. Jurkat-LAT-GFP cells were stimulated with compounds **1a**, **1d**, **1e**, **1f** and **1g** (1 and 10 μM) for 15 min, and the phosphorylation of both the NF-κB inhibitor IκBα, and the JNK were investigated by Western blots using specific mAbs. Prostratin (**1a**) and compound **1d** only at the 10 μM concentration were able to induce IκBα degradation and JNK1 + 2 activation (Fig. 4C, compare lanes 4 and 6 to lane 1). In contrast, those biochemical pathways were clearly activated when the cells were treated with 1 μM concentration of the compounds **1e**, **1f** and **1g** (Fig. 4C, compare lanes 7, 9 and 11 to lane 1). Additionally, we found that phorbol-12-hydroxy-13-acetate (**1b**) and phorbol 13-butyrate (**1c**) were ineffective to induce the activation of the signalling pathways investigated (data not shown). The degradation of IκB proteins occurs after signal-induced phosphorylation of IκB proteins at specific serine residues, a reaction catalysed by IKKs present in the IκB kinase complex (IKC) [27]. We therefore investigated if the induction of IκBα degradation by prostratin (**1a**) and P-13S (**1g**) is also due to the activation of kinase activity of the IKC. Endogenous IKC was isolated by immunoprecipitation with an anti-IKKγ Ab, and its activity was analyzed by immune complex kinase assays using recombinant IκBα protein as substrate. Fig. 4D shows that stimulation of Jurkat-LAT-GFP cells with prostratin (**1a**) and P-13S (**1g**) induced both the activation of IKC and the phosphorylation of IκBα (Fig. 4D, compare lanes 2, 3 to lane 1).

3.4. Activation of latent HIV-1 by prostratin (1a), phorbol 13-hexanoate (1d) and P-13S (1g) requires activity of classical PKC isoforms

Previous studies on prostratin-induced HIV-1 activation have suggested that both classical [15] and novel PKCs [26] are involved in this response. To further assess the ERK and PKC dependence of prostratin (**1a**) and phorbol-13 monoesters (**1d**–**1g**)-mediated antagonism of HIV latency, we pretreated Jurkat-LAT-GFP cells with medium or the chemical inhibitors Gö6976 (classical PKCs inhibitor), Gö6850 (classical and novel PKCs inhibitor), Gö6983 (pan-PKC inhibitor), rottlerin (PKCδ inhibitor) and PD 98059 (MEK inhibitor) at the indicated concentrations. Gö6976, Gö6850 and Gö6983 strongly inhibited GFP expression induced by Prostratin and the phorbol-13 monoesters **1d** and **1g**, further implicating a PKC-dependent signaling step in this response (Fig. 5A). TNFα induction of the latent HIV provirus was unaffected by any of these inhibitors, thus excluding non-specific toxic effects of these drugs (data not shown). In contrast, the PKCδ inhibitor rottlerin did not affect phorbol-induced GFP expression, ruling out the involvement of this PKC in HIV-1 reactivation, at least in Jurkat-LAT-GFP cells. Given the effects of both classical and novel PKCs inhibitors on activation of latent HIV-1 by prostratin (**1a**), phorbol 13-hexanoate (**1d**) and P-13S (**1g**), we next explored the biochemical pathways involved in NF-κB and AP-1 activation by Western blots. As shown in Fig. 5B, Prostratin (**1a**) and P-13S (**1g**)-induced IκBα degradation and JNK phosphorylation were completely inhibited by the presence of PKC inhibitors that target classical PKCs (Fig. 5B, compare lanes 3–5 to lane 2). Rottlerin did not affect PMA-induced JNK activation, but clearly prevented the activation of this MAPK induced by prostratin (**1a**) and P-13S (**1g**) (Fig. 5B, compare lane 7 to lane 2). These results indicate that different phorbol-based compounds activate distinct signal pathways to antagonise HIV-1 latency, and that the contribution of the novel PKCδ to this activity is negligible.

It has been shown that the ERK pathway is activated by the PKC pathway, and is involved in HIV-1 activation [28]. Thus, we studied this pathway in Jurkat-LAT-GFP cells stimulated with compounds **1a**, **1d** and **1g**. Cells were preincubated with the MEK inhibitor PD 98059, and then stimulated with the various phorboids. PD 98059 partially inhibited GFP expression induced by these compounds (**1a**, **1d**, **1g**), further implicating an ERK-dependent signaling pathway in this response (Fig. 5A). Accordingly, PD 98059 also prevented prostratin (**1a**) and P-13S (**1g**)-induced ERK activation (Fig. 5B, compare lane 6 to lane 2). Altogether, these results suggest that HIV-1 reactivation in response to non-tumor promoter phorbol esters can be mediated by both the NF-κB and the ERK pathways.

3.5. Effect of PKC activators on the translocation and activation of PKCα-GFP and PKCδ-GFP

The translocation of PKC from the cytoplasm to subcellular localizations is the hallmark for PKC activation [29], and their isozyme-specific functions may result in part from differences in subcellular localization [30,31]. There is a general agreement that only phorbol esters inducing a sustained PKC transloca-

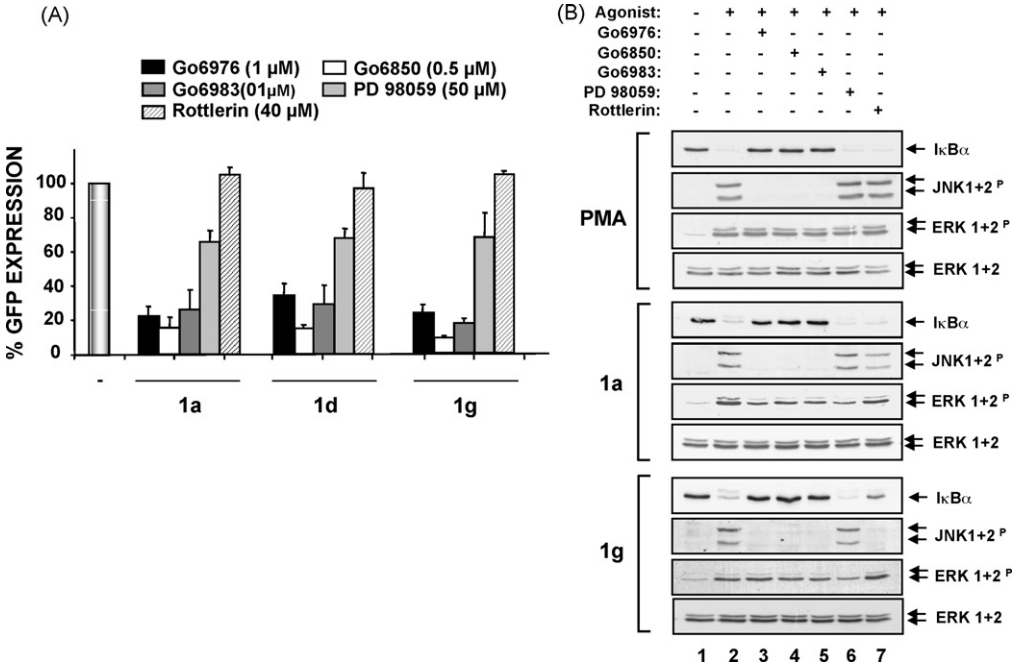


Fig. 5 – Prostratin, phorbol 13-hexanoate, and phorbol 13-stearate antagonize HIV-1 latency through a classical PKC-dependent pathway. (A) Jurkat LAT-GFP cells were pretreated with the indicated inhibitors for 30 min at the indicated dose, and then stimulated with the agonists (10 μM) for 6 h. The percentage of GFP+ cells was measured by flow cytometry. Results are represented as percentage of activation ± S.D. compared to cells treated with agonists in the absence of the chemical inhibitors (100% activation). (B) The cells were pretreated with the inhibitors as indicated and then stimulated with the agonists for 15 min. Total cell extracts were analyzed by Western blot with specific antibodies.

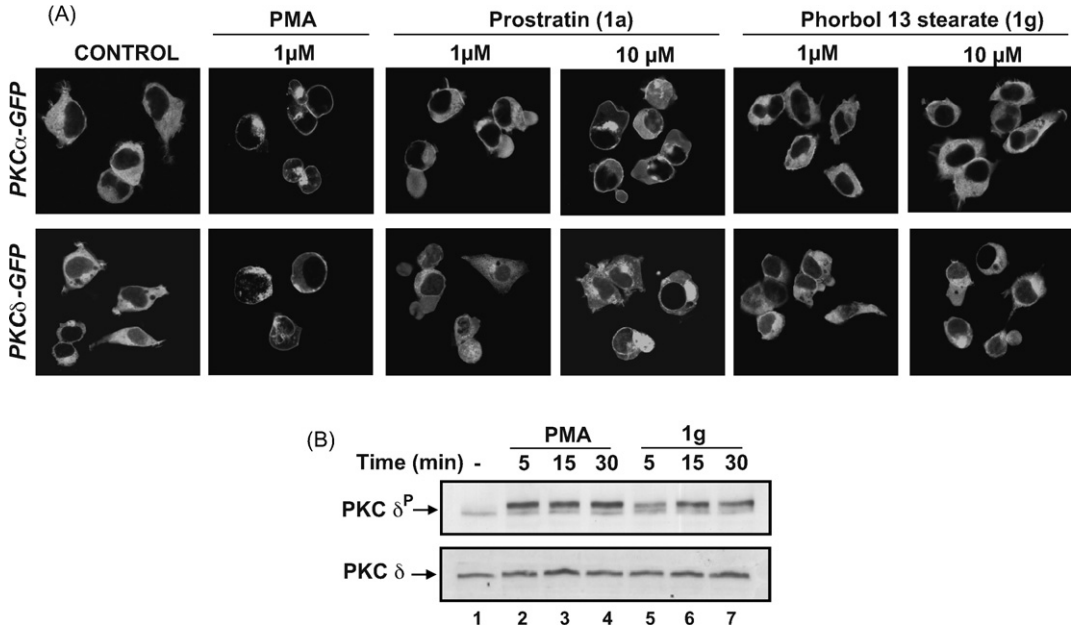


Fig. 6 – Effects of PMA and P-13S on PKCα-GFP and PKCδ-GFP expressed in CHO-K1 cells (A). Fluorescent images of CHO-K1 cells expressing PKCα-GFP and PKCδ-GFP proteins treated at 37 °C for 10 min with either PMA (1 μM), prostratin (1 and 10 μM) or P13-S (1 and 10 μM). Three additional experiments gave similar results. (B) PMA and phorbol-13 stearate phosphorylate PKCδ. Jurkat LAT-GFP cells were incubated with either PMA or P-13S (10 μM) for the indicated period of time. Total PKCδ and phosphorylated PKCδ (Thr505) were analyzed using specific antibodies by Western blots.

tion to the cell membrane are endowed with tumor promoter activity. Thus, while the tumor promoter 12-deoxyphorbol 13-tetradecanoate closely resembled PMA in its translocation pattern, its antihyperplastic and antipromoting 12-deoxyphorbol 13-phenylacetate analog caused punctuate intracellular accumulation of PKCδ on the nuclear membrane [31]. In order to explore the specificity of PKC α- and δ-GFP translocation in response to prostratin (**1a**), P-13S (**1g**) and PMA, we used CHO-K1 cells transiently transfected with constructs containing the full length open reading frame of either PKCα or PKC δ fused to the GFP gene. In the absence of stimuli, PKCα and δ were scattered in the cytoplasm. When a single dose of the tumor promoter PMA was added, both PKC isotypes were mainly translocated to the plasma membrane. Prostratin induced a transient translocation of PKCα to the plasma membrane and a perinuclear translocation of PKCδ. Conversely, P-13S induced only a little plasma membrane translocation of PKCα-GFP, without apparently affecting the cellular distribution of PKCδ (Fig. 6A). Similar results were found after 20 min of exposure to either PMA or P-13S (data not shown). In general PKCs are also regulated by phosphorylation and upon activation PKCδ exhibits phosphorylation of the activation loop (Thr-505) [32], which has been suggested to be required for the activity of PKCδ [33]. Thus, we studied by using a specific antibody anti-phospho PKCδ (Thr505) the effects of P-13S on PKCδ activation and phosphorylation, and we found that both PMA and P-13S activated PKCδ with a similar kinetic (Fig. 6B, compare lanes 2–7 to lane 1). Altogether our results strongly suggest that PMA, prostratin and P-13S differ substantially in their biological activities (activation vs. subcellular translocation).

3.6. *P-13S downregulates HIV receptors in peripheral blood lymphocytes*

The anti-HIV activity of phorbol esters has been linked to downregulation of HIV-1 receptors thus interfering with the viral entry step [9,16]. To analyze the effects of P-13S on cellular surface in primary T cell surface antigens, $CD3^+$ T cells were isolated from healthy volunteer donors, treated with either prostratin (10 μM) or P-13S (1 μM) and incubated for 24 h. The cells were then analyzed by FACS for CD4, CXCR4, CD69 and CD25 expression. Fig. 7 demonstrates several-fold CD4 and CXCR4 down-regulation in response to prostratin and P-13S. Therefore P13-S is also at least ten fold more potent than prostratin to downregulate HIV-1 receptors. Similar results were found when the activation antigens CD69 and CD25 were measured in peripheral T cells stimulated with either prostratin or P-13S (Fig. 7). However, none of these compounds showed mitogenic activity in human peripheral T cells (data not shown).

4. Discussion

The identification of potent natural or synthetic PKC agonists lacking tumor-promoter and cellular proliferative activities has opened new research avenues for the treatment of HIV-1. HIV-1 latency represents a major hurdle to the complete eradication of the HIV from patients under HAART regimens [5,6]. One solution would be the reactivation of the latent reservoirs in presence of HAART to prevent spreading of the infection by the newly synthesized viruses [7]. In this sense, a

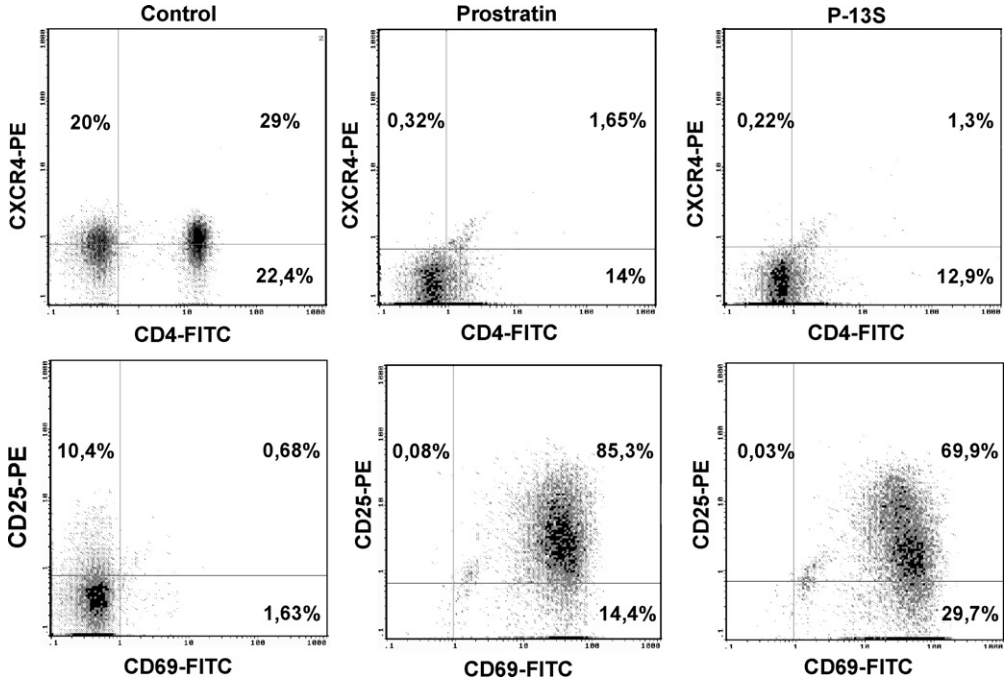


Fig. 7 – Effects of Prostratin and P-13S on cell surface antigen expression in primary T cells. Purified T cells were treated with either prostratin (10 μM) or P-13S (1 μM) for 24 h and the expression of CD4, CXCR4, CD69 and CD25 detected by flow cytometry as described in Materials and Methods. The results are representative of three experiments.

recent study shows that the therapy with HIV protease inhibitors (PIs) should be compatible with an inductive therapy for latent reservoir reduction/elimination in association with HAART regimens [34].

In this study we have synthesized novel 12-hydroxyphorbol-13 monoesters and explored the mechanism by which these compounds and the non-tumor promoter PKC activators, prostratin and bryostatin, antagonized HIV-1 latency. The capacity of prostratin to behave as an *in vivo* agent to purge latent HIV-1 proviruses [35] has raised considerable interest, owing to a potential clinical application in combination with HAART to eradicate HIV-1 infection. However, relatively high concentrations of prostratin are required to reactivate HIV-1 latency and it has been suggested that prostratin may have negative side effects and therefore it is unlikely that high-doses or long-term treatment would be well tolerated [26]. Since the PKC-dependent activation of the NF-κB and AP-1 pathways are well known mechanisms to reactivate HIV-1 latency, the identification of novel PKC activators lacking tumor-promoter activity such as synthetic diacylglycerol lactones [11] and 12-deoxyphorbol 13-phenylacetate [12] are of special interest for the clinical development of drugs that antagonize HIV-1 latency.

In addition to the above limitation of prostratin, its development as a drug has been hampered by its very limited availability by isolation. Prostratin is a derivative of 12-deoxyphorbol. While this polyol is not more available than prostratin itself, phorbol can be easily obtained in synthetically useful yields from a commercial source (Croton oil). We have therefore started an investigation aimed at discovering if prostratin-like properties could be induced in non-tumor promoting phorbol derivatives. To this purpose, a series of phorbol-13 monoesters was prepared and investigated for their capacity to antagonize HIV-1 latency. As a cellular model of HIV-1 latency we have used the Jurkat-LAT-GFP cells, which contain a full-length latent HIV provirus. This provirus contains GFP instead of Nef and transcriptional activation of the latent provirus can be easily detected by flow cytometry. While phorbol 13-acetate, a compound differing from prostratin only for the presence of a 12-hydroxyl, was totally inactive, activity could be induced by increasing the length of the acyl moiety, with the stearoyl analogue (P-13S) even outperforming the potency of prostratin as an HIV-1 latency antagonist by more than 10-fold. Activity was not due to a non-specific buffering of the increased polarity associated to the 12-hydroxyl, since the benzoate analogue of prostratin was inactive.

Since prostratin and phorbol 13-monoesters possess the critical O-4 and O-20 hydroxyls essential for binding the C1 domain of PKC, it does not seem unreasonable to assume these compounds can interact with PKC in the mainstream way of phorboid ligands. However, the presence of a polar 12β-hydroxyl in the upper moiety of the molecule might interfere with the translocation of PKCs to specific cell compartment, with an overall detrimental effect on the functional activity of these enzymes, since a hydrophobic complex is required for translocation to cell compartment other than plasma membrane and the activation of PKC substrates [12]. In accordance with this view, 12-deoxyphorbol derivatives bearing large lypophilic chains at the position 13, like 12-deoxyphorbol-13-tetradecanoate have been reported to be potent tumor promoters that translocate PKCs to plasma membrane [31]. On the other hand, the corresponding compound from the phorbol series (phorbol 13-myristate) (data not shown) as well

as P-13S, though potent antagonists of HIV-1 latency through a classical PKC-dependent pathway, failed to translocate PKCα and δ to the plasma membrane, and therefore may be devoid of tumor-promoting activity. This observation is in full accordance with the in vivo data reported in the pioneering investigations by Hecker in this area [36], and clearly indicate that the presence of a 12-hydroxyl, though compatible with interaction with PKCs, is detrimental for the transfer of the resulting complex to plasma membranes.

The discovery of a very promising HIV-1 purging activity of P-13S prompted us to investigate the mechanistic details by which this compound, in comparison to prostratin, reactivates HIV-1 latency. In our cell model both prostratin and P-13S could antagonize HIV-1 latency through a classical PKCs-dependent pathway and irrespective of the activation of the isoenzyme PKCδ. Moreover, the classical PKC inhibitor Gö6976 also inhibited IκBα phosphorylation and degradation and JNK activation induced by either prostratin or P-13S. Rottlerin (which is more selective for PKCδ) at the concentration tested did not affect P-13S-induced HIV-1 reactivation and NF-κB activation. Our results are in sharp contrast with those reported by Williams et al. using a similar model of HIV-1 latency and the same chemical PKC inhibitors. These authors showed that Gö6976 could not inhibit prostratin-induced HIV-1 reactivation, and similar results were obtained with the specific PKCθ inhibitor TER14867 suggesting that novel PKCs other than PKCθ are involved in prostratin signaling [26]. This discrepancy might be due to the parental Jurkat cells used to generate HIV-1 latently infected clones. In this sense, a critical role for PKCα in the HIV-1 antagonizing effects of prostratin has also been reported in Jurkat cells, suggesting that a coordinate action between PKCα and PKCθ is required for NF-κB activation and HIV-1 reactivation by this compound [15]. Jurkat-LAT-GFP express very low levels of PKCε (data not shown) and therefore our results suggest that in this cell line activated with either prostratin or P-13S, the novel PKCs are not critical for NF-κB activation and HIV-1 reactivation, but they are absolutely required for JNK activation (at least PKCδ).

Although PKCδ signaling and JNK activation are not required for P-13S-induced HIV-1 reactivation, we obtained evidence that activation of the ERK pathway may also be involved in prostratin signaling. In other latency models, it has been shown that the ERK pathway is involved in PMA- and cytokine activation of HIV-1 latency, while ERK cooperate with NF-κB to fully reactivate HIV-1 latency [28]. We found that, in addition to IKK activation, prostratin and P-13S also activated the ERK 1 + 2, while the MEK inhibitor PD 98059 partially prevented the HIV-1 antagonizing effects of these compounds. These results might be related to the ability of P-13S to relocalize RasGRP1 to the nuclear membrane and other cytoplasmic localizations (Marquez and Muñoz Unpublished results). RasGRP1 is a diacylglycerol and phorbol-binding protein that acts as a Ras exchange factor and therefore activates the Ras-Raf-ERK pathway [37]. RasGRP1 has been shown to connect PKCs to the ERK pathway, and both Rotllerin and Gö 6976 have been reported to block the phosphorylation of RasGRP-1 at threonine 184 [38,39]. Nevertheless, none of the chemical PKC inhibitors we used could clearly prevent prostratin and P-13S-induced ERK 1 + 2 phosphorylation. Therefore, it is possible that these phorboids directly bind and activate Ras-GRP-1 through a PKC-independent pathway, a hypothesis we are currently pursuing.

In summary, we have provided strong evidence that phorbol 13-monoesters containing medium or long aliphatic fatty acid chains are potent antagonists of HIV-1 latency, acting by activation classical PKCs and downstream triggering of the NF-κB pathway. These compounds do not translocate PKC to the plasma membrane, seemingly lack tumor promoter activity, downregulate the expression of the HIV-1 receptors CD4 and CXCR4 and therefore represent new lead compounds for the treatment of HIV-1 latency in combination with HAART.

Acknowledgements

The authors wish to thank Dr. P.M. Blumberg (NCI, Besthesda, MD, USA) for the PKCα-GFP and PKCδ-GFP plasmids. We thank Ms. Carmen Cabrero-Doncel for her assistance with the manuscript.

This work was supported by the Junta de Andalucía Grant P06-CTS-01353 (E.M.), the FIS grant PI040526 (E.M.) and by the ISCIII-RETIC RD06/006.

REFERENCES

[1] Barbaro G, Scozzafava A, Mastrolorenzo A, Supuran CT. Highly active antiretroviral therapy: current state of the art, new agents and their pharmacological interactions useful for improving therapeutic outcome. Curr Pharm Des 2005;11:1805–43.
[2] Blankson JN, Persaud D, Siliciano RF. The challenge of viral reservoirs in HIV-1 infection. Annu Rev Med 2002;53:557–93.
[3] Davey Jr RT, Bhat N, Yoder C, Chun TW, Metcalf JA, Dewar R, et al. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. Proc Natl Acad Sci USA 1999;96:15109–14.
[4] Persaud D, Zhou Y, Siliciano JM, Siliciano RF. Latency in human immunodeficiency virus type 1 infection: no easy answers. J Virol 2003;77:1659–65.
[5] Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci USA 1997;94:13193–7.
[6] Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med 1999;5:512–7.
[7] Kulkosky J, Pomerantz RJ. Approaching eradication of highly active antiretroviral therapy-persistent human immunodeficiency virus type 1 reservoirs with immune activation therapy. Clin Infect Dis 2002;35:1520–6.
[8] Fraser C, Ferguson NM, Ghani AC, Prins JM, Lange JM, Goudsmit J, et al. Reduction of the HIV-1-infected T-cell reservoir by immune activation treatment is dose-dependent and restricted by the potency of antiretroviral drugs. AIDS 2000;14:659–69.
[9] Kulkosky J, Culnan DM, Roman J, Dornadula G, Schnell M, Boyd MR, et al. Prostrat activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART. Blood 2001;98:3006–15.

[10] Warrilow D, Gardner J, Darnell GA, Suhrbier A, Harrich D. HIV type 1 inhibition by protein kinase C modulatory compounds. AIDS Res Hum Retroviruses 2006;22:854–64.
[11] Hamer DH, Bocklandt S, McHugh L, Chun TW, Blumberg PM, Sigano DM, et al. Rational design of drugs that induce human immunodeficiency virus replication. J Virol 2003;77:10227–36.
[12] Bocklandt S, Blumberg PM, Hamer DH. Activation of latent HIV-1 expression by the potent anti-tumor promoter 12-deoxyphorbol 13-phenylacetate. Antivir Res 2003;59:89–98.
[13] Zayed S, Hafez A, Adolf W, Hecker E. New tigliane and daphnane derivatives from Pimelea prostrata and Pimelea simplex. Experientia 1977;33:1554–5.
[14] Gustafson KR, Cardellina II JH, McMahon JB, Gulakowski RJ, Ishitoya J, et al. A nonpromoting phorbol from the samoan medicinal plant Homalanthus nutans inhibits cell killing by HIV-1. J Med Chem 1992;35:1978–86.
[15] Trushin SA, Bren GD, Asin S, Pennington KN, Paya CV, Badley AD. Human immunodeficiency virus reactivation by phorbol esters or T-cell receptor ligation requires both PKCalpha and PKCtheta. J Virol 2005;79:9821–30.
[16] Hezareh M, Moukil MA, Szanto I, Pondarzewski M, Mouche S, Cherix N, et al. Mechanisms of HIV receptor and co-receptor down-regulation by prostratin: role of conventional and novel PKC isoforms. Antivir Chem Chemother 2004;15:207–22.
[17] Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 2001;101:2353–64.
[18] Hall C, Lim L, Leung T. C1, see them all. Trends Biochem Sci 2005; 30:169–71.
[19] Slater SJ, Ho C, Kelly MB, Larkin JD, Taddeo FJ, Yeager MD, et al. Protein kinase Calpha contains two activator binding sites that bind phorbol esters and diacylglycerols with opposite affinities. J Biol Chem 1996;271:4627–31.
[20] Zhang G, Kazanietz MG, Blumberg PM, Hurley JH. Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. Cell 1995;81:917–24.
[21] Wang QJ, Fang TW, Fenick D, Garfield S, Bienfait B, Marquez VE, et al. The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C delta fused to green fluorescent protein. J Biol Chem 2000;275:12136–4.
[22] Sakai N, Sasaki K, Ikegaki N, Shirai Y, Ono Y, Saito N. Direct visualization of the translocation of the gamma-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein. J Cell Biol 1997;139:1465–76.
[23] Jordan A, Defechereux P, Verdin E. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. EMBO J 2001;20:1726–38.
[24] Sancho R, Calzado MA, Di Marzo V, Appendino G, Munoz E. Anandamide inhibits nuclear factor-kappaB activation through a cannabinoid receptor-independent pathway. Mol Pharmacol 2003;63:429–38.
[25] Hathcock KS. In vitro assays for mouse B and T cell function. Isolation and fractionation of lymphocytes. In: Coligan JS, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. Greene Publishing Associates and Wiley-Interscience; 1991. p. 3.2.1–3.
[26] Williams SA, Chen LF, Kwon H, Fenard D, Bisgrove D, Verdin E, et al. Prostratin antagonizes HIV latency by activating NF-kappaB. J Biol Chem 2004;279:42008–17.
[27] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000;18:621–63.
[28] Yang X, Chen Y, Gabuzda D. ERK MAP kinase links cytokine signals to activation of latent HIV-1 infection by stimulating a cooperative interaction of AP-1 and NF-kappaB. J Biol Chem 1999;274:27981–8.
[29] Tuthill MC, Oki CE, Lorenzo PS. Differential effects of bryostatin 1 and 12-O-tetradecanoylphorbol-13-acetate on the regulation and activation of RasGRP1 in mouse epidermal keratinocytes. Mol Cancer Ther 2006;5:602–10.
[30] Mochly-Rosen D, Henrich CJ, Cheever L, Khaner H, Simpson PC. A protein kinase C isozyme is translocated to cytoskeletal elements on activation. Cell Regul 1990;1:693–706.
[31] Wang QJ, Bhattacharyya D, Garfield S, Nacro K, Marquez VE, Blumberg PM. Differential localization of protein kinase C delta by phorbol esters and related compounds using a fusion protein with green fluorescent protein. J Biol Chem 1999;274:37233–9.
[32] Steinberg SF. Distinctive activation mechanisms and functions for protein kinase Cdelta. Biochem J 2004;384:449–59.
[33] Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science 1998;281:2042–5.
[34] Vandergeeten C, Quivy V, Moutschen M, Van Lint C, Piette J, Legrand-Poels S. HIV-1 protease inhibitors do not interfere with provirus transcription and host cell apoptosis induced by combined treatment TNF-alpha + TSA. Biochem Pharmacol 2007;73:1738–48.
[35] Hezareh M. Prostratin as a new therapeutic agent targeting HIV viral reservoirs. Drug News Perspect 2005;18:496–500.
[36] Hecker E. Cell membrane associated protein kinase C as receptor of diterpene ester co-carcinogens of the tumor promoter type and the phenotypic expression of tumors. Arznezimittel-Forsch 1985;35:1890–903.
[37] Mor A, Philips MR. Compartmentalized Ras/MAPK signaling. Annu Rev Immunol 2006;24:771–800.
[38] Roose JP, Mollenauer M, Gupta VA, Stone J, Weiss A. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. Mol Cell Biol 2005;25:4426–41.
[39] Zheng Y, Liu H, Coughlin J, Zheng J, Li L, Stone JC. Phosphorylation of RasGRP3 on threonine 133 provides a mechanistic link between PKC and Ras signaling systems in B cells. Blood 2005;105:3648–54.