Prostaglandin E₂ Up-regulates HIV-1 Long Terminal Repeat-driven Gene Activity in T Cells via NF-κB-dependent and -Independent Signaling Pathways*

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Replication of human immunodeficiency virus type-1 (HIV-1) is highly dependent on the state of activation of the infected cells and is modulated by interactions between viral and host cellular factors. Prostaglandin E₂ (PGE₂), a pleiotropic immunomodulatory molecule, is observed at elevated levels during HIV-1 infection as well as during the course of other pathogenic infections. In 1G5, a Jurkat-derived T cell line stably transfected with a luciferase gene driven by HIV-1 long terminal repeat (LTR), we found that PGE₂ markedly enhanced HIV-1 LTR-mediated reporter gene activity. Experiments have been conducted to identify second messengers involved in this PGE₂-dependent up-regulating effect on the regulatory element of HIV-1. In this study, we present evidence indicating that signal transduction pathways induced by PGE₂ necessitate the participation of cyclic AMP, protein kinase A, and Ca²⁺. Experiments conducted with different HIV-1 LTR-based vectors suggested that PGE₂-mediated activation effect on HIV-1 transcription was transduced via both NF-κB-dependent and -independent signaling pathways. The involvement of NF-κB in the PGE₂-dependent activating effect on HIV-1 transcription was further confirmed using a κB-regulated luciferase encoding vector and by electrophoretic mobility shift assays. Results from Northern blot and flow cytometric analyses, as well as the use of a selective antagonist indicated that PGE₂ modulation of HIV-1 LTR-driven reporter gene activity in studied T lymphoid cells is transduced via the EP₄ receptor subtype. These results suggest that secretion of PGE₂ by macrophages in response to infection or inflammatory activators could induce signaling events resulting in activation of proviral DNA present into T cells latently infected with HIV-1.

Infection with human immunodeficiency virus type-1 (HIV-1),¹ the etiologic agent of AIDS (1), leads to a progressive decline of CD4-expressing T cells resulting in impaired cellular immune functions. This infection is influenced by a complex interplay between viral and host factors, as well as by microbial agents termed cofactors. It has been postulated that such cofactors may be important in disease progression by enhancing cell-to-cell transmission or through up-regulation of HIV-1 expression in latently infected cells (2). Mycoplasma (3), mycobacteria (4), viruses (5, 6), and the protozoan parasite Leishmania (7, 8) may act as cofactors for the pathogenesis of HIV-1 infection either by directly modulating virus replication or by inducing a more profound immunosuppressive state. During coinfections, the inability of the host to develop an effective immune response may involve the participation of the immunosuppressive molecule PGE₂, an oxygenated polyunsaturated fatty acid that contain a cyclopentane ring structure.

PGE₂ are molecules that have been shown to modulate the immune response both in vitro and in vivo (9, 10). Macrophages, follicular dendritic cells, fibroblasts, and vascular endothelial cells synthesize PGE₂, while lymphocytes do not secrete this major product of arachidonic acid metabolism (11–14). A marked increase in PGE₂ production is generated in response to a variety of immunological stimuli including interleukin (IL)-1, tumor necrosis factor-α (TNF-α), antigen-antibody complexes, and lipopolysaccharide (15). Moreover, production of PGE₂ has been shown to be induced by infection with several pathogens such as Leishmania donovani (16), Leishmania major (17), Entamoeba histolytica (18, 19), Pseudomonas aeruginosa (20, 21), Staphylococcus epidermidis (22), Mycobacterium avium (23), herpes simplex virus type 1 (24), coxsackievirus virus (25), respiratory syncytial virus (26), and HIV-1 (27–30). PGE₂ has been implicated in decreasing T-cell proliferation, IL-2 production, and IL-2 receptor expression (15, 31–35). PGE₂ shifts the balance of the cellular immune response away from T-helper type 1 (Th1) favoring a Th₂ response which drives humoral responses toward the production of IgE (36). However, more recent findings have depicted PGE₂ as a pleiotropic molecule that can act both negatively or positively on the immune system (15).

An overproduction of PGE₂ (as high as 10⁻⁴ M) is seen in a number of disorders (e.g. allergy, hyper-IgE syndrome, Hodgkin lymphoma, trauma, sepsis, and transplantation), most of which are characterized by elevated Th2 and IgE re-

¹ The abbreviations used are: HIV-1, human immunodeficiency virus-1; PGE₂, prostaglandin E₂; IL, interleukin; TNF-α, tumor necrosis factor-α; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; IBMX, isobutylmethylxanthine; BAPTA, 1,2-bis(O-aminophenoxy)ethane-N,N,N',N' -tetraacetic acid; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PKA, protein kinase A; CAI, carboxyamidotriazole; NFAT, nuclear factor of activated T cells.
sponses (15, 36–38). As specified above, elevated levels of PGE$_2$ have also been reported in individuals infected with HIV-1 (27–30) and it has been postulated that this may contribute to the immunosuppressive state seen in such virally-infected patients (39). The mechanism(s) responsible for the enhanced prostaglandin formation is still undefined. The initial contact between the virus particle and its target cell might represent the crucial step leading to the production of PGE$_2$ by macrophages. This concept is supported by the finding that a significant production of endogenous PGE$_2$ is induced (20- to 40-fold increase) following incubation of primary human monocytes with the HIV-1 external envelope glycoprotein gp120 (40). However, in sharp contrast with this report, a previous study has demonstrated that interaction between gp120 and THP-1, a human monocytoid cell line, does not increase exogenous production of PGE$_2$ (39). It is important to specify that, unlike monocyte/macrophages, promonocoytoid THP-1 cells are not at a terminal stage of differentiation. In addition, a monomer form of gp120 was used in this study which might not parallel physiological conditions where gp120 is under a multimeric form (41). Depending on the cell type, binding of PGE$_2$ to one of its six different described receptors (EP$_1$, EP$_2$, EP$_3$, EP$_4$, EP$_{III}$, and EP$_{IV}$) can lead to activation of phospholipase C, phosphatidylinositol turnover, activation of adenylate cyclase via chola toxin-sensitive G$_{as}$ proteins and mobilization of intracellular Ca$^{2+}$ (42). Given that HIV-1 is highly dependent on intracellular signaling machinery for its life cycle, it is therefore possible that interaction of PGE$_2$ with its surface receptor(s) can modulate virus replication. A previous cellular study supports this postulate since PGE$_2$ was found to enhance HIV-1 replication in acutely infected T lymphoid cells (43).

The primary goal of the present work was thus to investigate the putative modulatory role of PGE$_2$ on the regulatory elements of HIV-1 (LTR) at both biochemical and molecular levels. For this purpose, we treated human T lymphoid cells stably and transiently transfected with different HIV-1 LTR-driven luciferase reporter gene vectors with concentrations of PGE$_2$ known to be found under physiological conditions. We then explored the intracellular second messengers participating in PGE$_2$-mediated signaling transduction pathway(s) and investigated DNA-binding transcriptional factor(s) and cell surface receptor(s) implicated in the PGE$_2$-dependent effect on HIV-1 transcription.

**EXPERIMENTAL PROCEDURES**

**Reagents**—PGE$_2$, phorbol 12-myristate 13-acetate (PMA), phytod-magglutinin (PHA), indomethacin and dibutyryl-cAMP were purchased from Sigma. H$_7$ was purchased from Seikagaku America Inc. (Tampa, FL). BAPTA/AM, IBMX, MDL-12,330A, and HA-1004 were purchased from BioMol (Plymouth Meeting, PA). The calcium inhibitor CAI was a generous gift from Dr. E. C. Kohn (National Institutes of Health, Bethesda, MD). AH 23848B was kindly provided by Dr. S. G. Lister (Glaxo Wellcome, United Kingdom). AH 23848B was kindly provided by Dr. S. G. Lister (Glaxo Wellcome, United Kingdom). AH 23848B was kindly provided by Dr. S. G. Lister (Glaxo Wellcome, United Kingdom). AH 23848B was kindly provided by Dr. S. G. Lister (Glaxo Wellcome, United Kingdom).

**Cells and Culture Conditions**—The parental lymphoid T cell line, Jurkat E6.1, was obtained from the American Type Culture Collection (ATCC, Rockville, MD), while 1G5 was supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institute of Health (Rockville, MD). 1G5 is a clonal cell line derived from Jurkat E6.1 cells which have been stably transfected with a luciferase gene driven by the HIV-1 LTR (44). The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.22% Na$_2$HPO$_4$, and were maintained at 37°C in a 5% CO$_2$ humidified atmosphere.

**Plasmids and Antibodies**—In our studies, we have used pLTR-LUC and pmsBLTR-LUC that have been kindly provided by Dr. K. Calame (Columbia University, NY). These molecular constructs contain the luciferase reporter gene under the control of wild-type (GOGACTTTCC) or NF-xB-mutated (CTACTTTCC) HIV-1, minimal LTR (453 to +80) (45). We have also used the pS-B-TATA-LUC vector which contains the minimal HIV-1 B region and a TATA box placed upstream of the luciferase reporter gene (46). This plasmid is a generous gift from Dr. W. C. Greene (The J. Gladstone Institutes, San Francisco, CA). The mouse monoclonal pNF-xB-LUC contains five (five copies of NF-xB-binding sites placed in front of the luciferase reporter gene (Stratagene, La Jolla, CA). NFAT-LUC contains the IL-2 minimal promoter with three tandem copies of NFAT-binding site placed upstream of the luciferase reporter gene (kindly provided by Dr. G. R. Crabtree, Howard Hughes Medical Institute, CA). Northern blot analyses were performed using human EP$_1$, EP$_2$, EP$_3$, EP$_4$, and EP$_{III}$ subtypes cDNA fragments. The cDNAs for human prostaglandin receptor EP$_1$ (1.3 kilobases), EP$_2$ (1.8 kilobases), and EP$_3$ (1.5 kilobases) were kind gifts from Dr. M. Abramovitz (Merck Frost, Qué, Canada). The cDNA for hEP$_4$ (1.1 kilobases) was generously provided by Dr. K. M. Kedzie (Allergan, Irvine, CA). The polyclonal antibody specific for EP$_3$ was obtained from Cayman Chemical (Ann Arbor, MI). This rabbit serum is directed against a synthetic peptide from the human EP$_3$ receptor.

**Modulation of HIV-1 LTR Activity by PGE$_2$**—In order to assess whether PGE$_2$ could modulate HIV-1 LTR activity, 1G5 cells (5 × 10$^6$) were either left untreated or treated with PHA (3 µg/ml), anti-CD3 antibody (clone OKT3 at 1 µg/ml), PMA (20 ng/ml), and TNF-α (2 ng/ml; R&D systems, Minneapolis, MN) in a final volume of 200 µl for 1 h at 37°C. In these experiments, the cells were harvested in the absence or presence of 100 nM PGE$_2$ for 24 h at 37°C. Dose-response experiments were done using a similar number of cells, washed once in phosphate-buffered saline (PBS, pH 7.4), and resuspended in 1 ml of fresh complete culture medium before incubation for 24 h at 37°C with PGE$_2$ at final concentrations of 1, 10, 100, and 1000 nM. Kinetic experiments were done by incubating 1G5 cells with 100 nM PGE$_2$ for 2, 6, 8, and 24 h. In some experiments, 1G5 cells were pretreated for 1 h at 37°C with second messenger inhibitors such as H$_7$, HA-1004, BAPTA/AM, indomethacin, MDL-12,330A, and IBMX at subcytotoxic and subcytostatic concentrations prior to treatment with 100 nM PGE$_2$ for 8 h at 37°C. The inhibitor CAI requires a pretreatment of at least 8 h for optimum inhibition and shows no acute interference with the growth properties of the cells (47). All experiments were performed three times and luciferase activity was evaluated for each quadruplicate sample by a modified version of a previously published procedure (48). Briefly, following the incubation period, 100 µl of cell-free supernatant were withdrawn from each well and 25 µl of cell culture lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 1% Triton X-100, and 10% glycerol) were added before incubation at room temperature for 30 min. In some experiments, cell extract (10 µl) was mixed with 100 µl of luciferase assay buffer (20 mM Tricine, 1.07 mM MgCl$_2$, 0.5 mM MgCl$_2$, 2.67 mM MgSO$_4$, 0.1 mM EDTA, 270 µM coenzyme A, 470 µM luciferin, 530 µM ATP, and 33.3 mM dithiothreitol) and the sample was read in the counting chamber of a standard liquid scintillation counter equipped with a single-photon monitor software (Beckman Instruments, Fullerton, CA). Total photo-events were measured over a 30-s time lapse.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared by the DEAE-dextran—DEAE-dextran method (49). In brief, 1G5 cells (10$^6$) were either left untreated or were treated with PGE$_2$ (100 nM) or TNF-α (2 ng/ml) for 30 min at 37°C. The incubation of cells with the stimulating agents was terminated by the addition of ice-cold PBS and nuclear extracts were prepared according to the microscale preparation protocol (50). Sedimented cells were resuspended in 400 µl of cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol, and...
0.2 mM phenylmethylsulfonyl fluoride). After 10 min on ice, the lysate was vortexed for 10 s and the samples were centrifuged for 10 s at 12,000 × g. The supernatant fraction was discarded and the pellet was resuspended in 100 μl of cold buffer B (20 mM HEPESS-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. Cellular debris were removed by centrifugation at 12,000 × g for 2 min at 4°C and the supernatant fractions were stored at −70°C until used. Ten micrograms of nuclear extracts were used to perform electrophoretic mobility shift assay. Protein content was determined by the commercial BCA Protein Assay Reagent (Pierce, Rockford, IL). Nuclear extracts were incubated for 30 min at room temperature in 15 μl of buffer of a binding solution (100 mM HEPESS, pH 7.9, 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM dithiothreitol, 5 mM EDTA, 250 mM NaCl, 2 μg of poly(dI-dC), 10 μg of nuclelease-free bovine serum albumin fraction V) containing 1 ng of 59P-5'-end-labeled double-stranded (dsDNA) oligonucleotide. Double-stranded DNA (100 ng) was labeled with γ32P-ATP and T4 polynucleotide kinase in a kinase buffer (New England Biolabs, Beverly, MA). This mixture was incubated for 30 min at 37°C and the reaction was stopped with 5 μl of 0.2 mM EDTA. The labeled oligonucleotide was extracted with phenol/chloroform and passed through a G-50 spin column. The double-stranded DNA oligonucleotide, which was used as a probe, contained the consensus NF-κB-binding site corresponding to the sequence 5'-ATGTGAGGGGACTTTC-3' and was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Ca). DNA-NF-κB complexes were resolved from free labeled DNA by electrophoresis in native 4% (w/v) polyacrylamide gel containing 50 mM Tris-HCl (pH 8.5), 200 mM glycine, and 1 mM EDTA. The gel was subsequently dried and autoradiographed.

**Northern Blot Analysis**—Total RNA was extracted by the Trizol method (51, 52) from 1G5 and Jurkat E6.1 cells. Fifteen micrograms of total RNA were separated on formaldehyde-agarose gel (1% agarose, 1% formaldehyde gel buffer MOPS, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, 2.2% formaldehyde). RNA was transferred to Hybond-N nylon membranes (Amersham) by capillary action using 10 × SSC (3 M NaCl, 0.3 M sodium citrate). RNA was fixed to the membrane by UV exposure and hybridized with radiolabeled probes for the EP receptors (EP1, EP2, EP3, and EP4) at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 μg/ml denatured fragmented salmon sperm DNA. Blots were washed and autoradiographed at −70°C.

**Flow Cytometry**—Cell surface expression of hEP4 receptor was evaluated by flow cytometry as follow. Cells lines 1G5 and Jurkat E6.1 (5 × 106) were washed once in PBS containing 2% fetal bovine serum (PBS pH 7.4 + 2% fetal bovine serum (PBSA)). Cells were then resuspended in 100 μl of PBSA to which was added 0.5 μg of polyclonal rabbit anti-hEP4 antibody, vortexed gently, and incubated for 30 min on ice. Cells were subsequently washed with PBSA and resuspended in 100 μl of PBSA containing fluorescein isothiocyanate-labeled chicken anti-rabbit IgG antibody (0.5 μg total) and further incubated for 30 min on ice. Cells were finally centrifuged and resuspended in 1% paraformaldehyde in PBS before being analyzed by flow cytometry (EPICS XL, Coulter Corp., Miami, FL).

**Experiments with the EP4 Receptor-specific Antagonist AH 23848B**—Assays with AH 23848B were performed by incubating 1G5 cells with 30 μM AH 23848B for 1 h at 37°C (53). After this pretreatment, cells were incubated with 100 nM PGE2 for 8 h at 37°C. Experiments were performed three times and luciferase activity was monitored as described above.

**RESULTS**

**Modulation of HIV-1 LTR-driven Gene Expression in T Cells by PGE2**—In order to assess whether PGE2 could affect the regulatory elements of HIV-1, we initially set out a dose-response experiment which was performed with PGE2 in a 24-h incubation period. The results showed a steady increase of HIV-1 LTR activity in 1G5 cells starting at as little as 1 nM concentration of PGE2 (Fig. 1A). In these experiments, PGE2 was reconstituted in absolute ethanol to get a stock solution of 1 mM which was serially diluted in complete culture medium to get our working dilutions (1, 10, 100, and 1000 nM). Incubation of 1G5 cells with the concentration of ethanol corresponding to the one used with 1000 nM PGE2 resulted in a 1.5-fold increase of HIV-1 LTR dependent activity, while no effect was seen with the equivalent ethanol concentration for 100 nM PGE2 (data not shown). Therefore, subsequent experiments were carried out using 100 nM PGE2 to avoid any putative influence of ethanol on PGE2-mediated enhancement of HIV-1 LTR activity. Ketin analyses were next performed to determine the optimal incubation time for this PGE2-mediated HIV-1 LTR-driven activation. The maximal positive effect of PGE2 was seen 8 h after the initiation of treatment (fold enhancement of 7.4) (Fig. 1B). Although this type of kinetic might be reminiscent of degradation of PGE2, it is unlikely since similar kinetic of time-dependent HIV-1 LTR-driven luciferase activity were measured with activators such as TNF-α, PHA, and PMA (data not shown). Moreover, preincubation of PGE2 in complete culture medium for 24 h at 37°C resulted in equal fold-induction of HIV-1 LTR-driven luciferase activity in 1G5 cells as compared with incubation with fresh PGE2 (data not shown), which demonstrates that PGE2 is fairly stable under these experimental conditions. Taken together, these data indicate that activation of HIV-1 regulatory elements by PGE2 was rapid and transient, thereby suggesting that the effect was direct and was resulting from PGE2-mediated signal transduction events.

Given that PGE2 is generally seen as a down-modulator of T-cell activation, we were next interested in determining the action of PGE2 on typical pathways known to lead to HIV-1 LTR activation in T cells. 1G5 cells were hence stimulated with various HIV-1 LTR activators in the absence or the presence of PGE2 for 8 h. These stimuli were shown, as expected, to act as potent inducers of HIV-1 LTR activity (fold increase over un-
treated 1G5 cells: PHA, 20.8; OKT3, 16.8; PMA, 76.4; and TNF-α, 17.7) (Fig. 2). Again, a marked up-regulation of HIV-1 LTR-dependent luciferase activity was also seen when 1G5 cells were incubated with PGE2 alone (10.9-fold increase over untreated 1G5 cells). A PGE2-mediated activating effect on HIV-1 LTR was also present with all stimuli used in this set of experiments (fold increase over 1G5 cells treated with each stimulus in the absence of PGE2: PHA, 1.8; OKT3, 2.3; PMA, 1.2; and TNF-α, 4.3). It was thus apparent that PGE2 specifically synergized with TNF-α in activating HIV-1 LTR activity. These results clearly indicated that PGE2 could further increase the overall positive effect mediated by various HIV-1 LTR-activating agents and thus confirmed that PGE2 could be considered by itself as a potent inducer of HIV-1 LTR transcription in T cells.

The biosynthesis of prostaglandins is known to be regulated at different levels. The arachidonic acid, the precursor form, is stored in membrane phospholipids prior to its release into cells by phospholipase A2. Free arachidonic acid is then metabolized by cyclooxygenase to an intermediate that leads to the formation of prostaglandins (54). To exclude any effect by metabolized by cyclooxygenase-1 (0.4, 0.8, 2.0, and 10.0 µM) and cyclooxygenase-2 (150 µM) we pretreated 1G5 cells with indomethacin suggesting that luciferase activity could be detected with indomethacin (55). To mimic PGE2-induced HIV-1 LTR activation in T cells, 1G5 cells were pretreated with HA-1004, a serine/threonine kinase inhibitor that preferentially inhibits PKA (Ki = 2.3 µM) and PKG (Ki = 1.3 µM) over PKC (Ki = 40.0 µM) (56). The PGE2-mediated activation of HIV-1 transcription was almost completely abrogated by a pretreatment with HA-1004 at concentrations sufficient to inhibit both PKA and PKG, but not PKC (1 and 5 µM) (Fig. 3B). To clearly discriminate between PKA and PKG in the PGE2-induced signaling cascade, 1G5 cells were pretreated with a specific inhibitor of adenylate cyclase (MDL-12, 330A) (57–59). Activation of HIV-1 LTR-mediated reporter gene expression by PGE2 was totally abolished by concentrations of MDL-12,330A sufficient to completely inhibit cAMP activity (Fig. 3C). Results from these experiments hence demonstrated that PKA was an essential intracellular second messenger participating in the PGE2-dependent up-regulating effect on HIV-1 LTR activity in T cells.

Adenylate cyclase transforms adenosine triphosphate (ATP) in cyclic adenosine monophosphate (cAMP), which is necessary for PKA activity. Knowing that PKA activation is negatively modulated by phosphodiesterase due to transformation of cAMP to 5′-AMP, cells were pretreated with IBMX, an inhibitor of phosphodiesterase activity (60). It is hence presumed that PGE2-induced activation of PKA should be sustained for longer periods of time in cells treated with IBMX thus resulting in a greater stimulation of HIV-1 LTR-dependent gene activity. The increase in intracellular cAMP levels caused by the presence of IBMX indeed led to a dose-dependent enhancement of HIV-1 LTR-driven luciferase activity in cells treated with PGE2 (Fig. 3D). This PKA/cAMP-dependent activation of HIV-1 transcription is in agreement with previous studies (61, 62). It should be noted that treatment of 1G5 cells with IBMX alone (1, 5, and 10 µM) had no effect on HIV-1 LTR-dependent reporter gene activity (data not shown).

The implication of Ca2+ in this process was next investigated by pretreating 1G5 cells with increasing concentrations of BAPTA/AM (1, 5, and 10 µM), an intracellular Ca2+ chelator (63). The capacity of PGE2 to influence HIV-LTR activity was monitored as described above. Data from this experiment suggested that Ca2+ was partly involved in this process as the maximal subcytotoxic concentration of BAPTA/AM used (10 µM) could not totally eliminate PGE2-mediated activating effect on HIV-1 transcription (Fig. 3E). Finally a newly described inhibitor of calcium mobilization, carboxyamidotriazole (CAI) (47), was used to reinforce the implication of Ca2+ in the PGE2-induced activation of HIV-1 LTR. Data obtained from this set of experiments confirmed that Ca2+ is indeed an important component of the PGE2-initiated signaling cascade which culminates in activation of HIV-1 LTR-dependent gene expression (Fig. 3F). Altogether, the use of specific inhibitors allowed us to demonstrate that PKA, cAMP, and Ca2+ are all involved to some degree to PGE2-dependent positive effect on the regulatory elements of HIV-1.

To mimic PGE2-induced HIV-1 LTR activation in T cells, 1G5 cells were next treated for 8 h with dibutyryl-cAMP, a cAMP analog, along with the calcium ionophore, ionomycin. Results showed that treatment of 1G5 cells with both chemical compounds was not sufficient to trigger HIV-1 LTR activity (data not shown). However, a dose-dependent significant increase in HIV-1 LTR-driven luciferase activity was seen upon the addition of PMA, ionomycin, and increasing concentrations of dibutyryl-cAMP (25, 50, 100, and 200 µM) (Fig. 4). It should be specified that PMA alone was strongly activating HIV-1 LTR-driven gene activity because this agent is recognized as one of the most potent activator of NF-κB (64), a pleiotropic transcription factor complex known as a good inducer of HIV-1 expression (65). Therefore, these data suggested that up-regulation of

**Fig. 2. Activation of HIV-1 LTR-driven luciferase activity by several stimuli in the absence or presence of PGE2.** 1G5 cells were either left untreated (control) or treated with PHA (3 µg/ml), OKT3 (1 µg/ml), PMA (20 ng/ml), or TNF-α (2 ng/ml) in the absence (□) or presence (▲) of 100 nM PGE2 for 8 h. Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the means (± S.D.) of four determinations and are representative of three independent experiments.
HIV-1 LTR activity requires the activation of the transcription factor NF-κB in addition to cAMP- and calcium-dependent signaling pathways.

**NF-κB-dependent and -independent Signaling Pathways Are Involved in Activation of HIV-1 LTR by PGE_2**—To define the region(s) required for the activation of HIV-1 LTR transcription by PGE_2 at the molecular level, the parental Jurkat E6.1 cell line was transiently transfected with different HIV-1 LTR-driven luciferase constructs carrying either the full-length (pLTR-LUC) or modified versions of the HIV-1 LTR promoter (pmκBLTR-LUC and pκB-TATA-LUC). These latter constructs either contained the complete regulatory elements of HIV-1 mutated at the two NF-κB-binding sites (pmκBLTR-LUC) or the minimal HIV-1 κB region and a TATA box (pκB-TATATATA-LUC). Transiently transfected Jurkat E6.1 cells were then stimulated with TNF-α, PHA/PMA, or PGE_2 for 8 h. In these experiments, stimulation with TNF-α leads to the activation of NF-κB (66), while the combination of PHA and PMA can enhance HIV-1 LTR activity via both NF-κB-dependent and -independent signaling pathways (data not shown). As shown in Fig. 5A, we observed a 13.7-, 83.3-, and 12.1-fold increase in luciferase activity for the full-length HIV-1 LTR (pLTR-LUC) and pmκBLTR-LUC constructs, respectively. With the molecular construct pmκBLTR-LUC, as expected, no increase in HIV-1 LTR-driven gene activity was detected with TNF-α, while PHA/PMA was still inducing HIV-1 LTR-dependent luciferase activity (8.5-fold increase). Interestingly, the luciferase-encoding vector mutated at both NF-κB-binding sites of the LTR was still responding to PGE_2, although at a slightly lower level compared with the wild-type HIV-1 LTR construct (3.8-versus 12.1-fold). This experiment was repeated several times and gave consistent results. Therefore, data from experiments conducted with pLTR-LUC and pmκBLTR-LUC were indicating that PGE_2-mediated positive effect on HIV-1 LTR-driven activity required both NF-κB-dependent and -independent signal transduction pathways.

To confirm that nuclear translocation and activation of NF-κB was indeed induced by PGE_2, cells were transiently

![Fig. 3](image-url) Identification of second messengers implicated in PGE_2-mediated up-regulation of HIV-1 LTR activity. 1G5 cells were incubated for 1 h with H7 (1, 5, 10, and 20 μM) (panel A), HA-1004 (1, 5, 10, and 20 μM) (panel B), MDL-12,330A (50, 100, and 250 μM) (panel C), IBMX (1, 5, and 10 μM) (panel D), BAPTA/AM (1, 5, and 10 μM) (panel E), and for 16 h with CAI (0.1, 1.0, and 10 μM) (panel F) prior to treatment for 8 h with 100 nM PGE_2. Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the means (± S.D.) of four determinations and are expressed as fold induction relative to basal luciferase activity in untreated control cells (considered as 1). These results are representative of three independent experiments.
HIV-1 following its binding to the NF-κB-transactivating protein Tat in transcriptional activation of 1G5 cells were either left untreated (control) or treated for 8 h with ionomycin (1 μM), PMA (20 ng/ml), and increasing concentrations of dibutyryl-cAMP (0, 25, 50, 100, and 200 μM). Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the means (± S.D.) of four determinations and are expressed as fold induction relative to basal luciferase activity in untreated control cells (considered as 1).

transfected with pκB-TATA-LUC. It should be specified that this vector allows monitoring of HIV-1 activation almost exclusively via NF-κB-dependent mechanism. Indeed, this vector is made of the minimal HIV-1 NF-κB-binding domains and a TATA box driving the luciferase reporter gene (46). In this case, a 15.4-fold increase in HIV-1 LTR-dependent reporter gene activity was seen in cells treated with PGE2, thereby confirming that NF-κB is directly involved in the PGE2-dependent activating effect on the regulatory elements of HIV-1. To further substantiate the participation of NF-κB in PGE2-mediated up-regulation of HIV-1 LTR transcription, Jurkat E6.1 were transiently transfected with pNF-κB-LUC, a vector made of five consensus binding sites for NF-κB, prior to incubation with PGE2. The implication of NF-κB in the PGE2-mediated activating effect on HIV-1 transcription was again clearly shown using this κB-driven reporter gene construct (17.5-fold increase) (Fig. 6B). The involvement of NF-κB was also examined by mobility shift assays. Results shown in Fig. 6 revealed the presence of a band specific for NF-κB that is induced following the treatment for 1 h with either PGE2 or TNF-α (lanes 3 and 4, respectively). The specific band for NF-κB was eliminated by competition experiment with unlabelled probe for NF-κB. These results were thus reinforcing the notion that PGE2 is up-regulating HIV-1 LTR dependent activity also through a NF-κB-dependent mechanism.

ALFA Subtype in the Studied T Lymphoid Lineages—
PGE2 is known to bind to specific protein receptors on a large array of target cells.

Previous cDNA cloning and pharmacologic experiments have identified six different PGE2 receptors (EPs) (68). Northern blot analyses were then performed to evaluate PGE2 receptor(s) expression on the T lymphoid cell lines used in the present study. The cDNAs for hEP1, hEP2, hEP3, and hEP4 were hybridized with total RNA from 1G5 and Jurkat E6.1 cells. Results indicated that the hEP4 gene was expressed on both 1G5 and Jurkat E6.1 cell lines, while EP1, EP2, and EP3 subtype receptors were not expressed (data not shown). Flow cytometry analysis was also carried out with a polyclonal antibody specific for EP4 receptor and confirmed its presence on the surface of Jurkat E6.1 (Fig. 7A) and 1G5 (Fig. 7B) cells. The identity of the prostaglandin receptor on T lymphoid 1G5 cells was directly addressed using a subtype selective pharmacologic antagonist. For this purpose, 1G5 cells were pretreated with AH 23848B (30 μM), a selective antagonist of human EP4 receptor, prior to the addition of PGE2. AH 23848B was found to abrogate PGE2-mediated up-regulation of HIV-1 LTR activity in 1G5 cells (Fig. 8). Altogether these results indicate that the hEP4 receptor is involved in the PGE2-mediated activating effect on HIV-1 transcription in T lymphoid cells.

![Graph](image88x575to258x729)

**Fig. 4.** Activation of HIV-1 LTR-driven luciferase activity by a combination of dibutyryl-cAMP, ionomycin, and PMA. 1G5 cells were either left untreated (control) or treated for 8 h with ionomycin (1 μM), PMA (20 ng/ml), and increasing concentrations of dibutyryl-cAMP (0, 25, 50, 100, and 200 μM). Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the means (± S.D.) of four determinations and are expressed as fold induction relative to basal luciferase activity in untreated control cells (considered as 1).

![Graph](image354x387to508x729)

**Fig. 5.** NF-κB-dependent and -independent activation of HIV-1 LTR by PGE2. A, Jurkat E6.1 cells were transiently transfected with pLTR-LUC, pmsBLTR-LUC, or pκB-TATA-LUC and were either left untreated or were treated for 8 h with TNF-α (2 ng/ml), PHA/PMA (3 μg/ml and 20 ng/ml, respectively), or PGE2 (100 nm). B, Jurkat E6.1 cells were transiently transfected with pκB-TATA-LUC and were either left untreated or treated for 8 h with TNF-α (2 ng/ml), PHA/PMA (3 μg/ml and 20 ng/ml, respectively), and PGE2 (100 nm). Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the means (± S.D.) of four determinations and are expressed as fold induction relative to basal luciferase activity in untreated control cells (considered as 1). These results are representative of three independent experiments.

![Graph](image27311)

**Fig. 6.** Phorbol ester-dependent and -independent activation of HIV-1 LTR by PGE2. A, Jurkat E6.1 and 1G5 cells were either left untreated or were treated for 8 h with TNF-α (2 ng/ml), PHA/PMA (3 μg/ml and 20 ng/ml, respectively), or PGE2 (100 nm). B, Jurkat E6.1 and 1G5 cells were transiently transfected with pκB-TATA-LUC and were either left untreated or were treated for 8 h with TNF-α (2 ng/ml), PHA/PMA (3 μg/ml and 20 ng/ml, respectively), and PGE2 (100 nm). Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the means (± S.D.) of four determinations and are expressed as fold induction relative to basal luciferase activity in untreated control cells (considered as 1). These results are representative of three independent experiments.

![Graph](image27311)

**Fig. 7.** Activation of HIV-1 LTR activity by PGE2 in the T lymphoid cell lines used in the present study. The cDNAs for hEP1, hEP2, hEP3, and hEP4 were hybridized with total RNA from 1G5 and Jurkat E6.1 cells. Results indicated that the hEP4 gene was expressed on both 1G5 and Jurkat E6.1 cell lines, while EP1, EP2, and EP3 subtype receptors were not expressed (data not shown). Flow cytometry analysis was also carried out with a polyclonal antibody specific for EP4 receptor and confirmed its presence on the surface of Jurkat E6.1 (Fig. 7A) and 1G5 (Fig. 7B) cells. The identity of the prostaglandin receptor on T lymphoid 1G5 cells was directly addressed using a subtype selective pharmacologic antagonist. For this purpose, 1G5 cells were pretreated with AH 23848B (30 μM), a selective antagonist of human EP4 receptor, prior to the addition of PGE2. AH 23848B was found to abrogate PGE2-mediated up-regulation of HIV-1 LTR activity in 1G5 cells (Fig. 8). Altogether these results indicate that the hEP4 receptor is involved in the PGE2-mediated activating effect on HIV-1 transcription in T lymphoid cells.
Activation of HIV-1 LTR Activity by PGE$_2$

**FIG. 6.** Nuclear translocation and activation of NF-κB by PGE$_2$. Jurkat E6.1 cells were either left untreated or were incubated for 30 min with either 100 nM PGE$_2$ or 2 ng/ml TNF-α (positive control). The nuclear extracts were next incubated with a 32P-end-labeled synthetic double-stranded NF-κB probe. Lanes 1 and 2 are negative controls containing no extracts or extracts from untreated cells, respectively. Lane 3 represents a positive control containing cells stimulated with TNF-α (2 ng/ml), while lane 4 are cells treated with PGE$_2$. Lanes 5 and 6 represent a 100 X competition with the unlabeled probe for NF-κB with PGE$_2$ and TNF-α, respectively. The position of the specific complex bound by the κB site probe is indicated by an arrow on the left side.

**DISCUSSION**

Immune and inflammatory responses are triggered by microorganisms such as bacteria, viruses, and protozoan, all known to be potential opportunistic pathogens in HIV-1-positive patients. The formation and production of elevated levels of inflammatory mediators such as PGE$_2$ is a hallmark of the HIV-1 infection (28–30). Prostaglandins play a role in disease exacerbation by directly altering T-cell functions or macrophage activation. Although it was thought that PGE$_2$ is primarily an immunosuppressive molecule that acts as a downregulator of many aspects of B- and T-cell function and proliferation, recent findings support a role for PGE$_2$ as a potentiator of immunoglobulin class switching and cytokine and cytokine receptor synthesis (15). This PGE$_2$-dependent positive effect on the immune response and the observation that higher levels of PGE$_2$ are detected in HIV-1-infected individuals (2–5-fold increase) have been the compelling force for our investigation. Knowing that PGE$_2$ is a good inducer of cAMP and that a 4-fold increase in intracellular levels of cAMP is seen in asymptomatic HIV-1-seropositive subjects as compared with uninfected controls (69), it was thus of prime importance to study the putative effect of PGE$_2$ on the regulatory elements of HIV-1.

We therefore asked whether the proinflammatory PGE$_2$ molecule had the ability to modulate HIV-1 transcription in T cells. This particular cell type was chosen since T cells are considered to be a major cellular reservoir for HIV-1 in the human peripheral blood (70). In this report, we present evidence indicating that PGE$_2$ up-regulates HIV-1 LTR-driven reporter gene expression in human T cells (Fig. 1). Our results are indicative of an optimal signal after 6 to 8 h of treatment. This is very similar to time kinetics of HIV-1 LTR activation by PMA, PHA, and TNF-α agents which directly act on the HIV-1 promoter. Although we cannot refute a possible indirect mechanism for HIV-1 LTR activation by PGE$_2$ which would involve production of cytokines, time course experiments suggested that a more direct process might be at the basis of the effect of PGE$_2$ on HIV-1 LTR. Interestingly, a specific synergistic HIV-1 LTR activation was observed using both PGE$_2$ and TNF-α (Fig. 2). This might be accounted by the fact that the effect exerted by TNF-α is exclusively via NF-κB, while PGE$_2$ acts also on region(s) other than NF-κB in the HIV-1 LTR (see below). Previously described synergistic activation by NF-κB and other factors have been reported (66, 67). The induction of HIV-1 LTR activity by PGE$_2$ thus support a previous cellular study showing a 2.5-fold increase in virus production following the addition of exogeneous PGE$_2$ to MT-4 cells acutely infected with HIV-1 (43).

In the present study, the involvement of specific intracellular second messengers in PGE$_2$-mediated up-regulation of HIV-1 LTR activity has been dissected using several signal transduction inhibitors. Experiments with indomethacin, a potent inhibitor of the cyclooxygenase pathway and thus of PGE$_2$ production, suggests that the PGE$_2$-induced HIV-1 LTR transactivation is mediated by cyclooxygenase-derived prostaglandins, possibly both PGE$_2$ and PGE$_{3,4}$. The involvement of these molecules in the activation of HIV-1 LTR was further confirmed by using the selective cyclic nucleotide phosphodiesterase inhibitors, which inhibit intracellular cAMP turnover. However, the role of PGE$_2$ in HIV-1 LTR transactivation seems to be rather complex, since it has been shown that PGE$_2$ is able to inhibit HIV-1 LTR transcription in some cell lines, while it activates HIV-1 LTR in others.

**FIG. 7.** Cytometry analysis of PGE$_2$ receptor subtype 4 on studied cell lines. Flow cytometric analyses were performed using a saturating concentration of polyonal anti-human EP$_4$ antibody in combination with fluorescein isothiocyanate-labeled chicken anti-rabbit IgG and fluorescein isothiocyanate-labeled goat anti-chicken IgG antibodies (panel A, Jurkat E6.1 cells; and panel B, 1G5 cells). The solid lines represent background fluorescence.

**FIG. 8.** Pharmacological study of the PGE$_2$ receptor with the selective antagonist AH 23848B. 1G5 cells were either left untreated or were treated with increasing concentrations of the selective antagonist of human EP$_4$, AH 23848B (0, 5, 15, and 30 μM) for 1 h prior to incubation for 8 h with PGE$_2$ (100 nM). Next, luciferase activity was monitored as described under “Experimental Procedures.” Results shown are the means (± S.D.) of four determinations and are expressed as fold induction relative to basal luciferase activity in untreated control cells (considered as 1). These results are representative of three independent experiments.
duction, suggest that only exogeneous PGE\(_2\) plays a role in the activation of HIV-1 LTR-driven gene expression. These results were expected based on studies that T cells had a limited capacity to metabolize arachidonic acid to prostaglandins (71–73). An earlier report indicated that interaction between PGE\(_2\) and an adenylate cyclase-coupled stimulatory receptor leads to activation of adenylate cyclase, hydrolysis of ATP, enhanced turnover of intracellular cAMP, and binding to PKA (74). Our findings are clearly supportive of this signaling cascade since we found that PGE\(_2\)-induced enhancement of HIV-1 LTR dependent activity requires the participation of adenylate cyclase, cAMP, and protein kinase A (Fig. 3, A–D). Using MT-4 cells, another group has shown that elevation of cAMP levels resulted in HIV-1 replication (62). It is also well known that cAMP-dependent pathways regulate the immune effector functions of lymphocytes and macrophages. For example, during immune response, cAMP exhibits positive regulatory effects at low concentrations whereas inhibitory effects are seen at high concentrations (75). Many of the earlier studies have shown that PGE\(_2\) interaction with T cells in vitro resulted in an elevation of the cAMP level (35) and that such elevated intracellular cAMP levels were responsible for the proliferative disturbances in T cells (76–78). Data from our experiment with the calcium chelator BAPTA/AM and the calcium ionophor CAII are suggestive of the importance of Ca\(^{2+}\) in the PGE\(_2\)-induced activation of HIV-1 transcription (Fig. 3, E and F). However, given that there is no published report indicating Ca\(^{2+}\) influx through the EP\(_4\) receptor, our results with BAPTA/AM and CAII, two inhibitors of intracellular calcium mobilization, lead us to postulate that PGE\(_2\) could generate calcium release from intracellular storage organelles. All these results were supported by data shown in Fig. 4 indicating that up-regulation of HIV-1 LTR requires the implication of cAMP and calcium, as well as the participation of the NF-\(\kappa\)B transcription factor.

Several agents known as potent activators of HIV-1 transcription (e.g., PMA, PHA, TNF-\(\alpha\), and anti-CD3 antibody) are all acting through a common mechanism, namely via the nuclear translocation of the transcription factor NF-\(\kappa\)B which binds to the enhancer region of the HIV-1 LTR (79). This transcription factor is sequestered in the cytoplasm due to its physical association with the inhibitor named IxB. NF-\(\kappa\)B is a pleiotropic transcription factor that controls the expression of a wide variety of genes, including cytokines such as IL-1, IL-2, IL-6, IL-8, interferon-\(\beta\), and TNF-\(\alpha\), as well as known genes for some cell adhesion molecules including ICAM-1 and VCAM-1. Its importance in the regulation of HIV-1 gene expression has been stated in numerous studies (80). Results from mobility shift assays suggest that the PGE\(_2\)-mediated effect on HIV-1 LTR activity is due to activation of the transcription factor NF-\(\kappa\)B by PGE\(_2\). This is in agreement with the previous demonstration that PGE\(_2\) activates NF-\(\kappa\)B in the macrophage-like cell line J7/74 (81). The fact that we have noticed that both NF-\(\kappa\)B and Ca\(^{2+}\) are key elements in the PGE\(_2\) effect on HIV-1 transcription is of interest considering that calcineurin, a Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase, has been reported to activate NF-\(\kappa\)B through the inactivation of IxB (82). Moreover, researchers had earlier found that cAMP-mediated enhancement of PKA might be involved in the dissociation of IxB from NF-\(\kappa\)B (79). Recent studies have revealed that NF-\(\kappa\)B is regulated through phosphorylation of the p65 subunit by PKA which is directly regulated by intracellular levels of cAMP (83). Our experiments hence support the notion that PGE\(_2\) might be activating the transcription factor NF-\(\kappa\)B via cAMP/PKA and calcium signaling pathways in human T lymphoid cells. However, our experiments were performed with \(\kappa\)B-driven reporter gene constructs (p\(\kappa\)B-TAPA-LUC and p\(\kappa\)B-LUC) and HIV-1 LTR-based vectors (pLTR-LUC and p\(\kappa\)mBLTR-LUC), furthermore, suggest that NF-\(\kappa\)B-binding regions and another element(s) in the HIV-1 LTR are involved in the activation of HIV-1 LTR-dependent transcription induced by PGE\(_2\).

NFAT is an immediate-early activation factor that plays a crucial role in T-cell activation and commitment processes through its control of IL-2 gene activation (84). Based on the demonstrated synergistic effect between NFAT and NF-\(\kappa\)B on the activation of HIV-1 transcription (67) and the proposed PGE\(_2\)-induced NF-\(\kappa\)B-independent pathway, we looked at the putative role of NFAT in the effect of PGE\(_2\). We found that NFAT was not involved in PGE\(_2\)-dependent activation of HIV-1 LTR-driven luciferase activity. These data were expected considering that PGE\(_2\) has been reported to inhibit NFAT activity (85). PGE\(_2\) could have the capacity to modulate several signal transduction pathways through its effect on transcription factors regulated by cAMP such as the cAMP response-element binding factor, the activating protein-1 (38), and Sp1 (86). The involvement of these three transcription factors in the observed NF-\(\kappa\)B-independent activation of HIV-1 LTR mediated by PGE\(_2\) is currently under investigation.

Finally, by Northern blot assays, flow cytometric analyses, and pharmacological studies, we demonstrated that studied T lymphoid cell lines (Jurkat E6.1 and 1G5) express the EP\(_4\) receptor subtype on their surfaces and that EP\(_1\), EP\(_2\), and EP\(_3\) receptors seem not to be expressed (Figs. 7 and 8 and data not shown). This finding is in accord with previous studies that have found by Northern blot analysis that the EP\(_4\) gene is expressed on T lymphoid cells such as Molt-4, KM-3, and Jurkat E6.1 (87–89). It has been demonstrated that EP\(_4\) receptors are coupled to adenylate cyclase via a stimulatory G protein (G\(_\alpha\)) and that such activation results in an enhancement of intracellular cAMP levels (53, 68). Interestingly, PGE\(_2\) has been shown to lead to an increase in intracellular cAMP levels partly via the EP\(_4\) receptor (90), a finding which lend credence to the potential implication of the EP\(_4\) receptor in the PGE\(_2\)-induced up-regulation of HIV-1 LTR activity.

Because of their intrinsic intracellular obligatory parasitic form of life, viruses depend heavily on cell metabolic machinery for their replication. Thus, changes in cellular metabolism might influence the viral life cycle. The experiments reported here highlight the positive action of PGE\(_2\), a powerful cAMP-inducing agent, on the regulatory elements of HIV-1. The presented data suggest that elevated levels of PGE\(_2\) detected in HIV-1-infected persons or induced by opportunistic pathogens might actively participate to immunological disturbances associated with AIDS and modify the pathogenesis of this retroviral disease by inducing a higher viral load. Finally, high concentrations of PGE\(_2\) (up to 100 \(\mu\)M) found in seminal fluids of HIV-1-infected persons might directly enhance virus replication and facilitate viral transmission during sexual activities (40).

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