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PHORBOL ESTER-MEDIATED INDUCTION OF HIV-1 FROM A CHRONICALLY INFECTED PROMONOCYTE CLONE: BLOCKADE BY PROTEIN KINASE INHIBITORS AND RELATIONSHIP TO TAT-DIRECTED TRANS-ACTIVATION

Jeffrey Laurence,* Santosh K. Sikder,* Sanjiv Jhaveri,*
and Jane E. Salmon+

*Department of Medicine, Laboratory for AIDS Virus Research, Division of Hematology-Oncology, New York Hospital-Cornell Medical Center, New York, NY 10021

+The Department of Medicine, The Hospital for Special Surgery, New York, NY 10021

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Potent inhibitors of protein kinases C and A, including 1-(5 isoquinolinyl sulfonyl) 2-methyl piperazine (H7), staurosporine, and 2-aminopurine, depressed phorbol ester-induced HIV-1 virion production and HIV-specific transcripts by >90% in chronically infected promonocytic cells. Suppression was dose-dependent and occurred at concentrations that had little effect on cell growth. These effects appeared to be specific to activation of the PKC-diacylglycerol system. They did not alter IUdR-mediated induction of HIV. In addition, PMA enhancement of an HIV-LTR driven reporter gene was not blocked by H7 in the presence or absence of exogenous tat, at concentrations capable of inhibiting upregulation of virus at the cellular level. Insight into the biochemical mechanisms of these processes is critical to understanding interactions of HIV with the immune system, and may eventually uncover new therapeutic strategies.

Induction of HIV replication from chronically infected T lymphocytes and monocytes can be initiated through signals as divergent as herpesvirus exposure, monokine and lymphokine treatment, perturbation of the CD3/T cell receptor pathway, and PMA exposure (1-3). Activation of protein kinases appears to be a key step in the mechanism of these agents (3,4). For example, NF-kB is one enhancer protein capable of upregulating HIV replication, as PMA-regulated phosphorylation of its inhibitory factor, IkB, enables the active moiety to bind to sequences in the HIV LTR (5). However, PMA responsive elements other than for NFkB have been identified in viral sequences, shared with HIV, that affect diverse transcription factors (6,7). Proteins encoded by certain DNA viruses also augment

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expression from the HIV-LTR enhancer, utilizing a variety of cis-acting elements (8). These findings suggest multiple pathways that can be used to activate HIV, distinct from NF-kB or the tat gene product.

In this study we have concentrated on chronic HIV-1 infection in the monocyte and its relationship to protein kinases and tat. We present further evidence for divergent pathways for regulation of latent HIV, and explore their relationship to PKC inhibition.

MATERIALS AND METHODS

Cells. U1.1A cells were subcloned, by limiting dilution, from U1.1, a clone of U937.3 promonocytic cells infected with the lymphadenopathy-associated virus (LAV) strain of HIV (9). They contain, on average, two proviral copies of HIV-1, as determined by Southern analysis using probes from plasmids PBH5-2 and PBH8-1, which together encompass the entire HIV-1 provirus.

Reagents. Three protein kinase inhibitors of varying specificities were utilized. Staurosporine (Kamiya Biochem. Co., Thousand Oaks, CA) has an inhibition constant (Ki) for PKC of 0.0007μM, which is at least 10-fold lower than for the cyclic-AMP and cyclic-GMP dependent kinases (10,11). H7 (Sigma) has an equivalent Ki for PKC and PKA of approximately 6μM (10), as does 2-aminopurine (12) (Sigma).

Viral assays. HIV antigens were quantitated in supernatants by an ELISA-based assay for viral p24 core protein as previously described (13). The sensitivity of this assay is $\leq$60pg/ml.

The ability of the tat transcription unit of HIV to enhance the expression of a reporter gene, chloramphenicol acetyl transferase (CAT), when CAT is linked to the LTR of HIV was measured as previously detailed (14).

In situ hybridization for HIV structural and regulatory transcripts were performed using a linearized plasmid, pIIIextat, containing HIV tat and env, labelled with digoxigenin-11-dUTP. Procedures for DNA labelling by the random primer method, p-formaldehyde cell fixation, hybridization and detection have been detailed elsewhere (15,16).

RESULTS

Cultures of U1.1A cells were exposed to varying concentrations of two molecules capable of substituting for diacylglycerol, PMA and PDB (4 B-phorbol-12,13-dibutyrate), as well as two analogs, phorbol 13-acetate and 4B-phorbol, which do not activate protein kinases and do not alter inositol lipid metabolism (17). Culture supernatants were assayed for HIV p24 core antigen at 48h. A 4-20 fold increase in viral antigen occurred in the presence of PMA or PDB, while phorbol 13-acetate and 4B-phorbol had little or no effect (Table I).

The effect of PK inhibition on phorbol ester-mediated induction of HIV in U1.1A was evaluated using three different inhibitors. None led to a change in basal levels of HIV antigen. DNA synthetic responses were

Table I. Effect of phorbol esters on induction of HIV from chronically infected U1.1A cells *

Phorbol Ester	Concentration (ng/ml)	HIV-1 p24 core antigen ($pg/10^4$ cells)
None	-	2920
PMA	5	13,040 ± 1240
PMA	50	32,440 ± 4920
PMA	500	11,200 ± 600
PDB	5	31,880 ± 4920
PDB	50	59,400 ± 6320
PDB	500	46,840 ± 2800
phorbol 13-acetate	5	2480
phorbol 13-acetate	50	3600
phorbol 13-acetate	500	1040
4B-phorbol	5	4640
4B-phorbol	50	5040
4B-phorbol	500	3800

* Cells were plated at 1 x 10^4/microwell in 0.2 ml culture medium together with the appropriate concentration of phorbol ester. Supernatants were harvested 48h later and tested for p24 antigen by an ELISA-based antigen capture system. PMA and PDB data represent the mean ±SD of three separate experiments. Phorbol 13-acetate and 4B-phorbol data represent pooled samples of triplicate wells.

inhibited in a concentration-dependent manner, both in the absence and presence of PMA, but no alteration in vital dye exclusion capacity occurred at doses below the maximum utilized. Maximal suppression of approximately 30% of baseline response occurred with 100 μM H7, 100 μM 2-aminopurine, and 0.05 μM staurosporine. A dose-dependent inhibition of viral core antigen by H7, 2-aminopurine, and staurosporine was noted, with staurosporine the most potent on a molar basis. This resulted in a 60-80% inhibition of HIV using 50μM H7 or 2-aminopurine, and a >70% inhibition with 0.001μM staurosporine (Table II). These concentrations had little effect on cell proliferation.

A parallel effect on HIV specific transcripts was sought by in situ hybridization for tat and env mRNAs in cells exposed to PMA in the presence of varying concentrations of staurosporine. As shown in Fig. 1, approximately 0.5% of chronically infected U1.1A cells expressed HIV-specific mRNA, rising to >85% 24 h after exposure to 5 mg/ml PMA. Staurosporine in concentrations as low as 0.001 M led to complete blockade of HIV induction.

We attempted to relate the U1.1A/phorbol ester effect and its modulation by the various inhibitors used specifically to protein kinases

Table II. Effect of protein kinase inhibitors on induction of HIV-1 from U1.1A cells by PMA*

Expt.	PKC Inhibitor Concentration PMA	Agent	μM	HIV-1 activity+ [p24] core Ag (pg/10^4 cells)	Inhibition %
A	-	-	-	1,880	-
	+	-	-	14,660 ± 1,080	
	+	H7	10	13,540 ± 650	7.6
	+	H7	50	5,840 ± 2,590	60.2
	+	H7	75	2,150 ± 730	85.3
	+	H7	100	1,070	92.7
B	-	-	-	1,080	-
	+	-	-	13,770	-
	+	2-aminopurine	10	17,930	0
	+	2-aminopurine	50	2,890	79.0
	+	2-aminopurine	75	710	94.8
	+	2-aminopurine	100	640	95.4
C	-	-	-	63	-
	+	-	-	13,920	-
	+	staurosporine	0.0001	9,920	28.9
	+	staurosporine	0.001	3,760	73.3
	+	staurosporine	0.01	<60	100
	+	staurosporine	0.05	<60	100

*Cells were plated at 1 x 10^4/microwell in 0.2 ml culture medium together with the appropriate concentration of protein kinase inhibitor for 1 h, followed by addition of 5 ng/ml PMA. Supernatants were harvested 48 h later and p24 Ag concentration measured.

+ Values for p24 Ag represent the mean ±SD of two experiments for experiment A, and the mean of pooled triplicate wells for experiments B and C.

by employing another HIV inducer, IUdR, which does not affect these enzymes (1). U1.1A was exposed to IUdR (50 ug/ml) for 96h and culture supernatants analyzed for p24. HIV was upregulated by IUdR in the presence or absence of H7, with doses as high as 100uM having no effect in this system (Table III).

We subsequently employed a transient transfection assay to study the relationship of phorbol ester induction of HIV to tat-mediated trans-activation. As shown in Table IV, conversion of radiolabelled chloramphenicol to its acetylated forms was stimulated by PMA when the HIV-LTR-CAT plasmid was transfected into U1.1A cells. PDB was an even more potent stimulant in this system, while phorbol 13-acetate and 4B-phorbol had no effect (data not shown). This stimulation was much less pronounced in the absence of exogenous tat (Table IV), and was clearly synergistic with tat. The latter finding was redemonstrated in uninfected U937 cells

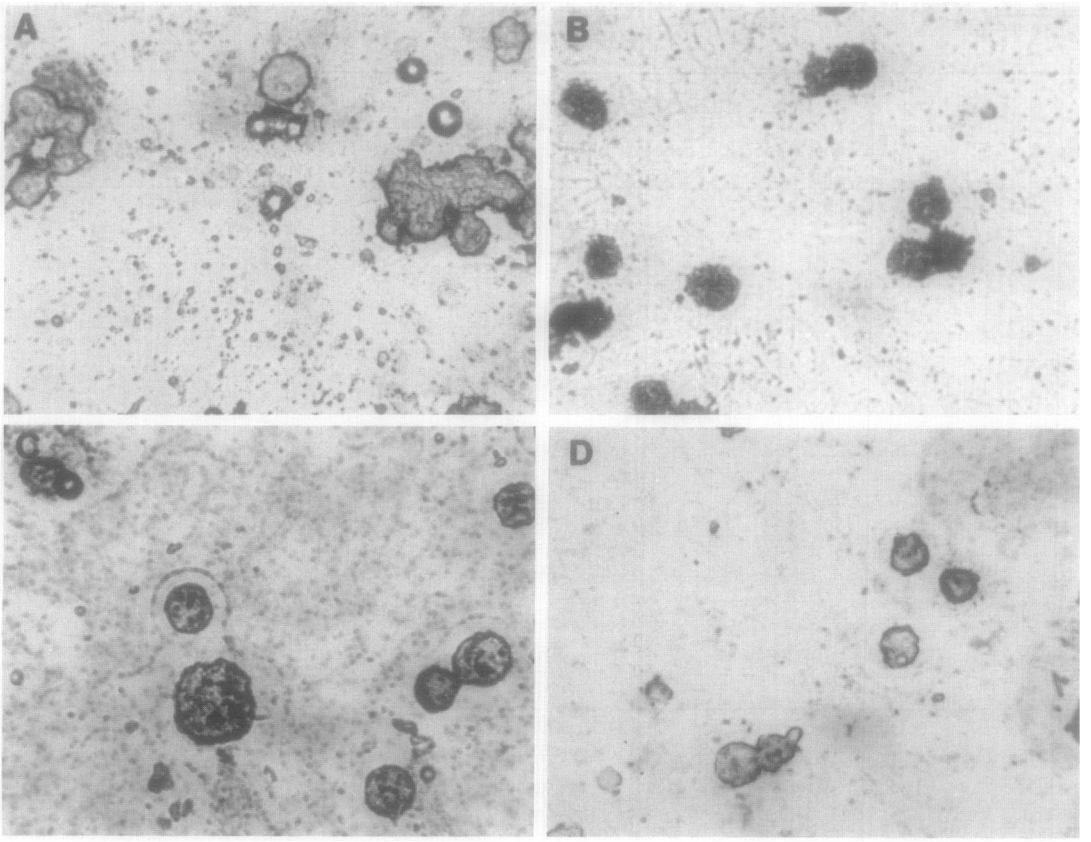

Fig. 1 U1.1A cells after hybridization with a digoxigenin-labeled HIV-tat-env probe in the presence of PMA (5 ng/ml) and varying concentrations of staurosporine. Blue-black precipitates are formed at sites of RNA accumulation in the cytoplasm. A: control, buffer only; B: PMA; C: PMA + 0.001 uM staurosporine; D: PMA + 0.01 uM staurosporine.

Table III. Effect of the Protein Kinase Inhibitor H7 on Induction of HIV-1 U1.1A Cells by Iododeoxyuridine*

Condition	IUdR	H7	HIV-1 activity [p24] core Ag (pg/10^4 cells)	HIV-1 activity Mean change (%) from control
1	-	-	3,584	-
	-	-	1,540	
2	+	-	14,618	+623.8
	+	-	17,346	
3	+	+	15,314	+683.6
	+	+	19,712	

*Cells were plated at 1 x 10^4/microwell in 0.2 ml culture medium together with buffer of 100 uM H7 for 1-2 h at 37°C, followed by addition of 50 ug/ml IUdR. Supernatants were harvested after a 16 h incubation and p24 antigent concentration assessed. Two separate experiments are represented for each condition.

Table IV. Effect of the protein kinase inhibitor H7 on upregulation of HIV-LTR-CAT activity by PMA in U1.1A cells in the presence and absence of exogenous tat*

Exp't.	PMA concentration (ng/ml)	H7 concentration (uM)	Plasmids transfected HIV-LTR/CAT	Plasmids transfected tat	CAT activity	Inhibition %
A	-	-	+	-	1.1	-
	50	-	+	-	17.2	-
	50	10	+	-	16.8	2.3
	50	50	+	-	20.0	0
	50	100	+	-	29.6	0
B	-	-	+	+	29.4	-
	50	-	+	+	86.6	-
	50	10	+	+	87.7	-
	50	50	+	+	92.7	0
	50	100	+	+	92.5	0
C	-	-	+	+	12.2 ± 2.3	-
	5	-	+	+	31.2 ± 1.4	-
	5	10	+	+	31.8 ± 5.2	0
	5	50	+	+	26.9 ± 0.4	13.8

*2 x 10^6 U1.1A cells per condition were exposed to DNA from each plasmid for cotransfections or salmon sperm DNA for controls (tat) negative cultures). 2ug of DNA were used in experiments A and C, and 1ug in experiment B. Phorbol esters were present at the start of transfection and throughout the 48 h culture period.

+CAT activity represents the conversion of [^{14}C]chloramphenicol into its acetylated forms. Each value is the mean ±SD of two acetylation assays, run on separate TLC plates but utilizing the same pool of cell lysate.

(Table V). Introduction of tat gave a 4-fold enhancement of CAT in the absence of PMA. PMA itself led to a 4-fold enhancement of LTR-CAT, but tat plus PMA caused a >70-fold increase in trans-activation.

Table V. Effect of the Protein Kinase Inhibitor H7 on PMA-mediated Upregulation of HIV-LTR-CAT Gene Activity in Uninfected U937 Cells

PMA	H7 concentration (uM)	Plasmids transfected HIV-LTR/CAT	Plasmids transfected tat	Relative CAT activity	Inhibition %
-	-	+	-	1.0	-
+	-	+	-	3.8	-
-	-	+	+	4.2	-
+	-	+	+	74.0	-
+	50	+	+	64.0	13.5
+	100	+	+	57.0	23.0

*Cultures were established and co-transfections performed as described in the legend to Table IV, except that uninfected U937.3 cells were used in place of their chronically infected counterpart, U1.1A. PMA concentration was 50ng/ml. Relative CAT activity was calculated by normalizing the percent conversion of [^{14}C]chloramphenicol in the absence of tat, 0.38%, to 1.

In terms of PKC inhibition, doses of H7 (Tables IV and V) or staurosporine (data not shown) capable of blocking HIV induction by 60-90% had little to no effect on PMA-mediated upregulation of HIV-LTR directed CAT activity, either in latently infected U1.1A cells, or in uninfected, parental U437 cells (Table V).

DISCUSSION

We have demonstrated that phorbol ester-mediated induction of HIV from a chronically infected cell is dependent on protein kinase activity, most probably PKC, and is susceptible to protein kinase inhibitors. We have also investigated its relationship to pathways associated with tat-mediated trans-activation.

We were able to distinguish the phorbol ester induction effect from another pathway of HIV rescue, mediated through the halogenated pyrimidine IUdR. Only the former appeared to be protein kinase dependent. While protein kinases consist of a family of structurally related proteins that differ in tissue distribution, cofactor dependency, and substrate specificity, all subtypes are inhibited by H7 and 2-aminopurine, bind PDB with similar affinity, and are activated by similar concentrations of PDB (18), permitting generalization of the phenomenon to this family of proteins. The dose of H7 which inhibited 50% of viral replication is between 10 and 50μM, which correlates with its Ki for PKC and PKA. The IC-50 for staurosporine was approximately 0.0005μM. Given the latter's increased specificity for PKC over other protein kinases (10,11), the effects observed were most likely related to this enzyme.

We also explored the relationship between the phorbol ester effect and tat-mediated trans-activation. Two major differences were apparent: the absence of significant inhibition of PMA-mediated activation of the HIV-LTR by H7, in the presence or absence of tat, at concentrations which block HIV induction; and the synergism between PMA-mediated activation of the HIV-LTR and tat activity. This synergism is consistent with the existence of two distinct modes of activation, and would be unexpected if both agents worked in an identical manner.

Our inhibition results are consistent with those from other cell systems. In one study, concentrations of H7 >200μM were required before inhibition of PMA-driven, HIV-LTR-CAT/tat interaction was seen (7), while in another, $\geq$100μM was used (19). These data, obtained with such high concentrations of H7, should not be used to define a PKC-linked phenomenon, given the lack of specificity of H7 for PKC, its inhibition of DNA synthetic responses or cytotoxicity, and the fact that these are far above the IC-50 for protein kinases.

These data also argue against the involvement of a cyclic AMP-mediated kinase in the PMA/tat trans-activation phenomenon. A cAMP-dependent PKA pathway has been identified in the activation of a related retrovirus, human T cell lymphotropic virus type I (HTLV-I) (20). PKC can, in turn, either enhance or antagonize the effects of PKA (20). However, the fact that H7 had no effect on tat-mediated trans-activation in our system, while it blocks both PKC and PKA with similar Ki's, and the fact that adenyl-cyclase regulators such as foreskolin and 8-bromo-CAMP have no effect on the HIV-LTR (20), suggest that PKA does not have a role in the regulation of chronic HIV infection.

There are, however, several caveats which pertain to this model. It is possible that actively replicating cells respond differently than chronically infected but non-transformed human monocytes. Preliminary data with human peripheral monocytes indicate that similar types of induction can be obtained in such cells derived directly from HIV seropositive carriers using PMA in concert with lipopolysaccharide (Ho, J. and J. Laurence, unpublished observations), or in acutely infected peripheral monocytes using various cytokines (21). Whether analogs of PMA or other PKC activators could induce chronic HIV-1 in vivo is unclear, but it is testable in transgenic murine models bearing HIV-LTR-CAT and tat constructs (22). The present findings that regulation of HIV in a monocyte line is mediated in part through PKC may suggest a possible approach to pharmacological intervention with anti-PKC agents in clinical situations.

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