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COMPARISON OF EFFECTS OF PROTEIN KINASE C INHIBITORS ON PHORBOL ESTER-INDUCED CD4 DOWN-REGULATION AND AUGMENTATION OF HUMAN IMMUNODEFICIENCY VIRUS REPLICATION IN HUMAN T CELL LINES

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SUMMARY: The potent protein kinase C inhibitors staurosporine, H-7, and UCN-01 were investigated for their effects on 12-0-tetradecanoylphorbol-13-acetate(TPA) -mediated CD4 down-regulation and on the augmentation of human immunodeficiency virus (HIV) expression. Staurosporine was the most effective TPA inhibitor for both of these actions. Because of its high cytotoxicity, the effect of H-7 on augmentation of HIV expression could not be determined. UCN-01 had no cytotoxic effect, but caused only little inhibition of the augmentation of HIV expression.

It is known that 12-0-tetradecanoylphorbol-13-acetate(TPA) can bind with and activate protein kinase C (PKC) (1-4). Recent evidence has shown that TPA down-regulation of the CD4 molecule, which is known to be the receptor for human immunodeficiency virus (HIV) (5,6), occurs in T cells and thymocytes in association with phosphorylation of the CD4 molecule (7-9). We have previously reported that TPA facilitated HIV replication in MOLT-4/HIV cells, a human T cell line chronically infected with HIV (10). Recently, it has been demonstrated that the effect of TPA on HIV is closely associated with activation of the HIV enhancer (11), and that the effect is blocked by H-7, a PKC inhibitor (12). However, these results were obtained from studies at the transcriptional level using a transfection technique. It has not been demonstrated whether or not PKC inhibitors can block TPA-mediated augmentation of HIV replication in HIV-infected cells.

Among a number of PKC inhibitors, staurosporine has been shown not to block TPA-mediated ornithine decarboxylase induction, whereas H-7 efficiently inhibited it (13). However, due to the strong cytotoxic action of H-7, it is

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unclear whether its inhibitory effect was actually due to inhibition of PKC activity or not. We examined the effects of three PKC inhibitors on TPA-induced CD4 down-regulation and on the enhancement of HIV expression. The characteristics of each compound are discussed in this report.

MATERIALS AND METHODS

Cells: Cells of the human T cell line MOLT-4 (14) and MOLT-4/HIV cells (15) (MOLT-4 cells chronically infected with HIV) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (complete medium).

Chemicals: TPA (Sigma, Chemical Co., St. Louis, MO) and H-7 (Seikagaku Kogyo, Tokyo, Japan) were stored at -20°C as stock solutions in complete medium of 1ug/ml and 1mM, respectively. Staurosporine and UCN-01 were kindly supplied by Kyowa Hakko Co., Ltd. Tokyo, Japan, and were stored at -20°C at a concentration of 1 ug/ml in complete medium.

Flow cytometric analysis: Staining of CD4 and CD8 molecules on the cell surface was conducted by using the Simultest T Helper/Suppressor Test (Becton Dickinson Immunocytometry Systems, Mountain View, CA). In brief, 5 x 10^5 cells were treated with 20 µl of Simultest T Helper/Suppressor at 4°C for 30 min. After incubation, cells were extensively washed with phosphate-buffered saline (pH 7.4) containing 2% fetal bovine serum and resuspended in 500 µl of the solution. They were then analyzed by laser flow cytometry (Epics Profile: Coulter Electronics Inc., Hialeah, FL).

Assay for HIV p24 antigen: HIV p24 (gag) antigen, in a cell-free culture supernatant, was quantitated by a commercially available enzyme immunoassay kit (Abbott laboratories, North Chicago, IL), according to the manufacturer's instructions.

Determination of viable cell numbers: Viable cell numbers were determined microscopically by the exclusion of 0.1% trypan blue dye.

RESULTS AND DISCUSSION

H-7 (16), staurosporine (SS) (17), and UCN-01 (18) have been reported to block the activity of PKC efficiently, with Ki values (concentration of half maximal inhibition) of 6.0 µM, 2.7 nM, and 4.1 nM, respectively. To compare their effects on the TPA-induced down-regulation of the CD4 molecule, MOLT-4 cells were pre-treated with various concentrations of these blockers for 30 min before incubation with 10 ng/ml of TPA. Figure 1 shows the flow cytometric analysis for cell surface CD4 and CD8 molecules after TPA treatment for 1 hr both in the presence and absence of 20-fold Ki for PKC of each chemical. CD4 molecules on the untreated cells had a mean relative fluorescence intensity (MRFI) of 56. This was reduced to 15 by TPA (10 ng/ml) treatment (unstained:2.7), whereas the MRFI for CD8 changed little under these conditions. SS almost abolished TPA-mediated CD4 down-modulation (MRFI of 49), while H-7 and UCN-01 inhibited very little (MRFI of 21 in either case). Essentially similar results were obtained with another T cell line, CCRF-CEM

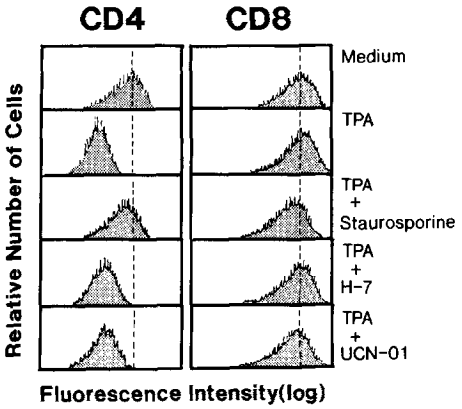

Figure 1. Effect of PKC inhibitors on TPA-mediated CD4 down-modulation. MOLT-4 cells were treated with 10 ng/ml TPA for 1 hr in the presence or absence of various inhibitors (20-fold Ki). Inhibitors were added 30 min before TPA. CD4 and CD8 fluorescence was determined as described in "Materials and Methods". Representative data of five separate experiments are shown.

(data not shown). Next, we examined the effect of these PKC inhibitors on TPA-mediated augmentation of HIV expression in MOLT-4/HIV cells. As shown in Figure 2(A), the level of HIV p24 antigen (HIV gag gene product) in cell-free culture fluid 24 hr after treatment with 10 ng/ml TPA was 5.3-fold greater than without TPA. It is clear from this figure that H-7 at concentrations 20-fold over the Ki ("20-fold Ki") effectively inhibited the TPA-induced expression of p24. Twenty-fold Ki SS was not as effective as H-7 but the production of p24 was reduced to less than twice that of the TPA-untreated control. UCN-01 was less effective than either H-7 or SS. TPA alone caused a 25% decrease in viable cell numbers compared with medium alone, while 20-fold Ki and 10-fold Ki H-7 markedly decreased viable cell numbers compared with TPA alone (Fig. 2B). In contrast, SS did not show such an effect. UCN-01 showed no additional cytotoxic effect at any concentration tested, but had only a slight inhibitory effect on the enhancement of HIV expression by TPA. These results suggested that the decrease in p24 production with high concentrations of H-7 might be explained by its cytotoxicity. Table 1 shows the effect of each PKC inhibitor alone on the growth of MOLT-4/HIV cells. Ten-fold Ki H7 was apparently cytotoxic after 24 hr and 20-fold SS showed cytotoxicity by 48 hr, whereas UCN-01 had no cytotoxic effect at least within 48 hr.

Thus, the effects of the three PKC inhibitors on proliferating cells differed at concentrations causing PKC inhibition in a cell-free system. SS had the most potent anti-TPA effect in the present cell culture systems. H-7 was not suitable for these studies because of its relatively high cytotoxicity, while UCN-01 was both less cytotoxic and less potent in its anti-TPA effect.

It has been reported that H-7 preferentially blocks various signals through the PKC-dependent pathway, including T cell activation in association with activation of the nuclear factor kB (NF-kB) (11,12), HIV- and TPA-induced phosphorylation of the CD4 molecule (19), and interleukin-6

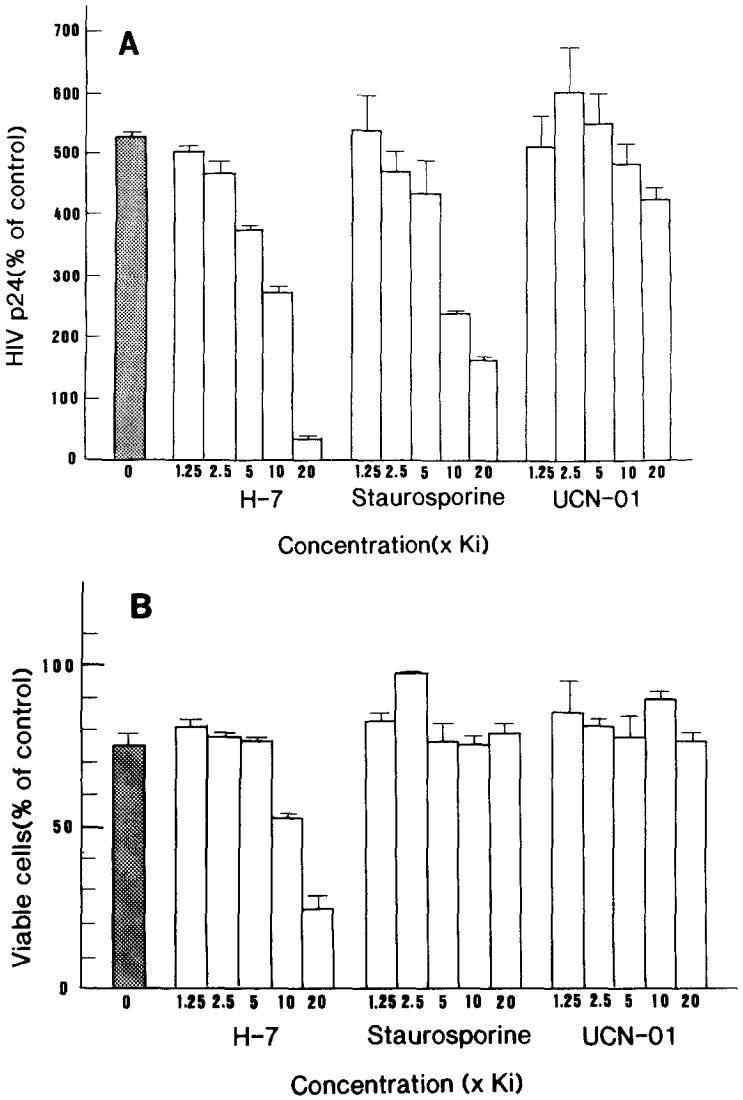

Figure 2. Effect of PKC inhibitors on cell growth and HIV expression in TPA-treated MOLT-4/HIV cells. MOLT-4/HIV cells (6×10^5 /ml) were treated with 10 ng/ml of TPA for 24 hr in the presence (open column) or absence (dotted column) of various concentrations of PK inhibitors, which were added 30 min before TPA. Then HIV p24 levels (A) and viable cell numbers (B) were determined as described in "Materials and Methods". In control cultures containing neither TPA nor PK inhibitor, we detected 87 ng/ml of HIV p24 in cell-free supernatant and a viable cell number of 13.3×10^5 /ml, and each of them was expressed as 100%. Each value represents the mean ± SD (n=2).

gene expression enhancement (20). In addition, H-7 has been shown to inhibit HIV infection in peripheral T-lymphocytes by 80% (19). In these experiments H-7 was used at concentrations of 10-100 uM, similar to those that we used. The differences in the efficacy of the anti-TPA action of these PKC inhibitors might be due to the variations in permeability or stability of these inhibitors. The differences in the results we obtained for CD4 down-

Table 1
Effect of protein kinase inhibitors on growth of MOLT-4/HIV cells

concn.[2] (x Ki)	% of control viable cell number[1]					
	24 hr			48 hr		
	H-7	SS	UCN-01	H-7	SS	UCN-01
20	20±2	94±3	99±12	7±1	75± 6	99± 4
10	68±1	98±5	101± 4	49±3	95± 4	96±10
5	90±7	99±3	97± 7	89±0	108± 4	97± 1
2.5	106±8	107±1	96± 2	98±6	94±10	97±10
1.25	106±4	104±8	108± 6	95±1	95± 9	98± 2

Cells were seeded (6 x 10^5 cells/ml) and then incubated in the presence or absence of a variety of concentrations of protein kinase inhibitors for 48 hr. Viable cell numbers were determined as described in "Materials and Methods."
[1] The control was incubated in medium alone. The viable cell numbers 24 and 48 hr after culture were 116 x 10^4/ml and 226 x 10^4/ml, respectively. Values represent the mean ± S.D. (n=2).
[2] concentration.

modulation compared with other authors might be due to the different cells used.

Little is known about the role of PKC systems in HIV-infected cells. From this point of view, PKC inhibitors are useful for elucidating the mechanism of HIV expression as well as determining other cellular functions of PKC, such as T cell activation. For this purpose, potent, selective and low cytotoxicity PKC inhibitors are required.

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