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Dual Activity of Pyrrolidine Dithiocarbamate on κB-Dependent Gene Expression in U937 Cells: I. Regulation by the Phorbol Ester TPA

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ABSTRACT. Pyrrolidine dithiocarbamate (PDTC) has been widely used as an inhibitor of the nuclear factor-κB (NF-κB) signalling pathway. Here, we show that κB-dependent reporter gene expression induced by low concentrations of 12-O-tetradecanoylphorbol-13-acetate (TPA) is potentiated by PDTC in the human promonocytic U937 cell line. The stimulatory effect of PDTC on κB-dependent gene expression was shown with a 4 × κB chloramphenicol acetyltransferase construct and required an intact κB element in the human immunodeficiency virus long terminal repeat (HIV-1 LTR). Unexpectedly, an HIV-1 LTR construct with a mutation of the activator protein 2 (AP-2) binding site located between the two κB elements was unresponsive to the stimulatory effect of PDTC with TPA. The stimulation or inhibition of κB-dependent gene expression was dependent on PDTC pre-treatment and the concentration of TPA. No stimulatory effect on HIV-1 LTR activity was observed with the metal chelator dipyridyl or the anti-oxidant *N*-acetyl-L-cysteine. These results are consistent with the hypothesis that PDTC treatment potentiated κB-dependent gene expression in a manner dependent on the concentration of TPA. CELL SIGNAL 11;7:479–489, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Phorbol ester, Dithiocarbamates, NF-κB, HIV-1 LTR, Gene regulation

INTRODUCTION

The biological effects of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) are mediated by a calcium and phospholipid-dependent protein kinase C (PKC). Evidence that PKC is a family of structurally related isotypes, differing in tissue expression, substrate specificity, subcellular distribution and activation requirements emphasised the critical role of this enzyme in signal transduction [1].

TPA was shown to induce a signal transduction cascade important in the regulation of gene expression at the transcriptional level through transcription factors, such as nuclear factor-κB (NF-κB) [2, 3]. The NF-κB transcription factor is composed of homodimeric and heterodimeric complexes of related proteins of the Rel superfamily [4, 5]. NF-κB is sequestered in the cytoplasm in a latent form stabilised by the inhibitory subunit IκB-α [6]. Diverse stimuli acting through the phosphorylation of IκB-α define a pathway for the translocation of NF-κB into the nucleus, where it binds to the κB sequence motif of target genes [5]. A large number of studies showed that the transcriptional activity of the human immundeficiency virus long terminal repeat (HIV-1 LTR) is dependent on two κB sequence elements [4, 6]. Studies with specific NF-κB subunits showed functional protein–protein interactions at the level of the HIV-1 LTR, between NF-κB family members [7] and Sp1; [8], activator protein 2 (AP-2) [9], AP-1 [10], p53 [11] and CAAT enhancer binding protein (C/EBP) [12]. Thus, protein–protein interactions at the transcriptional level define an additional site of gene regulation where our knowledge of the mechanisms responsible for the integration of multiple signals is limited.

In the present study, we observed a relation between pyrrolidine dithiocarbamate (PDTC) pre-treatment and the concentration of TPA that demonstrated both a stimulatory and an inhibitory activity on HIV-1 LTR and κB-dependent gene expression in the U937 cell line. We investigated the mechanism responsible for the stimulatory effect because the anti-oxidant activity of PDTC has been shown in a variety of different systems [13–15] to suppress the activation of NF-κB and stimulate AP-1 activity. In this paper, we describe evidence for the unexpected finding that PDTC stimulated κB-dependent gene expression.

MATERIALS AND METHODS

Cell Culture and Reagents

U937 cells were obtained from the ATCC; they were expanded, and frozen stocks were prepared. Cells were routinely grown for approximately 1 month before new cells were established from frozen stocks. U937 cells were grown in RPMI

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1640 supplemented with 10% FBS, 2 mM L-glutamine (GIBCO BRL, Life Technology Inc., Grand Island, NY), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Sigma Co., St. Louis, MO), ($RPMI_{10}$). TPA, PDTC, diethyl dithiocarbamate (DDTC), dipyridyl (DPY) and *N*-acetyl-L-cysteine (NAC) were from Sigma Co. TPA was dissolved in DMSO to produce a 1-mg/mL master stock. A working stock solution of 100 μg/mL was prepared in DMSO and used to prepare all other dilutions in $RPMI_{10}$ for the treatment of cells. Stock solutions of PDTC and NAC were dissolved in phosphate buffered saline (PBS), and pH was adjusted to 7.2 to produce a 1 M stock.

Electroporation of U937 Cells and Chloramphenicol Acetyltransferase (CAT) ELISA

U937 cells (4×10^7) were washed once in PBS and then once in serum-free RPMI 1640 ($RPMI_0$), and the cell pellet was resuspended in 0.4 mL of $RPMI_0$. The cell suspension (0.4 mL) was then transferred to the electroporation cuvette (Gene Pulser cuvette with 0.4-cm electrode; Bio-Rad, Hercules, CA). Cells were electroporated at 250 μF and 300 V with 10 μg of the pHIVCAT plasmid (obtained from P. Berg, Stanford University, Stanford, CA); the construction of this plasmid was previously described [16]. A pHIVCAT construct containing mutated κB sites [17] and a 4 × κBCAT construct containing four κB binding sites [18] were obtained from G. Nabel (University of Michigan, Ann Arbor, MI). In some experiments, cells were electroporated with 10 μg of the p4 × AP-1CAT plasmid. The expression of this construct was shown to be dependent on four AP-1 (four tandem oligonucleotides composed of 5′-GATCG TGACTCAGCGCG-3′; AP-1 site underlined) recognition sequences (CAT gene is placed downstream of a human metallothionein IIA minimal promoter). The 4 × AP-1 CAT plasmid was originally named $p(AP\text{-}1)_4MCAT2$ by V. Baichwal, University of California, Berkeley, CA [19]. Ten minutes after electroporation, cells were diluted into $RPMI_{10}$ medium and aliquoted 1 mL per well (8×10^5 cells/mL) into 24-well multiwell plates. Twenty-four hours later, cells were pre-treated with PDTC or NAC or DPY for 1 h by adding 1 mL of a 2× solution, then stimulated with TPA by adding 220 μL of a 10× solution. Twenty-four hours later, cells were harvested, cell extracts were prepared by three cycles of freeze-thawing in 0.25 M Tris, pH 7.8, and protein was determined by the method of Bradford (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA). The amount of CAT protein in cellular extracts was determined with the use of a CAT ELISA kit (Boehringer Mannheim, Indianapolis, IN) and a MAXline microplate reader (Molecular Devices, Menlo Park, CA). The amount of CAT protein was expressed in nanograms per millilitre and normalised to 100 μg of cellular protein.

Wild-Type and Mutant HIV-1 LTR CAT Plasmids

To evaluate the sequence elements required for a stimulatory effect of PDTC with TPA, HIV-1 LTR constructs with site-specific mutations in the κB or AP-2 sites were used. U937 cells (4×10^7) were electroporated with 10 μg each construct, and, 24 h postelectroporation, duplicate wells were treated with 0, 0.25, 0.5, 1 or 10 ng/mL of TPA, alone or after a 1-h pre-incubation with PDTC (200 μM). CAT expression was determined after 24 h by measuring CAT activity by the conversion of radiolabelled [^{14}C]chloramphenicol (Amersham Life Science, Inc., Arlington Heights, IL) into its acetylated derivatives by thin-layer chromatography [20]. The CAT ELISA could not be used, because of the lower activity of the mutant constructs. The wild-type HIV-1CAT plasmid and mutant constructs were obtained from G. Nabel (University of Michigan, Ann Arbor, MI). HIV-1 CAT with wild-type κB elements (5′-GGGACTTT CCGCTGGGGACTTTCC-3′; κB elements underlined) or mut-κB (**TCT**ACTTTCCGCTG**TCT**ACTTTCC; bold face type indicates the mutated sites) were described previously [17]. The site-directed mutagenesis of the HIV-1CAT plasmid was described for the mut-3′κB (GGGACTTTCC GCT**AGATCT**TTTCC) or mut-3′κB + 5′Sp1 [GGGA CTTTCCGCT**AGATCT**TTTCC; the 5′Sp1 site (underlined) was changed from GAGGCGTGGCC to GA**ACT CGAG**CC] [21] or mut-AP-2 (GGGACTTTCC**ATATG** GGACTTTCC) construct [9]. In some experiments, triplicate samples of cells were treated and changes in CAT activity were evaluated by statistical analysis by Student's *t*-test for paired samples. Values of $P < 0.05$ were accepted as significantly different. CAT activity values were expressed as percent conversion of [^{14}C]chloramphenicol into its acetylated derivatives. The amount of TPA-induced CAT activity from each construct was normalised per 200 μg of cellular protein and an 8-h CAT enzyme reaction.

Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from 10^7 cells by a rapid procedure [22]. For measuring NF-κB DNA binding activity, a κB oligonucleotide consisting of the 3′κB sequence from the HIV-1 LTR (5′-GATCCAGAGGGGACTTTCCAA GAGG-3′; κB site underlined) or HIV oligonucleotide consisting of the 5′ and 3′κB sequence from the HIV-1 LTR (5′-GATCACAAGGGACTTTCCGCTGGGGACTTT CCAAGAGG-3′; κB site underlined) was used for the electrophoretic mobility shift assay (EMSA). For measuring AP-1 DNA binding activity, a previously described AP-1 oligonucleotide (5′-GATCCAAAAAAGCATGAGTCA GACACC-3′; AP-1 site underlined) was used [23]. Oligonucleotides were annealed with the complementary strand to create the 5′-overhanging ends (*Bam H1*), which allowed labelling by Klenow polymerase in the presence of dNTPs, and with [^{32}P]dCTP (3000 Ci/mmol; Amersham) for EMSA. The labelled double-stranded oligonucleotide probes were purified on push columns (Stratagene, La Jolla, CA). The typical binding reaction of 20 μL contained 10,000 cpm of [^{32}P]labelled double-stranded oligonucleotide, 10 μg of nuclear extract in buffer C (20 mM HEPES-KOH, pH 7.9; 420

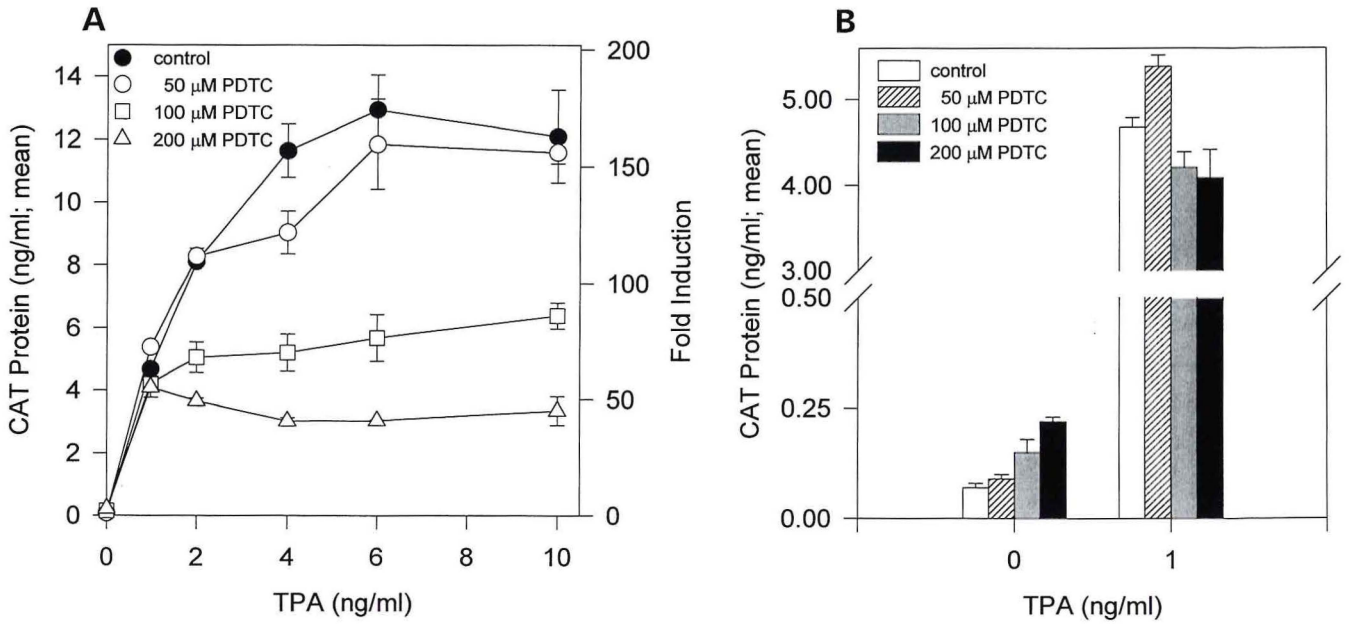

FIGURE 1. The effect of PDTC on TPA-induced HIV-1 LTR activity is dependent on the concentration of TPA. The human promonocytic cell line U937 was electroporated with the pHIVCAT plasmid, pre-treated for 1 h with the indicated concentrations of PDTC and then stimulated with TPA for 24 h. CAT protein was determined after a 24-h incubation, as described in the Materials and Methods section. The results shown are from a representative experiment and represent the (A) mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample standard deviation of duplicate cultures (data also shown as the fold induction with the data normalised to the untreated control). (B) Effect of PDTC on HIV-1 LTR activity with 0 or 1 ng/mL of TPA. Note change in scale from that of part A.

mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 25% glycerol) and final concentrations of 15 mM HEPES-KOH, 105 mM NaCl, 0.4 mM $MgCl_2$, 0.1% NP-40, 1.3 mM DTT, 0.05 mM PMSF,1 mg/mL BSA, 0.1 mg/mL poly dI-dC and 6.25% glycerol. Poly dI-dC was used to eliminate non-specific binding. Specific binding was demonstrated by competition with a 100- to 200-fold excess of unlabelled double-stranded oligonucleotide competitor. After incubation for 20 min at room temperature, samples were analysed on a 4% native acrylamide gel run at 170 V for 1.5 h in 0.5× TBE buffer. Gels were dried and visualised by autoradiography.

RESULTS

Effect of PDTC on TPA-Induced HIV-1 LTR Activity

A transient expression assay was used to investigate the effect of PDTC pre-treatment on TPA-induced HIV-1 LTR-directed expression of the chloramphenicol acetyltransferase reporter gene in the U937 cell line. U937 cells were electroporated with the pHIVCAT plasmid; 24 h after electroporation, cultures were pre-treated with 0, 50, 100 or 200 μM concentrations of PDTC for 1 h, then various concentrations of TPA were added, and CAT protein was determined after an additional 24-h incubation. Figure 1A shows the effect of PDTC and TPA treatment on CAT protein expression (data normalised to 100 μg of protein) or as the fold induction (data normalised to the untreated control). In U937 cells, the HIV-1 LTR-directed CAT expression was highly responsive to TPA. As shown in Fig. 1A, 1.0 ng/mL of TPA increased the CAT protein level 67-fold (from 0.07 ± 0.01 to 4.68 ± 0.11 ng/mL). With increasing concentrations of TPA, the induction reached a maximum of >180-fold (Fig. 1A). PDTC, in a concentration-dependent manner, inhibited the TPA-stimulated increase in CAT protein. However, as shown in Fig. 1B, PDTC alone, in a concentration-dependent manner, stimulated the level of CAT protein 3.1-fold (from 0.07 ± 0.01 to 0.22 ± 0.01 ng/mL with 200 μM PDTC) over the untreated control. The stimulatory effect of PDTC was unexpected. Interestingly, there was little stimulatory or inhibitory effect of PDTC pre-treatment on the level of CAT protein in the samples treated with 1 ng/mL of TPA (Fig. 1B). These data provided evidence for a threshold, regulated by TPA, important for the stimulatory effect of PDTC.

We next evaluated the effect of PDTC pre-treatment with ≤1-ng/mL concentrations of TPA on HIV-1 LTR-directed CAT expression (Table 1). TPA or PDTC treatment for 24 h had no significant effect on cellular protein (Table 1). PDTC pre-treatment increased a marginal increase in CAT protein induced by 0.25 ng/mL of TPA over the untreated control value from 1.5- to 11-fold with 200 μM PDTC. Interestingly, the stimulatory effect of PDTC decreased with increasing concentrations of TPA. Moreover, 200 μM PDTC inhibited the amount of CAT protein induced by TPA in a manner consistent with the hypothesis of a threshold, regulated by TPA, important for the stimula-

TABLE 1. Effect of PDTC on TPA-induced HIV-1 LTR and 4 × κBCAT activity

Treatment[a]		Cell protein[b] (mg/sample)		HIV-1CAT CAT protein[c] (ng/mL)			4 × κBCAT CAT protein (ng/mL)		
PDTC (μM)	TPA (ng/mL)	Mean	SD	Mean	SD	Fold[d]	Mean	SD	Fold
0	0.00	0.28	0.02	0.04	0.02	1.0	0.02	0.01	1.0
	0.25	0.26	0.01	0.06	0.01	1.5	0.17	0.01	8.5
	0.50	0.26	0.03	0.23	0.04	5.8	0.94	0.23	47.0
	0.75	0.24	0.01	0.93	0.07	23.3	2.59	0.14	129.5
	1.00	0.22	0.02	2.64	0.61	66.0	3.38	0.16	169.0
	10.0	0.20	0.03	9.22	0.39	230.5	11.41	1.13	570.5
50	0.00	0.22	0.01	0.05	0.01	1.3	0.03	0.01	1.5
	0.25	0.23	0.01	0.12	0.02	3.0	0.45	0.03	22.5
	0.50	0.22	0.02	0.59	0.15	14.8	2.10	0.21	105.0
	0.75	0.22	0.01	1.81	0.23	45.3	3.59	0.06	179.5
	1.00	0.20	0.02	3.98	0.54	99.5	4.14	0.01	207.0
	10.0	0.19	0.01	10.32	0.76	258.0	8.35	0.94	417.5
100	0.00	0.26	0.03	0.07	0.01	1.8	0.05	0.01	2.5
	0.25	0.24	0.01	0.27	0.02	6.8	0.56	0.15	28.0
	0.50	0.20	0.02	1.20	0.07	30.0	1.34	0.13	67.0
	0.75	0.18	0.02	2.39	0.04	59.8	2.08	0.11	104.0
	1.00	0.19	0.02	3.05	0.03	76.3	2.28	0.20	114.0
	10.0	0.25	0.04	4.19	0.05	104.8	2.60	0.06	130.0
200	0.00	0.25	0.01	0.09	0.03	2.3	0.07	0.01	3.5
	0.25	0.24	0.01	0.44	0.02	11.0	0.41	0.03	20.5
	0.50	0.22	0.01	1.13	0.04	28.3	1.15	0.07	57.5
	0.75	0.18	0.03	1.77	0.23	44.3	1.21	0.22	60.5
	1.00	0.20	0.03	1.75	0.06	43.8	1.53	0.30	76.5
	10.0	0.25	0.03	2.18	0.50	54.5	1.72	0.47	86.0

[a] U937 cells were electroporated with the pHIVCAT or p4 × κBCAT plasmid, pre-treated for 1 h with the indicated concentrations of PDTC and then stimulated for 24 h; CAT protein was determined after a 24-h incubation.

[b] Cellular protein was determined in duplicate from duplicate samples ($n = 4$).

[c] The values represent the mean CAT protein (ng/mL) determined by CAT ELISA and normalized to 100 μg of cellular protein ± the sample standard deviations.

[d] The values represent the fold induction with the data normalized to the untreated control.

tory effect of PDTC. These data demonstrated that the stimulation or inhibition of HIV-1 LTR-dependent gene expression was dependent on PDTC pre-treatment and the concentration of TPA.

Effect of PDTC on TPA-Induced κB-Dependent Gene Expression

To determine whether the stimulatory effect observed with PDTC in combination with low concentrations of TPA was unique to the HIV-1 LTR, we evaluated the effect of PDTC with a 4 × κBCAT construct containing four κB binding sites. U937 cells were electroporated with the p4 × κBCAT plasmid and pre-treated with PDTC for 1 h, TPA was added, and CAT protein was determined after a 24-h incubation. As shown in Table 1, pre-treatment with 200 μM PDTC in the absence of TPA showed approximately a 3.5-fold increase over the untreated control. Treatment with 0.25 ng/mL of TPA stimulated CAT protein 8.5-fold over the untreated control, and PDTC increased the effect of 0.25 ng/mL TPA approximately 3-fold (Table 1). In general, the stimulatory effect of PDTC with the 4 × κBCAT construct was similar to that observed with the HIV-1 LTR. These data show that pre-treatment with PDTC potentiated the effect of low concentrations of TPA on the level of CAT protein with both the HIV-1 LTR and 4 × κBCAT constructs.

Effect of PDTC on TPA-Induced CAT Activity of Wild-Type and Mutant HIV-1 CAT Constructs

To evaluate the sequence elements required for the stimulatory effect of PDTC with TPA, HIV-1 LTR constructs with site-specific mutations in the κB or AP-2 site were used (see the Materials and Methods section). Cells were electroporated with 10 μg of each construct; 24 h postelectroporation, duplicate wells were treated with 0, 0.25, 0.5, 1 or 10 ng/mL of TPA, alone or after a 1-h pre-incubation with PDTC (200 μM). CAT expression was determined after 24 h by measuring CAT activity by the conversion of radiolabelled [^{14}C]chloramphenicol into its acetylated derivatives by thin-layer chromatography. CAT activity was measured because the lower level of CAT expression from the mutant constructs prevented the use of the CAT ELISA. By measuring CAT enzyme activity, we found no stimulatory effect of PDTC in the absence of TPA treatment with the wild-type or mutant constructs (Table 2). This finding suggests that the CAT ELISA may be a more sensitive assay for de-

TABLE 2. Effect of PDTC on TPA-induced CAT activity of wild-type and mutant HIV-1 LTR constructs

Treatment[a]		CAT activity[a]									
		HIV-1(wt)[c]		Mut-κB		Mut-3′κB		Mut-3′κB + 5′Sp1		Mut-AP-2	
±PDTC (200 μM)	TPA (ng/mL)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
−	0.00	4.65	1.23	0.21	0.02	9.20	0.24	1.46	0.10	2.17	0.14
+		3.69	0.23	0.19	0.04	9.68	0.64	1.92	0.04	2.06	0.86
−	0.25	5.13	0.11	0.23	0.03	11.36	0.40	1.86	0.06	4.69	0.38
+		10.43	0.57	0.32	0.05	26.16	0.80	4.20	0.26	4.95	0.55
−	0.50	16.65	4.45	0.35	0.02	20.00	1.12	3.20	0.46	9.77	1.24
+		22.40	0.30	0.35	0.05	30.80	1.20	6.78	1.18	5.00	0.79
−	1.00	41.75	2.45	0.39	0.02	39.44	2.80	6.64	0.10	25.39	0.64
+		29.30	0.70	0.40	0.06	30.08	1.20	6.98	1.08	6.11	0.14
−	10.0	84.30	0.50	1.33	0.08	209.60	2.40	27.80	0.10	72.86	2.31
+		31.75	0.75	0.47	0.04	29.92	1.04	5.78	0.10	5.56	1.56
PDTC (μM)[d]											
0	0.00					3.81	0.36			2.36	0.66
50						3.39	0.12			2.24	0.35
100						3.97	0.54			3.33	1.12
200						4.05	1.07			2.59	0.36
0	0.25					3.39	0.38			2.87	0.26
50						5.15	0.05	*0.01*[e]		4.52	0.46
100						6.69	0.58	*0.03*		5.82	0.98
200						6.53	0.30	*0.004*		4.13	1.13

[a] CAT activity values represent the mean ± the sample standard deviations from duplicate samples. The percent conversion of [^{14}C] chloramphenicolin into its acetylated derivatives was normalized to 200 μg of cellular protein and an 8-h CAT enzyme reaction.

[b] U937 cells were electroporated with 10 μg of the indicated plasmid, pre-treated for 1 h with the indicated concentrations of PDTC, and then stimulated with various concentrations of TPA for 24 h; CAT activity was determined after a 24-h incubation.

[c] HIV-1 CAT with wild-type κB elements (5′-GGGACTTTCGCTGGGGACTTTCC-3′; κB elements underlined) or mut-κB (TCTACTTTCCGCTG TCTACTTTCC) or mut-3′κB (GGGACTTTCCGCTAGATCTTTTCC) or mut-3′κB + 5′Sp1 (GGGACTTTCCGCTAGATCTTTTCC; the 5′Sp1 site (underlined) was changed from GAGGCGTGGCC to CAACTCGAGCC) or mut-AP-2 (GGGACTTTCCATATGGGACTTTCC); boldface type represents the mutated sites.

[d] Cells were treated as indicated and triplicate samples were harvested for CAT assay.

[e] Statistical analysis by Student's *t*-test for paired samples. Values of $P < 0.05$ are shown.

tecting small changes in basal CAT expression. However, samples pre-treated with 200 μM PDTC and stimulated with 0.25 ng/mL of TPA showed a marked increase in CAT expression from the wild-type, mut-3′κB and mut-3′κB + 5′Sp1 constructs (Table 2). Notably, with these constructs, the stimulatory effect of PDTC markedly decreased with increasing concentrations of TPA, consistent with the previous results by measuring CAT protein by the CAT ELISA. In agreement with results in Jurkat T cells [21], there was little effect of the mutation of the 3′κB element in the mut-3′κB construct on the induction of HIV-1 LTR-dependent CAT protein by TPA. In Jurkat T cells, the mutation of the 3′κB element in combination with its adjacent Sp1 site in the mut-3′κB + 5′Sp1 construct completely prevented induction by TPA [21]. In U937 cells, the response of the mut-3′κB + 5′Sp1 construct to TPA was markedly decreased compared with the wild-type, mut-3′κB and mut-AP-2 constructs. Significantly, the stimulatory effect of PDTC was not observed with an HIV-1 LTR construct containing mutations in both NF-κB binding sites (mut-κB, Table 2). The low basal activity and poor TPA response of the mut-κB construct are consistent with a requirement for the NF-κB binding site in mediating the stimulatory effect of PDTC. Unexpectedly, a construct that changed the wild-type κB elements from 5′-GGGACTTTCCGCTGGGGACTTTCC-3′ (κB elements underlined) to 5′-GGGACTTTCCATATGGGACTTTCC-3′ (site-specific mutations in boldface type) by a mutation in the AP-2 binding site was unresponsive to the stimulatory effect of PDTC with 0.25 ng/mL of TPA. Significantly, the mut-AP-2 construct remained inducible by TPA, in agreement with the results in Jurkat T cells [9].

Additional studies were done for statistical analysis with cells electroporated with the mut-3′κB or mut-AP-2 construct treated in triplicate and CAT assays performed from individual samples. PDTC pre-treatment induced a significant difference ($P < 0.05$) in CAT expression from the mut-3′κB construct with samples stimulated with 0.25 ng/mL of TPA (Table 2). There was no significant difference in CAT expression from the mut-3′κB construct by PDTC without stimulation by TPA. There was no significant difference in CAT expression from the mut-AP-2 construct by PDTC with or without 0.25 ng/mL of TPA (Table 2).

Effect of PDTC on TPA-Induced NF-κB DNA Binding Activity

In other studies, we showed that PDTC induced NF-κB DNA binding activity in mouse L cells that was additive with the response with tumour necrosis factor-α (TNF) [24]. To determine if there was an effect of PDTC on NF-κB DNA

binding activity in the U937 promonocytic cell line, we evaluated nuclear extracts after a 1-h treatment by electrophoretic mobility shift analysis. As shown in Fig. 2A, NF-κB DNA binding activity to the κB probe was induced by TPA treatment. The lower band represents a non-specific DNA binding complex, as shown by its ability to be competed by oligos containing the κB, AP-1 or SP1 binding site in both control and TPA-treated nuclear extracts. As shown by others, the upper band represents the TPA-induced NF-κB DNA binding activity. The TPA-induced binding complex was supershifted by the p65 antibody; the p50 antibody had a weak but detectable effect resulting in a slight smearing (Fig. 2B). These data suggest that p65 (RelA) is the major form of NF-κB in U937 nuclear extracts prepared after a 1-h TPA treatment. A 1-h PDTC treatment did not stimulate NF-κB DNA binding activity (Fig. 2B and C) but inhibited the induction of NF-κB by 10 ng/mL of TPA (Fig. 2C). The results shown in Fig. 2D demonstrated that TPA-induced NF-κB DNA binding activity was only weakly detectable after 1 h with 2 ng/mL of TPA. The concentration-dependent induction of NF-κB DNA binding activity by TPA appeared to require TPA concentrations ≥4 ng/mL. To measure the activation of NF-κB by low concentrations of TPA, we evaluated the effect of a 1-, 2-, 6- or 24-h treatment with 1 or 10 ng/mL of TPA. NF-κB DNA binding activity was weakly detectable by EMSA in nuclear extracts only after a 6-h incubation and not detectable 24 h after treatment with 1 ng/mL of TPA. Treatment with 10 ng/mL of TPA resulted in the activation of NF-κB DNA binding activity at 1–6 h, which was not detectable after 24 h of TPA treatment (data not shown). EMSAs were next done with an HIV probe to determine if two κB binding sites were required for the detection of a binding complex affected by PDTC. As shown in Fig. 2E and F, the NF-κB DNA binding activity to the HIV probe was induced by a 1-h treatment with TPA. The binding complex was supershifted by p65, a slight smearing was observed with the p50 antibody and the pattern was unaffected by PDTC treatment. As shown in Fig. 2E, but more clearly in Fig. 2F, a non-specific band present in the untreated extracts co-migrated with the TPA-induced NF-κB DNA binding activity. The detection of the TPA-induced NF-κB DNA binding activity to the κB (Fig. 2B) or HIV (Fig. 2F) probe required TPA concentrations ≥4 ng/mL. These data show that PDTC pre-treatment potently inhibited the induction of NF-κB binding to the κB or HIV probe.

Because PDTC has been shown to induce AP-1 activity, we next evaluated the form of TPA-induced AP-1 DNA binding complex by EMSA with antibodies to c-Jun, JunB, c-Fos or p65. As shown in Fig. 2G, only the c-Fos antibody supershifted the upper band; the lower band was a non-specific DNA binding complex (data not shown). Interestingly, the concentration-dependent induction of both NF-κB (Fig. 2D and F) and AP-1 (Fig. 2H) DNA binding activity by TPA appeared to require TPA concentrations ≥4 ng/mL.

Stimulation of AP-1 DNA Binding and AP-1-Dependent Reporter Gene Activity by PDTC

PDTC induced AP-1 DNA binding activity, as measured by EMSA, in nuclear extracts after a 1-h treatment of U937 cells (data not shown) and, in a concentration dependent manner, PDTC pre-treatment potentiated the effect of TPA on AP-1-dependent (4 × AP-1CAT) reporter gene expression (Fig. 3).

Effect of PDTC Pre-incubation Time on TPA-Induced HIV-1 LTR Activity

To further define the conditions required for the stimulatory effect of PDTC on κB-dependent gene expression, we next evaluated the effect of increasing the pre-incubation time with PDTC on the potentiation of κB-dependent CAT expression observed with low concentrations of TPA. U937 cells were electroporated with the pHIVCAT plasmid and pre-treated with 200 μM PDTC for 1, 4 or 17 h before 0 h (defined as 24 h postelectroporation). TPA was added at 0 h, and CAT protein was determined after an additional 24-h incubation (Fig. 4). The stimulatory effect of 200 μM PDTC appeared maximal after a 1-h pre-incubation. Increasing the pre-incubation time from 1 to 4 or 17 h had little effect on the amount of TPA-induced CAT protein.

Metal Chelator DPY and Antioxidant NAC Inhibit TPA-Induced HIV-1 LTR Activity; Stimulatory and Inhibitory Activity of Dithiocarbamates

PDTC has metal-chelating in addition to anti-oxidant properties. To evaluate the chemical property of PDTC that was responsible for the stimulatory effect, we next examined the effect of the metal chelator dipyridyl on TPA-induced HIV-1 LTR activity in U937 cells. U937 cells were electroporated with the pHIVCAT plasmid and pre-treated with DPY at 50, 100 or 200 μM for 1 h; TPA was added, and CAT protein was determined after a 24-h incubation. The results (Fig. 5A) demonstrated that DPY only inhibited the induction of CAT protein by TPA. The inhibitory effect was greater with concentrations of TPA ≥0.75 ng/mL. To determine whether the stimulatory effect of PDTC was due to an anti-oxidant effect, we evaluated the effect of the anti-oxidant NAC on TPA-induced HIV-1 LTR activity. Samples pre-treated with 1, 10, 20 or 50 mM NAC showed only a concentration-dependent reduction in TPA-induced CAT expression (Fig. 5B). These data suggest that the metal-chelating or anti-oxidant properties of PDTC were unlikely to be responsible for the stimulatory activity of PDTC.

Dithiocarbamates such as PDTC are well known to exert pro-oxidant [25] and anti-oxidant [14, 15] effects. To determine if the stimulatory effect was unique to dithiocarbamates, we examined the effect of diethyldithiocarbamate on TPA-induced HIV-1 LTR activity (Fig. 5C). Samples pre-treated with 0, 50, 100 or 200 μM DDTC resulted in a stimulatory effect with TPA, which, like that of PDTC, was dependent on the concentration of TPA. Interestingly, pre-

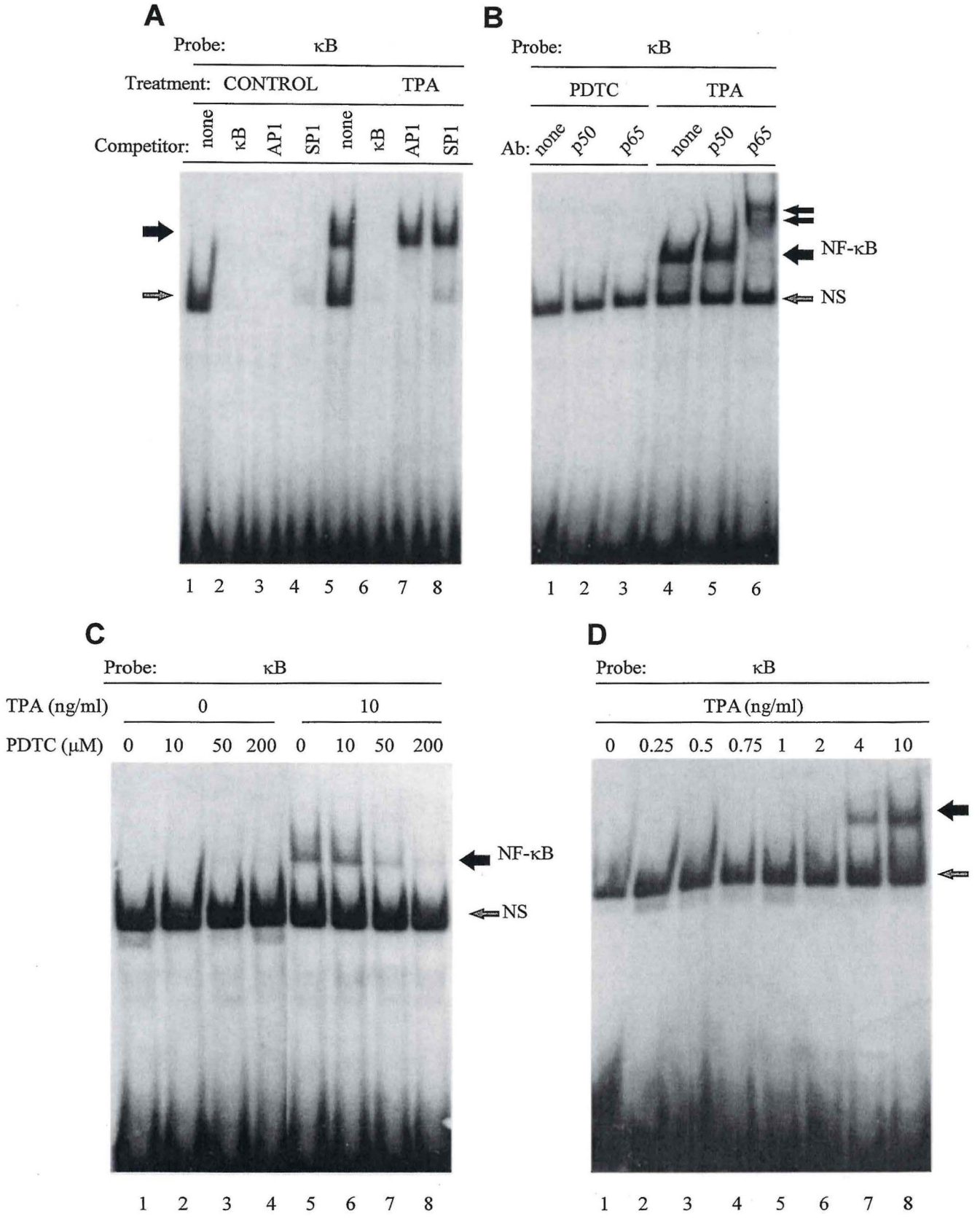

FIGURE 2. Effect of TPA on NF-κB and AP-1 DNA binding activity. Nuclear extracts were prepared from cultures treated as indicated for 1 h. (A–D) NF-κB DNA binding activity to the κB probe. (A) NF-κB binding in control (lanes 1–4) or TPA-treated (lanes 5–8) samples. DNA binding specificity shown by competition with oligos containing the κB, AP-1 or SP-1 binding site (non-specific binding (NS) indicated by the small shaded arrow). (B) No effect of 200 μM PDTC (lanes 1–3) versus strong effect of TPA (lanes 4–6) on the activation of NF-κB activity measured with the κB probe. The TPA-induced NF-κB DNA-binding complex was supershifted weakly by the p50 and strongly by the p65 antibody (doublet of arrows points to the p65-supershifted doublet (lane 6). (C) PDTC inhibits the induction of NF-κB by TPA. (D) The effect of TPA concentration on the induction of NF-κB DNA binding activity to the κB probe.

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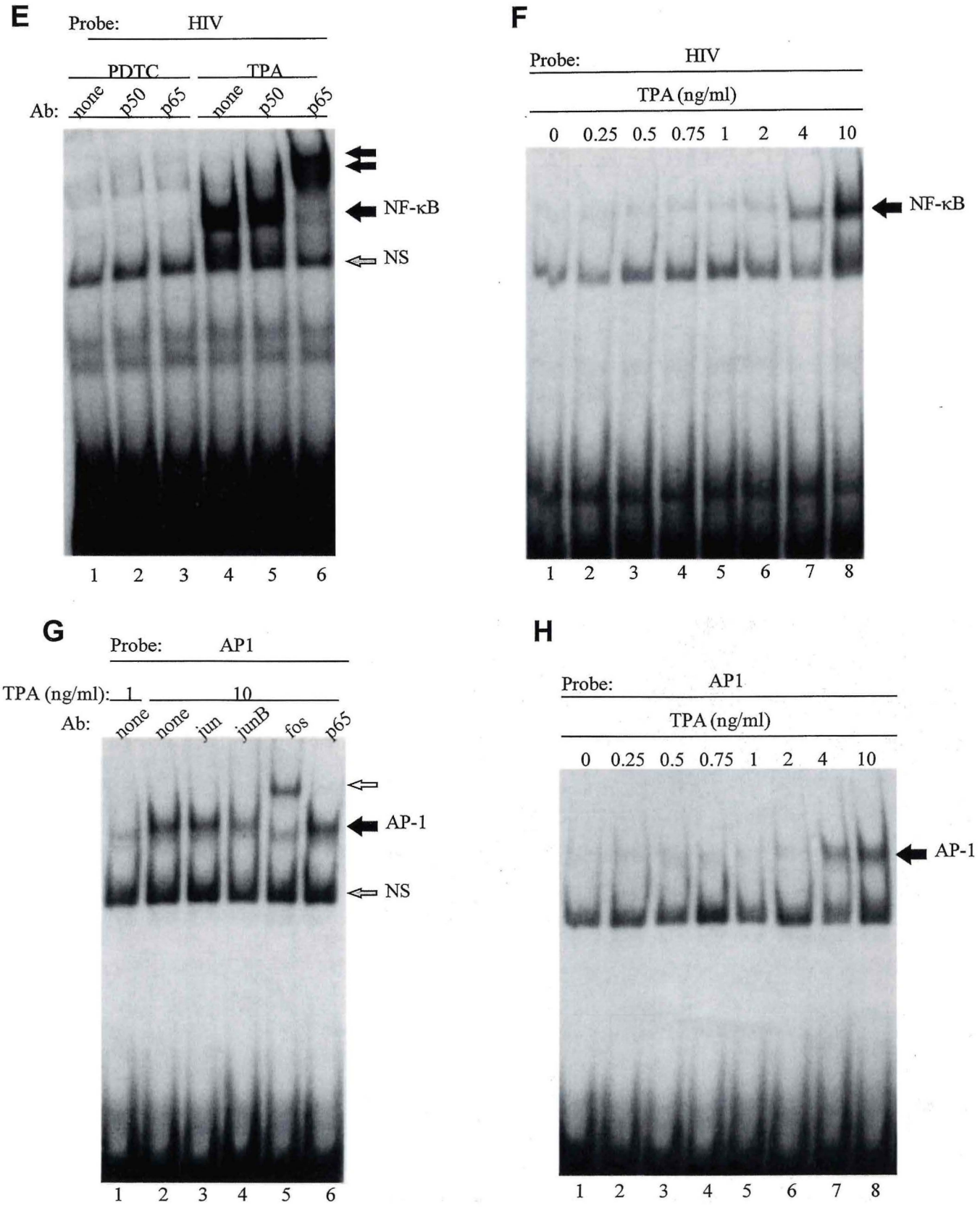

FIGURE 2. *Continued*: (E–F) NF-κB binding to the HIV probe with two κB binding sites. (E) No effect of 200 μM PDTC (lanes 1–3) versus strong effect of TPA (lanes 4–6) on the activation of NF-κB activity measured with the HIV probe. The TPA-induced NF-κB DNA-binding complex was supershifted weakly by the p50 and strongly by the p65 antibody (doublet of arrows points to the p65-supershifted doublet (lane 6). (F) The effect of TPA concentration on the induction of NF-κB DNA binding activity to the HIV probe. (G–H) AP-1 DNA binding activity. (G) TPA-induced AP-1 DNA binding activity was supershifted by the c-Fos (lane 5, upper white arrow) and not by p65 antibody (lane 6). (H) The effect of TPA concentration on the induction of AP-1 DNA binding activity in the same nuclear extracts that were used to measure NF-κB by the κB or HIV probe.

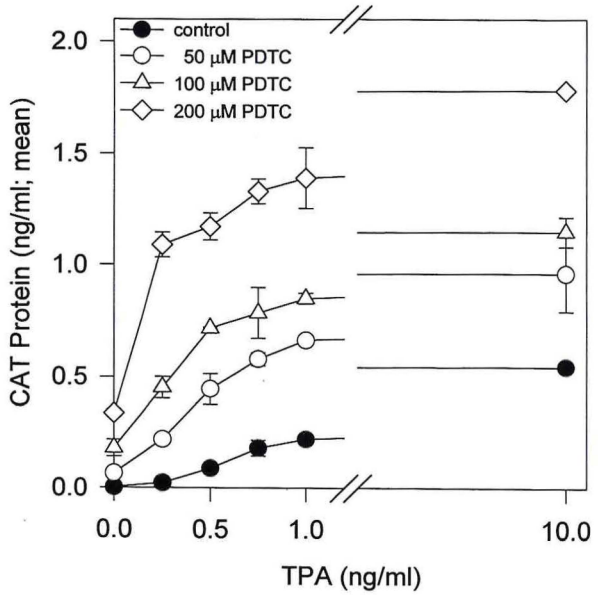

FIGURE 3. Stimulation of 4 × AP-1CAT activity by PDTC. Cells were electroporated and treated as described in Figure 1. The results are from a representative experiment and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample standard deviation of duplicate cultures.

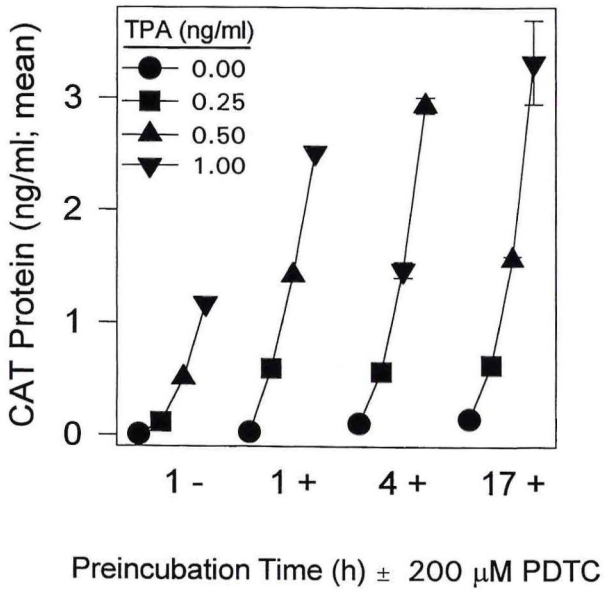

FIGURE 4. Effect of PDTC pre-incubation time on TPA-induced HIV-1 LTR activity. U937 cells were electroporated and treated as previously described, but the PDTC pre-incubation time was varied for 1, 4 or 17 h before the addition of various concentrations of TPA at 0 h (defined as 24 h postelectroporation). The results are from a representative experiment and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample standard deviation of duplicate cultures.

treatment with PDTC was a more potent inhibitor with 10 ng/mL of TPA than DDTC on HIV-1 LTR activity (data not shown). This difference between DDTC and PDTC may be due to the greater stability of PDTC [26].

DISCUSSION

In this report, a transient expression assay was used to define the experimental conditions required for demonstrating the stimulatory effect of PDTC on HIV-1 LTR- and κB-dependent gene expression induced by low concentrations of TPA. Significantly, both the HIV-1 LTR and 4 × κB-directed CAT reporter constructs were stimulated by the combination of PDTC and low concentrations of TPA. These data suggested that a functional interaction between NF-κB and κB enhancer elements was required, because the stimulatory effect of PDTC was not observed with an HIV-1 LTR reporter construct containing two mutated κB binding sites. We evaluated the sequence elements required for the stimulatory effect of PDTC on the induction of HIV-1 LTR-mediated gene expression by low concentrations of TPA. Unexpectedly, the stimulatory effect of PDTC with TPA was dependent on an intact AP-2 site located between the κB elements in the LTR. Moreover, the same mutation decreased the stimulatory effect of PDTC with TNF-α [35]. Interestingly, mutations that disrupt this AP-2 binding site decreased the basal levels of transcription from the HIV-1 LTR without affecting TNF-α induction in Jurkat T leukemia cells [9].

Other studies also showed that the basal activity of the HIV-1 LTR reporter construct was slightly increased by PDTC; see Fig. 6, lanes 11–13 in [23]. As shown here, the U937 cellular background appears to provide an appropriate environment for further studies to define the signalling pathway responsible for this effect. To measure the activation of NF-κB by low concentrations of TPA, we evaluated the effect of a 1-, 2-, 6- or 24-h treatment with 1 ng/mL of TPA. NF-κB DNA binding activity was weakly detectable by EMSA in nuclear extracts only after a 6-h incubation and not detectable 24 h after treatment with 1 ng/mL of TPA (Miller, unpublished results). This suggests that the CAT ELISA is markedly more sensitive than the NF-κB DNA binding assay for detecting small changes in HIV-1 LTR-dependent CAT activity. These data do not address, but bring into question, whether the stimulatory effect of PDTC with low concentrations of TPA was mediated through NF-κB released by the loss of IκB-α, because the rapid degradation of IκB-α was shown by a number of studies to be stimulated by TPA and inhibited by PDTC. Alternatively, the stimulatory effect of PDTC may be mediated by a signalling pathway resistant to the inhibitory effect of PDTC. Consistent with this idea, NF-κB DNA binding activity induced by TNF-α was unaffected by PDTC [35].

In this report, we have evaluated the hypothesis that PDTC treatment potentiated κB-dependent gene expression in a manner dependent on the concentration of TPA. Because dithiocarbamates such as PDTC are well known to exert pro-oxidant [25] and anti-oxidant effects [14, 15, 27], we also examined the effect of DDTC on TPA-induced HIV-1 LTR activity. As shown here, DDTC treatment resulted in a stimulatory effect with TPA, which, like that of

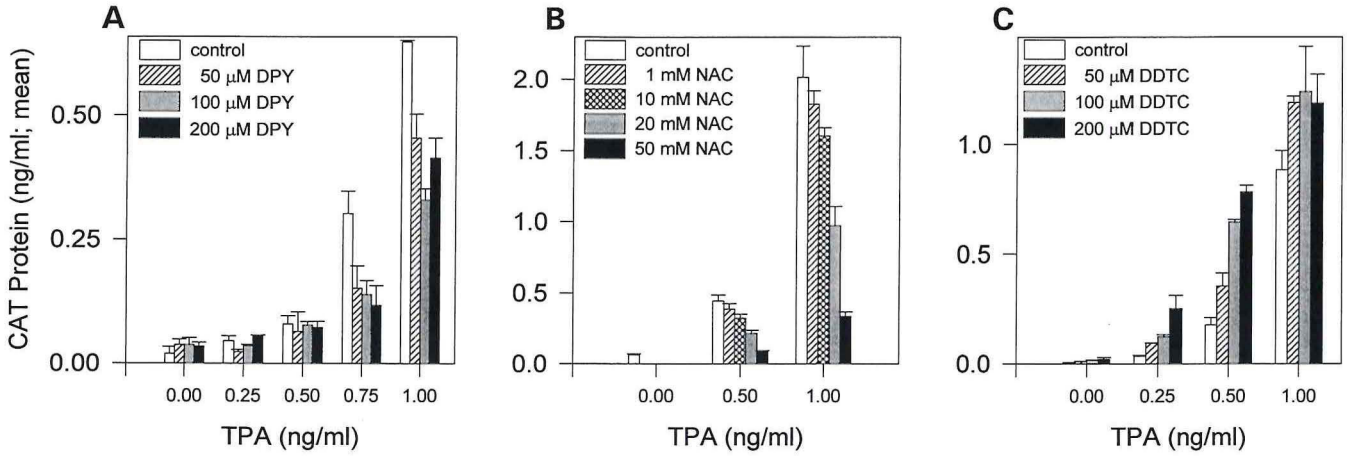

FIGURE 5. The stimulatory effect of PDTC on HIV-1 LTR activity is independent of metal-chelating or anti-oxidant activity. U937 cells were electroporated and treated as previously described; however, the 1-h pre-treatment was done with (A) the metal chelator DPY or (B) the anti-oxidant NAC or (C) the dithiocarbamate DDTC. The results are from three separate electroporations and represent the mean CAT protein value (normalised to 100 μg of cellular protein) ± the sample standard deviation of duplicate cultures for DPY or DDTC treatment. Replicate cultures ($n = 6$) were pre-treated with the indicated concentrations of NAC. The data points represent the mean ± SD. In the absence of TPA, NAC treatment resulted in undetectable CAT protein (data not shown).

PDTC, was dependent on the concentration of TPA. Consistent with an oxidant effect, DDTC was shown to stimulate TNF-α [28, 29]. Interestingly, pre-treatment with PDTC was a more potent inhibitor with 10 ng/mL of TPA than DDTC on HIV-1 LTR activity. This difference between DDTC and PDTC may be due to the greater stability of PDTC in solution [26].

Studies with the metal chelator DPY and the anti-oxidant NAC suggested that metal-chelating or anti-oxidant properties of PDTC were unlikely to be responsible for the stimulatory activity of PDTC in U937 cells. Speculation that the stimulatory effect of dithiocarbonates may be mediated through a mechanism involving redox control is supported by evidence demonstrating (1) the importance of small changes in the intracellular glutathione and glutathione disulphide (GSSG) levels in NF-κB regulation [30] and (2) a correlation between an increase in GSSG and inhibition of NF-κB activation by PDTC [31]. Significantly, the authors observed that NF κB and AP-1 activation were potentiated by the reducing agent dithiothreitol if added to cell cultures 1 h after the phorbol ester, indicating that a shift of redox conditions may support optimal oxidative activation with minimal inhibition of DNA binding. More recent evidence for a molecular cascade of redox regulation of AP-1 showed a direct interaction between thioredoxin (TRX) and a DNA repair enzyme, redox factor (Ref-1), which potentiated TPA-induced AP-1 activity [32]. Significantly, TPA translocated TRX into the nucleus, where an interaction with Ref-1 promoted the direct activation of AP-1 by Ref-1.

AP-1 DNA binding activity and AP-1-dependent transcriptional activity were shown to be stimulated by PDTC [14, 15, 27]. The stimulatory effect on AP-1 activity was found to be independent of PKC activation and to require the newly synthesised AP-1 components *c-jun* and *c-fos* [14, 15]. Although these effects were attributed to the anti-oxidant activity of PDTC, more recent studies indicate that this conclusion may not be correct, because some effects of PDTC may be due to an oxidant effect [33]. PDTC was shown to promote cell growth arrest and the expression of cell surface molecules linked with myeloid differentiation, such as CD11c in U937 cells [34]. Significantly, an AP-1 site in reporter constructs directed by the CD11c promoter was required for the response to PDTC. Our evidence that AP-1 DNA binding activity and AP-1-dependent transcriptional activity were induced by PDTC in U937 cells raised the hypothesis that PDTC-induced AP-1 activity may be implicated in the stimulatory effect of PDTC on κB-dependent gene expression. An important role of AP-1 in mediating the effect of PDTC on the regulation of the NF-κB signalling pathway is plausible because of evidence of protein–protein interactions between AP-1 and NF-κB family members [10].

Although NF-κB dependent gene expression is widely recognised to be regulated by a balance between positive and negative signals and multiple levels of control have been demonstrated in the regulation of NF-κB by distinct stimuli, our understanding of the molecular mechanisms in signal integration of the the NF-κB signalling pathway is limited. As shown here and in the accompanying paper, the U937 model system provides a cellular environment where the TPA and TNF-α signalling pathways regulating NF-κB function are parallel pathways with selective sites of regulation by PDTC.

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References

1. Hug H. and Sarre T. F. (1993) *Biochem. J.* **291**(Pt. 2), 329–343.
2. Griffin G. E., Leung K., Folks T. M., Kunkel S. and Nabel G. J. (1991) *Res. Virol.* **142**, 233–238.

3. Schmitz M. L., dos Santos Silva M. A. and Baeuerle P. A. (1995) *J. Biol. Chem.* **270**, 15576–15584.
4. Baeuerle P. A. and Henkel T. (1994) *Annu. Rev. Immunol.* **12**, 141–179.
5. Baeuerle P. A. and Baltimore D. (1996) *Cell* **87**, 13–20.
6. Baldwin A. S. Jr. (1996) *Annu. Rev. Immunol.* **14**, 649–683.
7. Schmid R. M., Perkins N. D., Duckett C. S., Andrews P. C. and Nabel G. J. (1991) *Nature* **352**, 733–736.
8. Perkins N. D., Agranoff A. B., Pascal E. and Nabel G. J. (1994) *Mol. Cell. Biol.* **14**, 6570–6583.
9. Perkins N. D., Agranoff A. B., Duckett C. S. and Nabel G. J. (1994) *J. Virol.* **68**, 6820–6823.
10. Stein B., Baldwin A. S. Jr., Ballard D. W., Greene W. C., Angel P. and Herrlich P. (1993) *EMBO J.* **12**, 3879–3891.
11. Gualberto A., Hixon M. L., Finco T. S., Perkins N. D., Nabel G. J. and Baldwin A. S. Jr. (1995) *Mol. Cell. Biol.* **15**, 3450–3459.
12. Ruocco M. R., Chen X., Ambrosino C., Dragonetti E., Liu W., Mallardo M., De Falco G., Palmieri C., Franzoso G., Quinto I., Venuta S. and Scala G. (1996) *J. Biol. Chem.* **271**, 22479–22486.
13. Schreck R., Rieber P. and Baeuerle P. A. (1991) *EMBO J.* **10**, 2247–2258.
14. Meyer M., Schreck R. and Baeuerle P. A. (1993) *EMBO J.* **12**, 2005–2015.
15. Meyer M., Pahl H. L. and Baeuerle P. A. (1994) *Chem. Biol. Interact.* **91**(2–3), 91–100.
16. Feng S. and Holland E. C. (1988) *Nature* **334**, 165–167.
17. Nabel G. and Baltimore D. (1987) *Nature* **326**, 711–713.
18. Nabel G. J., Rice S. A., Knipe D. M. and Baltimore D. (1988) *Science* **239**, 1299–1302.
19. Seto E., Mitchell P. J. and Yen T. S. (1990) *Nature* **344**, 72–74.
20. Gorman C. M., Moffat L. F. and Howard B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
21. Perkins N. D., Edwards N. L., Duckett C. S., Agranoff A. B., Schmid R. M. and Nabel G. J. (1993) *EMBO J.* **12**, 3551–3558.
22. Andrews N. C. and Faller D. V. (1991) *Nucleic Acids Res.* **19**, 2499.
23. Schreck R., Meier B., Mannel D. N., Droge W. and Baeuerle P. A. (1992) *J. Exp. Med.* **175**, 1181–1194.
24. Miller S. C., Taylor A., Watanabe K., Mok K. and Torti F. M. (1997) *Exp. Cell Res.* **230**, 9–21.
25. Nobel C. I., Kimland M., Lind B., Orrenius S. and Slater A. F. (1995) *J. Biol. Chem.* **270**, 26202–26208.
26. Topping R. J. and Jones M. M. (1988) *Med. Hypotheses* **27**, 55–57.
27. Schulze-Osthoff K., Los M. and Baeuerle P. A. (1995) *Biochem. Pharmacol.* **50**, 735–741.
28. East C. J., Abboud C. N. and Borch R. F. (1992) *Blood* **80**, 1172–1177.
29. Schmalbach T. K., Datta R., Kufe D. W. and Sherman M. L. (1992) *Biochem. Pharmacol.* **44**, 365–371.
30. Droge W., Schulze-Osthoff K., Mihm S., Galter D., Schenk H., Eck H. P., Roth S. and Gmunder H. (1994) *FASEB J.* **8**, 1131–1138.
31. Galter D., Mihm S. and Droge W. (1994) *Eur. J. Biochem.* **221**, 639–648.
32. Hirota K., Matsui M., Iwata S., Nishiyama A., Mori K. and Yodoi J. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 3633–3638.
33. Pinkus R., Weiner L. M. and Daniel V. (1996) *J. Biol. Chem.* **271**, 13422–13429.
34. Aragones J., Lopez-Rodriguez C., Corbi A., del Arco P. G., Lopez-Cabrera M., de Landazuri M. O. and Redondo J. M. (1996) *J. Biol. Chem.* **271**, 10924–10931.
35. Watanabe K., Kazakova I., Furniss M. and Miller S. C. (1999) *Cell. Signal.* **11**, 371–383.