Selective Inhibition of Mitogen-induced Transactivation of the HIV Long Terminal Repeat by Carboxyamidotriazole

CALCIUM INFLUX BLOCKADE REPRESSES HIV-1 TRANSCRIPTIONAL ACTIVATION*

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Carboxyamidotriazole (CAI) is a calcium influx inhibitor that has both antiproliferative and antimetastatic activities. Pretreatment of human T-cells with micromolar concentrations of CAI causes a near complete inhibition of calcium-regulated mitogen-induced transcription from the human immunodeficiency virus (HIV) long terminal repeat (LTR). This inhibition is selective since other mitogen-activated gene regulatory elements, such as the 12-O-tetradecanoylphorbol-13-acetate response element, are not influenced by the drug. HIV LTR transcription inhibition is maximal at 1.0 μM CAI, requires a pretreatment interval of at least 8 h for optimum inhibition, and shows no acute interference with the growth properties of the cells.

Moreover, the inhibition is rapidly reversible upon removal of the drug from the medium. Studies to identify enhancer elements within the HIV LTR that are functionally sensitive to low-dose long-term pretreatment with CAI indicate that the NF-xB-binding sites are among the major targets of drug action. In vitro DNA binding studies with nuclear extracts prepared from mitogen-induced T-cells stimulated in the presence of CAI indicate that the drug differentially influences the calcium-regulated downstream signal transduction pathways necessary for specific NF-xB DNA binding activity at the two xB sites within the HIV LTR. Studies with ionomycin and thapsigargin show that repression is specific for selected modes of inducible calcium entry and indicate that, in T-cells, a major mechanism of CAI action is to modulate calcium influx at a level that is proximal to the regulated release of calcium from intracellular stores. Measurement of calcium influx in CAI-treated cells reveals a dramatic and reversible inhibition of mitogen-induced calcium influx. These results indicate that CAI can be an important and effective pharmacological tool for analysis of the calcium-dependent modulation of HIV LTR transcription.

The clinical progression of disease resulting from infection with human immunodeficiency virus (HIV)† type 1 is controlled to a large extent by cellular processes that govern the transcriptional modulation of HIV gene expression. Most of this control is mediated through the gene regulatory region of the HIV genome, referred to as the HIV long terminal repeat (LTR) (1–4). A variety of host and virus-derived factors interact with this HIV promoter to influence transcriptional activity through a complex interplay of both positive and negative elements (2, 3).

The HIV LTR can be divided into three regions based on the viral and cellular regulatory proteins that bind them. These regions have been designated the modulatory region, the core region, and the Tat-responsive element (1–3). The modulatory region extends from residues −78 to −454 (relative to the start of transcription) and binds a variety of cellular proteins, including AP-1, chicken ovalbumin upstream factor, T-cell factor-1α, members of the nuclear receptor superfamily, and multiple members of the c-Rel-related transcription factors including NF-xB. Within the modulatory element, the most influential positive regulatory elements are the two tandemly repeated NF-xB elements located from residues −77 to −108. The region from residues −110 to −454 within the modulatory element has been often referred to as the negative regulatory element since large deletions in the intervening sequences often lead to up-regulation of viral production. The core element is required for both basal and activated transcriptional activity and binds chiefly to the transcription factors Sp1 and TFIIID. Finally, the Tat-responsive element region is indispensable for the viral transactivator Tat function, and it binds both Tat and other host cell factors.

HIV transcription is highly inducible in T-cells through signal transduction pathways that share significant overlap with those signaling events that are important for activated expression of T-cell lymphokines such as interleukin-2 (5, 6). Consequently, many of the mitogenic agents that induce interleukin-2 production, by augmenting specific calcium- and mitogen-activated protein kinase-dependent signaling events crucial for T-cell immune activation, also cause significant transcriptional activation of HIV LTR gene expression. These overlapping signal transduction requirements between T-cell lymphokine gene expression and HIV LTR transactivation forge an inextricable link between T-cell activation and inducible HIV gene expression. Although significant advances in our understanding of the control of interleukin-2 gene expression in T-cells have been made over the past few years, a detailed understanding of the precise mechanisms that govern mitogen-induced activation of the HIV LTR in T-cells remains obscure.

This report describes the selective inhibition of mitogen-induced HIV LTR transactivation in transiently transfected...
Jurkat T-cells by low-dose treatment with carboxamidotriazole (CAI). CAI reversibly inhibits HIV promoter activity by >90% at a concentration of 1 μM. This effect is highly selective at this low CAI concentration since the transactivation of other highly mitogen-inducible cis-elements, such as the 12-O-tetradecanoylphorbol-13-acetate response element (TRE), is unaltered. Systematic comparison by transfection analysis of the activity of specific HIV cis-regulatory elements linked to heterologous promoters shows that CAI exerts its predominant influence on the region of the HIVLTR containing NF-xB regulatory elements. Assessment of the in vitro DNA binding activity present in nuclear extracts derived from mitogen-induced T-cells shows selective repression of NF-xB DNA binding by CAI pretreatment. Calcium influx measurements in Jurkat T-cells, in combination with the differential effects of CAI on receptor-independent inducers of calcium influx, show that CAI specifically and reversibly disrupts the signal transduction events that must occur proximally to the release of calcium from internal stores.

EXPERIMENTAL PROCEDURES

Materials—Carboxamidotriazole was obtained through the Developmental Therapeutics Program, NCI, National Institutes of Health (Bethesda, MD). Ionomycin was from Calbiochem. PMA was from Sigma. Thapsigargin was from Molecular Probes, Inc. Cyclosporine was from Sandoz. SK&F 96365 was from BIOMOL Research Labs Inc. [3H]Chloramphenicol was from Amersham Corp. Acetyl-CoA was from Sandoz. SK&F 96365 was from BIOMOL Research Labs Inc. (Bethesda, MD). Ionomycin was from Calbiochem. PMA was from the Developmental Therapeutics Program, NCI, National Institutes of Health, Bethesda, MD.

Cell Culture and Transfection Assays—Jurkat T-cells were cultured in RPMI 1640 medium, 10% fetal calf serum, and 100 units/ml penicillin/streptomycin at 37°C in 5% CO2. Cell viability was determined by the trypan blue exclusion assay. Cells were transiently transfected by electroporation as described (7). CAT activity in extracts from transiently transfected cells was determined as described (10). Briefly, CAT activity was quantitated by excising radiolabeled spots of acetylated CAT reporter plasmid (see Fig. 4 and “Results”) and were a generous gift from Dr. U. Siebenlist (NIAID, National Institutes of Health, Bethesda, MD).

Preparation of Nuclear Extracts—Jurkat cells were suspended at 1×106 cells/ml and pretreated for 8–16 h as indicated with 10 μM CAI, followed by either no additions or 1–2 μg/ml PHA and 50–100 μg/ml PMA. Cells were harvested 8 h later by centrifugation at 1000×g at 4°C and washed three times with ice-cold phosphate-buffered saline. Nuclear and cytosolic extracts were prepared from the cell pellets as described previously (11).

Electrophoretic Mobility Shift Assays—EMSA analysis was carried out on 4 or 8% acrylamide gels with 5'-endlabeled duplex oligonucleotides as described previously (11, 12). The oligonucleotides used in this study were as follows: GALV-TRE, 5'-GATCCCGAGAAATAGATGAG-GA7TCCGCTGGGG-3'; 5'-HIV-xB, 5'-GATTCGAGTTGCTCAGAAGGCTTTCCCGTGGG-3'; 3'-HIV-xB, 5'-GATTCGAGTTGCTCAGAAGGCTTTCCCGTGGG-3'; and NFAT, 5'-GATTCACCCACAGAGGAATTTGTTCCTCATACAGGCGCT-3'.

Determination of Calcium Influx by Flow Cytometry—Intracellular calcium was assessed by quantitating the changes in fluorescence intensity of cells loaded with the Ca2+-sensitive fluorescent dye Fluo-3 as described previously (13). Briefly, Jurkat cells (at a density of 2×105 cells/ml) were incubated for 30 min at 37°C with 1 μM Fluo-3-acetoxyethyl ester (Molecular Probes, Inc.) in MeSO containing 37.5 mg/ml Pluronic F-127 (Molecular Probes, Inc.) to facilitate Fluo-3 incorporation. After loading, cellular fluorescence at 530 nm was measured by flow cytometry (FACScan, Becton Dickinson Advanced Cellular Biology, San Jose, CA) at room temperature. Histograms containing between 1000 and 1500 cells were acquired in log mode at 30-s (from 0 to 30 min) and 1-min (from 4 to 10 min) intervals, and the mean fluorescence intensity was converted to linear scale.

Western Blot Analysis—Western blot analyses were performed as described previously (14). Cross-reactive proteins were visualized by the ECL detection system (Amersham Corp.) according to the manufacturer’s specifications. Relative amounts of cross-reactive polypeptides were determined from densitometric tracings of ECL-exposed films following capture and quantitation by 1D Main image analysis software (AAB Inc.). Antibodies—Antibodies to Sp1 were purchased from Santa Cruz. Antibodies to p65 used in supershifting EMSA experiments have been previously described (15). Antibodies to IκB, p65, and c-Rel used for immunoblot analysis were from Santa Cruz. Supershifting anti-c-Rel antibodies were a generous gift from Dr. U. Siebenlist.

RESULTS

CAI Selectively Inhibits Mitogen-induced Transactivation of the HIV LTR—There is an extensive overlap between the signal transduction requirements for transactivation of T-cell cytokine promoters and for transcriptional activation of the HIVLTR (5, 6). As a result, several studies have evaluated drugs known to specifically repress the transcriptional activation of the interleukin-2 promoter, such as cyclosporin A and FK506, for their efficacy in inhibiting the transcriptional activation of the HIVLTR (9, 16, 17). Cyclosporin A (CSA) has indeed been found to have some utility in repressing both transactivation and replication of HIV promoters (17). Such findings have, in part, formed the basis for clinical studies designed to assess the possible role of cyclosporine and its analogues as therapeutic antiretroviral agents (18–20). Since CAI was found to be a highly effective calcium active agent that could potentially repress the signal transduction events critical for proliferation in numerous cell lines (21–23), studies were undertaken to evaluate the possibility that CAI could be used to repress mitogen-induced transactivation of the HIVLTR in the human Jurkat T-cell line.

Mitogen-dependent transactivation of the HIVLTR was assessed in vitro in human Jurkat cells transiently transfected with a plasmid reporter vector containing the chloramphenicol gene 3' of the entire HIVLTR promoter (residues −768 to +71) (see Fig. 4, left panel). As shown in Fig. 1A, prior treatment of Jurkat cells with 1 μM CAI resulted in a >90% reduction in LTR transactivation after PHA/PMA mitogenic stimulation. This inhibition was selective, as mitogen-dependent activation of Jurkat cells transiently transfected with a heterologous promoter containing three tandem copies of a 12-O-tetradecanoylphorbol-13-acetate response element (Fig. 1B) was not affected by CAI pretreatment.

CAI Inhibition of Mitogen-dependent HIVLTR Transactivation Is Dose-dependent and Maximal after 8 h of Pretreatment—CAI inhibition of PHA/PMA induction of the HIVLTR was dose-dependent, with half-maximal inhibition (K) at a concentration of ~0.5 μM (Fig. 2A). At a low dose (~2 μM), the repression of transactivation by CAI was highly selective for the LTR since transactivation of the TRE was virtually unaffected. However, at CAI concentrations >2 μM, the effects of CAI appeared to be less specific since significant inhibition of transactivation (>60%) was observed at these doses. An assessment of the time dependence of inhibition at the low concentrations (~1.0 μM) of CAI was demonstrated by the pretreatment time course for HIVLTR inhibition shown in Fig. 2B. Maximum inhibition of mitogen-induced HIVLTR transactivation with 1 μM CAI required a pretreatment interval of at least 8 h prior to stimulation. Under identical conditions, TRE transactivation remained completely unchanged (Fig. 2B).
**FIG. 1.** CAI selectively inhibits PHA/PMA induction of transactivation of the HIV LTR. A, Jurkat T-cells were transfected with 5 μg of the HIV LTR promoter-reporter vector pHIV-LTR-CAT, stimulated in the presence or absence of 1 μM CAI, and assayed for CAT activity as described under “Experimental Procedures” by thin-layer chromatography (left panel). Converted and unconverted [14C]chloramphenicol was excised from thin-layer chromatograms and counted by liquid scintillation (right panel). CAT activity is presented as relative conversion. Each transfection was performed in duplicate. B, Jurkat T-cells were transfected with 2.5 μg of pGALV-TRE-CAT reporter plasmid containing three tandem copies of a consensus TRE linked to a basal c-fos promoter (8) (left panel). CAT activity was determined as described for A (right panel). Each transfection was performed in duplicate. The results shown were comparable in at least two to three separate experiments and are displayed as mean ± S.E.

**FIG. 2.** Dose- and time-dependent inhibition of mitogen-induced transactivation of the HIV LTR by CAI. A, Jurkat T-cells were transfected with 5 μg of pHIV-LTR-CAT (●) or 2.5 μg of pGALV-TRE-CAT (○) reporter construct and then preincubated for 16 h with 0, 0.25, 0.5, 1.0, 1.5, 2.0, 5.0, or 10 μM CAI prior to stimulation with 2 μg/ml PHA and 50 ng/ml PMA. Cells were then harvested, and CAT activity was determined as described under “Experimental Procedures.” B, Jurkat T-cells were transfected with 5 μg of pHIV-LTR-CAT (●) or 2.5 μg of pGALV-TRE-CAT (○) and then pretreated with 1 μM CAI for 0, 1, 4, 8, and 12 h prior to harvesting. Cells were assayed for CAT activity as described for A. Transfections were done in duplicate for each point, and results are representative of two separate experiments. The results shown were comparable in two separate experiments and are displayed as mean ± S.E.
FIG. 3. CAI does not alter cell viability during transfection experiments, and CAI repression of HIV LTR transactivation is reversible. A, cells were withdrawn, and cell viability was determined by trypan blue exclusion at different stages in a typical transfection. Arrows indicate times at which cells were electroporated, when 10 μM CAI was added, and when the cells were harvested. B, after electroporation with 5 and 2.5 μg of pHIV-LTR-CAT and pGALV-TRE-CAT, respectively, Jurkat T-cells were incubated with 10 μM CAI for 8 h. Cells were then changed into fresh drug-free medium prior to stimulation with PHA/PMA. Cells were harvested and assayed for CAT activity as described in the legend to Fig. 1.

FIG. 4. CAI shows predominant inhibitory activity at the κB enhancer site within the HIV LTR. Left panel, shown is a schematic representation of the heterologous promoter-reporter vectors used in this study. Open rectangles indicate inserted LTR sequences (see “Results”). Horizontal lines indicate vector sequences. Right panel, 5 μg each of pHIV-LTR-CAT, pHIV-κB-CAT, pHIV-Sp1-CAT, and pHIV-κB/Sp1-CAT were transfected into Jurkat T-cells. The cells were subsequently left unstimulated or were stimulated with PHA/PMA in the presence or absence of 1 μM CAI. CAT activity was normalized to show percent maximal activity for each individual reporter vector. Percent inhibition of each reporter construct is show on the right. The data represent duplicate transfection for each point and are represented as mean ± S.E.

Effects of CAI Pretreatment on Jurkat Cells Are Reversible and Do Not Alter Cell Viability—Fig. 3A shows a representative cell viability profile for the Jurkat cells throughout the course of the in vitro transactivation protocol. The effect of CAI pretreatment on cell viability was indistinguishable between unstimulated and stimulated cells treated in the absence of the drug. Significant differences in viability were noted between stimulated and unstimulated cells secondary to a phorbol ester-induced apoptosis of the transfected cells, but these differences showed negligible influence by CAI. When, prior to stimulation, the cells were changed into fresh “drug-free” medium following 8 h of pretreatment with 10 μM CAI, the inhibition of HIV LTR transactivation was completely reversed (Fig. 3B).

NF-κB Enhancer Region Is the Major cis-Regulatory Element Targeted by CAI within the HIV LTR—It has long been established that the κB/Sp1 elements of the modulatory region of the HIV LTR are the most potent host factor-responsive enhancers in the HIV promoter (24–27). This region was therefore evaluated directly for its sensitivity to inhibition by low doses of CAI. The inhibitory effect of 16 h of pretreatment of Jurkat T-cells with 1 μM CAI was assessed on a series of heterologous reporter constructs containing the full-length LTR (residues −768 to +71), the tandemly repeated κB sites alone (residues −105 to −79), the three tandem Sp1 sites alone (residues −78 to −46), or the combined tandem κB/Sp1 sites (residues −105 to −46) (Fig. 4, left panel). Low-dose CAI treatment was 93% effective in inhibiting the transactivation of the HIV LTR, −63% effective in inhibiting the transactivation of the HIV-κB heterologous promoter, somewhat less effective (46.5%) in inhibiting the activation of the combined κB/Sp1 enhancer, and completely ineffective in inhibiting the basal activity of the isolated Sp1 enhancer elements (Fig. 4, right panel).

CAI Inhibits NF-κB Enhancer DNA Binding Activity in Nuclear Extracts Derived from CAI-treated Jurkat T-cells—The κB/Sp1 region of the HIV LTR has been shown to interact avidly with members of the dimeric Rel family of transcription factors (κ-Rel, p65, p50, and p52). The κB enhancer elements are present as two nonequivalent tandem copies that are distinct because of their flanking sequences (25) (Fig. 5A). DNA binding at both the 5′-κB and 3′-κB sites was induced >10-fold in response to mitogenic signals delivered by PHA/PMA. Similarly, inducible DNA binding to both κB sites was significantly inhibited in cells pretreated with CAI (Fig. 5B). The primary inducible DNA-binding complexes in T-cells are composed of dimeric mixtures of Rel family dimers containing either p65 or c-Rel (28). Antibody supershift experiments demonstrated that the inducible DNA-binding complexes contained some proportion of both p65 and c-Rel (Fig. 5C). The inducible DNA-binding
CAI Inhibits the Nuclear Accumulation of c-Rel and p65 in Activated Jurkat T-cells—NF-κB-dependent transcription is regulated to a large extent by both the transcriptional control of c-Rel expression and the IkB-mediated cytoplasmic sequestration of κB dimers, containing c-Rel and p65 (28, 29). Activation of NF-κB occurs through the induction of IkB degradation, which releases the active p65 or c-Rel complexes to translocate to the nucleus (28). Immunoblot analysis of nuclear extracts from Fig. 5 showed CAI-dependent inhibition of c-Rel and p65 nuclear accumulation (Fig. 6A). The nuclear accumulation of c-Rel was consistently much more sensitive to CAI than was the accumulation of p65 (Fig. 6A, compare top and middle panels). In addition, the reduction of cytosolic IkB, following PHA/PMA stimulation, was partially blocked by CAI (Fig. 6A, bottom panel).

The nuclear translocation of p65 and c-Rel was measured in extracts in which Jurkat cells were stimulated in the presence and absence of CAI, followed by the simultaneous preparation of nuclear and cytosolic extracts. Immunoblot analysis of these paired extracts showed that the increased nuclear appearance of c-Rel after stimulation was blocked by CAI (Fig. 6B). Interestingly, the reciprocal depletion of cytoplasmic c-Rel upon stimulation appeared unchanged with CAI treatment. This results suggests that CAI may influence the nuclear accumu-
lation of c-Rel by blunting both its synthesis (i.e. its mRNA production) and its translocation (29). As illustrated in Fig. 6B, the nuclear accumulation of p65 was variably affected by CAI. CAI appeared to produce a more significant repression of p65 translocation in the T-cell extract shown in Fig. 6B than in the extracts shown in Fig. 6A. There was also a partial CAI inhibition of the reciprocal depletion of cytoplasmic p65 upon T-cell stimulation (Fig. 6B, bottom right panel).

**CAI Inhibition of Mitogen Induction of HIV LTR Transactivation Is More Potent than SK&F 96365 and Cyclosporin A Inhibition**—CSA and its analogues have previously been shown to have some effect on mitogen-induced transactivation of the HIV LTR (5, 9). The primary mechanism of action of CSA occurs through an immunophilin-dependent inhibition of the calcium-controlled activation of the calmodulin-dependent phosphatase, calcineurin, and subsequent downstream events linked to the translocation of c-Rel/B-related transcription factors to the nucleus (31). SK&F 96365 is an agent that inhibits numerous calcium-regulated cellular events (32). Pretreatment of cells with 1.0 μM SK&F 96365 produced much weaker repression of HIV LTR transactivation than did pretreatment with CAI (Fig. 7A). Similarly, when cells were treated with CSA concentrations sufficient to fully inhibit calcineurin activity (33), CSA-dependent repression of the HIV LTR was also much less effective compared with CAI (Fig. 7, compare A and B).

**CAI Inhibition of HIV LTR Transactivation Is Dependent on the Mode of Mitogen-dependent Calcium Mobilization**—T-cell activation in the presence of the lectin PHA requires the presence of extracellular calcium (34). The mitogenic pathway by which the HIV LTR induces transactivation in T-cells is dependent on the triggered rise in intracellular calcium that occurs after PHA-mediated ligation and/or cross-linking of glycosylated T-cell surface receptors, including the T-cell/CD3 receptor. This pathway is mediated by the phospholipase C-dependent production of inositol 1,4,5-triphosphate, which triggers a release of calcium from intracellular stores (35). The rise in intracellular calcium is biphasic in nature and is characterized by an initial rapid rise in intracellular calcium due to the depletion of intracellular calcium stores. This initial “spike” in intracellular calcium is followed by a plateau phase secondary to the influx of extracellular calcium via calcium release-activated channels (36). Although the HIV LTR and the isolated kB sites can be effectively activated with PHA alone in Jurkat T-cells, the activation has been known to occur synergistically in the presence of phorbolester esters such as PMA (9, 37, 38). HIV LTR-kB activation in the presence of PHA is only partially repressed by CSA, yet the synergistic transactivation that occurs in the presence of both PHA and PMA is insensitive to CSA (9). In contrast, transactivation of the HIV LTR in the presence of PHA either alone or with PMA was significantly repressed by 1 μM CAI (Fig. 7, B (left panel) versus A (right panel)). Interestingly, the transactivation induced by PMA added together with either ionomycin or thapsigargin (agents that increase intracellular calcium through surface receptor-independent pathways) (39, 40) was completely insensitive to the effects of 1.0 μM CAI (Fig. 7B, middle and right panels). These findings suggest that CAI may interfere with calcium-dependent signal transduction events driven by receptor-mediated control of calcium influx.

**PHA-induced Calcium Influx in Jurkat T-cells Is Reversibly Inhibited by 1 μM CAI**—The selective inhibition by CAI of receptor-dependent mitogenic stimulation by PHA indicated that CAI may act at the level of a receptor-operated calcium influx. To test this possibility, PHA/PMA-induced calcium influx was measured in control and CAI-treated (1 or 10 μM) Jurkat cells using calcium-sensitive fluorescent dye-loaded cells (Fig. 8A). CAI added at 1 and 10 μM was effective in completely abolishing the PHA/PMA-induced increase in intracellular calcium. Interestingly, this inhibition was reversible if the cells were changed into drug-free medium after pretreatment with CAI (Fig. 8A, C). Thus, pretreatment of cells with low doses (1 μM) of CAI can cause a selective and reversible inhibition of HIV LTR transactivation by interfering with the signal transduction events that mediate receptor-dependent increases in intracellular calcium.

**CAI Inhibits the Nuclear DNA Binding Activity and Transcriptional Activation of NFAT**—NFAT is a transcription factor that is well recognized for its requirement for calcium-dependent signaling during T-cell activation (41, 42). In fact, it is commonly employed as a downstream indicator of activated T-cell receptor signaling (42). As expected, both the DNA binding activity (Fig. 8B, left) and transcriptional activation by NFAT (Fig. 8B, right) were repressed in activated T-cells by prior treatment with CAI.
CAI is a potent drug with antimetastatic, antiproliferative, and antiangiogenic properties (43, 44). This study provides evidence that 8 h of pretreatment of T-cells with 1 μM CAI causes a selective reversible inhibition of mitogen-dependent transactivation of the HIV LTR. Under these conditions, this inhibition occurs without altering the transactivation of other mitogen response gene regulatory elements such as the TRE. The region of the HIV LTR containing the κB enhancer element has been found to be the primary target through which CAI represses HIV LTR transactivation. This finding is further substantiated by the observation that nuclear factors that bind the HIV-κB elements are reduced in nuclear extracts from CAI-treated cells. Interestingly, 1 μM CAI repression of HIV LTR transactivation in Jurkat T-cells is highly dependent on the nature of the calcium-mobilizing mitogen stimulation. PHA-dependent induction, where intracellular calcium is increased via signal transduction pathways involving extracellular ligation of T-cell surface receptors, such as CD3, is effectively inhibited by 1 μM CAI. In contrast, mitogenic agents that increase intracellular calcium through receptor-independent mechanisms, such as ionomycin and thapsigargin, are not inhibited by CAI. Finally, direct measurement of calcium influx targeting by CAI is further highlighted by the observation that CAI inhibits both the appearance of NFAT DNA binding activity and transactivation in activated Jurkat T-cells.

It is not clear why CAI requires an 8-h pretreatment interval to have full effect. This requirement could reflect simply the time required for the drug to gain full entry into the cell or interact with cell-surface molecules. The rapid reversibility of the effects of CAI (<15 min, the time required to change the cells into a drug-free medium) argues against a simple model based on slow diffusion through the lipid bilayer. CAI may

**FIG. 7.** CAI is more effective than cyclosporin A and SK&F 96365 against HIV LTR transactivation and shows differential inhibition of PHA-induced versus ionomycin- and thapsigargin-induced transactivation of the HIV LTR. A: left panel, Jurkat T-cells were transfected with 5 μg of pHIV-LTR-CAT and then incubated with 1 μM SK&F 96365 for 16 h prior to stimulation with PHA/PMA. Cells were then harvested and assayed for CAT activity as described under “Experimental Procedures.” Right panel, Jurkat T-cells, transfected with 5 μg of pHIV-LTR-CAT, were incubated with 100 ng/ml cyclosporin A 2 h prior to stimulation as described previously (9). Cells were then harvested and processed for CAT activity as described above. The data represent averages of duplicate transfections and are shown as mean ± S.E. B: left panel, Jurkat T-cells were transfected with 5 μg of pHIV-LTR-CAT and either left unstimulated or stimulated with either 1 μg/ml PHA alone or 1 μg/ml PHA with 50 ng/ml PMA with or without a 16-h pretreatment with 1 μM CAI, as indicated. Cells were harvested and assayed for CAT activity as described above. Middle panel, Jurkat T-cells were transfected with 5 μg of pHIV-LTR-CAT and either left unstimulated or stimulated with 1 μM ionomycin plus 100 ng/ml PMA with or without a 16-h pretreatment with 1 μM CAI, as indicated. Cells were harvested 16 h later and processed for CAT activity as described above. Right panel, Jurkat T-cells were transfected with 5 μg of pHIV-LTR-CAT and either left unstimulated or stimulated in the presence of 140 nM thapsigargin plus 100 ng/ml PMA with or without 1 μM CAI. Cells were harvested, and CAT activity was quantitated as described above. The data represent the mean of two independent transfections and are shown as mean ± S.E.
show preferential affinity for calcium channels in the “open” conformation over the “closed” conformation, and the time requirement for inhibition may simply reflect the time required to bind to a stochastic distribution of open channels over the 8 h. In such a scenario, the rapid reversibility of the CAI inhibition could be due to a low affinity interaction (micromolar) between CAI and its target receptor or merely reflect very rapid dissociation and association rates for stochastically available binding sites. Because CAI has a high protein binding activity (45), the lengthy duration of pretreatment could reflect the time required for the drug to partition between the protein-rich culture medium and the cell surface. Still other interpretations include the possibility that CAI may be metabolized intracellularly to a freely and rapidly diffusible active product that accrues to threshold levels during the pretreatment period.

A role for calcium in NF-κB regulation has been suggested by numerous recent studies (9, 29, 46–48). Several of these studies have indicated that individual Rel family members display differential requirements for calcium-dependent signaling (9, 29, 46). The general consensus from these studies has been that c-Rel activity is much more strongly influenced by calcium signaling than is RelA (p65), whose calcium dependence is more variable. In fact, c-Rel has been shown previously to have activation characteristics that are indistinguishable from those of NFAT (47). These characteristics include a requirement for activation by both phorbol ester and calcium-mobilizing agents and a susceptibility to repression by the immunophilin class of calcineurin inhibitors including FK506 and cyclosporin A. Our finding that the nuclear translocation of c-Rel is much more dramatically affected by CAI treatment than that of p65 is consistent with these previous observations. The differential sensitivity of p65 and c-Rel to calcium blockade is likely due to the fact that c-Rel induction is also controlled transcriptionally by multiple active NF-κB enhancer elements in its promoter (49). Accordingly, small IκB-mediated changes in the nuclear levels of p65 or c-Rel would be expected to actively impinge on c-Rel transcription, thus reducing the total amount of cellular c-Rel, as shown in Fig. 6B.

Interestingly, pentoxifylline, a vasoactive anti-inflammatory drug, has also been shown to preferentially influence the induction of c-Rel over other Rel family members (46). This finding correlates well with the ability of pentoxifylline to inhibit transactivation of the HIV LTR (50, 51). However, unlike pentoxifylline, CAI is also a very potent inhibitor of NFAT induction (Fig. 8B). A simple and logical conclusion that can be drawn from the functional distinctions between the actions of these different classes of drugs is that pentoxifylline exerts its effect at a point in the calcium-dependent signaling pathway that is distal to the effects of the immunophilins, while the action of CAI occurs proximal to both pentoxifylline and FK506/CSA (i.e. at the level of calcium influx). Recent reports have implicated a role for NFAT in the transcriptional regulation of the HIV LTR through the κB site (52). Although a direct transcriptional role for NFAT at the HIV-κB enhancer elements has not been clearly demonstrated, the ability of CAI to repress
both c-Rel- and NFAT-dependent transactivation clearly underlies its potency as an HIV LTR transcriptional inhibitor. Although it is clear that the c-sites are the major targets of CAI action, we cannot rule out a possible effect of CAI on other cis-regulatory elements within the HIV LTR. Under the conditions used in this study, deletion of the c-sites reduces the mitogen inducibility of the HIV LTR to <0.6-fold above background (data not shown), thus making accurate assessment of possible CAI inhibition at other non-c-sites impractical.

The precise mechanism through which CAI is able to inhibit calcium influx into Jurkat T-cells remains an area of active investigation. Prior studies have provided clear evidence that, at high concentrations, CAI can inhibit calcium entry and other downstream events in diverse cell types (22, 44, 53). In addition, significant inhibition of tyrosine phosphorylation of phospholipase C-γ has been demonstrated (54). Thapsigargin acts by inhibiting the Ca²⁺-ATPase pump on the endoplasmic reticulum, thus allowing calcium release into the cytoplasm (40). It has also been proposed that depletion of internal calcium stores by ionophore may also elicit calcium influx (39). The fact that thapsigargin- or ionomycin-activated cells are resistant to low-dose (1 μM) pretreatment with CAI indicates that CAI may target events that occur prior to the depletion of internal calcium stores. Eligible targets include the protein-tyrosine kinase-dependent events set in play at the cytoplasmic face of the plasma membrane following ligation of T-cell surface receptors (30, 55–57).

It is likely that CAI can blunt multiple calcium-dependent signaling events during mitogenic stimulation. Although highly selective at low concentrations, a higher concentration of CAI produces a broader inhibition of downstream effectors of mitogenic signaling. As noted in this study, 10-fold higher concentrations of CAI can effectively inhibit activation of the TRE (Fig. 2). The mechanism of this level of inhibition remains to be determined. It is important to note that this higher level of CAI is not toxic since the effects are fully reversible (Fig. 3). CAI is likely to have effects at more distal events in the multiple signal pathways triggered by mitogenic stimulation. The potency of CAI action and its ready reversibility will make it a very effective pharmacological tool for dissecting the intricate web of signal transduction events critical to cell growth, proliferation, and differentiation. The selective efficacy of CAI action in repressing HIV LTR transactivation highlights the usefulness of calcium entry as a viable therapeutic target in the development of antiretroviral strategies.

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