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Cyclosporin A Enhances the Calcium-Dependent Induction of AP-1 Complex and *c-fos* mRNA in a T Cell Lymphoma

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The immunosuppressant cyclosporin A (CsA) exerts its pharmacologic actions by inhibiting calcineurin function. Here, we investigated the effect of CsA on the DNA-binding activity of the transcription factor, AP-1, in YAC-1 cells. We found that elevation of intracellular Ca^{2+} by ionomycin increased AP-1 DNA-binding activity in these cells. CsA treatment upregulated the ionomycin-induced, but not the basal AP-1 DNA-binding activity. In contrast, a CsA analog, MeVal[4]CsA, that does not inhibit calcineurin, failed to enhance ionomycin-induced AP-1 DNA-binding activity. This activity was shown to involve c-Fos, c-Jun and JunB. CsA consistently augmented ionomycin-induced *c-fos* mRNA expression and more variably that of *JunB.* Therefore, calcineurin negatively regulates Ca^{2+}-stimulated AP-1 activity principally at the *c-fos* induction level. By inhibiting calcineurin, CsA shifts the balance between positive and negative AP-1 regulation. Since AP-1 controls the transcription of many genes, this finding may have implications for both the immunosuppressive and toxic effects of CsA.

Cyclosporin A (CsA), currently used for the prophylaxis of organ transplant rejection (1, 2), exerts its immunosuppressive activity by inhibiting the production of lymphokines including IL-2, IL-3, IL-4, GM-CSF, TNF-α and interferon (IFN)-γ in activated T cells (3). This action is mediated through binding of CsA to an intracellular protein with peptidyl-prolyl-*cis-trans* isomerase activity, termed cyclophilin (Cyp) (4, 5). The Cyp-CsA complex thus formed is capable of interacting with and blocking the enzymatic activity of the Ca^{2+}/calmodulin-dependent protein serine/threonine phosphatase, calcineurin (6) which plays a role in dephosphorylating the nuclear factor of activated T cells (NF-AT) (7). By preventing NF-AT dephosphorylation, the Cyp-CsA complex inhibits the nuclear translocation and transcriptional activity of NF-AT and thereby blocks the activation of genes that depend on this factor (7).

Although the best characterized effects of CsA concern its suppression of lymphokine gene transcription, it has become clear that the drug can also regulate the expression of other genes in T cells as well as non-T cells (8-10). Defining the mechanisms of this modulation of non-lymphokine genes is an important issue as it can explain some of the deleterious side effects of CsA, including neurotoxicity and nephrotoxicity (11), that limit the drug's clinical utility.

We previously described a model in the YAC-1 murine T cell lymphoma, where CsA augments the expression of the Ly-6E antigen, a cell surface molecule unrelated to lymphokines and inducible by IFN-γ (12). In this system, the enhancing effect of CsA was found to depend on co-treatment of the cells with the Ca^{2+}-mobilizing ionophore, ionomycin. While investigating this phenomenon, we observed that ionomycin stimulates the DNA-binding activity of AP-1, a transcription factor known to play a central role in regulating gene expression

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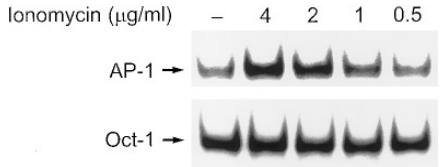

FIG. 1. Induction of AP-1 DNA-binding activity by ionomycin in YAC-1 cells. The cells were incubated for 2 hrs in the absence or in the presence of various ionomycin concentrations. Nuclear extracts were prepared and DNA-binding activity of AP-1 or Oct-1 were analyzed by EMSA.

in a variety of cellular responses (13). We therefore examined the effect of CsA on this induction of AP-1 activity by ionomycin in YAC-1 cells.

MATERIALS AND METHODS

Cell cultures. The YAC-1 cell line (American Type Culture Collection, Rockville, MD) was propagated in log-growth phase (unless otherwise indicated, Fig. 5A). Prior to use, the cells were washed once and brought to a concentration of 2×10^5 cells/ml. The cells were incubated in a volume of 50 ml (EMSA) or 20 ml (mRNA analysis) at 37°C with different agents as indicated.

Nuclear protein extraction. After treatment, the cells were washed twice with PBS. Cell pellets were resuspended in 150 μl of buffer A (10 mM HEPES pH 7.5, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and incubated on ice for 10 min. Eight μl 10 % NP-40 was then added and nuclei were separated from the cytosol by centrifugation at 13000 g for 2 min. The nuclear pellets were resuspended in 100 μl of buffer B (25 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 20% glycerol, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin). After high speed centrifugation of the lysates for 5 min, the supernatants, referred to as nuclear extracts, were stored at −70°C until analysis. Protein concentration was measured using a Pierce (Rockport, IL) protein assay.

Electrophoretic mobility shift assay (EMSA). The DNA-binding reactions were performed on nuclear extracts using synthetic oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA). The amount of nuclear protein used in AP-1 and Oct-1 binding reaction was 10 μg and 5 μg respectively. The sequence of the AP-1 consensus oligonucleotide was 5′-CGCTTGATGACTCAGCCGGAA-3′. The specificity of AP-1 DNA-binding was assessed by competition analyses, using unlabeled oligonucleotide for the AP-1 DNA-binding site and an oligonucleotide containing mutations in the AP-1 site (CTCAG to CTTGG). The sequence of the Oct-1 consensus oligonucleotide was 5′-TGTCGTATG-CAAATCACTAGAA-3′. Oligonucleotides were 5′ end-labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN), and 0.1 ng of labeled oligonucleotide was used in each DNA-binding reaction. For the gel supershift experiments, nuclear extracts were incubated for 30 min with anti-AP-1 component or control antibodies (Santa Cruz Biotechnology) before addition of the labeled oligoprobes. EMSAs were carried out in 20 μl consisting of 12.5 mM HEPES pH 7.5, 100 mM NaCl, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.05 % NP-40, 250 μg/ml BSA, 4 μg poly(dI-dC) for AP-1 or 2 μg for Oct-1. Electrophoresis was performed in a preelectrophoresed 6% polyacrylamide native gel. Images of the dried gel and quantitation of protein-DNA complexes were obtained from 4-24 hr exposures in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RNA extraction and slot-blot analysis. Total cellular RNA was isolated using RNAzol (Cinna/Bioteck, Friendswood, TX). RNA samples were blotted onto nitrocellulose filters using a slot-blot apparatus (Bio-Rad, Hercules, CA). The cDNA probes for murine *c-fos, c-jun* and *junB* (provided by Dr. Michael Karin, La Jolla, CA) were labelled with [^{32}P]-dCTP to high specific activity using a random primed DNA labeling kit (Boehringer-Mannheim). RNA blots were prehybridized and hybridized with the probes as described. The blots were autoradiographed using Hyperfilm-ECL (Amersham, Arlington Heights, IL).

RESULTS AND DISCUSSION

Detection of Ca^{2+}-Stimulated AP-1 DNA-Binding Activity in YAC-1 Cells

The DNA-binding activity of AP-1 was determined by EMSA in nuclear extracts from YAC-1 cells that were incubated for 2 hrs at 37°C in the absence or in the presence of ionomycin at concentrations previously demonstrated to cause Ca^{2+} mobilization and a modulation of IFN-γ-induced Ly-6E expression (12). As shown in Figure 1, while untreated cells displayed a faint band of shifted AP-1 probe, a much stronger band was

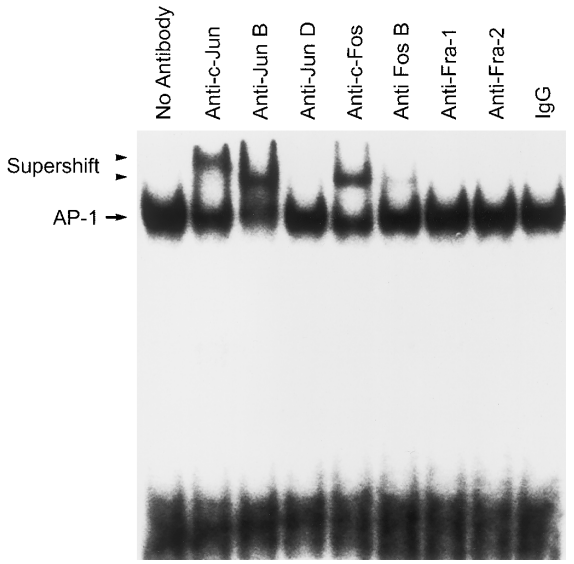

FIG. 2. Ionomycin-induced AP-1 complex contains c-Jun, Jun B and c-Fos proteins. Nuclear extracts from ionomycin-treated YAC-1 cells (2 μg/ml for 2 hrs) were incubated with 1 μl of each of the following antibodies: anti-c-Jun (lane 2), anti-Jun B (lane 3), anti-Jun D (lane 4), anti-c-Fos (lane 5); anti-Fos B (lane 6), anti-Fra-1 (lane 7), and anti-Fra-2 (lane 8) or serum from a nonimmunized rabbit (lane 9). Arrows indicate the mobility of the supershift bands.

detectable following ionomycin treatment, in a dose-dependent manner. This DNA-protein complex induced by ionomycin was specific for AP-1 as it was abolished by the addition of 100-fold molar excess of unlabeled AP-1 oligonucleotide probe but not by a mutant AP-1 oligonucleotide (Data not shown). Furthermore, in the same nuclear extracts, the level of Oct-1 DNA-binding activity was unaffected by ionomycin treatment (Fig. 1).

The present observation that ionomycin treatment alone is sufficient to induce strong AP-1 DNA binding activity in a T cell line is remarkable. Earlier studies in Jurkat T cells have indicated that although it synergizes with PKC activation mediated by PMA, ionomycin has no effect by itself in inducing AP-1 (14-16). In other cell types however, such as neurons, Ca^{2+}-signaling was found sufficient to promote AP-1 DNA-binding activity (17). Therefore, the Ca^{2+}-dependency of AP-1 activity induction may be cell type-specific.

There exist at least two types of AP-1 protein complex: Jun homodimer and Fos/Jun heterodimer (13). Hence, it was important to determine the composition of the AP-1 complex induced by ionomycin. Incubation of nuclear extracts with antibodies to Fos family proteins prior to the DNA-binding assay produced supershift bands that indicated the presence of c-Fos, but not Fos B, Fra-1 or Fra-2 in this complex (Figure 2, lane 5-9). Likewise, the use of antibodies to Jun family proteins demonstrated the involvement of c-Jun and JunB in the formation of the ionomycin-induced AP-1 complex (Figure 2, lane 2-4). Note that the monoclonal anti-c-Jun antibody produced a greater supershift than the other polyclonal antibodies in these assays and that preincubation with serum from non-immunized rabbit had no effect. Therefore, the AP-1 complex induced by ionomycin in YAC-1 cells contains the c-Fos, c-Jun and Jun B proteins. Such heteromeric AP-1 complexes usually function as effective transcriptional activators (13). However, further experiments using reporter gene constructs driven by AP-1 binding sites will be required to verify this point in YAC-1 cells.

	1	2	3	4	5	6	7	8	9	10
Ionomycin (1 μg/ml)	–	+	+	+	+	+	–	–	–	–
Cs A (μg/ml)	–	–	10	1	0.1	0.01	10	1	0.1	0.01

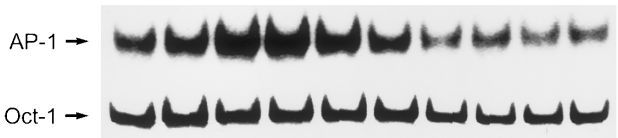

FIG. 3. CsA increases ionomycin-induced AP-1 DNA-binding activity. YAC-1 cells were preincubated with the indicated concentrations of CsA for 30 min and further incubated for 2 hrs in the presence (lanes 2–6) or absence of ionomycin (1 μg/ml) (lanes 1, 7-10). Nuclear extracts were analyzed by EMSA with AP-1 and Oct-1 oligonucleotide probes.

CsA Augments Ca^{2+}-Stimulated AP-1 DNA-Binding Activity

Having established that ionomycin induces AP-1 DNA binding activity in YAC-1 cells, it was interesting to investigate the effect of CsA on this response. The cells were pretreated with various concentrations of CsA for 30 min and further incubated with or without 1 μg/ml ionomycin for 2 hrs. As shown in Figure 3, CsA enhanced AP-1 DNA-binding activity in the presence, but not in the absence of ionomycin. This effect of CsA was reproduced in four independent experiments, the increase of AP-1 DNA-binding activity compared to ionomycin alone varying from 1.7- to 3.8-fold, as determined by Phosphorimager quantitation. Antibody-based super-shift analyses similar to those presented in Figure 2 further demonstrated that the CsA + ionomycin-induced AP-1 complex also includes c-Fos, c-Jun and JunB proteins (data not shown). Moreover, the enhancing effect of CsA on AP-1 DNA-binding activity was clearly dose-dependent (Fig. 5), occuring at drug concentrations (0.1-10 μg/ml) that are known to suppress calcineurin phosphatase activity in cells (18). It was verified that such CsA treatment does block calcineurin activity in YAC-1 cells (data not shown).

To further determine the role of calcineurin inhibition in this effect of CsA on ionomycin-induced AP-1 activity, we used a CsA analog, $MeVal^4$-CsA that does not inhibit calcineurin even though it binds to Cyp and inhibits its peptidyl-prolyl-*cis-trans* isomerase activity (19). Furthermore, $MeVal^4$-CsA acts as an antagonist of CsA immunosuppression, due to its ability to compete with CsA for binding to intracellular Cyp (19). Figure 4 shows that at a concentration of 10 μg/ml, $MeVal^4$-CsA failed to increase ionomycin-stimulated AP-1 DNA-binding activity but blocked the enhancement caused by CsA. This rules out the possibilities that the enhancing action of CsA on ionomycin-stimulated AP-1 DNA-binding activity could result from non-specific effects or from an inhibition of the peptidyl-prolyl-*cis-trans* isomerase activity of Cyp (5). Instead, the action of CsA in this system must reflect an inhibition of calcineurin phosphatase activity, implying that calcineurin functions as a negative regulator of the AP-1 DNA-binding activity stimulated by Ca^{2+} in YAC-1 cells.

The effect of CsA on AP-1 induction was formerly investigated in the context of IL-2 gene activation. Using the Jurkat T cell line, it was found that CsA does not alter AP-1 DNA binding activity stimulated by T cell receptor ligation + PMA or ionomycin + PMA (14, 20). Likewise, AP-1 sequence-binding proteins were not inhibited by this drug in EL4 lymphoma cells (21). However, in primary human T cell cultures stimulated with PHA + PMA, the AP-1 DNA binding activity was reported to be suppressed by CsA (22). Our findings in YAC-1 cells therefore disclose a unique, previously unrecognized stimulating action of CsA on this activity. Again, these apparently discrepant observations may indicate cell type-and stimulus-specific differences in the regulation of AP-1 DNA binding activity.

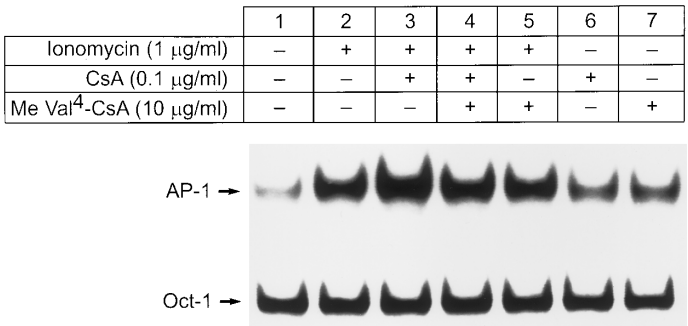

FIG. 4. MeVal[4]-CsA does not increase ionomycin-induced AP-1 DNA binding activity but blocks the enhancing effect of CsA. YAC-1 cells were preincubated for 30 min in the absence (lane 1, 6. 7) or presence of 100 ng/ml CsA (lanes 3, 4, 6) with (lane 4) or without 10 μg/ml MeVal[4] -CsA (lane 3) or with 10 μg/ml MeVal[4] -CsA alone (lane 5, 7) and further incubated for 2 hrs without (lane 1, 6, 7) or with 1 μg/ml ionomycin (lanes 2-5). Nuclear extracts were analyzed by EMSA for DNA-binding activity of AP-1 or Oct-1. The DNA-binding activitiy of nuclear extract from untreated cells is shown in lane 1.

CsA Reproducibly Augments Ionomycin-Induced Expression of c-fos mRNA

The negative regulation of AP-1 DNA-binding activity by calcineurin might reflect alterations in the phosphorylation events that affect DNA-binding of the AP-1 complex (23) and/or a down-regulation of ionomycin-driven generation of AP-1 components. In favor of the latter possibility, we found that the CsA-mediated enhancement of AP-1 DNA-binding activity was partially inhibited by actinomycin D and cycloheximide (data not shown), suggesting a requirement for *de novo* RNA and protein syntheses in this enhancement.

To evaluate the hypothesis that calcineurin/CsA affect AP-1 activity in YAC-1 cells in part by modulating the generation of AP-1 components, we examined the expression of these components at the mRNA level. Since the gel supershift experiments demonstrated that the ionomycin-induced AP-1 complex regulated by CsA contains c-Fos, c-Jun and JunB proteins, we specifically examined these components. YAC-1 cells were treated with ionomycin in the absence or presence of CsA and mRNA expression was analyzed after 30 min of incubation. As shown in Figure 5A, YAC-1 cells displayed little basal expression of *c-fos* mRNA but ionomycin induced the accumulation of this mRNA which was further augmented by CsA co-treatment. Similar results were obtained in four additional experiments. A kinetics study of *c-fos* mRNA expression indicated that CsA markedly prolongs the duration of this expression induced by ionomycin (Fig. 6). In contrast, *c-Jun* mRNA was already present in untreated YAC-1 cells and this constitutive expression was unchanged by ionomycin or ionomycin + CsA (Fig. 5A, B). The mRNA for *JunB* was also not affected by incubation with ionomycin or ionomycin + PMA in overgrown cells that expressed a high basal level of this mRNA (Fig. 5A). In contrast, under log-phase growth conditions where basal *junB* mRNA level was low, an enhancement by CsA in presence of ionomycin was observed (Figure 5B). Therefore, a dampening of Ca^{2+}-dependent induction of *c-fos* and, to a lesser extent *junB* is likely to contribute to the down-regulation of AP-1 DNA-binding activity by calcineurin. However, the potential role of translational or post-translational (e.g. phosphorylation) modifications of AP-1 components in this effect remains to be determined.

The expression of *c-fos* has been shown to be transiently induced by a variety of agents in numerous cell types (13). In several instances (24-26), a rise in intracellular Ca^{2+} was found sufficient for this induction, operating both at the initiation of transcription and through relief

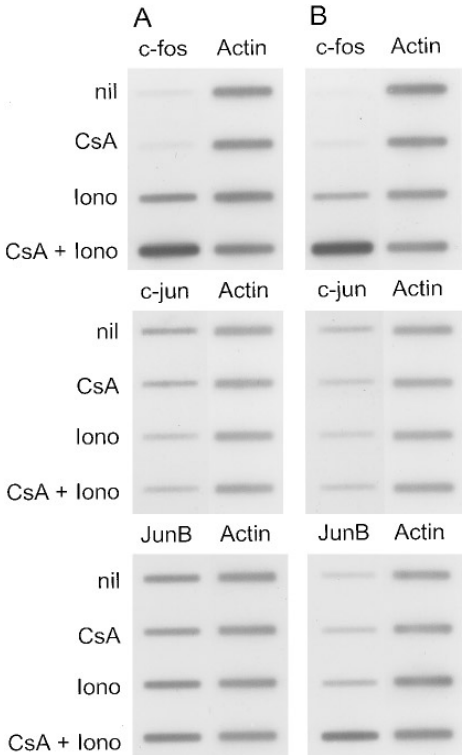

FIG. 5. Effects of ionomycin and CsA on the expression of mRNA for *c-fos, c-jun* and *Jun B.* YAC-1 cells were preincubated for 30 min in the absence or in the presence of CsA (1 μg/ml) and were treated with ionomycin (1 μg/ml) or left untreated for another 30 min. Total cellular RNA was extracted, blotted onto nitrocellulose filter and hybridized with [^{32}P]-labeled cDNA probes for *c-fos, c-jun* or *Jun B.* After autoradiography, the blots were stripped and rehybridized with [^{32}P]-labeled *β-actin* cDNA probe followed by autoradiography. A: Cells were overgrown, B: Cells were in log-phase growth.

of a block of transcriptional elongation (26). It will be interesting to determine whether such a dual mechanism of *c-fos* regulation is involved in YAC-1 cells, and if so, at which step might calcineurin intervene. On the other hand, the mechanisms of regulation of *c-jun* and *JunB,* are probably distinct from those of *c-fos.* For example, Ca^{2+} signaling was found to

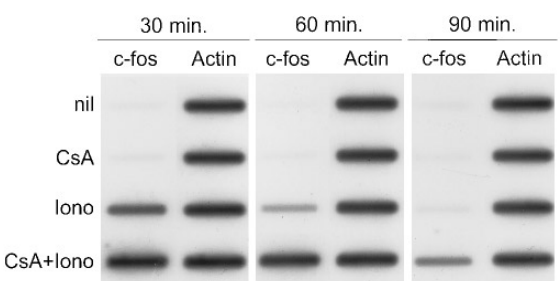

FIG. 6. Time-course of *c-fos* mRNA induction by ionomycin without or with CsA. YAC-1 cells were preincubated for 30 min in the absence or in the presence of CsA (1 μg/ml) and were treated with ionomycin (1 μg/ml) or left untreated for 30 to 90 min. Total cellular RNA was extracted and analyzed for *c-fos* and *β-actin* transcripts as in Figure 5.

induce *c-fos* and *c-jun*, but not *junB* in a salivary cell line (24). Nevertheless, under certain conditions, *JunB* appears to be modulated in parallel with *c-fos* through Ca^{2+}-dependent/CsA-sensitive mechanisms in our system.

If calcineurin is a negative regulator of Ca^{2+}-dependent AP-1 activity and *c-fos* mRNA induction in YAC-1 cells, then what is the positive regulator of this pathway? One possible candidate is Cam kinase IV which was recently reported to mediate *c-fos* reporter gene transcription and AP-1 induction in Jurkat cells (27). We are currently investigating this possibility.

The unique regulation of Ca^{2+}-dependent AP-1 DNA-binding activity by CsA in YAC-1 cells may relate to the Ca^{2+}-dependent enhancement of Ly-6E induction caused by CsA in these cells (12). One may speculate that an upregulation of AP-1 activity also plays a role in other situations where CsA enhances the expression of certain genes, such as IP10 (8), IL-6 (9) and TGF-β (10). Interestingly, an elevation of AP-1 DNA-binding that is dependent on calcium was recently demonstrated in the kidney of CsA-treated rats (28), suggesting that the present findings may have implications for the mode of nephrotoxicity of CsA (11). Further analysis of the YAC-1 cell model should therefore be useful to explore the role of AP-1 activity in CsA-mediated immunosuppressive and toxic effects.

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