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Inhibition of CREB- and cAMP response element-mediated gene transcription by the immunosuppressive drugs cyclosporin A and FK506 in T cells

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Abstract The clinically important immunosuppressant drugs cyclosporin A and FK506 (tacrolimus) inhibit in T-cells calcineurin phosphatase activity and nuclear translocation of the cytosolic component of the transcription factor nuclear factor of activated T-cells (NF-ATc) that is involved in the induction of early genes during T-cell activation. This effect has been proposed to explain at least part of the immunosuppressive effect of these drugs. Previous studies in pancreatic islet cell lines have shown that cyclosporin A and FK506 through inhibition of calcineurin interfere also with the function of the transcription factor cAMP response element binding protein (CREB) that is activated by cAMP and calcium signals and binds to cAMP/calcium response elements (CRE). By transient expression of CRE-reporter genes or GAL4-CREB fusion proteins, the present study shows that inhibition of CREB/CRE-directed transcription by cyclosporin A and FK506 occurs in a great variety of cell types including in cell lines derived from tissues in which adverse effects of the immunosuppressants develop. CREB activity and CRE-mediated transcription was blocked by these drugs also in Jurkat T-cells. When taken together with recent evidence for an essential role of CREB in T-cell activation and proliferation, the present results suggest that inhibition of CREB/CRE-directed transcription may be a molecular mechanism of the immunosuppressive effect of cyclosporin A and FK506.

Key words Cyclosporin A · FK506 · cAMP response element · CREB · Transcription · Immunosuppressive drugs · T-cells

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Introduction

Cyclosporin A and FK506 (tacrolimus) are powerful immunosuppressive drugs, which are widely used to prevent graft rejection following organ transplantation. The therapeutic application of cyclosporin A and FK506 is, however, limited by untoward effects that are shared by both drugs, including nephrotoxicity, hypertension, neurotoxicity and impaired glucose tolerance (Mason 1990; Japanese FK 506 Study Group 1993; Burke et al. 1994). Cyclosporin A and FK506 repress an early step in T-cell activation. According to current understanding, inhibition of calcineurin phosphatase activity and thereby inhibition of nuclear translocation of the cytosolic component of the transcription factor nuclear factor of activated T-cells (NF-ATc) may explain at least part of the immunosuppressive effect of cyclosporin A and FK506. In combination with activating protein 1 (AP-1), NF-ATc binds to specific DNA control elements and activates the transcription of early genes, such as those encoding interleukin 2 (IL-2) and other cytokines (Schreiber and Crabtree 1992; Morris 1994; Szamel et al. 1993; Ockenfels et al. 1996).

Cyclic AMP response element binding protein (CREB) is a ubiquitously expressed transcripition factor that is phosphorylated at serine 119 and thus activated by increases in cellular cAMP levels or increases in the cytosolic free calcium concentration (Meyer and Habener 1993; Ghosh and Greenberg 1995; Schwaninger et al. 1993a, b, 1995). CREB thereby confers cAMP and calcium responsiveness to genes that carry a CREB binding site, cAMP response element (CRE), with the consensus octamer motif TGACGTCA (Meyer and Habener 1993; Ghosh and Greenberg 1995). Studies in pancreatic islet cell lines have shown that cyclosporin A and FK506 inhibit CRE-directed transcription after activation by membrane depolarization-induced calcium influx or an elevation of cellular cAMP levels (Schwaninger et al. 1993b, c, 1995). The effective concentrations are consistent with the reported affinities of both drugs to their distinct immunophilin re-

ceptors and are similar to those concentrations effective in T-cells. At the same concentrations, these agents also inhibited calcineurin phosphatase activity in islet cells (Schwaninger et al. 1993b, c, 1995). When inhibition of calcineurin by FK506 or cyclosporin A was reversed by rapamycin or overexpression of calcineurin, CRE-dependent transcription was disinhibited (Schwaninger et al. 1993b, c, 1995). Using a GAL4-CREB fusion protein, it was shown that after activation by cAMP or calcium cyclosporin A and FK506 inhibit the transcriptional activity of CREB without blocking its phosphorylation on serine 119 (Schwaninger et al. 1993b, 1995). Thus, through inhibition of calcineurin phosphatase activity, cyclosporin A and FK506 block, in islet cells, CREB/CRE-directed transcription.

In those previous studies, the inhibition by cyclosporin A and FK506 was found in pancreatic islet cell lines, that express the peptide hormones glucagon or insulin (Schwaninger et al. 1993b, c, 1995; Eckert et al. 1996). To examine whether inhibition of CREB/CRE-directed transcription by cyclosporin A and FK506 is specific for pancreatic islet cells or takes place in a variety of other cells as well, in the present study the effect of cyclosporin A and FK506 on CREB/CRE-directed transcription was investigated in several distinct cells.

Methods

Plasmid construction. The plasmid 5xGal4E1BLuc was prepared by subcloning the *Hind*III/*Kpn*I fragment from the plasmid G5E1BCAT (Liu and Green 1990) into the *Hind*III–*Kpn*I sites of pXP2 (Nordeen 1988). The plasmid 4xPubdT109Luc was prepared by subcloning the *Hind*III/*Bgl*II fragment from the plasmid 4xPubdCAT (Brabletz et al. 1991) into the *Hind*III–*Bgl*II sites of pXP2 (Nordeen 1988). The constructs were confirmed by sequencing. The plasmids 4xSomCRET81Luc (Schwaninger et al. 1995), pZ1 (Schwaninger et al. 1993a), and pSG424 (Sadowski and Ptashne 1989) have been described before.

Cell culture and transfection. The following nonimmune cell lines were grown in the media given: pig kidney LLC-PK_1-$FBPase^+$ cells (Gstraunthaler and Handler 1987) (obtained from Dr. G. Gstraunthaler, Innsbruck, Austria), DMEM containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, and 5 mM glucose; neuroblastoma (mouse) – glioma (rat) hybrid NG108-15 cells (Klee and Nirenberg 1974) (obtained from Dr. G. Thiel, Köln, Germany), DMEM containing 10% FCS, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine; mouse neuroblastoma NS20Y cells (Amano et al. 1972) (obtained from Dr. G. Thiel, Köln, Germany), DMEM containing 10% FCS; human choriocarcinoma JEG-3 cells, DMEM containing 10% FCS; human hepatocellular carcinoma HepG2 cells, DMEM containing 10% FCS; mouse pituitary gonadotrope αT3-1 cells (Windle et al. 1990) (obtained from Dr. P. L. Mellon, La Jolla, CA), DMEM containing 5% FCS and 5% horse serum; growth hormone- and prolactin-producing rat pituitary GH_3 cells (obtained from Dr. H. J. Steinfelder, Göttingen, Germany), DMEM containing 10% FCS, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1 mM sodium pyruvate; AtT20 mouse pituitary corticotrope cells (obtained from ATCC, Rockville, MD), Ham's F-10 medium containing 15% horse serum and 2.5% FCS; αTSH mouse thyrotrope cells (Akerblom et al. 1990) (obtained from Dr. P. L. Mellon, La Jolla, CA), DMEM containing 5% FCS and 5% horse serum; rat adrenal pheochromocytoma PC12 cells (obtained from DSM, Braunschweig, Germany), RPMI 1640 containing 10% horse serum and 5% FCS; smooth muscle A7r5 cells derived from fetal rat thoracic aorta (obtained from Drs. A. Lückhoff and G. Schultz, Berlin, Germany), DMEM containing 10% FCS. All media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were transfected with the reporter plasmid 4xSomCRET81Luc either by the calcium phosphate precipitation method (5–10 μg of reporter plasmid/6-cm dish; LLC-PK_1-$FBPase^+$, NG108-15, NS20Y, JEG-3, HepG2, PC12 and A7r5 cells) or in suspension by the DEAE-dextran method (2–5 μg of reporter plasmid/6-cm dish; GH_3, AtT20, αT3-1, and αTSH cells).

The human leukemic T-cell line Jurkat E6.1 was maintained in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Jurkat cells were transiently transfected with 6 μg of reporter plasmid/6-cm dish. The plasmid Rous sarcoma virus-chloramphenicol acetyltransferase (1 μg/6-cm dish) was added as a second reporter to check for transfection efficiency. When indicated, 6 μg of the expression vectors pZ1 or pSG424 were cotransfected per 6-cm dish. Jurkat cells were transfected by the DEAE-dextran method with chloroquine treatment.

Cell extracts (Schwaninger et al. 1993a) were prepared 48 h after transfection. Cells were stimulated with forskolin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), high KCl, A23187 or phytohemagglutinin (PHA) for 6 h before harvest. FK506 or cyclosporin A were added 1 h before stimulation. The chloramphenicol acetyltransferase assay (Schwaninger et al. 1993a) and the luciferase assay were performed as previously described (Schwaninger et al. 1993a).

Calcineurin phosphatase assay. After incubation of the cells with cyclosporin A (5 μM), FK506 (167 nM) or solvent for 1 h, hypotonic lysates were prepared and calcineurin phosphatase activity was measured as described (Fruman et al. 1992; Schwaninger et al. 1993c, 1995).

Immunoblots. Immunoblots from cell lysates using either anti-CREB antiserum (Waeber et al. 1991) or anti-phosphoCREB antibodies (Ginty et al. 1993) were performed as described (Schwaninger et al. 1995).

Materials. Luciferin, forskolin, A23187, TPA, and PHA-P were purchased from Sigma. FK506 (Fujisawa) was dissolved in ethanol. A stock solution of cyclosporin A (Sandoz) (10 mg/ml) was prepared in ethanol with 20% Tween 80 and further diluted in medium. Controls received the solvent only.

Results

Effect of cyclosporin A and FK506 on calcineurin phosphatase activity and CRE-directed transcription in nonimmune cells

CRE-directed transcription was measured by transient transfection of the luciferase reporter gene that was placed under the transcriptional control of four copies of a well characterized CRE, which is known as a high affinity CREB binding site and mediates responsiveness to both cAMP and calcium (Schwaninger et al. 1995). Figure 1 shows the effects obtained in LLC-PK_1 cells, a pig kidney cell line of renal proximal tubular epithelial cells (Gstraunthaler and Handler 1987). Treatment with FK506 (167 nM) inhibited calcineurin phosphatase activity by 90% (Fig. 1). CRE-directed transcription was stimulated by the adenylate cyclase activator forskolin (10 μM) about 5-fold (Fig. 1). FK506 had no effect on CRE-directed transcription both under basal conditions and after stimulation by forskolin (Fig. 1). Similar results, i.e. no change in

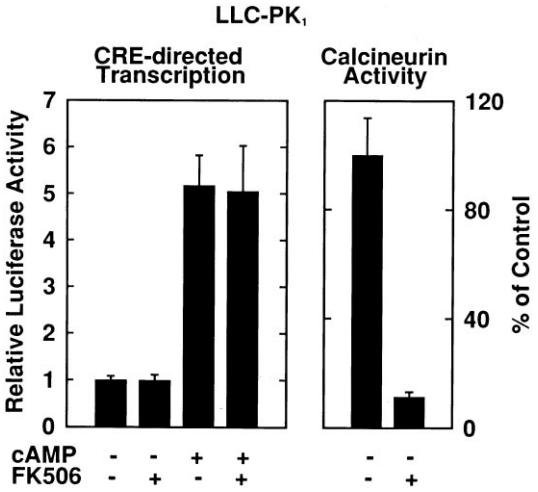

Fig. 1 Effect of FK506 on calcineurin phosphatase activity and CRE-directed transcription in LLC-PK_1 cells. For transcription experiments, the cells were transfected with the reporter plasmid 4xSomCRET81Luc and stimulated by forskolin (10 μM; cAMP) in the absence or presence of FK506 (167 nM). Luciferase activity is expressed relative to the mean value measured in each experiment in the untreated cells. Calcineurin activity is expressed as percentage of activity in untreated cells. Values are means ± SEM of four independent experiments, each done in triplicate

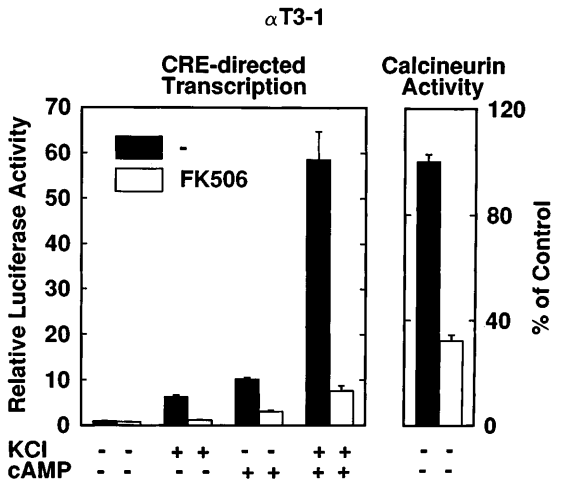

Fig. 2 Effect of FK506 on calcineurin phosphatase activity and CRE-directed transcription in αT3-1 cells. For transcription experiments, the cells were transfected with the reporter plasmid 4xSomCRET81Luc and stimulated by high potassium-induced membrane depolarization (KCl, 45 mM), forskolin (10 μM; cAMP) or both in the absence or presence of FK506 (167 nM). Luciferase activity is expressed relative to the mean value measured in each experiment in the untreated cells. Calcineurin activity is expressed as percentage of activity in untreated cells. Values are means ± SEM of three independent experiments, each done in duplicate

Table 1 Effect of FK506 and cyclosporin A on calcineurin activity and CRE-directed transcription in various cell lines. For transcription experiments, the cells were transfected with the reporter plasmid 4xSomCRET81Luc and stimulated by forskolin (10 μM) in the absence or presence of FK506 (167 nM) or cyclosporin A (CsA, 5 μM). Luciferase activity is expressed as percent of the mean value measured in each experiment in untreated cells. Calcineurin activity is expressed as percentage of activity in untreated cells. Values are means ± SEM of three to five independent experiments, each done in duplicate

		CRE-directed transcription		Calcineurin activity
		–	Forskolin	–
NG108	–	100 ± 3	219 ± 23	100 ± 8
	FK506	204 ± 41	359 ± 67	23 ± 2
NS20Y	–	100 ± 6	390 ± 57	100 ± 6
	FK506	112 ± 20	441 ± 54	36 ± 6
JEG	–	100 ± 10	488 ± 46	100 ± 9
	FK506	125 ± 3	739 ± 91	11 ± 2
	CsA	88 ± 4	387 ± 90	13 ± 5
HepG2	–	100 ± 6	238 ± 28	100 ± 13
	FK506	121 ± 6	355 ± 73	14 ± 4
	CsA	134 ± 12	322 ± 73	22 ± 3

CRE-directed transcription after stimulation by cAMP in spite of inhibition of calcineurin phosphatase activity by FK506 (167 nM) or cyclosporin A (5 μM), were obtained in the cell lines NG108, NS20Y, JEG, and Hep G2 (Table 1). As inhibition of CREB/CRE-directed transcription by the calcineurin phosphatase inhibitors cyclosporin A and FK506 requires the expression and activation of a kinase that phosphorylates the calcineurin substrate (Schwaninger et al. 1993b, c, 1995), we cannot exclude that under different conditions cyclosporin A and FK506 could block CRE-mediated transcription in these cell lines.

Different results were obtained in several other cell lines. Figure 2 shows the effects in αT3-1 cells, a mouse cell line of the pituitary gonadotrope lineage (Windle et al. 1990). CRE-directed transcription was stimulated by high potassium-induced membrane depolarization and calcium influx as well as by forskolin (10 μM) (Fig. 2). Calcium and cAMP stimulated CRE-mediated transcription synergistically. While CRE-mediated transcription was stimulated 6.3-fold by KCl and 10.2-fold by forskolin, the combination of the two induced a 59-fold increase in transcription (Fig. 2). cAMP and calcium have been shown by several studies to synergistically stimulate CRE-directed gene transcription (Schwaninger et al. 1993a, 1995). Parallel to an inhibition of calcineurin phosphatase activity, FK506 markedly inhibited CRE-directed transcription after stimulation by membrane depolarization, cAMP and a combination of the two (Fig. 2). Treatment of the cells with cyclosporin A produced a similar inhibition (Table 2). Cyclosporin A and FK506 did not change basal transcriptional activity (Fig. 2 and data not shown).

FK506 and cyclosporin A inhibited calcineurin phosphatase activity and CRE-directed transcription also in various other nonimmune cell types (Table 2). As in αT3-1 gonadotrope cells, FK506 and cyclosporin A were effective in other phenotypically distinct pituitary cell lines. After stimulation by membrane depolarization, cAMP or both, marked inhibition of CRE-directed transcription by FK506 and cyclosporin A was found in the growth hor-

Table 2 Inhibition of CRE-directed transcription by FK506 and cyclosporin A in various nonimmune cell types

Cell line	Stimulus	Inhibition (%) by	
		FK506	Cyclosporin A
GH_3	KCl	[a]	[a]
	cAMP	86.8 ± 1.3	82.0 ± 2.5
	KCl + cAMP	94.9 ± 0.9	92.7 ± 1.2
AtT20	KCl	86.6 ± 9.1	87.5 ± 14.2
	cAMP	39.9 ± 3.8	29.0 ± 3.1
	KCl + cAMP	61.3 ± 1.6	49.7 ± 1.5
αT3-1	KCl	97.2 ± 2.6	96.5 ± 1.4
	cAMP	77.5 ± 2.3	64.7 ± 10.0
	KCl + cAMP	88.7 ± 1.3	86.6 ± 2.5
αTSH	KCl	[a]	[a]
	cAMP	15.4 ± 6.8	28.8 ± 1.9
	KCl + cAMP	46.2 ± 2.2	45.8 ± 5.1
PC12	KCl	47.5 ± 18.1	n.d.
	cAMP	0	n.d.
	KCl + cAMP	0	n.d.
A7r5	KCl	112.8 ± 6.0	103.0 ± 8.9
	cAMP	[b]	[b]
	KCl + cAMP	72.8 ± 7.1	59.2 ± 4.2

The indicated cell lines were transfected with the reporter plasmid 4xSomCRET81Luc and stimulated by high potassium-induced membrane depolarization (KCl, 45 mM), forskolin (10 μM; cAMP) or both in the absence or presence of FK506 (167 nM) or cyclosporin A (5 μM). The values show the percent decrease in the stimulation of transcription in the presence of FK506 or cyclosporin A compared with the stimulation in the absence of FK506 or cyclosporin A. The stimulation of transcription in the absence of FK506 or cyclosporin A (controls) by KCl, cAMP, and KCl + cAMP was (fold stimulation) 0.8 ± 0.1, 28.2 ± 4.1, and 68.3 ± 14.5 (GH_3 cells), 1.9 ± 0.4, 92.0 ± 1.9, and 290.9 ± 26.2 (AtT20 cells), 1.1 ± 0.1, 34.4 ± 5.1, and 122.3 ± 5.0 (αTSH cells), 2.9 ± 0.7, 10.3 ± 1.6, and 35.8 ± 5.8 (PC12 cells), 2.1 ± 0.3, 1.4 ± 0.2, and 3.8 ± 0.8 (A7r5 cells), respectively.

[a] no stimulation of transcription by KCl in controls

[b] no stimulation of transcription by forskolin in controls. n.d., not determined. Values are means ± SEM of three to five independent experiments, each done in duplicate. Calcineurin activity was inhibited by FK506 and cyclosporin A in all cell lines tested

mone- and prolactin-producing GH_3 cells as well as in AtT20 mouse pituitary corticotrope cells, whereas in the αTSH mouse thyrotrope cell line the inhibition by the immunosuppressants ranged from 15 to 46% (Table 2). In the neuronal pheochromocytoma cell line PC12, FK506 inhibited CRE-directed transcription by about 50% only after stimulation by membrane depolarization (Table 2). In the vascular smooth muscle cell line A7r5 derived from fetal rat thoracic aorta, FK506 and cyclosporin A inhibited CRE-directed transcription completely when stimulated by membrane depolarization and by 60 to 75% when transcription was further enhanced by adding forskolin (Table 2).

In the above experiments, cyclosporin A and FK506 were used at concentrations (5 μM and 167 nM, respectively) that were found in previous experiments in pancreatic islet cell lines to produce a maximum inhibition of calcineurin phosphatase activity and CRE-directed transcription (Schwaninger et al. 1993b, c, 1995). A concentration-response curve in GH_3 cells showed that after stimulation by forskolin (10 μM) CRE-mediated transcription was inhibited by cyclosporin A and FK506 in a concentration-dependent manner (not shown). The IC_{50} value for cyclosporin A was about 50 nM and for FK506 about 1 nM.

Effect of cyclosporin A and FK506 on CREB/CRE-directed transcription in the T-cell line Jurkat

Cyclosporin A and FK506 exert their major therapeutic effects by inhibiting T-cell activation (Schreiber and Crabtree 1992; Morris 1994). We therefore studied CREB/CRE-directed transcription in the T-cell line Jurkat, which was used in previous investigations to demonstrate the inhibition by cyclosporin A and FK506 of calcineurin phosphatase activity and NF-AT-directed transcription (Schreiber and Crabtree 1992; Morris 1994; Clipstone and Crabtree 1992). Confirming previously published data (Schreiber and Crabtree 1992; Clipstone and Crabtree 1992), transcription directed by an NF-AT site from the IL-2 gene promoter was markedly enhanced by the combined stimulus protein kinase C-activating phorbol ester plus calcium ionophore (Fig. 3); treatment of the cells with cyclosporin A prevented transcriptional activation of the NF-AT site (Fig. 3). In parallel with the inhibition of NF-AT-directed transcription, cyclosporin A blocked calcineurin activity (not shown). CRE-directed transcription was not detectably activated by TPA/A23187, but was stimulated by forskolin and even more so by forskolin in combination with TPA/A23187 (Fig. 3) consistent with a recent report (Brindle et al. 1995). Cyclosporin A inhibited the activation of CRE-directed transcription by 60–75% (Fig. 3). A similar inhibition was produced by FK506 (167 nM) (not shown).

In addition to CREB, multiple transcription factors can bind to CREs (Meyer and Habener 1993; Ghosh and Greenberg 1995). To examine whether cyclosporin A and FK506 interfere with the action of CREB, a GAL4-CREB fusion protein was used. This consists of the DNA-binding domain of the yeast transcription factor GAL4 (amino acids 1–147) fused to the transactivation domain of CREB-327 (amino acids 1–261) (Fig. 4). Together with a reporter plasmid containing the luciferase reporter gene under the control of 5 copies of a GAL4-binding site (5xGal4E1BLuc), the GAL4-CREB fusion protein allows a specific functional analysis of the CREB transactivation domain without interference from endogenous CRE-binding proteins (Schwaninger et al. 1993a). Cotransfection of the reporter plasmid 5xGal4E1BLuc and the expression plasmid pZ1, which encodes the GAL4-CREB fusion protein (Schwaninger et al. 1993a), resulted in transcriptional activity that was stimulated about 3-fold by cAMP plus TPA/A23187 (Fig. 4). The transcriptional activity observed was conferred by the CREB transactivation do-

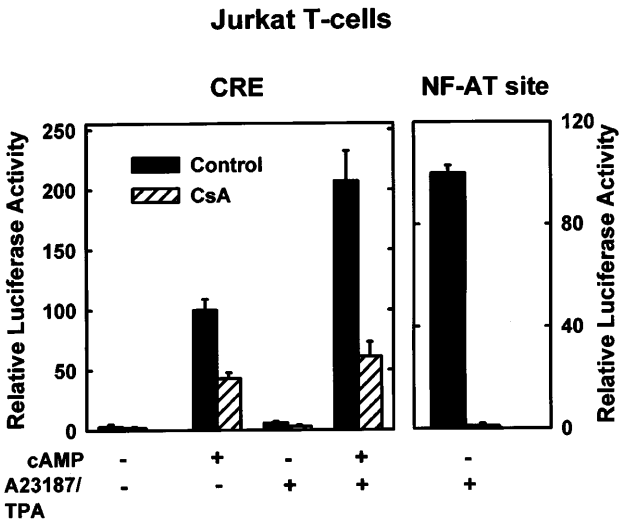

Fig. 3 Inhibition of CRE-directed transcription by cyclosporin A in Jurkat T-cells. The cells were transfected with the reporter plasmid 4xSomCRET81Luc (CRE) or 4xPubdT109Luc (NF-AT site) and stimulated by forskolin (10 μM; cAMP), A23187 (5 μM) plus TPA (300 nM) or both in the absence or presence of cyclosporin A (CsA, 5 μM). Luciferase activity is expressed as percent of the mean value measured in each experiment in forskolin- (CRE) or A23187/TPA-treated cells (NF-AT site) in the absence of CsA. Values are means ± SEM of three experiments, each done in triplicate

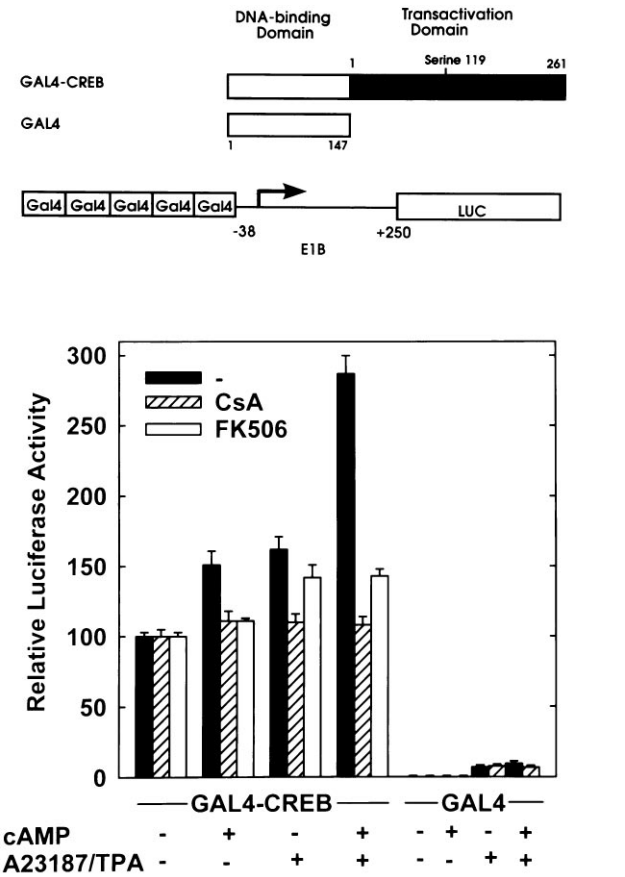

Fig. 4 Inhibition of CREB activity by cyclosporin A and FK506 in Jurkat T-cells. A luciferase reporter gene under the control of GAL4-binding sites (plasmid 5xGal4E1BLuc) was transfected into Jurkat T-cells together with an expression vector encoding a GAL4-CREB fusion protein (plasmid pZ1) or together with an expression vector encoding GAL4(1–147) (plasmid pSG424). The cells were stimulated with forskolin (10 μM; cAMP), A23187 (5 μM) plus TPA (300 nM) or both in the absence or presence of cyclosporin A (CsA, 5 μM) or FK506 (167 nM). Luciferase activity is expressed as percent of the mean value measured in each experiment in untreated controls expressing GAL4-CREB. Values are means ± SEM of four independent experiments, each done in duplicate

main, as virtually no activity was detected after cotransfection of the plasmids 5xGal4E1BLuc and pSG424 (Fig. 4), which encodes only the GAL4 DNA-binding domain (Sadowski and Ptashne 1989). Treatment of the cells with cyclosporin A and FK506 did not change basal transcriptional activity of the GAL4-CREB fusion protein, but almost completely prevented its activation by cAMP plus TPA/A23187 (Fig. 4). Similar results were obtained, when the calcium ionophore A23187 was replaced by the lectin PHA used in combination with TPA as another mitogenic stimulus. GAL4-CREB activity was stimulated 5.7-fold by cAMP plus TPA/PHA and this induction was almost abolished by cyclosporin A (data not shown). These results indicate that cyclosporin A and FK506 block the activation of CREB in stimulated Jurkat T-cells. Forskolin-stimulated GAL4-CREB activity was inhibited also in GH_3 cells (not shown).

The above experiments demonstrate inhibition of CREB/CRE-directed transcription in Jurkat T-cells by two different compounds (cyclosporin A and FK506) that block calcineurin phosphatase activity through distinct intracellular receptors (Schreiber and Crabtree 1992), suggesting that cyclosporin A and FK506 inhibit the stimulation of CREB transcriptional activity through inhibition of calcineurin. CREB activity is directly regulated by protein kinase A phosphorylation of a single serine residue, serine 119 of CREB-327 (corresponding to serine 133 in CREB-341) (Meyer and Habener 1993). The same serine residue is a substrate also for calcium-dependent (Ghosh and Greenberg 1995) and Ras-dependent (Xing et al. 1996) kinases and its phosphorylation is required for CREB activation (Meyer and Habener 1993). It was, therefore, tested whether cyclosporin A changes the phosphorylation of CREB at serine 119. The phosphorylation of CREB at serine 119 was investigated by immunoblots of Jurkat T-cell lysates using an antibody that recognizes CREB phosphorylated at serine 119 but fails to recognize CREB that is not phosphorylated at this site (Ginty et al. 1993; Schwaninger et al. 1995). Stimulation of Jurkat T-cells with cAMP plus TPA/A23187 resulted in the phosphorylation of CREB (Fig. 5, compare lane 1 to lane 7, the arrow indicates phosphoCREB). Cyclosporin A did not inhibit the phosphorylation of CREB at any time point examined (from 15 min to 6 h) (Fig. 5). The decrease in signal intensity observed at 2, 4, and 6 h in the absence of cyclosporin A was even somewhat delayed in the presence of cyclosporin A (Fig. 5), consistent with a recent study in cultured hippocampal neurons (Bito et al. 1996). Thus,

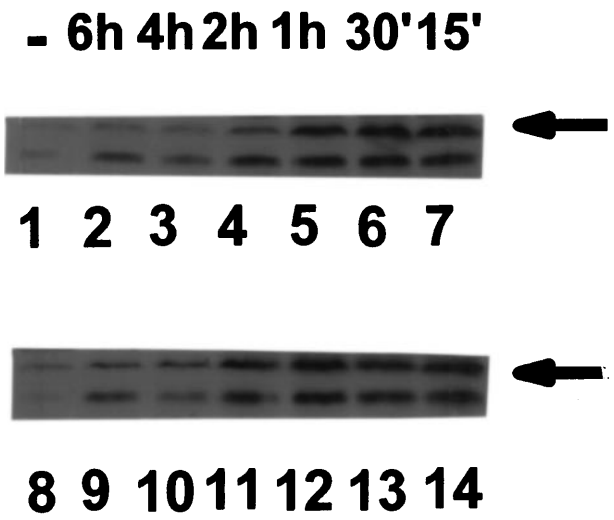

Fig. 5 Cyclosporin A does not inhibit the phosphorylation of CREB at serine 119 induced by stimulation of Jurkat T-cells. The phosphorylation of CREB at serine 119 was investigated by immunoblotting with an antibody specific for the serine-119-phosphorylated form of CREB. Immunoblot of a typical experiment. Jurkat T-cells were treated for 7 h with cyclosporin A (5 μM) (*lanes* 8–14) or the solvent (*lanes* 1–7). Cells were stimulated by forskolin (10 μM), A23187 (5 μM), and TPA (300 nM) for 15 min (*lanes* 7 and 14), 30 min (*lanes* 6 and 13), 1 h (*lanes* 5 and 12), 2 h (*lanes* 4 and 11), 4 h (*lanes* 3 and 10), or 6 h (*lanes* 2 and 9) or were left unstimulated (*lanes* 1 and 8). The *arrow* indicates serine-119-phosphorylated CREB. In addition to serine-119-phosphorylated CREB (a band of 43 kDa comigrating with CREB as stained by another anti-CREB antiserum which recognizes both phosphorylated and unphosphorylated forms of CREB) a band of 38 kDa was detected in these blots as has been reported previously (Ginty et al. 1993; Schwaninger et al. 1995). This protein could correspond to ATF-1, which is highly homologous to CREB and seems to cross-react with the anti-phosphoCREB antiserum (Ginty et al. 1993; Schwaninger et al. 1995). A single experiment is shown which is representative of 4 experiments

cyclosporin A inhibits the induction of CREB/CRE-directed transcription in Jurkat T-cells without reducing the phosphorylation of CREB at serine 119 in response to Jurkat T-cell stimulation.

Discussion

The present study shows that inhibition of CREB/CRE-directed transcription by the immunosuppressants cyclosporin A and FK506 is not restricted to pancreatic islet cells but is observed in a variety of cell types. Included are cell lines that are derived from tissues in which immunosuppressive or adverse effects of the immunosuppressants develop. Inhibition of CREB/CRE-directed transcription represents thus a novel molecular mechanism of cyclosporin A and FK506 action, which could underlie pharmacological effects, both desired and undesired.

The adverse effects of cyclosporin A and FK506, which are structurally unrelated, are very similar (Kahan 1989; Japanese FK 506 Study Group 1993; Suthanthiran and Strom 1994; Mason 1990) suggesting a shared underlying cause also for the toxic effects. In animal models of cyclosporin A – induced acute neurogenic vasoconstriction (Lyson et al. 1993) or nephrotoxicity (Dumont et al. 1992), evidence has been presented which indicates that toxicity may also be linked to immunophilin-mediated inhibition of calcineurin and inhibition of gene transcription (Morris et al. 1992). Activation of CRE-mediated transcription was inhibited by cyclosporin A and FK506 in pituitary, neuronal pheochromocytoma, and vascular smooth muscle (this study) as well as pancreatic islet cell lines (Schwaninger et al. 1993b, c, 1995). Both drugs have toxic side effects in related tissues (Mason 1990; Stephanou et al. 1992; Davenport and Hodson 1992; Kahan 1989; Japanese FK 506 Study Group 1993; Leszczynski et al. 1993). The relationship, if any, between these adverse effects and the inhibition of CREB/CRE-directed transcription in pituitary, neuronal pheochromocytoma, pancreatic islet and vascular smooth muscle cell lines is at this time unclear. However, transcriptional inhibition by both cyclosporin A and FK506 in cell lines that are derived from tissues in which shared adverse effects of the immunosuppressants develop, is suggestive and warrants further investigation.

Cyclosporin A and FK506 are powerful suppressors of the immune system, most notably of T-cells. Inhibition of CREB activity and CRE-mediated transcription by cyclosporin A and FK506 was observed also in Jurkat T-cells. This cell line faithfully mimics the early stages of T-cell activation and has been used to demonstrate the effect of cyclosporin A and FK506 on NF-AT-directed transcription (Schreiber and Crabtree 1992; Clipstone and Crabtree 1992). CREB is phosphorylated on serine-119 in response to T-cell activation signals such as TPA/A23187 (Brindle et al. 1995; Barton et al. 1996). This phosphorylation is required but not sufficient for transcriptional activation (Brindle et al. 1995; this study) as has been reported before, under different conditions, in PC12 (Thompson et al. 1995) and pancreatic islet cells (Schwaninger et al. 1995). However, mitogenic stimuli such as TPA/A23187 and TPA/PHA (this study) or stimulation of the T-cell receptor complex with the monoclonal antibody OKT3 (Brindle et al. 1995) synergize with cAMP to stimulate CREB activity and CRE-mediated transcription, although other costimuli may induce CREB activity during antigenic stimulation of T-cell activation (Suthanthiran and Strom 1994), in which CREB seems to play a critical role. In transgenic mice that express a dominant-negative form of CREB under the control of the T-cell-specific CD2 promoter/enhancer, T-cell development was normal but T-cells displayed a profound proliferative defect characterized by markedly decreased IL-2 production, G1 cell-cycle arrest and subsequent apoptotic death in response to a number of different activation signals (Barton et al. 1996). These findings indicate that CREB becomes phosphorylated and activated during T-cell stimulation and is required for normal IL-2 production and T-cell proliferation. When the results of the present study, showing inhibition of CREB/CRE-directed transcription by cyclosporin A and FK506

in Jurkat T-cells, are taken together with the evidence for an essential role of CREB in T-cell activation and proliferation, they strongly suggest that inhibition of CREB/CRE-directed transcription is a molecular mechanism through which cyclosporin A and FK506 exert the immunosuppressive effect.

Because of its calcineurin-dependent nuclear translocation, the transcription factor NF-ATc is a well defined functional target of cyclosporin A and FK506. Through the calcium- and probably calcineurin-dependent activation of mitogen-activated protein kinases which phosphorylate the Jun activation domain, JNK1 and JNK2, the activation of transcription factors including NF-IL-2 and AP-1 may, under certain conditions, also be cyclosporin A/FK506 sensitive (Su et al. 1994). Whereas the protein synthesis-independent induction of NF-κB by TPA is not suppressed by cyclosporin A or FK506, calcium-dependent activation of NF-κB binding site multimers, which requires new protein synthesis, was found to be drug sensitive (Mattila et al. 1990). Although the pharmacological significance of these findings is unclear and the alterations observed may reflect secondary rather than causative events in the drugs' action, they suggest that multiple transcription factors could be affected. Since the transcription factor CREB is required for normal induction of IL-2 production and T-cell proliferation (Barton et al. 1996), the inhibition of CREB activity in T-cells by cyclosporin A and FK506 as shown by this study suggests a prominent role of CREB among other transcription factors in immunosuppression by cyclosporin A and FK506.

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