Stimulation of the Ca^{2+}-mediated *egr-1* and c-*fos* expression in murine erythroleukaemia cells by cyclosporin A

András SCHAEFER*[1], Mária MAGÓCSI†, Anette FANDRICH* and Hans MARQUARDT*

*Department of Toxicology, Hamburg University Medical School and Fraunhofer Department of Toxicology and Environmental Medicine, Grindelallee 117, D-20146 Hamburg, Germany, and †National Institute of Haematology, Blood Transfusion and Immunology, H-1113 Budapest, Daróczi út 24, Hungary

The Ca^{2+}-induced expression of the primary response genes *egr-1* and c-*fos* was investigated in the murine erythroleukaemia cell line ELM-I-1. Exposure of the cells to the Ca^{2+}-ionophore A23187 led to a rapid transient rise in *egr-1* and c-*fos* mRNA production followed by an increase in Egr-1 and c-Fos protein levels as well as an increase in Egr-1 and activator protein 1 (AP-1) DNA-binding activity. Preincubation of the cells with KN-62, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinases, strongly decreased the Ca^{2+}-mediated expression of *egr-1* and c-*fos*. In contrast, treatment with cyclosporin A, which inhibits the Ca^{2+}/calmodulin-dependent protein phosphatase 2B or calcineurin, increased both *egr-1* and c-*fos* mRNA production and the DNA-binding activity of the Egr-1 and AP-1 transcription factors in response to the intracellular Ca^{+} concentration ($[Ca^{2+}]_i$)-increasing agents A23187 or cyclopiazonic acid. Enhancement of the Ca^{2+}-induced c-*fos* and *egr-1* expression by cyclosporin A was correlated with the capability of this agent to inhibit calcineurin phosphatase activity in ELM-I-1 cells. Studies on the phosphorylation state and DNA-binding activity of the cAMP response element-binding protein (CREB) did not demonstrate an early Ca^{2+}-dependent activation of this transcription factor, suggesting that the regulation of c-*fos* and *egr-1* expression by Ca^{2+} is not linked to CREB in the haematopoietic ELM-I-1 cells. The results indicate that calcineurin exerts negative regulatory effects on both *egr-1* and c-*fos* expression in murine erythroleukaemia cells, in addition to the calcineurin-mediated down-regulation of c-*myb* expression observed previously in this cell system. This study therefore emphasizes the important role of calcineurin as a negative modulator of gene expression in certain cell types.

INTRODUCTION

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has been established as an important regulator of gene expression in various cell systems. An increase in $[Ca^{2+}]_i$ leads to the activation of Ca^{2+}/calmodulin-dependent protein kinases, which in turn phosphorylate transcription factors involving cAMP response element-binding protein (CREB), CCAAT/enhancer-binding protein and serum response factor, and induce the expression of target genes [1–7]. Moreover, not only protein phosphorylation but also dephosphorylation through the Ca^{2+}/calmodulin-dependent, serine/threonine-specific protein phosphatase PP2B or calcineurin has been shown to be involved in the Ca^{2+}-mediated activation of gene expression. In T lymphocytes, dephosphorylation of transcription factor nuclear factor AT by calcineurin is required for the induction of interleukin 2 (IL-2) expression, which mediates T-cell activation; calcineurin represents a molecular target for the immunosuppressive agents cyclosporin A (CsA) and FK506 [8–12]. In addition to IL-2, calcineurin has been implicated in the induction of the expression of other cytokines such as IL-4 and granulocyte/macrophage colony-stimulating factor in lymphatic cell lines [13,14], in the induction of *NGFI-B* (nerve growth factor-induced gene) expression in PC12 phaeochromocytoma cells with neural characteristics [15], as well as in the cAMP response element (CRE)-mediated gene transcription in pancreatic islet cells [16,17].

In contrast with the involvement in transcriptional activation mechanisms, a negative regulatory role of calcineurin in gene expression has been also observed. The calcineurin inhibitor CsA enhances the expression of transforming growth factor β in human T cells [18] as well as in a murine renal cell line, MCT [19]. In the neural PC12 cells, both CsA and FK506 stimulate the Ca^{2+}-induced expression of the primary response gene *NGFI-A* [15], corresponding to *egr-1*, *zif-268*, *krox24* or *TIS8* [20]. The expression of c-*fos*, which is co-regulated with *egr-1* in several tissues [21,22], was not increased by calcineurin inhibitors in PC12 cells [15]. More recently, however, enhancement of c-*fos* expression by CsA has been reported in a T-cell lymphoma cell line after treatment with ionomycin [23] as well as with FK506 in electrically stimulated hippocampal neurons [24].

In previous studies we have demonstrated a Ca^{2+}-mediated down-regulation of c-*myb* mRNA levels in murine erythroleukaemia cells, which was abolished by CsA in correlation with the inhibition of calcineurin phosphatase activity [25]. This suggests that c-*myb* might also be a target for inhibitory effects of calcineurin on the gene expression. Here we report that the suppression of c-*myb* expression by calcineurin in murine erythroleukaemia cells is accompanied by a negative regulation of both *egr-1* and c-*fos* expression. These results underline the important role of calcineurin as a negative modulator of gene expression in certain cell types.

EXPERIMENTAL

Materials

A23187, 4-Br-A23187, ionomycin, cyclopiazonic acid (CPA), thapsigargin, 8-Br-cAMP, Bradford reagent and protease inhibitors were purchased from Sigma (St. Louis, MO, U.S.A.); okadaic acid was from Life Technologies (Gaithersburg, MD, U.S.A.); calyculin A, KN-62 and fura 2 acetoxymethyl ester were from Calbiochem (San Diego, CA, U.S.A.); poly(dI-dC) and Sephadex G-25 were from Pharmacia (Uppsala, Sweden);

Abbreviations used: AP-1, activator protein 1; $[Ca^{2+}]_i$, intracellular Ca^{+} concentration; CPA, cyclopiazonic acid; CRE, cAMP response element; CREB, CRE-binding protein; CsA, cyclosporin A; EMSA, electrophoretic mobility-shift assay; IL, interleukin; NGFI, nerve growth factor-induced gene; pCREB, phosphorylated CREB; PP, protein phosphatase.

[1] To whom correspondence should be addressed (e-mail aschaefe@uke.uni-hamburg.de).

T4 polynucleotide kinase was from Promega (Madison, WI, U.S.A.); CsA was from Sandoz (Basel, Switzerland); and FK506 was from Fujisawa (Tokyo, Japan).

[α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (4500 Ci/mmol) were obtained from ICN Pharmaceuticals (Irvine, CA, U.S.A.); α-minimal essential medium without nucleotides, horse serum and penicillin/streptomycin were purchased from Life Technologies (Gaithersburg, MD, U.S.A.); and analytical grade chemicals from Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany).

Cells and culture conditions

Erythropoietin-sensitive murine erythroleukaemia cells, line ELM-I-1 [26,27], were kindly provided by Professor W. Ostertag (Heinrich Pette Institute for Experimental Virology and Immunology, Hamburg, Germany). Cells were grown in α-minimal essential medium without nucleosides, supplemented with 10% (v/v) horse serum, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified air/CO_2 (19:1) atmosphere. For the experiments, exponentially growing cells were plated at $(6–8) \times 10^4$ cells/ml; approx. 16 h later the cells were treated with the test substances.

Northern blot analysis

Total cellular RNA from ELM-I-1 cells was isolated by the acid guanidium thiocyanate/phenol/chloroform method [28]. Northern blot analysis of the RNA fraction was performed as described previously [25]. For densitometric quantification, the autoradiograms of the blots were digitized with the GelPrint 2000i system, version 2.3, from Biophotonics Corp. (Ann Arbor, MI, U.S.A.) and analysed by the One-Dscan software from Scan-analytics (Billerica, MA, U.S.A.).

The following hybridization probes were used: human c-*myb* and β-actin cDNA probes, as described previously [25]; a 1.0 kb *Pst*I–*Pvu*II v-*fos* probe was obtained from Oncor (Gaithersburg, MD, U.S.A.), a 252 bp *egr-1* probe was synthesized by PCR in a Perkin Elmer GeneAmp PCR System 9600 as described [29].

Western blot analysis

Western blot analysis from whole ELM-I-1 cells or nuclear fractions [30] was performed as described previously [25]. The protein content of the samples was determined by the method of Bradford [31]. The following antibodies were used: anti-(Egr-1) and anti-(c-Fos), rabbit polyclonal IgG, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); anti-CREB and anti-(phosphorylated CREB) (anti-pCREB), rabbit polyclonal IgG, purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Electrophoretic mobility-shift assay (EMSA)

ELM-I-1 cells were treated with the test substances; at defined time points thereafter, nuclear fractions were prepared by the method of Antalis and Godbolt [30]. From the isolated nuclei, protein was extracted in the presence of the proteinase inhibitors aprotinin, leupeptin, antipain (each at 10 μg/ml) and PMSF (0.2 mM) as described [32]. The protein content of the extracts was estimated by the method of Bradford [31]. Binding reactions for EMSA were performed in 10 mM Tris/HCl (pH 7.5)/50 mM NaCl/1 mM $MgCl_2$/0.5 mM EDTA/0.5 mM dithiothreitol/4% (v/v) glycerol/50 μg/ml poly(dI-dC) with 5 μg of nuclear protein extracts and 1 μl [$(2–5) \times 10^4$ c.p.m.] of labelled oligonucleotide in a total volume of 15 μl. The reaction mixtures were incubated for 20 min at room temperature, and protein–DNA complexes were separated from free oligonucleotide on a 5% (w/v) native polyacrylamide gel. The specificity of DNA binding was assessed by competition analyses with unlabelled specific or non-specific oligonucleotides.

The following oligonucleotide probes were used: AP-1, CREB, Sp1 (Promega) and Egr-1 (Santa Cruz Biotechnology). The oligonucleotide probes were labelled with [γ-^{32}P]ATP with the use of the T4 polynucleotide kinase and purified on a Sephadex G-25 column.

Measurement of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ by fluorescence and the subsequent calculations were performed with the use of the fluorescent Ca^{2+} indicator fura 2 acetoxymethyl ester as described previously [33].

RESULTS

Calcium ionophore-induced *egr-1* and c-*fos* expression in ELM-I-1 cells

ELM-I-1 cells were treated with A23187 (1.5 μM) and the production of *egr-1* and c-*fos* mRNA was followed for up to 120 min by Northern blot analysis. Down-regulation of c-*myb* mRNA by A23187 [25] was studied comparatively and the levels of β-actin mRNA were determined as an internal control for RNA loading (Figure 1). The results showed a rapid increase in

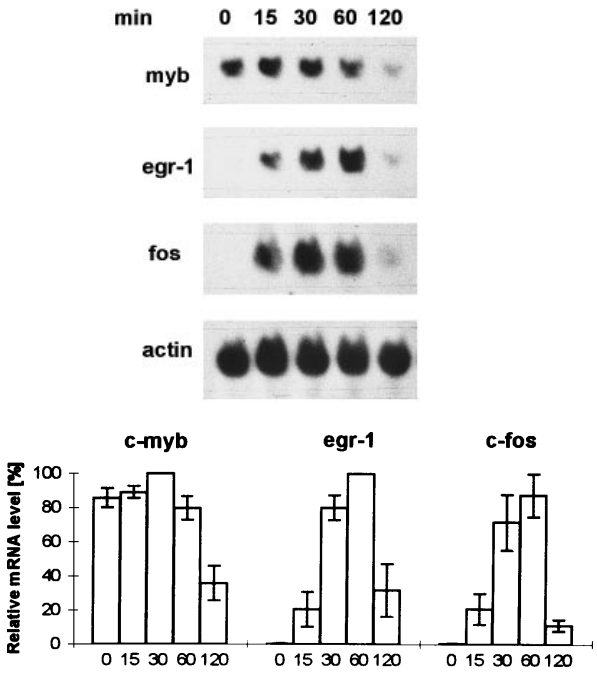

Figure 1 A23187-induced changes in c-*myb*, *egr-1* and c-*fos* mRNA levels in ELM-I-1 cells

Cells were exposed to A23187 (1.5 μM); at the indicated time points, cells were collected and total cellular RNA was isolated and tested by Northern blot analysis. Denatured RNA (40 μg) was size-fractionated on a 1% (w/v) agarose gel and capillary-transferred to a nylon filter. The blot was hybridized sequentially with the ^{32}P-labelled DNA probes. β-actin mRNA was determined as a control to verify the amount of RNA in each lane. Upper panel: autoradiograms from a representative experiment. Lower panel: autoradiograms were subjected to quantification by densitometry and the results for c-*myb*, *egr-1* and c-*fos* were normalized to those of β-actin, the unregulated control. The quantitative results are expressed as percentages of maximum intensity measured for a signal type on the same blot and represent means ± S.E.M. for three or four independent experiments.

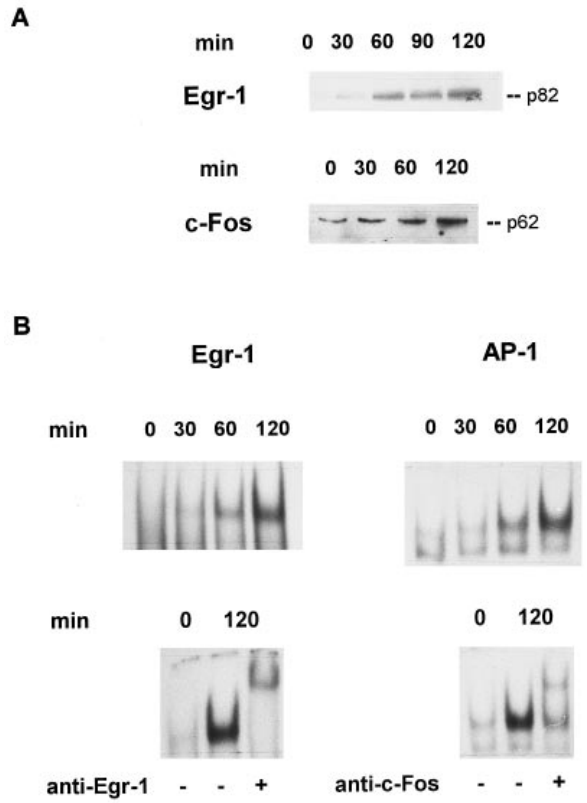

Figure 2 Egr-1 and c-Fos protein synthesis and DNA-binding activity of the transcription factors Egr-1 and AP-1 in A23187-treated ELM-I-1 cells

Cells were exposed to A23187 (1.5 μM); at the indicated time points, Egr-1 and c-Fos protein levels as well as the activities of the Egr-1 and AP-1 transcription factors were analysed. (**A**) Western blot analysis of the protein levels. Total cellular protein (Egr-1) (60 μg) or nuclear protein extracts (c-Fos) (60 μg) were separated by electrophoresis on an SDS/12.5% (w/v) polyacrylamide gel, transferred to a nylon filter and analysed with polyclonal anti-(Egr-1) and anti-(c-Fos) antibodies. The blots were repeated with similar results. (**B**) EMSA analysis of Egr-1 and AP-1 DNA-binding activity. Protein (5 μg) from nuclear extracts was incubated with ^{32}P-labelled oligonucleotide probes at room temperature for 20 min and DNA–protein complexes were resolved on a 5% (w/v) native polyacrylamide gel. Specific DNA-binding was identified by competition with excess unlabelled oligonucleotides (results not shown). Lower panel: nuclear extracts were incubated with the oligonucleotides for an additional 10 min in the presence or absence of anti-(Egr-1) or anti-(c-Fos) antibodies (100 ng), as indicated. The autoradiograms show the specific bands detected in the assay and are representative of three independent experiments.

both *egr-1* and c-*fos* mRNA within 15 min. *Egr-1* and c-*fos* mRNA reached a maximum at 30–60 min and had strongly declined by 120 min, when the A23187-induced decrease in c-*myb* mRNA occurred.

To characterize further the expression of *egr-1* and c-*fos* in ELM-I-1 cells after treatment with A23187, we studied the kinetics of the synthesis of Egr-1 and c-Fos proteins as well as the DNA-binding activity of the Egr-1 and AP-1 transcription factors. Western blot analysis revealed an increasing production of Egr-1 and c-Fos proteins up to 120 min, which was correlated with enhanced Egr-1 and AP-1 DNA-binding activity as shown by EMSA (Figure 2). Incubation of the nuclear protein extracts for EMSA with the anti-(Egr-1) or anti-(c-Fos) antibodies used for Western blot analyses, diminished Egr-1 and AP-1 DNA-binding activity and led to the appearance of supershifted complexes, identifying a role of Egr-1 and c-Fos proteins in Egr-1 and AP-1 DNA-binding activity respectively (Figure 2B).

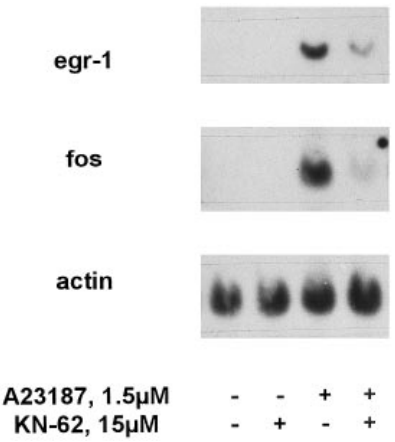

Figure 3 Effect of KN-62 on the A23187-induced expression of *egr-1* and c-*fos* mRNA in ELM-I-1 cells

Cells were preincubated with KN-62 (15 μM) for 30 min and then exposed to A23187 (1.5 μM) for an additional 30 min. Northern blot analyses of *egr-1*, c-*fos* and β-actin mRNA levels were performed as described in the Experimental section and in the legend to Figure 1. Autoradiograms from a representative experiment are shown here. The inhibitions (means $\pm$ S.E.M.) of A23187-induced *egr-1* and c-*fos* expression by KN-62 in three separate experiments were 89.4 $\pm$ 3.0% and 86.8 $\pm$ 6.6% respectively.

These results indicate that the expression of *egr-1* and c-*fos* as well as the activity of the gene products are regulated in Ca^{2+} ionophore-treated ELM-I-1 cells by similar kinetics.

Studies on the involvement of Ca^{2+}/calmodulin-dependent kinase in A23187-induced expression of *egr-1* and c-*fos*

To examine whether Ca^{2+}/calmodulin-dependent kinase is involved in the A23187-induced expression of *egr-1* and c-*fos* in ELM-I-1 cells, we studied the effect of KN-62, a selective inhibitor of different Ca^{2+}/calmodulin-dependent kinases [6,34,35]. Incubation of ELM-I-1 cells in the presence of KN-62 (15 μM) strongly decreased *egr-1* and c-*fos* mRNA production in response to A23187 treatment (Figure 3). This observation is in accord with previous results on PC12 cells [15] and suggests that the induction of *egr-1* and c-*fos* expression by Ca^{2+} is mediated mainly by Ca^{2+}/calmodulin-dependent kinase also in the haemopoietic cell line used in the present studies.

Because Ca^{2+}/calmodulin-dependent kinase might induce c-*fos* expression via the phosphorylation of CREB [1,3,24], and potential CRE sequences have also been found in *egr-1* promoter [20], we investigated whether the induction of *egr-1* and c-*fos* expression by A23187 in ELM-I-1 cells is correlated with a rapid activation of this transcription factor. However, Western blot analysis with antibodies that recognize total CREB or its phosphorylated form did not show an increase in CREB phosphorylation in cells exposed to 1.5 μM A23187 for 10 or 30 min (Figure 4A). Interestingly, relatively high levels of pCREB could be detected in unstimulated ELM-I-1 cells. High pCREB levels have recently been shown also in untreated lung epithelial cells [36]. In parallel experiments, an increase in CREB phosphorylation was observed by 0.5 mM 8-Br-cAMP, as well as in the presence of the serine/threonine-specific protein phosphatase inhibitors okadaic acid (25–100 nM) or calyculin A (1–3 nM) (results not shown). In accordance with the results of Western blot analyses, EMSA with oligonucleotide corresponding to CRE revealed a high DNA-binding activity of CREB in ELM-

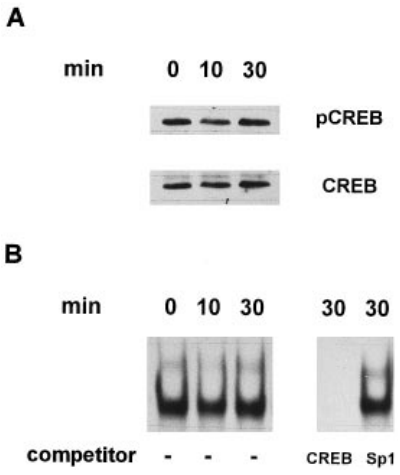

Figure 4 Studies on the phosphorylation state and DNA-binding activity of CREB in A23187-treated ELM I-1 cells

Cells were exposed to A23187 (1.5 μM) for 10 and 30 min. (**A**) Whole cell extracts were analysed by immunoblotting with specific antibodies recognizing pCREB or total CREB irrespective of phosphorylation state. (**B**) Nuclear extracts were prepared and assessed for CREB activity by EMSA. Specific DNA binding was evaluated with an excess of unlabelled oligonucleotide probe for CREB and for Sp1 (right panel). No other band could be detected in the assay. Western blot analysis and EMSA were performed as described in the Experimental section and in the legend to Figure 2. The results shown are representative of three independent experiments.

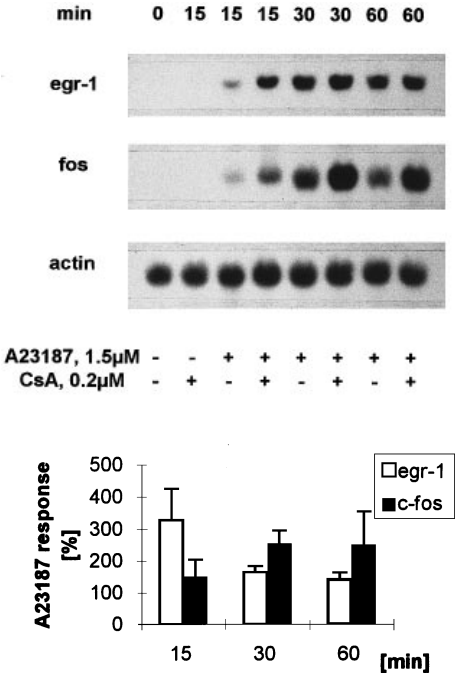

Figure 5 Effect of CsA on the A23187-induced expression of *egr-1* and c-*fos* mRNA in ELM-I-1 cells

Cells were preincubated with CsA (0.2 μM) for 30 min before the incubation was started with A23187 (1.5 μM). At the indicated time points, cells were harvested and the levels of *egr-1*, c-*fos* and β-actin mRNA were analysed as described in the Experimental section and in the legend to Figure 1. Quantitative data were calculated from four (15 and 60 min) or eight (30 min) separate experiments and are shown in the bottom panel. The results are expressed as percentages of the A23187 response in the presence of CsA at the indicated time points (means $\pm$ S.E.M.). At 15 min, stimulation of A23187-induced c-*fos* expression by CsA was not seen in one of four experiments. Statistical evaluation of the results at 30 min on the basis of eight experiments with a two-tailed *t*-test showed a significant stimulation of *egr-1* and c-*fos* mRNA expression by CsA ($P < 0.01$ in both cases). At this time point, CsA was significantly more active in stimulating c-*fos* expression ($P < 0.05$).

I-1 cells, which was not increased in response to 1.5 μM A23187 (Figure 4B).

Enhancement of the Ca^{2+}-induced expression of *egr-1* and c-*fos* mRNA by CsA

In further experiments, the effect of CsA on the Ca^{2+}-mediated expression of *egr-1* and c-*fos* in ELM-I-1 cells was investigated. In previous studies we characterized the inhibition of calcineurin phosphatase activity by CsA in this cell line [25]. The results in Figure 5 show the time course of induction of synthesis of *egr-1* and c-*fos* mRNA by A23187 (1.5 μM) in the presence and the absence of CsA (200 nM). Incubation with CsA enhanced the production of both *egr-1* and c-*fos* mRNA in response to A23187. The effect of CsA on the expression of *egr-1* was accentuated at a lower level of mRNA production 15 min after A23187 exposure. At 30 and 60 min, when a maximum in *egr-1* and c-*fos* expression was achieved, CsA was more active in increasing c-*fos* mRNA levels.

CsA, in concentrations between 3 and 12.5 nM, has been shown to inhibit calcineurin activity by 50 % in ELM-I-1 cells [25]. FK506, as also reported in mouse progenitor mast cells [37], did not inhibit calcineurin activity in ELM-I-1 cells up to a concentration of 1000 nM [25]. The results presented in Figure 6 demonstrate that CsA enhanced the A23187-induced c-*fos* mRNA production at a concentration as low as 10 nM, whereas 1000 nM FK506 did not show this effect. This was also seen in the *egr-1* mRNA levels, although the stimulatory effect of CsA on the expression of *egr-1* was weaker at 30 min (results not shown). These results therefore indicate a correlation between the inhibition of calcineurin phosphatase activity by CsA and the observed effects on the mRNA levels in ELM-I-1 cells.

Previous experiments demonstrated a down-regulation of c-*myb* expression in murine erythroleukaemia cells also with CPA (1.25–5 μM) and thapsigargin (0.5–2 nM), inhibitors of the Ca^{2+} pump in the endoplasmic reticulum [25,33]. Measurements of $[Ca^{2+}]_i$ in ELM-I-1 cells with the fluorescent indicator fura 2 showed, in accordance with previous data from F4-6 murine erythroleukaemia cells [33], that CPA and thapsigargin induced a net increase in $[Ca^{2+}]_i$ up to 400 nM at the above concentrations. In contrast, the increase in $[Ca^{2+}]_i$ by the Ca^{2+}-ionophores A23187 (as 4-Br-A23187) or ionomycin (each at 1.5 μM) was in the micromolar range (results not shown). When CPA (5 μM) was used in the experiments instead of A23187 (1.5 μM), the maximum production of *egr-1* and c-*fos* mRNA and the maximum duration of the up-regulation were decreased compared with those of A23187 (Figure 7). CsA exerted stimulatory effects on the expression of *egr-1* and c-*fos* also at this lower intracellular Ca^{2+} level. Similarly to the results obtained with A23187 at a lower level of mRNA production (Figure 5; 15 min), CsA was more active in stimulating *egr-1* expression in response to CPA.

Studies on the effect of CsA on Ca^{2+}-induced Egr-1 and AP-1 DNA-binding activity

The production and DNA-binding activity of transcription factors is regulated at several points [38]. To investigate whether the observed effects of CsA on *egr-1* and c-*fos* mRNA levels are correlated with similar changes in the activity of the corresponding transcription factors, we studied the DNA-binding

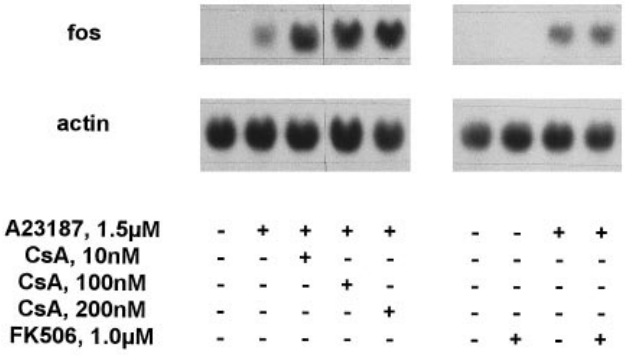

Figure 6 Studies on the effect of CsA and FK506 on the A23187-induced expression of c-*fos* mRNA in ELM-I-1 cells

Cells were incubated with CsA or FK506 at the indicated concentrations for 30 min and then exposed to A23187 (1.5 μM) for an additional 30 min. c-*fos* and β-actin mRNA levels were measured by Northern blot analysis as described in the Experimental section and in the legend to Figure 1. No detectable levels of c-*fos* mRNA were seen with 10–200 nM CsA in the absence of A23187 (results not shown). Quantitative results calculated from three independent experiments (percentages of A23187 response, means ± S.E.M.) were as follows: 230.1 ± 45.7% for 10 nM CsA and 122.0 ± 15.7% for 1000 nM FK506.

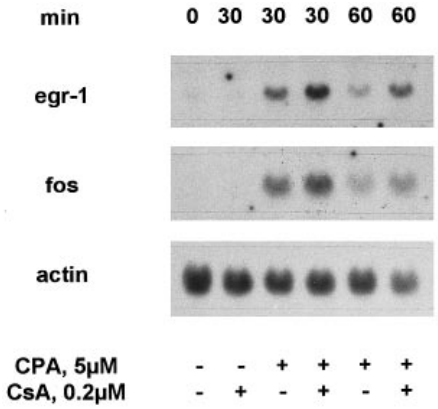

Figure 7 Effect of CsA on the expression of *egr-1* and c-*fos* mRNA induced by CPA

Cells were preincubated with CsA (0.2 μM) for 30 min; 5 μM CPA was then added and the cells were incubated for a further 30 and 60 min. Northern blot analysis of *egr-1*, c-*fos* and β-actin mRNA levels was performed as described in the Experimental section and in the legend to Figure 1. The results are representative of two independent experiments. In a third experiment the effect of CsA was tested only at 30 min after exposure to CPA. On the basis of three experiments, the CPA-induced *egr-1* and c-*fos* expression in the presence of CsA at 30 min were (means ± S.E.M.) 271.4 ± 54.4% and 164.2 ± 11.3% respectively.

activity of Egr-1 and AP-1 by using EMSA. DNA binding by the transcription factor Sp1, which proved to be very active in ELM-I-1 cells and did not respond to treatment with $[Ca^{2+}]_i$-increasing agents, was comparatively studied. Figure 8 shows the activity of Egr-1, AP-1 and Sp1 in nuclear extracts from ELM-I-1 cells treated with A23187 (1.5 μM) in the presence and the absence of CsA (200 nM). The DNA-binding activity of both Egr-1 and AP-1, but not that of Sp1, was enhanced in the the presence of CsA. A stimulation of Egr-1 and AP-1 activity by CsA was also seen when cells were exposed to CPA (5 μM), which induced only a slight increase in the activity of Egr-1 and AP-1 in the absence of CsA (results not shown).

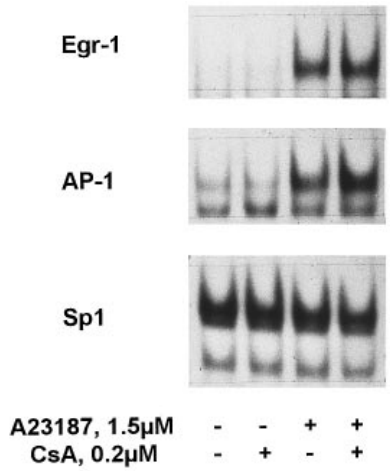

Figure 8 Effect of CsA on the DNA-binding activity of Egr-1, AP-1 and Sp1 transcription factors in A23187-treated ELM-I-1 cells

Cells were preincubated in the presence and in the absence of CsA (0.2 μM) for 30 min and then treated with 1.5 μM A23187; 2 h after exposure to A23187, cells were harvested and nuclear extracts were prepared and analysed by EMSA with Egr-1, AP-1 and Sp1 oligonucleotide probes as described in the Experimental section and in the legend to Figure 2. The autoradiograms show Egr-1, AP-1 and Sp1 DNA binding in the same samples. Other specific bands were not detected. The results were confirmed by repeated assays with samples of two independent experiments.

DISCUSSION

The present experiments demonstrate a Ca^{2+}-mediated expression of the primary response genes *egr-1* and c-*fos* in the murine erythroleukaemia cell line ELM-I-1. *Egr-1* and c-*fos* mRNA production and protein synthesis as well as the activity of the Egr-1 and AP-1 transcription factors showed similar kinetics in A23187-treated cells, supporting the notion that the expression of these genes might be co-regulated in different cell types [21,22]. In accordance with previous studies in PC12 phaeochromocytoma cells [15], the results show that the Ca^{2+}-dependent induction of *egr-1*/*NGFI-A* and c-*fos* expression is mediated mainly by a Ca^{2+}/calmodulin-dependent protein kinase (Figure 3). Ca^{2+}/calmodulin-dependent phosphorylation of the transcription factor CREB has been suggested as being responsible for the Ca^{2+}-induced expression of c-*fos* in cells of neural origin [1,3], and the Ca^{2+}/calmodulin-dependent kinase type IV seems to have a critical role in this process [6,24]. Although CRE is present in both c-*fos* and *egr-1* promoters [20], our experiments do not provide evidence for an early Ca^{2+}-dependent phosphorylation and activation of CREB in the murine erythroleukaemia cells studied (Figure 4). Similar observations were obtained in a T-cell hybridoma line, suggesting that the CREB-linked Ca^{2+}-signalling pathway is utilized in a cell-type-dependent fashion [39]. Serum response element (SRE)-dependent pathways have been also implicated in the Ca^{2+}-mediated transcriptional activation of c-*fos* [39–42]. It is interesting to note that Elk-1, a member of the ternary complex factor (TCF) family, has recently been identified as a substrate of calcineurin [43]. Further studies are necessary to identify the transcription factor(s) or associated proteins that mediate the Ca^{2+}-dependent induction of *egr-1* and c-*fos* expression in murine erythroleukaemia cells. In addition, transcriptional elongation controlled by an intragenic regulatory element of the c-*fos* gene

[44,45] might also be a target for the regulation of c-*fos* expression by Ca^{2+} [39,46,47]. It is not yet known whether an intragenic regulation of transcriptional elongation also operates in the *egr-1* gene.

This study is in accord with previous results on neuronal PC12 cells; those results demonstrated a negative regulatory role of calcineurin in the Ca^{2+}-mediated expression of *egr-1/NGFI-A* [15]. However, in contrast with the PC12 cells, in which the inhibition of calcineurin enhances *egr-1/NGFI-A* expression without influencing the expression of c-*fos*, in the murine erythroleukaemia cell line now studied, the expression of both *egr-1* and c-*fos* was increased by CsA in correlation with the inhibition of calcineurin phosphatase activity (Figures 5 and 6) [25]. Stimulation of c-*fos* expression and AP-1 DNA-binding activity by CsA has been demonstrated in a T-cell lymphoma cell line exposed to ionomycin [23]. In addition, FK506 enhances c-*fos* expression in hippocampal neurons after a short electrical stimulation [24]. The expression of *egr-1* was not studied in these experiments. The present study is therefore the first demonstration that calcineurin might negatively regulate the expression of both *egr-1* and c-*fos* in a cell system. *egr-1* and c-*fos* represent typical primary response genes induced by a variety of agents and as such could influence multiple aspects of cell regulation [20]. The stimulatory effect of calcineurin inhibitors on the Ca^{2+}-mediated expression of these genes suggests an important role for calcineurin as a negative modulator of gene expression in certain cell types. The experiments with the endoplasmic reticulum Ca^{2+} pump inhibitor CPA show that this negative regulation also operates under conditions of lower $[Ca^{2+}]_i$ (Figure 7). These results therefore imply that calcineurin might increase the threshold of induction of *egr-1* and c-*fos* expression by Ca^{2+}, allowing a moderate increase in $[Ca^{2+}]_i$ without activation of programmes in gene expression initiated by these primary response genes. In contrast, the perturbation of calcineurin activity might favour the Ca^{2+}-induced expression of *egr-1* and c-*fos*. Recent observations suggest that calcineurin couples Ca^{2+}-dependent protein dephosphorylation to the redox state of the cells and that it undergoes inactivation on elevated superoxide anion levels [48].

Although the present experiments suggest a negative regulation of *egr-1* and c-*fos* expression by calcineurin in ELM-I-1 cells, previous results indicated a calcineurin-mediated down-regulation of c-*myb* expression in this cell line [25]. The augmentation of the A23187-induced increase in *egr-1* and c-*fos* mRNA by CsA was evident within 15–30 min (Figure 5). In contrast, a decrease in c-*myb* mRNA levels was detected in ELM-I-1 cells 2 h after exposure to A23187 (Figure 1). However, given a half-life of c-*myb* mRNA of 1–3 h [49], the suppression of c-*myb* expression might occur concomitantly with the regulatory effects of calcineurin on *egr-1* and c-*fos*. This potential negative co-regulation of c-*myb*, *egr*-1 and c-*fos* expression by calcineurin suggests a functional role for this enzyme in the murine erythroleukaemia cells studied. The early down-regulation of c-*myb* expression in response to $[Ca^{2+}]_i$-increasing agents is associated with an induction of haemoglobin synthesis in this cell type, indicating that calcineurin regulates aspects of erythroid differentiation [25,33]. The negative modulation of *egr-1* and c-*fos* expression might therefore also be important for the induction of erythroid differentiation by $[Ca^{2+}]_i$-increasing agents. However, the involvement of a Ca^{2+}-signalling pathway in erythropoietin-induced terminal erythroid differentiation is not clear at present (reviewed in [50]).

Because calcineurin commonly seems to suppress *egr-1*, c-*fos* and c-*myb* expression in murine erythroleukaemia cells, this cell type might represent a useful model system for the study of mechanisms of the calcineurin-mediated negative regulation of gene expression. The substrates of calcineurin that might be responsible for the observed effects have yet to be defined. In *egr-1* and c-*fos* expression, Ca^{2+}/calmodulin-dependent protein kinase and calcineurin exert opposite regulatory effects. In hippocampal neurons, regulation of the phosphorylation state of CREB by Ca^{2+}/calmodulin-dependent kinase IV and calcineurin has been shown to control c-*fos* expression [24]. However, such a mechanism is unlikely in the regulation of c-*fos* and *egr-1* expression in the haemopoietic cell line studied, because the Ca^{2+}-induced expression of these genes was not correlated with an increase in CREB phosphorylation (Figure 4). In contrast, the high expression of c-*myb* in ELM-I-1 cells is apparently independent of Ca^{2+}/calmodulin-activated protein kinase [25], suggesting that calcineurin might mediate negative effects on gene expression without also antagonism towards a Ca^{2+}/calmodulin-dependent kinase.

Regulatory effects of calcineurin might be mediated in certain tissues by the serine/threonine-specific protein phosphatase PP1 via a protein phosphatase cascade [24,51,52]. Okadaic acid, a well-characterized inhibitor of PP1 and PP2A, has been widely used to identify these enzymes in cellular signalling pathways. This agent inhibits PP1 at higher concentrations than PP2A [51]. In PC12 cells, okadaic acid has been reported to enhance the A23187-induced expression of *NGFI-A/egr-1* and c-*fos* at concentrations of 300–500 nM [15]. Similar effects of okadaic acid on the Ca^{2+}-dependent expression of *egr-1* and c-*fos* mRNA could also be observed in the present experiments above 100 nM; however, these were not correlated with an increase in Egr-1 and AP-1 DNA-binding activity (results not shown). Moreover, in PC12 cells, 300–500 nM okadaic acid also enhanced the Ca^{2+}-induced expression of *NGFI-B*, which is inhibited and not stimulated by CsA and FK506 [15]. Therefore the effects of okadaic acid at higher concentrations favouring an inhibition of PP1 seem to be more complex and do not mimic the action of CsA in every respect. Further analyses are required for the elucidation of the potential role of PP1 in mediating the observed regulatory effects of calcineurin on gene expression.

In conclusion, the results of the present study suggest that calcineurin negatively regulates the expression of both *egr-1* and c-*fos* in murine erythroleukaemia cells in addition to the calcineurin-mediated suppression of c-*myb* expression previously reported in this cell type [25]. The exact mechanisms of the observed Ca^{2+}-dependent regulation of the expression of these genes remain to be determined.

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