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

Andrés SCHAEFER\(^1\), Mária MAGŐCSI\(^†\), 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, Dárdci 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 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\(^{2+}\) 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-myc 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\(^{2+}\)], 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 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 the 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 calcineurin, increased both c-fos mRNA levels in murine erythro-leukaemia cells, which was abolished by CsA in correlation with the inhibition of calcineurin phosphatase activity [25]. This suggests that c-myc might also be a target for inhibitory effects of calcineurin on the gene expression. Here we report that the suppression of c-myc expression by calcineurin in murine erythro-leukaemia 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.); calycin 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\(^{2+}\)], intracellular Ca\(^{2+}\) 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).

\[ \alpha^{32}P]CTP (3000 Ci/mmol) and \[\gamma^{32}P\]ATP (4500 Ci/mmol) were obtained from ICN Pharmaceuticals (Irvine, CA, U.S.A.); guanidium thiocyanate (v/v) 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 a 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) x 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 Scanaanalytics (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 PstI–PvuII 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-(c-Fos), rabbit polyclonal IgG, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); anti-(phosphorylated CREB) (anti-pCREB), rabbit polyclonal IgG, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); AP-1, CREB, Sp1 (Promega) and Egr-1 (Santa Cruz Biotechnology). The oligonucleotide probes were labelled with [\(\gamma^{32}P\)]ATP with the use of the T4 polynucleotide kinase and purified on a Sephadex G-25 column.

**Electrophoretic mobility-shift assay (EMSA)**

ELM-I-1 cells were treated with 1A23187 (1.5 \(\mu\)M); 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 mRNA 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.

**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 \(\mu\)M) 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 \(\mu\)M); 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.
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-myc 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).

These results indicate that the expression of egr-1 and c-fos as well as the activity of the gene products are regulated in Ca²⁺-ionophore-treated ELM-I-1 cells by similar kinetics.

**Studies on the involvement of Ca²⁺/calmodulin-dependent kinase in A23187-induced expression of egr-1 and c-fos**

To examine whether Ca²⁺/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 Ca²⁺/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 Ca²⁺ is mediated mainly by Ca²⁺/calmodulin-dependent kinase also in the haemopoietic cell line used in the present studies.

Because Ca²⁺/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 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-
In further experiments, the effect of CsA on the Ca\textsuperscript{2+} enhancement of the Ca\textsuperscript{2+}-mediated expression of egr-1 and c-fos mRNA by CsA 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 \mu M) 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.

Studies on the effect of CsA on Ca\textsuperscript{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 activity of CREB in A23187-treated ELM-I-1 cells.

Cells were preincubated with CsA (0.2 \mu M) for 30 min before the incubation was started with A23187 (1.5 \mu M). At the indicated time points, cells were harvested and the levels of egr-1, c-fos and \beta-actin mRNA were analysed as described in the Experimental section and in the legend to Figure 1. Quantitative data were calculated from four 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 (mean \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).

Studies on the effect of CsA on Ca\textsuperscript{2+}-induced expression of egr-1 and c-fos mRNA in ELM-I-1 cells

Cells were preincubated with CsA (0.2 \mu M) for 30 min before the incubation was started with A23187 (1.5 \mu M). At the indicated time points, cells were harvested and the levels of egr-1, c-fos and \beta-actin mRNA were analysed as described in the Experimental section and in the legend to Figure 1. Quantitative data were calculated from four 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 (mean \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).

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

In further experiments, the effect of CsA on the Ca\textsuperscript{2+}-mediated expression of egr-1 and c-fos mRNA 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 \mu M) 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 \mu M) and thapsigargin (0.5–2 nM), inhibitors of the Ca\textsuperscript{2+} pump in the endoplasmic reticulum [25,33]. Measurements of [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i} up to 400 nM at the above concentrations. In contrast, the increase in [Ca\textsuperscript{2+}]\textsubscript{i} by the Ca\textsuperscript{2+}-ionophores A23187 (as 4-Br-A23187) or ionomycin (each at 1.5 \mu M) was in the micromolar range (results not shown). When CPA (5 \mu M) was used in the experiments instead of A23187 (1.5 \mu M), 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\textsuperscript{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.
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 [Ca²⁺]-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 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).

**DISCUSSION**

The present experiments demonstrate a Ca²⁺-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 Ca²⁺-dependent induction of egr-1/NGFI-A and c-fos expression is mediated mainly by a Ca²⁺/calmodulin-dependent protein kinase (Figure 3). Ca²⁺/calmodulin-dependent phosphorylation of the transcription factor CREB has been suggested as being responsible for the Ca²⁺-induced expression of c-fos in cells of neural origin [1,3], and the Ca²⁺/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 Ca²⁺-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 Ca²⁺-signalling pathway is utilized in a cell-type-dependent fashion [39]. Serum response element (SRE)-dependent pathways have been also implicated in the Ca²⁺-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 Ca²⁺-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
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$^{2+}$] ($\text{Figure 7}$). 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$^{2+}$] 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-myc 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-myc mRNA levels was detected in ELM-I-1 cells 2 h after exposure to A23187 (Figure 1). However, given a half-life of c-myc mRNA of 1–3 h [49], the suppression of c-myc expression might occur concomitantly with the regulatory effects of calcineurin on egr-1 and c-fos. This potential negative co-regulation of c-myc, 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-myc expression in response to [Ca$^{2+}$]-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$^{2+}$]-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-myc 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-myc 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-myc 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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Ca²⁺-regulation of egr-1 and c-fos expression


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