Crosstalk during Ca\(^{2+}\) -, cAMP-, and glucocorticoid-induced gene expression in lymphocytes

Diane R. Dowd a,*, Jan S. Ryerse b, Paul N. MacDonald c, Roger L. Miesfeld d, Merideth C. Kamradt a

a The E.A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University Health Sciences Center, 1402 S. Grand Blvd., St. Louis, Missouri 63104, USA
b The Department of Pathology, Saint Louis University Health Sciences Center, St. Louis, Missouri 63104, USA
c The Department of Pharmacological and Physiological Sciences, Saint Louis University Health Sciences Center, St. Louis, Missouri 63104, USA
d The Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, USA

Received 22 November 1996; accepted 20 January 1997

Abstract

In the WEHI7.2 thymoma cell line, cAMP, glucocorticoids, or increases in cytosolic Ca\(^{2+}\) concentration lead to cell death by apoptosis. In the present study, we examined the effects of these compounds on cAMP response element (CRE)-mediated gene expression. Thapsigargin and A23187 were employed to increase cytosolic Ca\(^{2+}\) levels and induce apoptosis. Both compounds enhanced transcription from a CRE preceding apoptotic death. Moreover, the transcriptional response to the combination of forskolin and either thapsigargin or A23187 was synergistic mirroring the effect on cell death. Importantly, dexamethasone treatment, which causes an efflux of Ca\(^{2+}\) from the ER [1,2], induced transcription from a CRE alone or in synergy with forskolin. The increase in CRE-controlled gene expression correlated with a decrease in cell viability. Following treatment with forskolin, thapsigargin, or dexamethasone, the CRE binding protein (CREB) was phosphorylated at levels correlating with the level of induced gene expression. These data suggest that transcriptional crosstalk between independent signaling pathways occurs in lymphocytes, and CREB may play a central role in the mediation of CRE-dependent transcription by these diverse set of apoptotic agents. © 1997 Elsevier Science Ireland Ltd.

Keywords: cAMP; Ca\(^{2+}\); Glucocorticoids; Cyclic AMP response element binding protein; Apoptosis

1. Introduction

At specific stages during the development of a lymphocyte, the cell makes a commitment to differentiation or apoptotic cell death. This balance between proliferation and death maintains the precise balance of appropriate mature cells in the organism. Apoptosis of lymphocytes is often referred to as programmed cell death because there appears to be a genetic program controlling the cell’s fate and this program is activated by physiological signals. Morphologically, the cell exhibits characteristics such as cellular condensation, membrane blebbing and chromosomal condensation on the nuclear periphery (reviewed in [3]). Biochemically, an endonuclease is often activated which cleaves the DNA into nucleosomal sized fragments. Ca\(^{2+}\) fluxes may play a role in the apoptotic death of lymphocytes (reviewed in [4–6]). McConkey et al. [7] reported an influx of extracellular calcium during glucocorticoid-mediated apoptosis of primary lymphocytes. Moreover, in cultured WEHI7.2 and W7MG1 murine lymphoma...
cells, Lam et al. observed an efflux of Ca$^{2+}$ from the endoplasmic reticulum (ER) which may be critical to conveying the apoptotic effects of glucocorticoids [1,2]. The negative effects of glucocorticoids on cell viability and DNA integrity were mimicked by thapsigargin [1], a compound which causes a Ca$^{2+}$ efflux from the ER by inhibiting the ER-specific Ca$^{2+}$/ATPase [8,9]. Other evidence suggesting a role for calcium in lymphocyte apoptosis include the observations that the steady state levels of calmodulin RNA are increased during glucocorticoid-mediated apoptosis [10,11] and calmodulin inhibitors interfere with the apoptotic process [7,10]. Moreover, the expression of the calcium binding protein calbindin D$_{28K}$ in the WEHI7.2 cells attenuates death caused by a variety of agents, presumably by buffering calcium fluxes in the cells [12]. The role of calcium may be to activate proteins such as calmodulin, and degradative enzymes such as endonuclease, proteases, and lipases [5,13,14]. In addition, Wyllie et al. demonstrated that lymphocyte apoptosis induced by the calcium ionophore A23187 is inhibited by cycloheximide [15]. This dependence on macromolecular synthesis suggests that calcium may control apoptosis, in part, by altering gene expression. However, the role of Ca$^{2+}$ fluxes in lymphocyte transition has not been established. Precedence for such Ca$^{2+}$-mediated transcription is found in a number of other systems including fibroblasts, hepatoma cells, and neuronal cells [16–20]. Often, Ca$^{2+}$-mediated transcription occurs through cAMP response elements (CREs) and the associated transcription factor known as CRE binding protein, or CREB [21,22].

This study was undertaken to establish whether calcium fluxes alter gene expression in lymphocytes during the apoptotic process. Using thapsigargin and A23187 as model compounds for inducing cell death in a calcium-dependent manner, we examined their effects on the induction of a CRE-linked reporter gene. Moreover, because death induced by dexamethasone may involve Ca$^{2+}$ fluxes, we also examined the effect of this glucocorticoid on CRE-mediated gene expression alone, and in combination with cAMP. These studies establish that treatment with the apoptotic agents thapsigargin, forskolin, or glucocorticoids induce transcription from a CRE via the phosphorylation of CREB to its bioactive form. Co-treatment with either dexamethasone and forskolin or with thapsigargin and forskolin resulted in increases in the levels of CREB phosphorylation, CRE-mediated transcription, and death that were greater than with treatment by any of the agents alone. Therefore, in lymphocytes, cAMP, Ca$^{2+}$, and glucocorticoids may coordinate regulation of the expression of a subset of genes through the bioactivation of the CREB transcription factor.

2. Materials and methods

2.1. Materials

Dexamethasone, forskolin and A23187 were purchased from Sigma; thapsigargin was purchased from LC Laboratories. Dexamethasone and forskolin were dissolved in ethanol and stored at $-20^\circ$C in the dark; thapsigargin and A23187 were dissolved in DMSO and stored at 4°C in the dark.

2.2. Cell culture

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% iron supplemented calf bovine serum (Hyclone) at 37°C in an atmosphere of 6% CO$_2$ and 90% humidity. Viable cells were determined by trypan blue exclusion and counted with the use of a hemacytometer.

2.3. Electron microscopy

Logarithmically growing WEHI7.2 cells were harvested at low speed in a microcentrifuge and after discarding the supernatant were fixed with glutaraldehyde and postfixed with osmium tetroxide in sodium cacodylate buffer. After washing with distilled water, the tissue was stained en bloc with uranyl acetate, dehydrated through graded ethanols and propylene oxide, and embedded in Polybed resin. Thin sections were poststained with uranyl acetate and lead citrate and viewed and photographed with a JEOL 100 CX electron microscope.

2.4. Transient transfection assay

For these studies, the plasmids ptkCAT and pCRE(2)tkCAT were used as reporter constructs [23]. ptkCAT encodes the reporter gene chloramphenicol acetyl transferase and the thymidine kinase promoter. pCRE(2)tkCAT encodes an insertion of two copies of a simple CRE from the human glycoprotein hormone a-subunit gene [24,25]. Logarithmically growing cells were harvested, washed once with HBS buffer (5 mM KCl, 137 mM NaCl, 6 mM glucose, 21 mM HEPES (N-2-hydroxyethylpiperazine-N’2-ethane-sulfonic acid, pH 7.05) 0.7 mM Na$_2$HP0$_4$), and resuspended in HBS buffer at $1.25 \times 10^7$ cells per ml. Reporter plasmid (40 μg) was added per ml of cells and electroporation was carried out at 200 mV and 960 μF with a Gene Pulser (Bethesda Research Laboratories). Following electroporation, like samples were combined and realiquoted into 10 ml DMEM containing 10% calf bovine serum per point in the presence of the indicated compound(s). This allowed for normalization due to potential differ-
ences between cuvettes. At the indicated times, cell extracts were prepared and assayed for CAT activity as described [26].

2.5. Western blot

\[1 \times 10^7\] cells were treated with solvent control, 2.5 \(\mu\)M forskolin, 0.02 \(\mu\)M thapsigargin, 1 \(\mu\)M dexamethasone, or a combination for 3 h. The cells were harvested at 1000 \(\times\) g, resuspended in phosphate buffer saline (PBS), and boiled with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were sonicated and subjected to centrifugation at 13 600 \(\times\) g. Samples were separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane (Immobilon, Millipore). Immunoblots incubated with the anti-phosphorylated CREB primary antibody were processed as described [27]. Immunoblots incubated with the anti-CREB primary antibody (Santa Cruz, Biotechnology), which recognizes phosphorylated and nonphosphorylated CREB, were performed as recommended by the supplier. Antibody-antigen complexes were detected with a horse radish peroxidase conjugated goat-anti-rabbit IgG antibody (Kirkegard and Perry Laboratories). The blots were developed with Renaissance Western Blot Chemiluminescence Reagents (Dupont) and exposed to film for 10–15 min. Quantitation was done by densitometry.

3. Results

3.1. Morphological and biochemical characterization of thapsigargin-induced apoptosis in WEHI7.2 cells

Our previous work strongly implicated a direct role for calcium in apoptosis of WEHI7.2 lymphocytes [10,12]. In support of this, Lam et al. recently demonstrated that glucocorticoid treatment of WEHI cells results in a release of \(Ca^{2+}\) from estrogen receptor (ER) stores and this event may be required for apoptotic death [1,2]. Thapsigargin treatment, which leads to a dose-dependent release of \(Ca^{2+}\) from the ER in WEHI cells, mimicked the effects of glucocorticoids in causing DNA fragmentation and cell death, suggesting that the thapsigargin-induced death of lymphocytes is apoptotic in nature [1,28]. Indeed, thapsigargin induced cell death in the WEHI7.2 cells in a concentration dependent manner (Fig. 1), with a 97% decrease in cell number observed after 24 h of treatment with 0.1 \(\mu\)M thapsigargin. A loss in cell viability was accompanied by an increase in DNA fragmentation (data not shown). However, since DNA fragmentation is not the sole criterion for determining the nature of death (apoptosis or necrosis) [29], we examined morphological criteria to establish that thapsigargin-induced death in WEHI7.2 cells was apoptosis. Ultrastructural evaluation of WEHI7.2 cells treated for 8 h with 0.06 \(\mu\)M thapsigargin revealed chromosomal condensation on the nuclear periphery (Fig. 2A,B) and cytoplasmic blebbing (Fig. 2B), both of which are cytological changes characteristic of apoptosis. In contrast, treatment with DMSO vehicle alone had no effect on WEHI7.2 cell morphology (Fig. 2C). These observations indicate that treatment of the WEHI7.2 cells with thapsigargin leads to death by inducing apoptosis and supports the biochemical data demonstrating apoptosis-associated DNA fragmentation (Lam, 1993 no. 185; data not shown).

3.2. Calcium fluxes activate CRE-mediated gene expression

We previously observed cAMP-mediated gene expression in apoptotic WEHI7.2 cells [12,30]. Because \(Ca^{2+}\) and cAMP signaling pathways may converge at the transcriptional control of CRE-containing promoters [21,22], we tested the ability of thapsigargin to regulate transcription of a CRE-linked reporter gene. As depicted in Fig. 3A, thapsigargin treatment of WEHI7.2 cells resulted in a dose dependent increase in transcription from the reporter construct pCRE(2)tkCAT. This concentration-dependent effect of thapsigargin on gene expression correlates with its ability to induce apoptosis (Fig. 1). A similar transcriptional effect was observed with ionophore A23187 (Fig. 3B), another inducer of calcium fluxes and apoptosis [12,15,31,32]. This supports the hypothesis that the

![Fig. 1. Effect of thapsigargin on the growth of WEHI7.2. Cells were treated with the indicated levels of thapsigargin or DMSO vehicle for 24 h. Viable cells were determined by trypan blue exclusion, counted on a hemacytometer, and reported relative to the DMSO vehicle treated population. Shown is the mean of at least three independent experiments ± sem.](image-url)
thapsigargin induced gene expression through a calcium-dependent mechanism. In these studies, thapsigargin (t), A23187 (A) and forskolin (f) led to a 5.1-, 6.0-, and 5.2-fold induction in CRE-controlled CAT activity, respectively. Forskolin induces the cAMP pathway of signal transduction by activating adenylate cyclase [33], whereas thapsigargin and A23187 induce a Ca$^{2+}$ mediated transcriptional pathway [20,34]. The co-treatment of WEHI7.2 with thapsigargin and A23187 (tA) led to an additive 9.9-fold induction in CAT activity, while the co-treatment of either agent with forskolin resulted in synergistic activation. Specifically, thapsigargin and forskolin (tf) induced activation of the pCRE(2)tkCAT construct 29.6-fold while A23187 and forskolin (Af) increased CAT activity 24.5-fold. An increase in CAT activity from the ptkCAT control vector was not observed with any agent demonstrating that the Ca$^{2+}$ and cAMP-induced responses are mediated through the CRE (Fig. 3B). Interestingly, treatment of WEHI7.2 with low levels of both thapsigargin and forskolin resulted in more death than the same concentration of either agent alone (Fig. 4), thus correlating the level of CRE-mediated transcription (Fig. 3B) with death in these cases.

3.3. Dexamethasone activates transcription from a CRE

It has been reported that the release of Ca$^{2+}$ from ER stores may be an integral step in both thapsigargin and glucocorticoid-mediated death [1,28]. Because thapsigargin treatment activated CRE-dependent transcription in WEHI7.2 cells, we hypothesized that a similar transcriptional effect may occur following glucocorticoid treatment due to an efflux of Ca$^{2+}$ from the ER. As predicted, dexamethasone treatment of the WEHI cells resulted in a reproducible 2.4-fold induction of CRE-dependent transcription (Fig. 5A). Forskolin treatment led to a 6-fold induction while the treatment with both agents resulted in a 12-fold synergistic induction of CAT activity. This effect was dependent upon the glucocorticoid receptor (GR), as it was not observed in the GR-mutant cell line, WEHI7.418. This is consistent with the observation that glucocorticoid-mediated depletion of ER Ca$^{2+}$ stores is receptor dependent [1]. The specificity of the response for the CRE was also evident in that it was not observed with the ptkCAT vector lacking the CRE. The effects of glucocorticoids on CRE-mediated transcription were not explained by increases in intracellular cAMP. Over the time period of these experiments, cAMP levels remained unaltered following glucocorticoid treatment (data not shown). The apoptotic response of these cells to dexamethasone and forskolin was correlated with the level of CRE-linked gene expression. When these compounds were added to growing cells, the killing effect of the two agents together was greater than with either agent alone (Fig. 5B). A similar observation was reported for other WEHI7 derivatives [35].
3.4. Treatment with apoptotic agents results in the phosphorylation of the transcription factor CREB

Having demonstrated that dexamethasone, thapsigargin and forskolin led to an induction of CRE-controlled gene expression, we wanted to determine whether this was a result of CREB bioactivation. The transcriptional activity of CREB is associated with the phosphorylation of the protein at Ser133, and calcium and cAMP induce phosphorylation through calmodulin-dependent kinase and PKA, respectively [22,36–38]. To monitor the state of phosphorylation of CREB, an antibody was used which specifically recognizes phosphorylated Ser133 [27]. WEHI cells were treated with thapsigargin (t), forskolin (f), or a combination (tf) and analyzed by Western blot analysis (Fig. 6). An increase was observed in the amount of phosphorylated CREB after treatment with either agent (lanes 6 and 7), consistent with our previous observations of transcriptional induction. Moreover, the co-treatment with thapsigargin and forskolin led to a level of CREB phosphorylation greater than with either agent alone (lane 8). These data support our previous findings in which forskolin and thapsigargin synergistically activate CRE-mediated gene expression (Fig. 4B). In addition, WEHI7.2 cells were treated with dexamethasone (d), forskolin (f), or a combination (df), and Western blot analysis was performed using the antiphosphorylated CREB antibody. As assessed by densitometry of the resulting autoradiogram, a 1.8-fold increase in the levels of phosphorylated CREB after dexamethasone treatment was reproducibly observed (lane 2), whereas a 4.4-fold increase in the amount of phosphorylated CREB occurred after forskolin treatment (lane 3). The co-treatment with both forskolin and dexamethasone displayed an 8-fold increase in the level of phosphorylation (lane 4). The levels of CREB phosphorylation observed in this system are comparable with the magnitude of the transcriptional responses (Fig. 5).

In order to verify that the concentration of CREB protein remained constant while only the state of phosphorylation changed, we analyzed parallel samples for total CREB protein. Using a CREB-specific antibody
which detects both phosphorylated and nonphosphorylated CREB, we did not detect significant changes in CREB protein concentration (Fig. 6, lower panels). Likewise, these treatments did not cause a change in steady state levels of CREB mRNA by Northern blot analysis (data not shown).

![Fig. 6. Western blot analysis demonstrating the effect of forskolin, thapsigargin, and dexamethasone on CREB phosphorylation. Cells were treated with 2.5 μM forskolin (f), 0.02 μM thapsigargin (t), 1 μM dexamethasone (d), or a combination of agents (df or tf) for 3 h. Control samples (c) were incubated with solvent vehicle. Cells were lysed with SDS sample buffer and subjected to electrophoresis and Western blot analysis as described in experimental procedures. Upper panels were probed with the primary anti-phosphorylated CREB antibody and the arrow designates phosphorylated CREB. Lower panels labeled CREB were incubated with the anti-CREB antibody. Shown are representative experiments.](image)

4. Discussion

Our ongoing interest in the role of Ca$^{2+}$ during lymphocyte apoptosis prompted this examination of potential calcium-mediated events involved in the death process. Here, we used thapsigargin-treated WEHI7.2 cells as a model of Ca$^{2+}$-induced death [1]. By inhibiting the ER-specific Ca$^{2+}$/ATPase, thapsigargin causes a dose-dependent efflux of ER calcium stores in WEHI cells [2]. In agreement with this, we demonstrate that this agent induces death in a concentration-dependent fashion, and the dying cells exhibit the characteristic morphological and biochemical events that are indicative of apoptosis including membrane blebbing and DNA condensation on the nuclear membrane. These results confirm and extend the observations of Lam et al. wherein thapsigargin induced DNA fragmentation in WEHI cells [1]. Furthermore, in the present study we establish that one important downstream effect of thapsigargin treatment of the WEHI7.2 cell line is to enhance transcription from a CRE-regulated reporter construct. This transcriptional response to thapsigargin was mimicked by the Ca$^{2+}$ ionophore A23187 suggest-
ing that the response was a result of Ca\(^{2+}\) fluxes (Fig. 3). Therefore, Ca\(^{2+}\) elicited a transcriptional response during apoptosis of the WEHI7.2 cells in addition to the well established role in directly activating degradative enzymes. Our data demonstrating Ca\(^{2+}\)-mediated transcriptional control support the observation that the apoptotic effects of A23187 can be blocked by inhibitors of protein synthesis [15]. Fig. 6 strongly suggests that the transcriptional response we observed may be mediated by CREB since thapsigargin led to the phosphorylation of CREB on Ser133 and phosphorylation of this site is an indicator of transcriptional activity [36]. These observations are particularly significant because they suggest that calcium fluxes during apoptosis initiate a signaling cascade that ultimately activates CREB-controlled gene expression. Although we demonstrate that CREB-activation and CRE-mediated gene expression occur during apoptosis, additional studies are required to firmly link these two processes to the cell death mechanism. Identification and characterization of endogenous genes that are regulated by Ca\(^{2+}\) during the death process will help establish this vital link. The mechanism of death induced by Ca\(^{2+}\) and thapsigargin may be similar to that induced by glucocorticoids, as several studies suggest the glucocorticoid-induced apoptotic pathway is calcium-dependent [1,4,7,10,12,31,32,39]. Specifically, in addition to its well established role in regulating GR-dependent gene transcription associated with apoptosis, dexamethasone may also activate the Ca\(^{2+}\)-dependent transcriptional pathway in WEHI7.2 through a receptor-dependent efflux of calcium from ER stores [1]. We present evidence that dexamethasone activated CRE-regulated gene expression is also receptor dependent and propose that this event is mediated by Ca\(^{2+}\)-dependent phosphorylation of CREB at Ser 133. Thus, these events represent one point of crosstalk in the Ca\(^{2+}\)-, glucocorticoid-, and cAMP-induced signaling pathways in lymphocytes. It is of interest to note that when a combination of apoptotic agents were added to the cells simultaneously, the effect on CRE-dependent transcription was either additive (e.g., A23187 and thapsigargin), or synergistic (e.g., forskolin in the presence of thapsigargin, A23187, or dexamethasone). The additive response of thapsigargin and A23187 may reflect the activation of the same pathway (Ca\(^{2+}\)-dependent) while the synergistic-responses might result from activation of separate yet converging pathways mediated by the effects of Ca\(^{2+}\) and cAMP [18]. Alternatively, cAMP may modulate glucocorticoid-induced Ca\(^{2+}\) fluxes, although forskolin treatment alone does not alter cytosolic Ca\(^{2+}\) levels (data not shown). Whether the pathways are convergent or cAMP alters hormone-mediated Ca\(^{2+}\) fluxes, the end result is a synergism in CREB bioactivation and CRE-dependent transcription. A similar synergistic effect was observed during the Ca\(^{2+}\)- and cAMP-induced c-fos gene expression in PC12 cells, an effect which was mediated through a CRE promoter element [18]. The authors proposed that the pathways by which calcium and cAMP enhance c-fos gene expression converge via the activation of the transcription factor CREB. In this regard, Ca\(^{2+}\) fluxes were shown to lead to the activation of Ca\(^{2+}\)/calmodulin dependent kinases (CaMK), which in turn can phosphorylate and activate the CREB protein [21,22,37,40]. The localization of CaMK I and CaMK IV in the nucleus, and their ability to activate CREB-regulated gene expression, make them candidates for mediating the transcriptional effects seen [37,38,41–43]. In support of this hypothesis, Antoine et al. demonstrated that CaMK IV and forskolin act synergistically to induce c-fos expression in AtT20 corticotrophic cells [43]. In comparison, increases in intracellular cAMP lead to the well characterized activation of PKA, phosphorylation of CREB, and subsequent regulation of CRE-controlled genes [36,44]. Thus, through separate kinases, the Ca\(^{2+}\) and cAMP pathways converge on CREB as the common transcriptional activator. Our current studies suggest that CREB may serve a similar pivotal role in the responses of lymphocytes to glucocorticoids, Ca\(^{2+}\), or cAMP.

Other groups have observed a synergism in the apoptotic responses induced by glucocorticoids and cAMP [45,46], an effect which was attributed to a cAMP-mediated increase in GR levels as well as the capacity of the receptor to produce an apoptotic response. Moreover, Rangarajan et al. observed that in F9 cells, cAMP potentiates GR function by increasing the affinity of GR for its cognate hormone response element, presumably through a phosphorylation event [47]. Our observations of glucocorticoid mediated transcription of the pCRE(2)tkCAT reporter construct are unique in that they suggest an alternative mechanism of crosstalk between glucocorticoids and cAMP which may be acting independently or in conjunction with those reported previously. We propose that in addition to their traditional role in GR-mediated transcription, glucocorticoids also modulate Ca\(^{2+}\) levels in the cell which in turn regulate CRE-driven gene expression to some extent. This extends our previous work which suggested a convergence of apoptotic pathways [30,48]. It will be important in the future to define the mechanism of Ca\(^{2+}\) and glucocorticoid-induced phosphorylation of CREB and to determine if this response is specific for all lymphocytes, apoptotic lymphocytes or is a more general mechanism of apoptosis in a variety of cells.

**Acknowledgements**

We extend special thanks to M. Greenberg and D. Ginty for the antiphosphorylated CREB antibody and
technical advise and to P. Mellon for her generous gift of the ptkCAT and pCRE(2)tkCAT plasmids. We would also like to thank S. Gurwitch for technical assistance and critical reading of the manuscript. This work was supported by a grant from the NIH (AI 35910) to DRD.

References


