FEBS 17407

FEBS Letters 392 (1996) 129–136

Activation of NF-κB by ER stress requires both Ca^{2+} and reactive oxygen intermediates as messengers

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Received 24 June 1996

Abstract The eukaryotic transcription factor NF-κB is activated by a large variety of stimuli. We have recently shown that ER stress, caused by an aberrant accumulation of membrane proteins within this organelle, also activates NF-κB. Here, we show that activation of NF-κB by ER stress requires an increase in the intracellular levels of both reactive oxygen intermediates (ROIs) and Ca^{2+}. Two distinct intracellular Ca^{2+} chelators and a panel of structurally unrelated antioxidants prevented NF-κB activation by various ER stress-eliciting agents, whereas only antioxidants but not the Ca^{2+} chelators prevented NF-κB activation by the inflammatory cytokine TNF-α. Consistent with an involvement of calcium, the ER-resident Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid (CPA), which trigger a rapid efflux of Ca^{2+} from the ER, also potently activated NF-κB. Pretreatment with a Ca^{2+} chelator abrogated this induction. The Ca^{2+} chelator BAPTA-AM inhibited ROI formation in response to thapsigargin and CPA treatment, suggesting that the Ca^{2+} increase preceded ROI formation during NF-κB activation. The selective inhibitory effect of the drug tepoxalin suggests that the peroxidase activity of cyclooxygenases or lipoxygenases was responsible for the increased ROI production in response to Ca^{2+} release by thapsigargin.

Key words: Endoplasmic reticulum; NF-κB; Ca^{2+}; Radical oxygen intermediate

1. Introduction

The eukaryotic transcription factor NF-κB is a central mediator of the immune response. As such, it is activated by a large variety of pathological stimuli including bacterial lipopolysaccharide, viral proteins and inflammatory cytokines (for a review see [1]). Since these agents activate many diverse signal transduction pathways and second messenger systems, NF-κB must either be activated by several mechanisms or the various pathways must converge at one point to elicit a prototypic NF-κB-activating reaction.

In most cells, NF-κB is cytoplasmically retained in an inactive complex by binding to an inhibitory subunit, called IκB [2]. All NF-κB-inducing stimuli characterized to date cause the phosphorylation and subsequent degradation of IκB-α, thereby releasing active NF-κB. This consists of a heterodimer of members of the NF-κB/Rel family of transcription factors, most often p50 and p65 (RelA). Activated NF-κB rapidly translocates to the nucleus where it induces transcription of target genes, such as cytokines, immunoreceptors and adhesion molecules (for a detailed list see [1]). Both IκB-α phosphorylation and degradation are prevented by a large variety of structurally unrelated antioxidants [3–8]. Moreover, micromolar concentrations of H_2O_2 lead to NF-κB activation in some cell lines. Based on these observations, it was proposed that reactive oxygen intermediates (ROIs) serve as common second messengers in many NF-κB-activating conditions [9].

The precise signal transduction pathways leading to ROI production and subsequent NF-κB activation remain unknown for many stimuli. However, in a number of cases, the enzymes responsible for ROI production have been identified. TNF-mediated NF-κB activation in L929 cells requires an intact mitochondrial electron transport chain since addition of the complex I inhibitor rotenone or elimination of mitochondria blocks NF-κB activation by this cytokine [10]. In contrast, the complex III inhibitor antimycin A, which potentiates ROI production augmented NF-κB induction. It was proposed that mitochondrial ubiquinone generates the NF-κB-activating ROIs in response to TNF stimulation. Triggering of the CD28 receptor on T cells also results in the production of ROIs and NF-κB activation [11]. Inhibitors of 5-lipoxygenase block NF-κB activation by CD28, suggesting that this enzyme generates the ROIs in this pathway. Overexpression of the ROI-producing enzyme cyclooxygenase-1 was found to augment NF-κB induction by phorbol ester. This effect was dependent on the enzyme's peroxidase activity [12]

We have recently shown that NF-κB is activated by a variety of agents which perturb ER function [13]. Since ER stress activates NF-κB-dependent nuclear gene expression, a novel ER-nuclear signal transduction pathway could be defined. This pathway is pharmacologically distinct from the previously characterized unfolded protein response pathway. Using mutants of the adenovirus protein E3/19K we have identified the NF-κB-activating signal as the retention and subsequent accumulation of proteins in the ER, a condition we have termed ER overload [14].

Since the inactive NF-κB resides in the cytoplasm, ER overload must emit a signal that reaches NF-κB in the cytosol. We show here that both the efflux of Ca^{2+} from the ER and the subsequent generation of ROIs are required for ER overload-mediated NF-κB activation. NF-κB induction by ER stress is prevented by pre-incubation of cells with the intracellular Ca^{2+} chelators BAPTA-AM and TMB-8. Moreover, inhibition of the ER-resident Ca^{2+}-ATPase by thapsigargin or CPA, which causes rapid efflux of Ca^{2+} from the ER, potently induced NF-κB. This activation was blocked by BAPTA-AM. Antioxidants prevented NF-κB induction both in response to ER stress and thapsigargin or CPA stimulation, suggesting that ROIs are also required for NF-κB-mediated ER-nuclear signal transduction. Incubation of cells with BAPTA-AM inhibits ROI production following thapsigargin or CPA stimulation, suggesting that Ca^{2+} release precedes ROI formation

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PII S0014-5793(96)00800-9

in the NF-κB-mediated ER-nuclear signal transduction pathway.

2. Materials and methods

2.1. Cell culture and transfections

HeLa cells (ATCC no. CCL 2) and 293 cells (ATCC no. CRL 1573) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and 50 μg/ml penicillin-streptomycin (all from Gibco-BRL). HeLa and 293 cells were plated 12–16 h prior to transfection at a density of 10^6 cells per 60 mm dish. Transfections were performed using calcium phosphate precipitation as previously described [15]. The amounts of plasmids used are indicated in the figure legends. Tunicamycin, brefeldin A, 2-deoxyglucose, TPA, thapsigargin, *N*-acetyl-L-cysteine (NAC), butylated hydroxyanisole (BHA), rotenone, nordihydroguaiaretic acid (NDGA), dithoithreitol (DTT), pyrrolidine dithiocarbamate (PDTC), metyrapone, allopurinol, hydroxyurea, curcumin and indomethacin were purchased from Sigma Biochemicals. Cyclopiazonic acid, TMB-8 and 5,8,11,14-eicosatetraynoic acid (ETYA) were purchased from Calbiochem. The protein kinase C inhibitor bisindolylmaleimide was purchased from Boehringer Mannheim. BAPTA-AM was obtained from Molecular Probes. Tepoxalin was generously donated by Dr. T. Parks, Boehringer Ingelheim, Inc. TNF was a kind gift from Prof. Karl Decker, Freiburg.

2.2. Plasmids

The hemagglutinin expression vector contains the entire HA coding sequence of the influenza virus strain A/FPV/Rostock/34 (H7N1) cloned into the vaccinia virus insertion vector pSC11 and was a generous gift from Drs. W. Garten and H. Klenk (University of Marburg). The μ chain expression plasmid was a kind gift from Dr. M. Reth (Max Planck Institute, Freiburg). The E3/19K vector contains the *Eco*RI D fragment of adenovirus 2 in the pBluescript II KS vector [16]. The MHC class I K^d expression vector has been described elsewhere [17]. Both were kindly provided by Dr. H.-G. Burgert, Max Planck Institute, Freiburg.

2.3. Electrophoretic mobility shift assays

Total cell extracts were prepared using a high-salt detergent buffer (Totex) (20 mM HEPES, pH 7.9, 350 mM NaCl, 20% (w/v) glycerol, 1% (w/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% PMSF, 1% aprotinin). Cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma) and resuspended in four cell volumes of Totex buffer. The cell lysate was incubated on ice for 30 min, then centrifuged for 5 min at $13000 \times g$ at 4°C. The protein content of the supernatant was determined and equal amounts of protein (10–20 μg) added to a reaction mixture containing 20 μg BSA (Sigma), 2 μg poly(dI-dC) (Boehringer), 2 μl buffer D+ (20 mM HEPES, pH 7.9; 20% glycerin, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF), 4 μl buffer F (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1% PMSF) and 100000 cpm (Cerenkov) of a ^{32}P-labeled oligonucleotide in a final volume of 20 μl. Samples were incubated at room temperature for 25 min. Reactions for AP-1 contained 5 mM $MgCl_2$ in addition. NF-κB and AP-1 oligonucleotides (Promega) were labeled using γ-[^{32}P]ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Fermentas).

2.4. FACS analysis

Cells (1×10^6) were washed three times in phosphate-buffered saline (PBS) and harvested in PBS. DCFH diacetate (Fluka) was added to a final concentration of 5 μM and cells incubated at 37°C for 10 min.

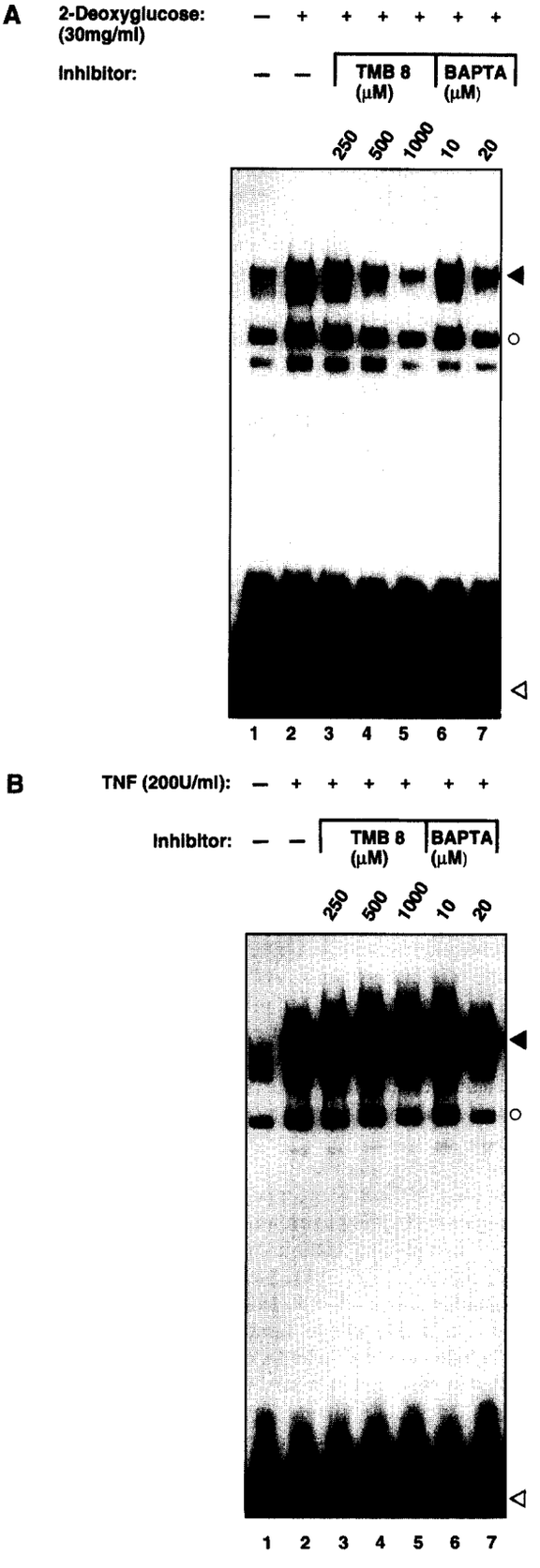

Fig. 1. Effect of Ca^{2+} chelators on NF-κB activation by 2-deoxyglucose and TNF. (A) HeLa cells were pretreated for 1 h with the indicated concentrations of TMB-8 (lanes 3–5) and BAPTA-AM (lanes 6,7). Subsequently, cells were stimulated with 30 mg/ml 2-deoxyglucose for 4 h (lanes 2–7). Unstimulated control cells are shown in lane 1. (B) Cells were pretreated with the same concentrations of TMB-8 and BAPTA-AM as in A, but then stimulated with 200 U/ml TNF for 1 h (lanes 2–7). Unstimulated control cells are again shown in lane 1. Total cell extracts were prepared and assayed in an EMSA using a high-affinity κB-binding site as a probe. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

Cells were scanned on a FACSSORT flow cytometer (Becton Dickinson) with excitation and emission settings at 495 and 525 nm, respectively. The results were analyzed with the Lysis II software programm.

3. Results

3.1. Ca^{2+} chelators abrogate NF-κB activation by 2-deoxyglucose but not TNF-α

We investigated the effect of the intracellular Ca^{2+} chelators BAPTA-AM and TMB-8 on the activation of NF-κB by 2-deoxyglucose, an inhibitor of *N*-glycosylation which elicits ER stress. Previously, we have shown that NF-κB activation by ER stress follows slow kinetics, most likely due to the gradual accumulation of protein within the ER [13], with maximal activation seen after 24 h. However, since Ca^{2+} chelators may not retain their activity over such a long period of time, HeLa cells were treated with 30 mg/ml 2-deoxyglucose for only 4 h after a preincubation of 1 h with various concentrations of the chelators. Thereafter, total cell extracts were prepared and assayed for NF-κB DNA binding activity using electrophoretic mobility shift assays (EMSA). Incubation of HeLa cells with 2-deoxyglucose for 4 h led to significant but sub-maximal NF-κB activation (Fig. 1A, lane 2). Pretreatment of cells with two different Ca^{2+} chelators prevented NF-κB activation by 2-deoxyglucose (Fig. 1A, lanes 3–7), suggesting that an increase in intracellular Ca^{2+} was required for NF-κB induction by the *N*-glycosylation inhibitor.

We tested whether BAPTA-AM and TMB-8 could also affect NF-κB activation by TNF-α. HeLa cells were pretreated for 1 h with the same concentrations of BAPTA-AM and TMB-8 used in the previous experiment, afterwards, cells were stimulated with 200 IU/ml TNF for 1 h. TNF strongly induced NF-κB DNA binding activity in HeLa cells (Fig. 1B, lane 2). In contrast to 2-deoxyglucose-mediated NF-κB activation, pretreatment with Ca^{2+} chelators did not significantly influence NF-κB activation by TNF (Fig. 1B, lanes 3–7).

3.2. Calcium chelators interfere with many ER stress-inducing conditions

In order to determine whether Ca^{2+} chelators also inhibit NF-κB activation in response to other ER stress-inducing compounds, HeLa cells were preincubated with 40 μM BAPTA-AM for 1 h and then stimulated with 50 μg/ml tunicamycin, 30 mg/ml 2-deoxyglucose, 250 μg/ml brefeldin A, 5 μM thapsigargin or 15 μM CPA for 4 h. Again, activation by these agents is sub-maximal due to the short treatment time. NF-κB activation by all these agents is inhibited by pretreatment with the Ca^{2+} chelator (Fig. 2), indicating that Ca^{2+} is required as a messenger for NF-κB activation by a number of structurally and mechanistically unrelated ER stress-eliciting compounds.

ER stress can also be elicited by the expression of integral membrane proteins which accumulate in the ER [13]. We have previously shown that the overexpression of influenza virus hemagglutinin [18], adenovirus E3/19K [14], immunoglobulin heavy chain [18] and MHC class I [14] causes NF-κB activation by ER overload. Here, we investigated the effect of the Ca^{2+} chelator TMB-8 on NF-κB activation by overexpression of these proteins. 293 cells were pretreated for 1 h with 1 mM TMB-8, which does not interfere with the DNA transfection

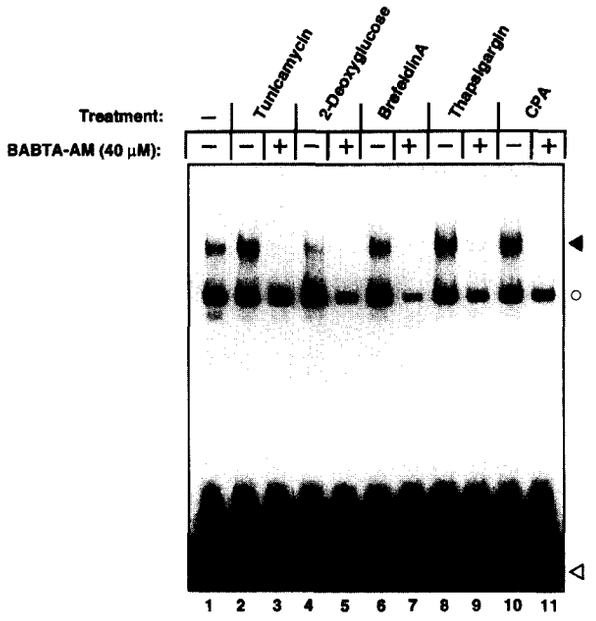

Fig. 2. Pretreatment with the Ca^{2+} chelator BAPTA-AM abrogates NF-κB activation by ER stress-inducing agents. HeLa cells were incubated with 40 μM BAPTA-AM for 1 h (lanes 3,5,7,9,11) or left untreated (lanes 2,4,6,8,10), after which they were stimulated with 50 μg/ml tunicamycin (lanes 2,3), 30 mg/ml 2-deoxyglucose (lanes 4,5), 250 μg/ml brefeldin A (lanes 6,7), 5 μM thapsigargin (lanes 8,9) or 15 μM CPA (lanes 10,11) for 4 h. Total cell extracts were prepared and assayed in an EMSA using a high-affinity κB-binding site as a probe. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

procedure, subsequent protein expression or TNF-mediated NF-κB activation [14]. Subsequently, cells were transfected with 6 μg of expression vectors for the various proteins. 4 h after transfection, total cell extracts were prepared and assayed for NF-κB DNA binding activity. The NF-κB activation induced by expression of all four proteins was inhibited by the Ca^{2+} chelator TMB-8 (Fig. 3). Thus, ER stress caused by both drugs and protein overexpression requires an increase in intracellular Ca^{2+} for activation of transcription factor NF-κB.

3.3. NF-κB activation by ER stress does not require protein kinase C

An increase in intracellular calcium may activate Ca^{2+}/phospholipid-dependent protein kinase C isozymes. Moreover, the Ca^{2+} chelator TMB-8 has been reported to have effects on phospholipid metabolism and protein kinase C [19]. We therefore assessed whether activation of protein kinase C played a role in ER stress-mediated NF-κB activation. 293 cells were pretreated for 1 h with various concentrations of the PKC inhibitor bisindolylmaleimide [20] and subsequently either transfected with 6 μg of E3/19K expression plasmid (Fig. 4A,B, lanes 2–5), stimulated with 200 IU/ml of TNF (lanes 6–9), or with 50 ng/ml of the active phorbol ester phorbol 12-myristate 13-acetate (TPA) (lanes 10–13). Transfected cells were harvested after 4 h, stimulated cells after 1 h. Total cell extracts were prepared and assayed for DNA binding activities of both transcription factors NF-κB (Fig. 4A) and AP-1 (Fig. 4B). Neither E3/19K-mediated nor TNF-stimulated NF-κB activation was altered by the protein

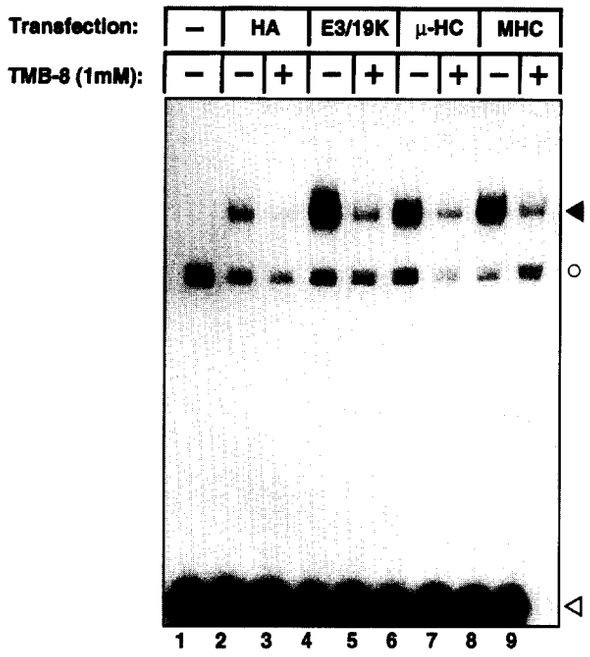

Fig. 3. The Ca^{2+} chelator TMB-8 prevents NF-κB activation by ER overload. 293 cells were pretreated for 1 h with 1 mM TMB-8 (lanes 3,5,7,9) or left untreated (lanes 2,4,6,8). Cells were then transiently transfected with 6 μg of either a influenza haemagglutinin expression vector (lanes 2,3), an E3/19K expression vector (lanes 4,5), a immunoglobulin μ-heavy chain expression vector (lanes 6,7) or an expression vector for MHC class I (lanes 8,9). 4 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes, the open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

kinase C inhibitor treatment (Fig. 4A, lanes 2–9). In contrast, the induction of AP-1 by TPA, which involves protein kinase C [21], was completely inhibited by 0.5 μM bisindolylmaleimide (Fig. 4B, lanes 10–13). E3/19K-mediated AP-1 activation was also sensitive toward PKC inhibition (Fig. 4B, lanes 6–9). In 293 cells, TPA did not induce NF-κB and TNF did not stimulate AP-1 (Fig. 4A, lane 10; Fig. 4B, lane 6). The results provide pharmacological evidence that NF-κB activation by ER overload is independent of protein kinase C.

3.4. *Antioxidants prevent NF-κB activation by ER overload*

It has been suggested that ROIs serve as common messenger for many NF-κB activating stimuli [9]. We therefore used a panel of structurally unrelated antioxidants to investigate the role of ROIs in NF-κB activation by ER overload. 293 cells were pretreated for 1 h with 30 mM *N*-acetyl-L-cysteine (NAC), 250 μM butylated hydroxyanisole (BHA), 5 μM rotenone, 40 μM nordihydroguaiaretic acid (NDGA), 100 μM pyrrolidine dithiocarbamate (PDTC) or 5 mM dithiothreitol (DTT). Subsequently, cells were transfected with 6 μg of E3/19K expression vector. Total cell extracts were prepared 4 h after transfection and analyzed for NF-κB DNA binding. All six antioxidants more or less strongly interfered with E3/19K-induced NF-κB activation (Fig. 5). The effect of PDTC is less pronounced because this compound does not retain its antioxidative activity over 4 h. Immunoprecipitations revealed that, with the exception of PDTC, these antioxidants did not interfere with transfection or E3 protein expression in these cells (data not shown). PDTC inhibited E3/19K expression by approx. 50%. Taken together, these data suggest that ROIs also play a role as messenger molecules in ER overload-mediated NF-κB activation.

3.5. *Inhibition of ER-resident Ca^{2+}-ATPase induces oxidative stress and NF-κB activation*

The compounds thapsigargin and CPA inhibit the ER-resident Ca^{2+}-ATPase, thereby causing a rapid efflux of Ca^{2+} from the ER lumen into the cytoplasm [22]. In contrast to ER stress-eliciting agents, such as 2-deoxyglucose, which act slowly [13], thapsigargin and CPA activate NF-κB with rapid kinetics, maximal activation occurring after 1 h of stimulation [14]. We therefore investigated whether these agents also require ROIs to activate NF-κB. This may indicate whether an increase in calcium precedes the generation of ROIs. HeLa cells were pretreated for 1 h with 100 μM of the antioxidant PDTC before stimulation with either 5 μM thapsigargin, 15 μM CPA or 50 ng/ml TPA. The antioxidant inhibited NF-κB activation by all three stimuli (Fig. 6), suggesting that the thapsigargin and CPA-induced Ca^{2+} release causes the production of ROIs which ultimately mediate NF-κB activation.

3.6. *Tepoxalin inhibits NF-κB activation by thapsigargin but not by TPA or TNF*

We tried to identify pharmacologically the enzyme responsible for ROI production in response to calcium increase by thapsigargin. To this end, HeLa cells were treated with inhibitors for a variety of ROI-producing enzymes and assayed for

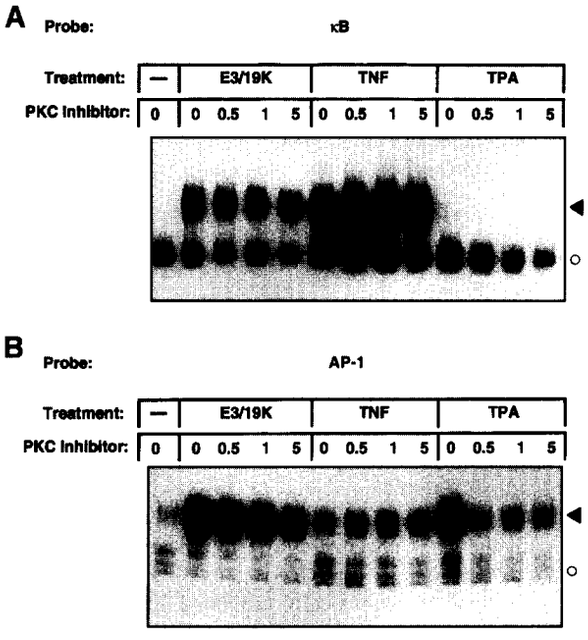

Fig. 4. E3/19K-induced NF-κB activation does not require protein kinase C. 293 cells were pretreated for 1 h with 0.5, 1 or 5 μM bisindolylmateimide as indicated and subsequently either transfected with 6 μg of E3/19K expression plasmid (lanes 2–5), stimulated with 200 U/ml of TNF (lanes 6–9), or treated with 50 ng/ml TPA (lanes 10–13). Transfected cells were harvested after 4 h, stimulated cells after 1 h. Total cell extracts were prepared and assayed for DNA binding of both transcription factors NF-κB (A) and AP-1 (B). Sections of fluorograms are shown. A filled arrowhead indicates specific NF-κB (A) or AP-1 (B) complexes and the open circles denote non-specific binding to the probes.

thapsigargin-induced NF-κB activation (Fig. 7). Neither the xanthine oxidase inhibitor allopurinol, nor the ribonucleotide reductase inhibitor hydroxyurea had any effect on the induction of NF-κB by thapsigargin (lanes 6–11). The P450 inhibitor metyrapone and the cyclooxygenase inhibitor indomethacin slightly reduced NF-κB activation, but only at very high concentrations, (lanes 5,17). However, these high doses far exceeded the IC_{50} required for enzyme inhibition in intact cells and, therefore, most likely reflected nonspecific effects of the compounds. Curcumin, an inhibitor of the lipoxygenase pathway [23], inhibited NF-κB activation at very low concentrations (20 μM, lane 12). However, this inhibition may be caused by curcumin's potent antioxidative potential [24]. We thus tested the lipoxygenase and cyclooxygenase inhibitor ETYA, an arachidonate analogue, for its ability to inhibit NF-κB activation by thapsigargin. At concentrations up to 300 μM, ETYA was unable to inhibit NF-κB induction (lanes 13–20). However, a second lipoxygenase and cyclooxygenase inhibitor, tepoxalin that specifically targets the intrinsic peroxidase activity of the enzymes [25], inhibited thapsigargin-mediated NF-κB activation at μM concentrations (Fig. 8, lanes 2–5). Tepoxalin had no effect on TPA- or TNF-mediated NF-κB activation (lanes 6–13). This suggests that the peroxidase activity within cxclooxygenases or lipooxygenases may be responsible for generating ROIs in response to increased intracellular calcium levels.

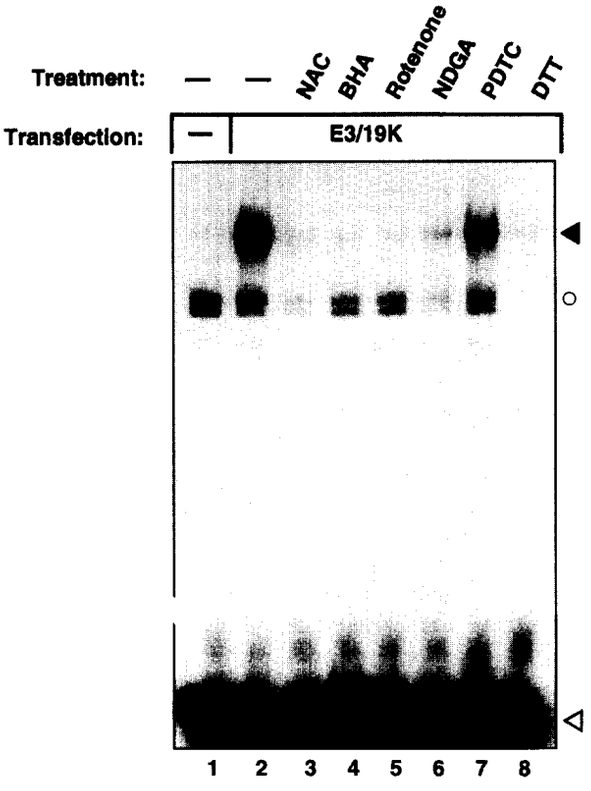

Fig. 5. E3/19K-mediated NF-κB activation is inhibited by various structurally unrelated antioxidants. 293 cells were left untreated (lanes 1,2) or treated for 1 h with 30 mM *N*-acetyl-L-cyteine (NAC, lane 3), 250 μM butylated hydroxyanisole (BHA, lane 4), 5 μM rotenone (lane 5), 40 μM nordihydroguaiaretic acid (NDGA, lane 6), 100 μM pyrrolidine dithiocarbamate (PDTC, lane 7) or 5 mM dithiothreitol (DTT, lane 8). Subsequently, cells were transfected with 6 μg of E3/19K expression vector (lanes 2–8). 4 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes, the open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

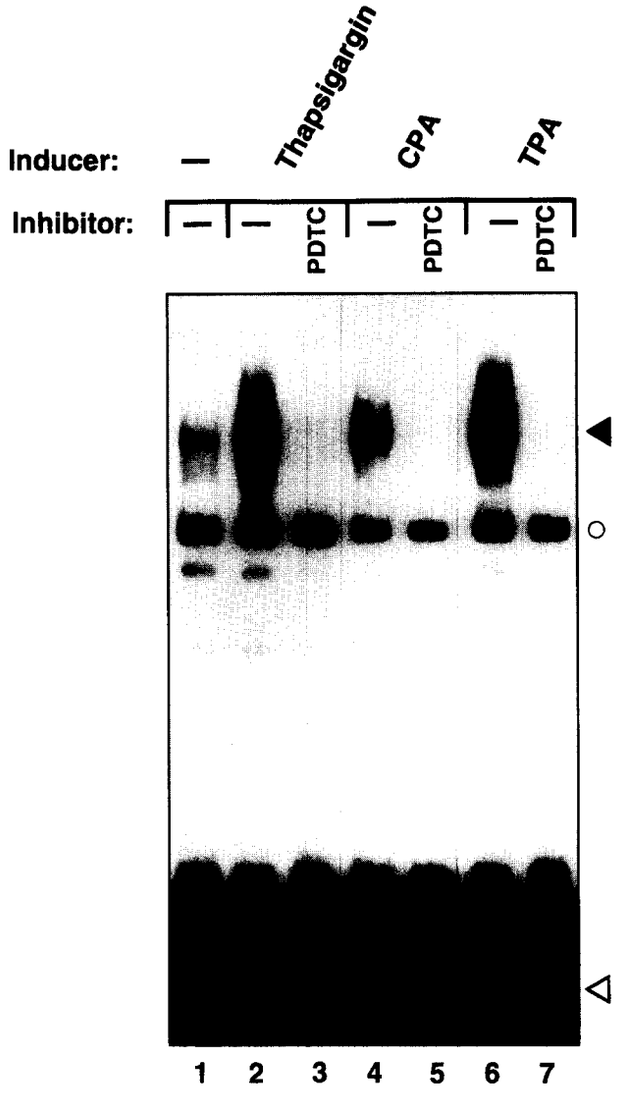

Fig. 6. NF-κB activation by inhibitors of the ER resident Ca^{2+}-ATPase is inhibited by the antioxidant PDTC. HeLa cells were pretreated for 1 h with 100 μM PDTC (lanes 3,5,7) or left untreated (lanes 1,2,4,6). Cells were then stimulated with 5 μM thapsigargin (lanes 2,3), 15 μM CPA (lanes 4,5) or 50 ng/ml TPA (lanes 6,7) for 1 h. Total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

3.7. Ca^{2+} release precedes ROI generation during thapsigargin and CPA treatment

So far, we have provided pharmacological evidence that ER stress-mediated NF-κB activation requires both Ca^{2+} release from the ER as well as the production of ROIs. However, it is not clear which of the two events comes first and whether one is a consequence of the other. We have addressed this question by investigating the effect of Ca^{2+} chelators, Ca^{2+}-ATPase inhibitors and antioxidants on the intracellular production of ROIs as determined by flow cytometry using the fluorescent dye DFCH [26]. HeLa cells were preincubated for 1 h with 30

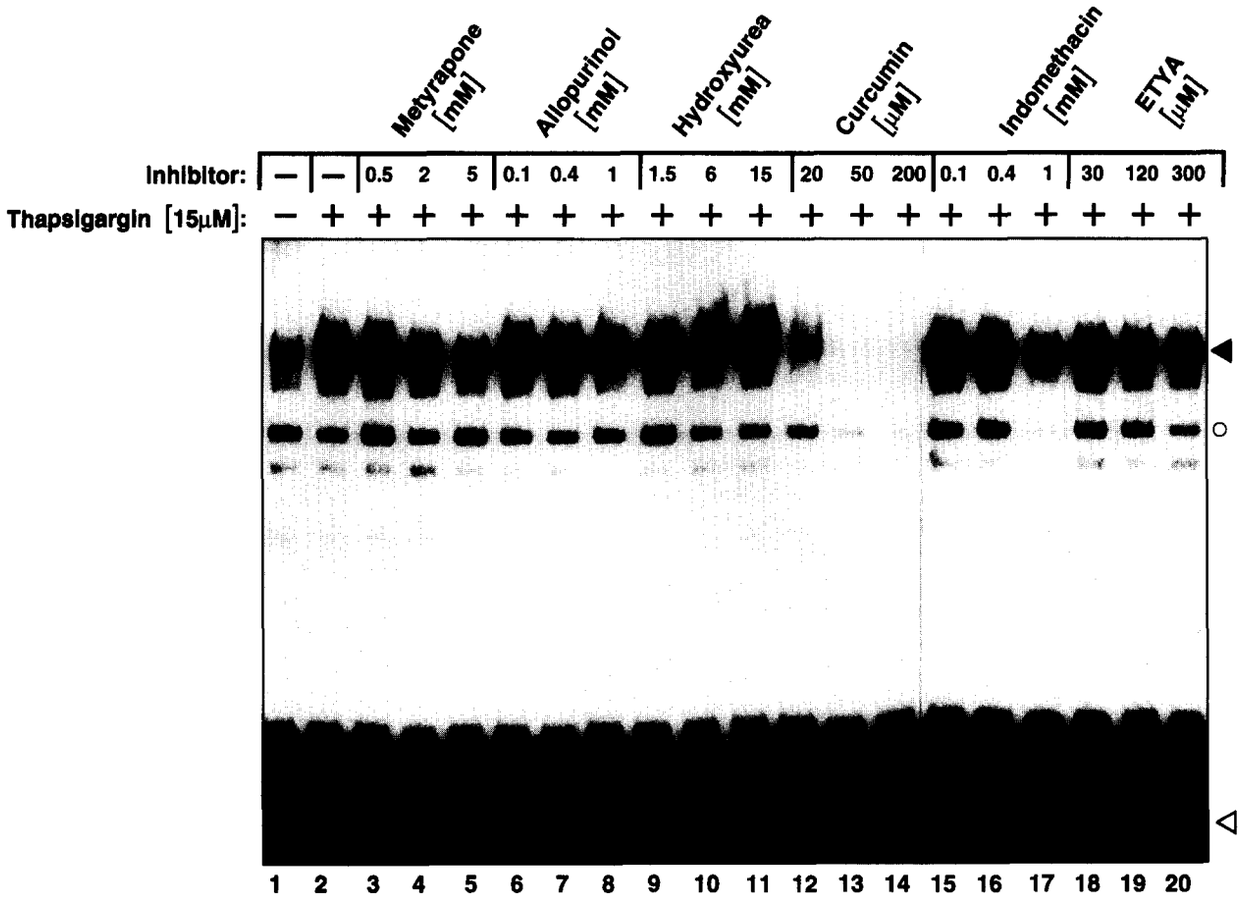

Fig. 7. Effect of various inhibitors of ROI producing enzymes on NF-κB activation by thapsigargin. HeLa cells were treated for 1 h with the p450 inhibitor metyrapone (lanes 3–5), the xanthine oxidase inhibitor allopurinol (lanes 6–8), the ribonucleotide reductase inhibitor hydroxyurea (lanes 9–11), curcumin, an inhibitor of the lipoxygenase pathway (lanes 12–14), the cyclooxygenase inhibitor indomethacin (lanes 15–17) or the lipoygenase and cyclooxygenase inhibitor ETYA, an arachidonate analogue (lanes 18–20), at the indicated concentrations. Afterwards, cells were stimulated for 1 h with 15 μM thapsigargin. Total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

μM of the Ca^{2+} chelator BAPTA-AM or 100 μM of the antioxidant PDTC. Cells were then stimulated with either 250 μM H_2O_2, 5 μM thapsigargin or 15 μM CPA for 1 h. Cells were then incubated with 5 μM DCFH for 10 min at 37°C and analyzed for ROI production by flow cytometry. As expected, treatment with H_2O_2 induces oxidative stress, which is reflected in a significant increase in mean fluorescence (Fig. 9). Thapsigargin and CPA treatment also increased the ROI production of HeLa cells showing that inhibition of Ca^{2+}-ATPase, i.e. Ca^{2+} efflux from the ER, causes oxidative stress. The antioxidant PDTC decreased intracellular ROI production in response to added H_2O_2, thapsigargin and CPA (Fig. 9). Interestingly, the Ca^{2+} chelator BAPTA-AM also reduced ROI content in response to all three stimuli (Fig. 9). In addition, both PDTC and BAPTA-AM lowered the basal level of ROIs in unstimulated HeLa cells. Of note, its chemical structure does not predict BAPTA-AM to have any antioxidative potential. These data show that the release of Ca^{2+} from the ER by inhibition of the ER-resident Ca^{2+}-ATPase leads to oxidative stress in HeLa cells and that this can be prevented by incubation with a Ca^{2+} chelator. Moreover, they suggest that Ca^{2+} release precedes the production of ROIs during ER stress.

4. Discussion

The disruption of ER function represents a severe stress to cells. This can occur during viral infection or the exposure to xenobiotics. We have previously shown that cells can respond to ER stress by inducing the transcription factor NF-κB [13]. Since active NF-κB translocates directly to the nucleus and induces gene transcription, this established a novel signal transduction pathway between the ER and the cell nucleus [13]. In the present study, we wished to determine the second messenger molecules which mediate the activation of NF-κB by ER stress. The observation that inhibition of the ER-resident Ca^{2+}-ATPase, which causes rapid release of Ca^{2+} from the organelle, potently activates NF-κB, suggested that Ca^{2+} release may mediate NF-κB activation by ER stress. We show here that two structurally unrelated Ca^{2+} chelators inhibit NF-κB activation in response to various ER stress inducing conditions. These include the inhibition of *N*-glycosylation (tunicamycin and 2-deoxyglucose), ER-Golgi fusion (brefeldin A), inhibition of the ER-resident Ca^{2+}-ATPase (thapsigargin and CPA) as well as the overexpression of four different proteins which are known to accumulate in the ER (Fig. 3). It therefore appears that the release of Ca^{2+} from the ER repre-

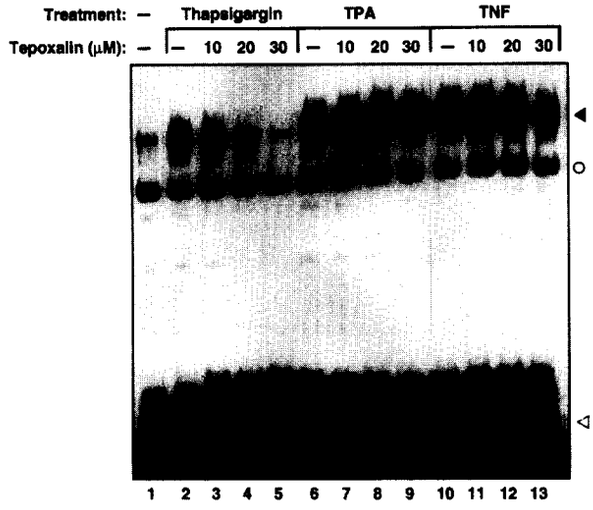

Fig. 8. The lipoxygenase and cyclooxygenase inhibitor tepoxalin prevents NF-κB activation by thapsigargin, but not by TPA or TNF. HeLa cells were preincubated for 1 h with various concentrations of tepoxalin as indicated. Subsequently, cells were stimulated with 15 μM thapsigargin (lanes 2–5), 50 ng/ml TPA (lanes 6–9) or 200 U/ml TNF (lanes 10–13) for 1 h. Total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes non-specific binding to the probe and the open arrowhead shows unbound oligonucleotide.

sents a general mechanism by which NF-κB is activated in response to ER stress.

With the exception of T lymphoid cells, Ca^{2+} has not been previously implicated as a messenger in NF-κB activation. In T cells, calcium ionophores are fairly weak activators of NF-κB on their own, however, they strongly synergize with phorbol esters [27]. In HeLa cells, calcium ionophores do not significantly induce NF-κB (data not shown). Activation of NF-κB by TNF is not affected by pretreatment of HeLa (Fig. 1B) or 293 cells (data not shown) with Ca^{2+} chelators. These data preclude that TMB-8 or BAPTA-AM are exhibiting a previously unrecognized antioxidative capacity. In addition, the structure of these Ca^{2+} chelators does not suggest that they possess antioxidative properties.

We show that, in addition to Ca^{2+}, the generation of ROIs is required to activate NF-κB in response to ER stress. Moreover, Ca^{2+} release from the ER causes oxidative stress in HeLa cells, which can be abrogated by pretreatment with a Ca^{2+} chelator. The observed decrease in steady state ROI levels in HeLa cells following treatment with BAPTA-AM (Fig. 9) suggests that the intracellular Ca^{2+} concentration modulates ROI homeostasis. An interrelationship between ROI levels and cellular Ca^{2+} concentrations has been previously reported in various systems. Treatment of several different cell types with H_2O_2 induces a considerable increase in cytosolic free calcium [28–30]. This is caused by the release of internal stores rather than by the influx of external Ca^{2+} because the effect is also seen in the absence of external Ca^{2+}. H_2O_2 was shown to access inositol triphosphate-sensitive Ca^{2+} stores because the oxidant no longer elicits Ca^{2+} release in cells which have been depleted of these stores by pretreatment with thapsigargin [29]. Interestingly, H_2O_2 and O_2^- generated by the hypoxanthine-xanthine oxidase system were shown to inhibit the ER-resident Ca^{2+}-ATPase in isolated ER membranes [31,32]. However, it remains to be determined whether H_2O_2 also exerts this effect in vivo. If this is the case, this may represent the mechanism by which ROIs mediate Ca^{2+} release.

Glucocorticoid-induced apoptosis of thymocytes involves both ROI production and a rise in intracellular Ca^{2+} [33]. Treatment of thymocytes with the antioxidant NAC prevented both the glucocorticoid-induced rise in intracellular Ca^{2+} and endonuclease activation. Interestingly, contrary to our observation in HeLa cells, in thymocytes pretreatment with BAPTA-AM failed to inhibit glucocorticoid-induced oxidative stress. However, the Ca^{2+} chelator quin 2 prevents H_2O_2-mediated DNA damage and cytotoxicity in thymocytes. Thus, many physiological processes require both Ca^{2+} and ROIs and each of the messengers can perpetuate the production or release of the other in a positive regulatory feedback. In order to elucidate the mechanism by which Ca^{2+} release leads to ROI production, the Ca^{2+} responsive ROI-generating enzymes need to be identified.

Here, we have tested a number of compounds that block known ROI-generating enzymes. While one inhibitor of cyclooxygenases and lipoxygenases, tepoxalin [34–36], inhibited NF-κB activation in response to thapsigargin, a second inhibitor of these enzymes, ETYA, had no effect. This may be explained by the different mechanism of action of the two inhibitors. Cyclooxygenase-1 (COX-1) is composed of a cyclase and a peroxidase activity. While the arachidonic acid analogue EYTA blocks substrate binding, tepoxalin was shown to inhibit the peroxidase activity of COX-1 [25,37]. It has been shown that tepoxalin does not act as an antioxidant, a possibility which must be considered for NF-κB inhibitors [38]. This is consistent with our observation that tepoxalin does not inhibit TPA- or TNF-induced NF-κB activation. In contrast, a previous study reported that tepoxalin inhibits TPA/ionomycin-mediated NF-κB stimulation in HeLa cells

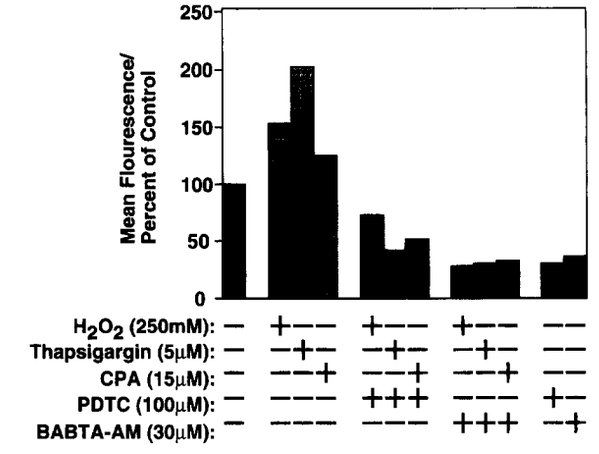

Fig. 9. Thapsigargin and CPA stimulation of HeLa cells causes oxidative stress, which is prevented by preincubation with the Ca^{2+} chelator BAPTA-AM. HeLa cells were pretreated for 1 h with either 100 μM PDTC or 30 μM BAPTA-AM as indicated. Cells were subsequently stimulated for 1 h with either 250 μM H_2O_2, 15 μM thapsigargin or 75 μM CPA. Cells were harvested in PBS as incubated with 5 μM of the flourescent dye DCFH for 10 min at 37°C. Cells were subjected to FACS analysis. Mean flourescence as a percent of untreated control cells is depicted. The experiment was performed four times with similar results. The average of two independent experiments is shown.

and slightly reduces TNF-mediated activation [38]. However, the effect on TNF-mediated induction was very small and non-specific binding was also decreased by tepoxalin treatment. We therefore submit that tepoxalin does not affect TNF-mediated NF-κB activation in HeLa cells. We also observed no effect of tepoxalin on TPA-mediated NF-κB stimulation. We cannot compare this result to the inhibition of TPA/ionomycin-induced activation reported by Kazmi et al. [38] since different stimuli and cells were used. However, it is interesting to speculate that tepoxalin may specifically interfere with Ca^{2+}-mediated NF-κB activation as induced by thapsigargin and ionophore treatment. The fact that Kazmi et al. did not observe any inhibition of PMA/ionophore mediated NF-κB stimulation by treatment with the cyclooxygenase inhibitor naproxen, the lipoxygenase inhibitor zileuton or a combination of both agents supports the idea that tepoxalin is selectively targeting the enzyme's peroxidase moiety.

The transcription factor dorsal, a NF-κB homologue, is responsible for the generation of dorso-ventral polarity in the fruitfly *Drosophila melanogaster*. Like NF-κB, dorsal is retained in an inactive cytoplasmic form by binding of an inhibitor protein, the IκB homologue cactus. It was recently shown that an increase of intracellular Ca^{2+} causes nuclear localization of transfected dorsal protein in Schneider cells [39]. It remains to be investigated whether Ca^{2+} also mediates this effect in the *Drosophila* embryo. However, it appears that Ca^{2+} may serve a more important role in NF-κB activation than previously recognized.

Acknowledgements: The authors thank Elmar Böhm for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ba-957/1-3) and the European Community Biotechnology Programme.

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