0026-895X/02/6101-177–185$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 61:177–185, 2002

Vol. 61, No. 1
1101/953850
Printed in U.S.A.

Evidence for Unique Calmodulin-Dependent Nuclear Factor-κB Regulation in WEHI-231 B Cells

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Received April 5, 2001; accepted October 5, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Immature B cells express constitutive nuclear factor-κB (NF-κB) activity and inhibition of this activity is associated with the induction of apoptotic cell death. Previous studies have implicated a calcium-dependent proteolysis of the NF-κB inhibitory protein IκBα as critical in the maintenance of constitutive NF-κB activity in these cells. We tested whether modulation of diverse calcium-dependent processes affects the maintenance of constitutive NF-κB activity in the WEHI-231 immature B cell line. Calmodulin inhibitors, but not calcineurin inhibition, blocked both IκBα turnover and the maintenance of constitutive NF-κB activity. Inhibition of NF-κB DNA binding activity by the calmodulin antagonist W13 also resulted in a loss of the expression of the NF-κB target gene, IκBα. However, prolonged inhibition of NF-κB activity for up to 8 h did not lead to apoptotic induction in the WEHI-231 cells. Moreover, removal of calmodulin inhibitors resulted in the reappearance of constitutive NF-κB activity and the renewed expression of the IκBα gene. Thus, calmodulin activity is a requirement for the continual turnover of IκBα and the maintenance of constitutive NF-κB function in WEHI-231 cells. In addition, our findings suggest that inhibition of NF-κB activity does not lead to the immediate onset of apoptosis, indicating that prolonged inhibition of NF-κB–dependent gene expression is required to cause apoptosis of WEHI-231 B cells.

Activation of the transcription factor NF-κB has been intensely studied in recent years and has received considerable attention as a paradigmatic signaling pathway. The versatility of the NF-κB transcription factors is underscored by the distinct types of NF-κB–activating agents as well as the great number of NF-κB–regulated genes whose products affect such broad cellular processes as apoptosis, immune response, inflammation, cell adhesion, and the cell cycle (Ghosh et al., 1998). Studies using genetic and biochemical approaches have identified several components of a signaling network that ultimately direct the degradation of NF-κB inhibitory proteins (IκBs), a prerequisite of NF-κB activation. In a majority of cell types NF-κB is kept inactive as a cytoplasmic complex with an IκB family member until an activating signal allows for the liberation and nuclear translocation of NF-κB. However, many instances in which NF-κB is constitutively activated have also been described. Such activation of NF-κB in the apparent absence of an inducing stimulus is generally attributable to either 1) the deregulation of one or more components in the signaling pathway, or 2) the normal developmental program of a cell. The deregulation of Rel/NF-κB or IκB protein activity has been implicated in aberrant cell growth, cell death, and oncogenesis (Luque and Gelinas, 1997). It is now known that many lymphoid malignancies as well as solid tumors display constitutively nuclear NF-κB activity, although the molecular mechanisms responsible for this activity are not altogether clear. Furthermore, as a part of their transformation process, the Epstein-Barr virus, the human T-cell leukemia virus, and hepatitis B virus constitutively activate NF-κB (Mosialos, 1997). Although the transforming abilities of NF-κB activity await further clarification, it is clear that activated NF-κB can have profound effects on a cell's tendency to undergo apoptosis (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996).

This work was supported by National Institutes of Health grant CA81065, a Howard Hughes Medical Institute fund through the University of Wisconsin Medical School, the Shaw Scientist Award from the Milwaukee Foundation (to S.M.), and a National Institutes of Health Predoctoral Training Grant T32-GM07215 (to S.D.S.).

ABBREVIATIONS: NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; Igκ, immunoglobulin κ light chain; TPCK, tosylphenylalanine chlormethylketone; PDTC, pyrrolidine dithiocarbamate; CsA, cyclosporin A; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; W12, *N*-(4-aminobutyl)-2-napthalenesulfonamide, HCl; W13, *N*-(4-aminobutyl)-5-chloro-2-napthalenesulfonamide, HCl; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction; AP-1, activator protein 1; CaM, calmodulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CI-I, calpain inhibitor I; LPS, lipopolysaccharide; TCR, T-cell receptor; CaN, calcineurin; PMA, phorbol 12-myristate 13-acetate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl.

Constitutive activity of NF-κB may also be established and maintained as a part of normal cellular development. Most notably, both B and T lymphocytes contain constitutively activated NF-κB at distinct stages during their development (Sen and Baltimore, 1986). In fact, NF-κB was first characterized based on its presence in the nucleus of several unstimulated B-cell lines (Sen and Baltimore, 1986). Within the context of B cells, several roles for constitutive NF-κB activity have been suggested, including the demethylation of chromatin surrounding the immunoglobulin κ light chain (Igκ) locus (Demengeot et al., 1995; Kirillov et al., 1996), the transcription and rearrangement of the Igκ gene (Scherer et al., 1996), the temporal regulation of the Oct-2 transcription factor (Bendall et al., 1997), and as a survival mechanism targeted for down-regulation leading to the apoptotic death of self-reactive immature B cells (Schauer et al., 1998).

Although many effects of constitutive NF-κB activity have been demonstrated in the B-lymphocyte life cycle, the underlying cause of this activity has not been established. We and others have shown previously that unlike most cases of inducible NF-κB activity, constitutive NF-κB levels in B cells are not affected by proteasome inhibitors (Phillips and Ghosh, 1997; Miyamoto et al., 1998). Conversely, free calcium (Ca^{2+}) chelating agents were shown to reduce the amount of DNA binding NF-κB in unstimulated B cells but could not inhibit the proteasome-dependent activation of NF-κB in either B or non-B cells (Miyamoto et al., 1998). Both upstream and downstream events associated with calcium regulation of NF-κB activity in B cells remain to be determined. In the present study, we have examined potential downstream components involved in proteasome-independent IκBα degradation and NF-κB activation in B cells. We found that calmodulin (CaM) inhibitors selectively reduce the active portion of NF-κB in B cells, concomitant with a block in IκBα protein turnover and a marked decrease in the message of a NF-κB–dependent target gene. However, the immunosuppressant drug cyclosporin A, which inhibits the Ca^{2+}/CaM-dependent phosphatase calcineurin, was unable to reduce NF-κB levels in unstimulated cells. Furthermore, inhibition of constitutive NF-κB activity through interference of CaM function in the B cells was reversible even after 6 h and did not result in a rapid onset of apoptosis. Our data suggest a unique role for Ca^{2+}/CaM activity in the maintenance of a constitutive pool of NF-κB in B cells and further suggest that antiapoptotic proteins whose synthesis is regulated by NF-κB may have relatively long half-lives in the context of B cells.

Materials and Methods

Cell Culture and Reagents. WEHI-231 and 70Z/3 cells were maintained in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 1250 U/ml penicillin G (Sigma Chemical, St. Louis, MO), 0.5 mg/ml streptomycin sulfate (Sigma Chemical), and 5×10^{-5} M β-mercaptoethanol in a 5% CO_2 humidified incubator. W231.Bcl-X_L cells were generated by retroviral infection of WEHI-231 cells with pLNLCA-flagBcl-X_L followed by selection in 1 mg/ml G418 (Invitrogen, Carlsbad, CA) and were subsequently maintained as described above in the added presence of 0.5 mg/ml G418. For splenocytes, whole spleens were isolated from C57BL/6 female mice approximately 70 days old. Individual cells were manually released, filtered, and red blood cells were removed by density-gradient centrifugation over LymphoPrep (Mediatech). The remaining cells were plated at 1 to 1.5×10^7/ml in the same media described above and allowed to recover for 2 to 3 h before treatment. Dimethyl sulfoxide, tosylphenylalanine chlormethylketone (TPCK), pyrrolidine dithiocarbamate (PDTC), cyclosporin A (CsA), and cycloheximide were obtained from Sigma Chemical. BAPTA-AM; calpain inhibitor I; *N*-(4-aminobutyl)-2-napthalenesulfonamide, HCl (W12); *N*-(4-aminobutyl)-5-chloro-2-napthalenesulfonamide, HCl (W13); and calmidazolium were from Calbiochem (San Diego, CA).

Electrophoretic Mobility Shift Assay. Cells were aliquoted in 12-well culture dishes at $\sim 2 \times 10^6$/ml and treated at 37°C, 5% CO_2 in a humidified incubator. After treatment the cells were pelleted, washed twice in ice-cold PBS, and stored at −70°C until further processing. Nuclear extract preparation and the conditions for EMSA have been described previously (Miyamoto et al., 1994b). Briefly, 2 to 3 μg of nuclear extract or 4 to 6 μg of whole cell extract was incubated on ice with 0.5 μg of poly dI-dC before addition of ^{32}P-labeled double-stranded oligonucleotide containing either the κB binding site from the Igκ intronic enhancer (5′-CTCAACAGAGGGGACTTTCCGAGAGGCCAT-3′), the NF-Y binding site (5′-TTTTCTGATTGGTTCTGGCGAGTTTGG-3′), or the AP-1 binding site (Promega, Madison, WI). Oligonucleotides used for competition assays were either the wild-type κB sequence or a mutated κB binding site (5′-TCAACAGAGCTCACTTTATGAGAGGCC-3′). The c-Rel antibody 5075 used for supershift analysis has been described previously (Inoue et al., 1992), and others are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): p65 (sc-372-G), RelB (sc-226), p50 (sc-114-G), and p52 (sc-297-G). Reactions were separated in a 4% native acrylamide gel, and the gels were dried and exposed to X-ray film.

Apoptosis Assays. For flow cytometric analyses, $\sim 10^6$ cells were first washed in PBS containing 1 mM EDTA and 0.1% BSA, resuspended in 100 μl of the PBS/EDTA/BSA solution plus 900 μl of chilled ethanol, and fixed overnight at 4°C. Cells were washed in phosphate citric acid buffer before being incubated overnight in PBS/EDTA/BSA containing 0.2 mg/ml RNase A and 50 μg/ml propidium iodide (Molecular Probes, Eugene, OR). Samples were processed on FACStar. DNA laddering assays were performed as described previously (Smith et al., 1989).

Western and Northern Blotting. Conditions for processing and probing Western blots were as described previously (Miyamoto et al., 1998). Rabbit anti-IκBα antibody (C21) was purchased from Santa Cruz Biotechnology, Inc., monoclonal anti-flag antibody was from Kodak IBI (New Haven, CT), and anti-α-tubulin antibody was purchased from Calbiochem. For Northern blots, total cellular RNA was prepared from $\sim 10^7$ cells/sample according to manufacturer's instructions for RNeasy (QIAGEN, Valencia, CA). From each sample 15 μg of total RNA was separated in a 1% formaldehyde-agarose gel, transferred to a GeneScreen nylon membrane (PerkinElmer Life Sciences, Boston, MA), and cross-linked with UV irradiation. DNA probes were labeled with [^{32}P]dCTP by random priming of cDNA fragments spanning IκBα (*Apa*I-Eco72I) or GAPDH (*Hin*dIII-*Pst*I).

Semiquantitative Reverse Transcription PCR. Total cellular RNA was prepared from treated or untreated cells as described above for Northern blotting. RT-PCR reactions for each treatment condition were performed with 5, 25, and 125 pg of total RNA with the Access RT-PCR kit according to instructions (Promega) and PCR products were fractionated on 1.5% agarose gels. The primer sequences for PCR amplification of Bcl-2, A1, and β-actin have been described previously (Tomayko and Cancro, 1998). Those used for PCR amplification of IκBα were sense, 5′-CCGCAGGAGGCGCCGCTG-3′, and antisense, 5′-GGTATTTCCTCGAAAGTCTCG-3′, and generated a product of 285 base pairs.

Results

Inhibitors of NF-κB Cause a Rapid Onset of Apoptotic Cell Death. The serine protease inhibitor TPCK as well as the antioxidant PDTC have previously been shown to

abrogate the NF-κB activity constitutively present in WEHI-231 cells (Miyamoto et al., 1994a; Wu et al., 1996). We have shown that an intracellular Ca^{2+} chelating agent, BAPTA-AM, is also able to effectively inhibit the DNA binding activity of NF-κB in these cells by stabilizing the IκBα inhibitor protein (Miyamoto et al., 1998). Because in the WEHI-231 cell line a loss of constitutive NF-κB activity is reported to lead to the rapid onset of apoptosis (Wu et al., 1996), we were interested in determining whether BAPTA-AM could induce apoptosis in these cells as is the case for TPCK and PDTC. The Bcl-2 family member Bcl-X_L protects WEHI-231 cells from apoptosis initiated by TPCK, PDTC, and a number of other apoptotic agents (Fang et al., 1995). Therefore, we introduced epitope-tagged Bcl-X_L into WEHI-231 cells and screened numerous clones for the stable expression of the exogenous Bcl-X_L protein to be included as a control. We selected clone 1.4 (Fig. 1A, lane 5) for our experiments and will refer to this line as W231.Bcl-X_L. To ensure that stable expression of the Bcl-X_L protein does not alter the specificity or composition of constitutive NF-κB in these cells, we used nuclear extract from W231.Bcl-X_L cells to perform the competition and supershift experiments shown in Fig. 1B. Consistent with the parental WEHI-231 cells, we were unable to detect p65, RelB, or p52. Rather, as in the WEHI-231 cell line, the upper, diffuse band distinguished by EMSA consists primarily of p50/c-Rel heterodimers as well as c-Rel homodimers, whereas the lower, sharper band is composed of p50 homodimers (Miyamoto et al., 1994b).

Inhibition of NF-κB activity in either the WEHI-231 or the W231.Bcl-X_L cell line was determined by gel shift analysis and is compared in Fig. 2A. Doses of TPCK and BAPTA-AM were chosen that strongly inhibit the DNA binding of the p50/c-Rel heterodimeric complex yet have little or no effect on the p50 homodimeric complex. Treatment of WEHI-231 or W231.Bcl-X_L cells with TPCK, PDTC, or BAPTA-AM strongly inhibited NF-κB activity irrespective of exogenous Bcl-X_L expression (Fig. 2A, compare lanes 3 and 10, 4 and 11, and 5 and 12). Incubation with a high concentration of the proteasome inhibitor CI-I is ineffective at blocking constitutive p50/c-Rel activity (Fig. 2A, lanes 6 and 13), which is consistent with previous observations (Miyamoto et al.,

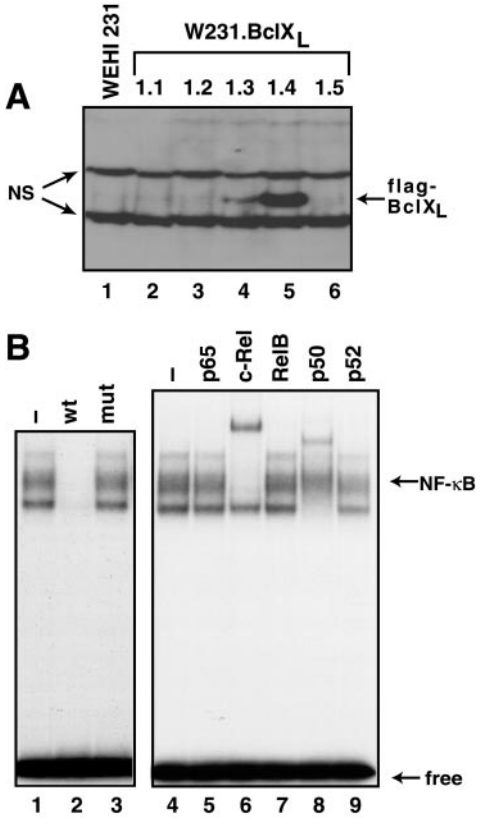

Fig. 1. Stable expression of flag-Bcl-X_L does not alter NF-κB activity in WEHI-231 cells. A, Western blot analysis of parental WEHI-231 (lane 1) and individual WEHI-231 clones stably expressing flag-Bcl-X_L (lanes 2–6) probed with anti-flag antibody. NS, nonspecific protein. B, nuclear extract from untreated W231. Bcl-X_L cells were used for gel shift analysis in the absence of competing oligonucleotide (lane 1) or in the presence of a 10-fold molar excess wild-type or mutant unlabeled κB oligonucleotide (lanes 2 and 3, respectively). In lanes 5 to 9, 200 ng of each of the indicated antibodies was added to the EMSA binding reactions. No antibody was added to lane 4.

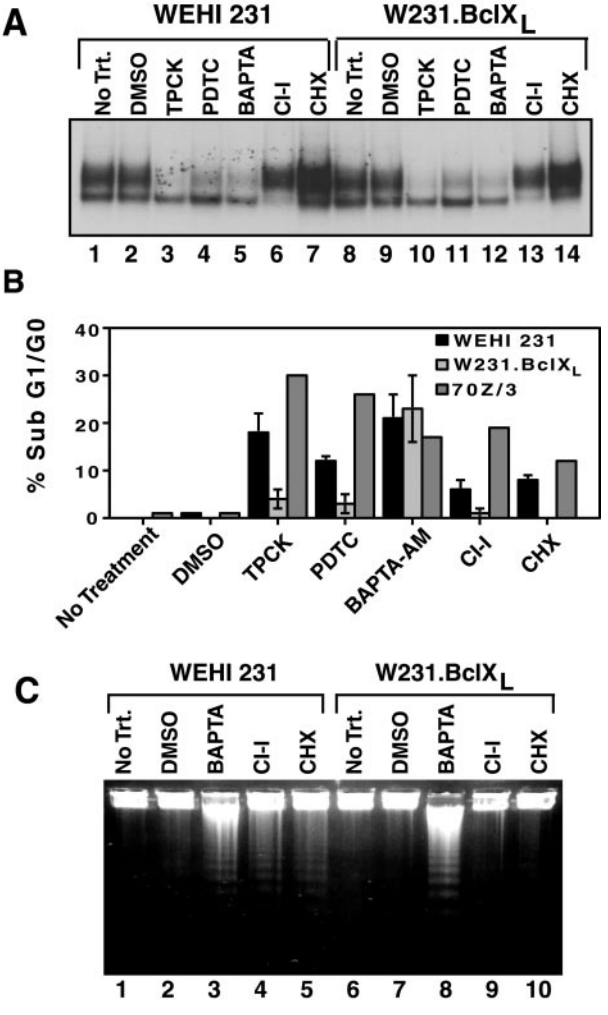

Fig. 2. Ca^{2+} chelator BAPTA-AM induces apoptosis independent of NF-κB inhibition or Bcl-X_L expression. A, parental WEHI-231 cells or W231.Bcl-X_L were either left untreated or treated with solvent at 0.1%, TPCK at 25 μM, PDTC at 25 μM, BAPTA-AM at 30 μM, CI-I at 50 μM, or 20 μg/ml cycloheximide for 4 h. A gel shift assay for κB-binding activity was performed using equal amounts of nuclear protein extract from each of the indicated treatment conditions. B, WEHI-231, W231. Bcl-X_L, or 70Z/3 cells were treated as outlined in B and assayed for sub-G_1/G_0 DNA content as described under *Materials and Methods*. The cells with sub-G_1/G_0 DNA content are represented as a percentage of the total cells assayed over one to three independent experiments. C, WEHI-231 or W231.Bcl-X_L cells were treated as described in B and DNA fragmentation was determined by agarose gel electrophoresis.

1998). The high concentration of CI-I used in this experiment resulted in a marked reduction of p50 homodimers, perhaps owing to the proteasome-dependent synthesis of the p50 molecule (Palombella et al., 1994). The prosurvival effects of NF-κB that have been characterized thus far seem to be mediated by the up-regulated expression of antiapoptotic gene transcripts. Therefore, as an additional control we used cycloheximide to intervene downstream of transcription by blocking protein synthesis in an attempt to mimic the effects of NF-κB withdrawal. It has been shown that inhibition of protein synthesis does in fact accelerate programmed cell death in both WEHI-231 cells and normal B cells (Illera et al., 1993; Quintans et al., 1994). When cycloheximide was added to the media an increase in NF-κB activity was observed (Fig. 2 A, lanes 7 and 14). This is consistent with the reported shorter half-life of the IκBα and IκBβ proteins relative to the NF-κB subunits, c-Rel, and p50 (Miyamoto et al., 1994b), which over time could lead to a larger pool of free NF-κB proteins.

Unstimulated 70Z/3 pre-B cells, in contrast to the WEHI-231 immature B cells, do not exhibit detectable amounts of activated NF-κB and are not dependent on constitutive NF-κB activity for their survival (Sen and Baltimore, 1986). Therefore, the 70Z/3 cell line was included in our apoptosis studies as a control for NF-κB–independent effects of the inhibitors. Figure 2B shows that, as expected, an incubation of only 4 h with TPCK or PDTC is sufficient to cause the appearance of cell death phenotypes in WEHI-231 cells and that this effect was largely blocked in the W231.Bcl-X_L cells (Wu et al., 1996). Treatment with BAPTA-AM also resulted in cell death of the WEHI-231 cells. However, in the case of BAPTA-AM, exogenous Bcl-X_L expression could not rescue the WEHI-231 cells from this fate. Strikingly, the 70Z/3 pre-B cell line, in which NF-κB activity is undetectable (Sen and Baltimore, 1986), was sensitive to cell death induced by TPCK, PDTC, and BAPTA-AM. To confirm apoptosis as the cause of death after BAPTA-AM treatment, a DNA laddering assay was performed in the WEHI-231 and W231.Bcl-X_L cells and is shown in Fig. 2C. Consistent with the flow cytometric analysis, BAPTA-AM treatment caused the nucleosomal fragmentation of genomic DNA in both WEHI-231 parental as well as W231.Bcl-X_L cells, whereas TPCK, PDTC, CI-I, and cycloheximide caused laddering only in the parental cells (Fig. 2C; data not shown). In addition, marked DNA laddering was also observed in 70Z/3 cells treated with TPCK, PDTC, BAPTA-AM, CI-I, and cycloheximide (data not shown). We conclude that BAPTA-AM treatment results in the apoptotic death of WEHI-231 cells and that this effect is not directly due to inhibition of NF-κB. Furthermore, because TPCK and PDTC also cause rapid apoptosis of 70Z/3 cells, these agents exert toxic effects that are independent of NF-κB inhibition yet sensitive to a Bcl-X_L protective pathway.

Inhibition of CaM Activity Results in a Decline of Constitutive NF-κB Activity. The observation that BAPTA-AM is a potent inducer of apoptosis in both the W231.Bcl-X_L and the 70Z/3 cell lines raises the possibility that constitutive NF-κB activity is not inhibited by calcium chelation per se, but rather as a result of cell death incurred by calcium chelation. Yet, CI-I and cycloheximide induce apoptosis in WEHI-231 cells, and under these conditions, NF-κB activity is either unchanged or increased, respectively, indicating that reduction of NF-κB activity after BAPTA-AM treatment in WEHI-231 cells is not likely to be a consequence of cell death processes. Therefore, to identify a potential downstream effector(s) of calcium in the regulation of constitutive NF-κB activity, we treated WEHI-231 cells with diverse inhibitors and the DNA binding of NF-κB was determined by EMSA (data not shown). Among those agents tested, treatment with CaM antagonists consistently led to a reduction in the constitutive activity of NF-κB in both the WEHI-231 and W231.Bcl-X_L cell lines.

To demonstrate the antagonistic action of the CaM inhibitor W13 toward CaM, a closely related but less potent inhibitor was used. The compound W12 is the dechlorinated homolog of W13 and this singular difference between the two compounds accounts for a 5-fold reduced binding affinity for CaM. Because of this, W12 is required at approximately 5-fold higher concentrations, both in vivo and in vitro, than is W13 to achieve comparable inhibition of CaM activity (Chafouleas et al., 1982). We therefore carried out a dose response in W231.Bcl-X_L cells of W13 in comparison with W12 over 3 h. W13 is approximately 5-fold more potent an inhibitor of NF-κB than is W12 (Fig. 3A, top). Protein from the same sample extracts was incubated with probe containing the AP-1 binding site to validate the integrity of the extracts (Fig. 3A, bottom). The specificity of the AP-1 binding complex was determined by competition EMSA with unlabeled AP-1 oligonucleotide (data not shown). To ensure that W13 does not directly interfere with the capacity of NF-κB to bind DNA, we added up to a 10-fold molar excess of W13 to the in vitro DNA binding reaction mixture and noted no difference in the measured activity of NF-κB (Fig. 3B, lanes 2–4). The structurally related compound W7 is also able to inhibit the DNA binding activity of NF-κB in these cells as well as the structurally unrelated CaM inhibitor calmidazolium in a dose-dependent manner (data not shown). Time course experiments demonstrated that the ability of W13 to inhibit NF-κB DNA binding activity waned by 7 h of treatment (Fig. 3D). To determine whether this observed loss of effectiveness was due to the metabolism of W13 we tested whether W13 could inhibit NF-κB activity after a 6-h incubation period in complete media. A comparison of the detectable NF-κB activity between lanes 3 and 4 of Fig. 3E indicates that the potency of W13 in inhibiting NF-κB is reduced after the prolonged exposure of W13 to complete cell media.

Pooled lymphocytes extracted from spleens exhibit constitutive NF-κB activity that is present almost entirely due to the B-cell population of splenocytes (Feuillard et al., 2000; Fields et al., 2000). To determine whether the W13-dependent inhibition of NF-κB observed in W231.Bcl-X_L cells is representative of primary B cells, splenocytes were harvested and treated with W13, and NF-κB activity was assessed by EMSA. As with the W231.Bcl-X_L cells, NF-κB activity in freshly isolated spleen cells was reduced in a dose-dependent manner (Fig. 3C, top, lanes 2 and 3), whereas unrelated transcription factor activity was not affected (Fig. 3C, bottom, lanes 2 and 3; others not shown). Therefore, perturbation of CaM activity in W231.Bcl-X_L cells as well as primary splenocytes disrupts the constitutive DNA binding activity of NF-κB.

CaM Inhibition by W13 Does Not Lead to Rapid Cell Death in WEHI-231 Cells. Because W13 treatment of unstimulated W231.Bcl-X_L cells effectively inhibits the consti-

tutive NF-κB DNA binding activity, we were next interested in examining whether W13 treatment also results in a rapid induction of apoptosis as is the case for TPCK, PDTC, and BAPTA-AM. To this end, we incubated 70Z/3, WEHI-231, and W231.Bcl-X_L cells in media containing W13 or BAPTA-AM for up to 6 h and assayed for the fragmentation of genomic DNA as an indication of apoptosis. As before, BAPTA-AM treatment led to a rapid onset of apoptosis in WEHI-231 and W231.Bcl-X_L cells (Fig. 4, lanes 6 and 11, respectively). Surprisingly, however, inhibition of CaM activity for up to 6 h by W13 was not able to induce apoptosis in any one of the cell lines tested (Fig. 4, lanes 2, 3, 7, 8, 12, and 13). Therefore, because interfering with CaM function results in a marked decrease in NF-κB activity but not apoptosis, we conclude that CaM inhibitors are comparably effective yet overall less toxic than other pharmacological compounds used to inhibit constitutive NF-κB activity in WEHI-231 cells. These studies also demonstrate that extensive inhibition of constitutive NF-κB activity does not lead to the rapid onset of apoptosis. Additionally, these data suggest that antiapopototic gene products synthesized in a NF-κB–dependent manner may have relatively long half-lives in WEHI-231 cells.

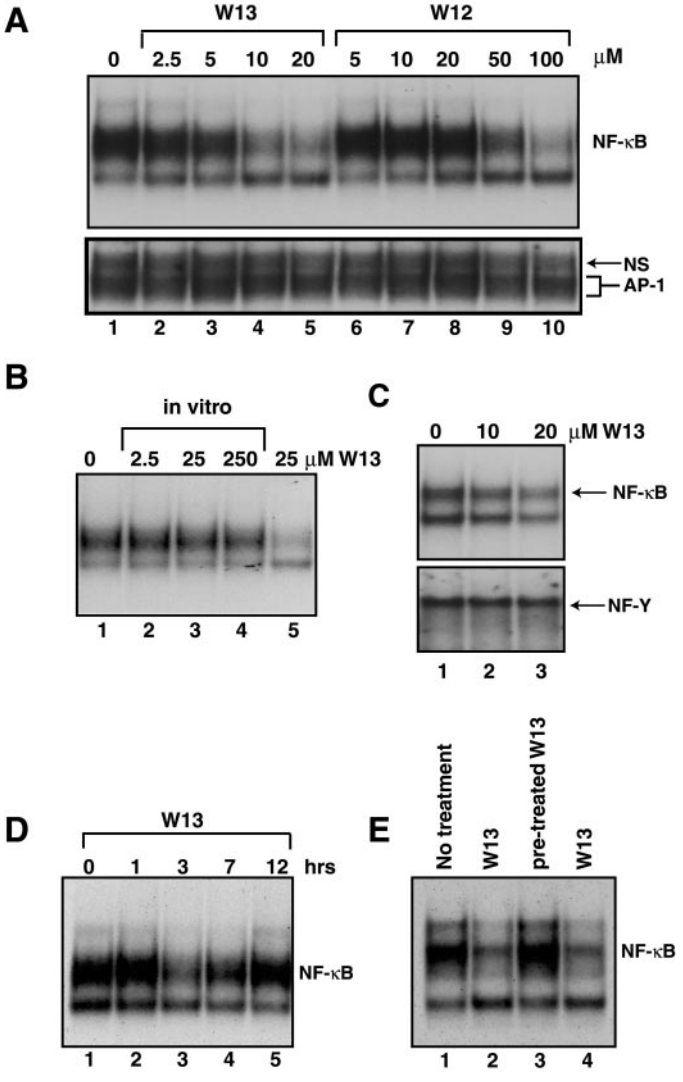

Fig. 3. W13 acts as a CaM antagonist to inhibit NF-κB activity. A, W231.Bcl-X_L cells were either left untreated or were treated with increasing concentrations of W13 or W12 for 3 h before termination. Nuclear extracts were prepared, and equal amounts from each sample were used for EMSA of both κB and AP-1 DNA binding activity. The diffuse band representing NF-κB (p50/c-Rel) is indicated in the top and that representing AP-1 in the bottom. The specificity of the AP-1 band was determined by competition EMSA with unlabeled AP-1 oligonucleotide (data not shown). B, W13 was added at the indicated amounts to samples from the same W231.Bcl-X_L cell nuclear extract preparation (lanes 1–4). The reactions were incubated at room temperature for 20 min before addition of radiolabeled κB probe and subsequent gel shift analysis. C, freshly extracted splenocytes were cultured in the presence of 0, 10, or 20 μM W13 for a period of 3 h. Total cell extracts were prepared from treated cells and examined by EMSA for DNA binding activity using a κB oligonucleotide (top) or NF-Y oligonucleotide (bottom). D, W231.Bcl-X_L cells were treated for the indicated times in the presence of 20 μM W13 and total cell extracts were analyzed by EMSA for NF-κB DNA binding activity. E, W13 was incubated either with W231.Bcl-X_L cells suspended in complete media or with complete media alone. After 6 h the cells were terminated (lane 2) and previously untreated W231.Bcl-X_L cells were resuspended with either fresh W13 (lane 4) or with the preincubated W13-containing media (lane 3) and terminated after 3 h. Total cell extracts were analyzed by EMSA.

Calmodulin Inhibition Reversibly Blocks NF-κB Target Gene Expression. Although our EMSA data indicate that CaM inhibition leads to a reduction of NF-κB DNA binding activity, it is unclear whether NF-κB function is equally inhibited. The gene encoding IκBα is among the target loci whose transcriptional activity is highly increased after NF-κB activation. Several κB sites have been identified within the promoter region of the IκBα gene, which provide a direct linkage between NF-κB activity and IκBα mRNA synthesis (Bail et al., 1993; Martin et al., 1993; Chiao et al., 1994). It follows then that one consequence of constitutive NF-κB activity in B cells is a relatively large amount of IκBα transcript (Miyamoto et al., 1994a). The half-life of IκBα mRNA is very short, approximately 15 to 20 min, which enables us to examine the quantity of IκBα message as an indication of the NF-κB transcriptional activity within the cell. When WEHI-231 cells were treated for increasing times with W13 the total amount of IκBα transcript dropped (Fig. 5A, middle, lanes 2–4) concomitantly with the decrease in the DNA binding capacity of NF-κB (Fig. 5, top, lanes 2–4). Removing W13 from the culture medium after 6 h allowed the WEHI-231 cells to completely recover constitutive NF-κB

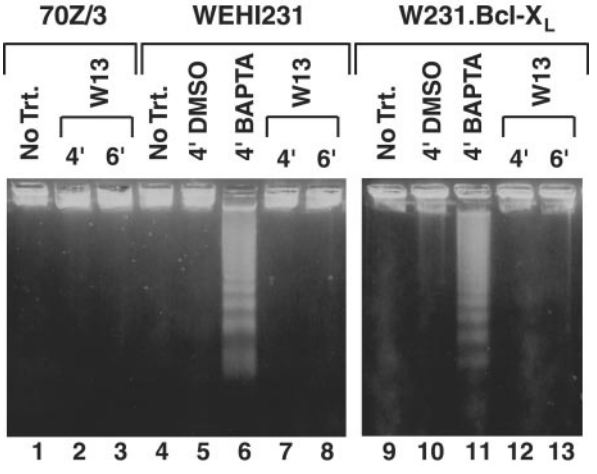

Fig. 4. W13 treatment does not induce apoptosis. WEHI-231, W231.Bcl-X_L, or 70Z/3 cells were left untreated for 6 h or, where indicated, were treated with 20 μM W13, 30 μM BAPTA-AM, or 0.1% dimethyl sulfoxide for either 4 or 6 h. After treatment the cells were assayed for nucleosomal DNA fragmentation by agarose gel electrophoresis.

activity (Fig. 5, top, lane 5). Importantly, IκBα mRNA was resynthesized to a total amount resembling the initial amount of transcript (Fig. 5A, middle, lane 5). RNA loading was relatively constant as indicated by the GAPDH signal (Fig. 5A, bottom).

To determine whether W13 treatment results in a general repression of transcription, more particularly at the IκBα locus, we treated the 70Z/3 pre-B cell line with LPS for 18 h followed by the addition of W13 to the culture medium. The cells were then processed to be assayed by EMSA for NF-κB activity and by Northern blot for IκBα transcript amounts as described above. The measured half-life of the IκBα transcript under these conditions was ~24 min (data not shown), which indicates that the IκBα locus must be continually transcribed to maintain constant levels of IκBα mRNA. Clearly, LPS alone caused an appreciable activation of NF-κB activity (Fig. 5B, top, lane 2) as well as a substantial increase in the amount of IκBα mRNA (Fig. 5B, middle, lane 2). Importantly, treatment of the stimulated 70Z/3 cells with W13 for up to 6 h was unable to cause a decline in the measurable amount of IκBα mRNA (Fig. 5B, middle, lanes 3 and 4), which is consistent with the inability of W13 to inhibit NF-κB activity under these conditions (Fig. 5B, top, lanes 3 and 4). These studies demonstrate that CaM activity is selectively necessary for the maintenance of constitutive NF-κB activity in B cells, which is under the control of a calcium-dependent process.

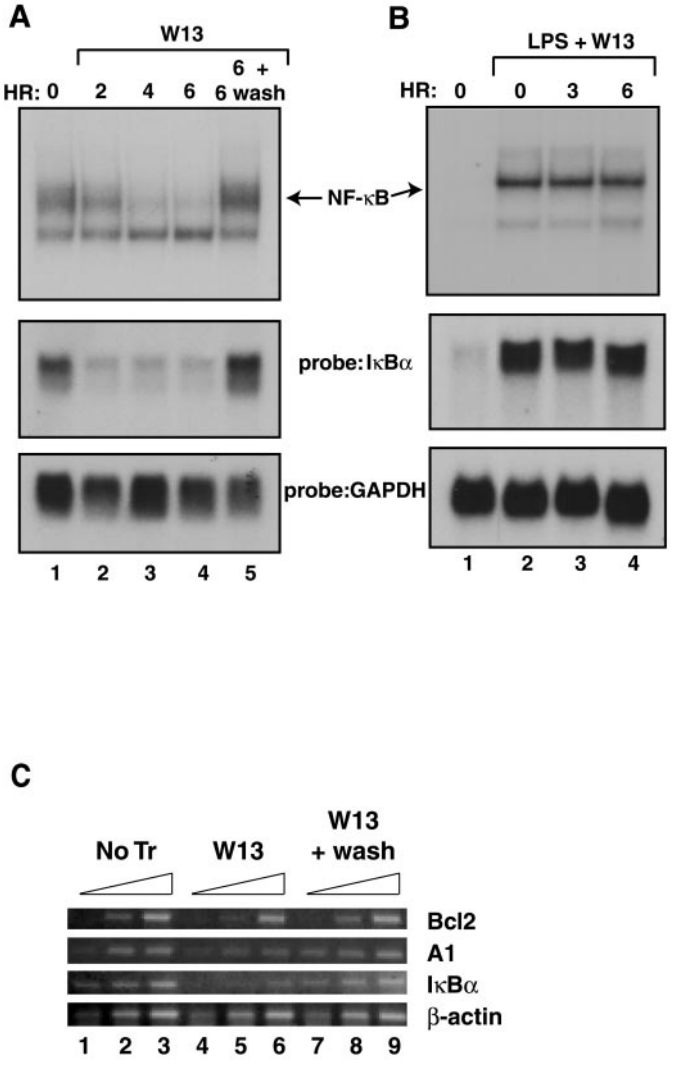

Fig. 5. W13 treatment causes a reversible decline in the IκBα gene transcript. A, W231.Bcl-X_L cells were either left untreated or were incubated with 20 μM W13 for 2, 4, or 6 h before being terminated. One sample was washed after a 6-h treatment with W13 and allowed to recover in complete media for an additional 6 h (lane 5). Top, EMSA prepared from whole cell extracts. From the same samples total RNA was isolated and 10 μg was used for Northern blot analysis in the bottom two for the determination of IκBα and GAPDH transcript levels. B, 70Z/3 pre-B cells were either left untreated or were stimulated with 10 μg/ml LPS for 18 h and then further treated with 20 μM W13 for 3 or 6 h. As in A, the top represents an EMSA prepared from whole cell extracts, and in the bottom two 10 μg of total RNA was probed for IκBα or GAPDH as indicated. C, total RNA was isolated from WEHI-231 cells that were untreated (lanes 1–3), incubated for 6 h with W13 (lanes 4–6), or washed and recovered for 6 h after a 6-h W13 treatment (lanes 7–9). The amount of input RNA per RT-PCR reaction was increased over 25-fold for each sample (1×, 5×, 25×).

The selectivity of W13 treatment allowed us to address which, if any, of the putative NF-κB–responsive antiapoptotic genes may exhibit disrupted regulation upon inhibition of NF-κB. To test for this, semiquantitative RT-PCR was performed using RNA isolated from untreated and W13-treated WEHI-231 cells (Fig. 5C). As expected, the level of total IκBα transcript dropped dramatically as a result of W13 treatment as well as that of the A1 antiapoptotic gene. The total amount of Bcl-2 changed only slightly and expression of neither Bcl-X_L nor the cellular inhibitor of apoptosis proteins was detectable under these conditions (data not shown).

Rapid Turnover of IκBα in WEHI-231 Cells Requires CaM Activity. Because the degradation of IκBα almost invariably precedes the activation of NF-κB, including the constitutively active NF-κB in WEHI-231 cells (Miyamoto et al., 1994a), we wanted next to determine the effect that W13 treatment has on the stability of the IκBα protein. Previously, others and ourselves have demonstrated that IκBα is unusually short-lived in WEHI-231 cells relative to non-B and other cell types (Rice and Ernst, 1993; Miyamoto et al., 1998; Doerre and Corley, 1999). The half-life of IκBα was tested by blocking protein synthesis with cycloheximide and terminating cells at successive time points. Both IκBα and α-tubulin as a loading control were probed against in the same blot. In the absence of W13 and consistent with previous studies, the IκBα protein is rapidly degraded in the WEHI-231 cells with an approximate half-life of under 1 h (Fig. 6A, lanes 1–4). However, the addition of W13 completely blocked the degradation of IκBα even up to 3 h after treatment (Fig. 6A, lanes 5–7). When an equal concentration of W12 was added in addition to cycloheximide over 3 h, a very weak effect on the turnover of IκBα was observed (Fig. 6A, compare lanes 4 and 8), consistent with its relatively weak CaM inhibitory activity. The basal turnover of IκBα in the 70Z/3 pre-B cell line was not affected by W13 treatment (data not shown). Moreover, signal-dependent degradation of IκBα is also not affected with W13 or W12 treatment (Fig. 6B, compare lane 2 with lanes 4 and 6). Therefore, CaM activity is critical for the accelerated degradation of IκBα and the maintenance of constitutive NF-κB function in WEHI-231 cells.

Constitutive NF-κB Activity Requires Calmodulin but Not Calcineurin Activity. A dependence on Ca^{2+} for NF-κB activation been demonstrated for various inducers of NF-κB, most notably the T-cell receptor (TCR). In this case an intracellular Ca^{2+} increase after stimulation is a requisite

for activating the Ca^{2+}/CaM-dependent phosphatase calcineurin (CaN), which in turn is necessary for activating NF-κB (Mattila et al., 1990; Frantz et al., 1994; Steffan et al., 1995). Because we have shown a Ca^{2+}/CaM dependence of constitutive NF-κB activity in a B-cell line, we were interested in assessing whether this activity was also CaN-mediated as is the case in T cells after TCR stimulation. Signaling through the TCR can be simulated through the synergistic effect of Ca^{2+} ionophore and phorbol ester added simultaneously. Treatment of the W231.Bcl-X_L cell line with phorbol-12-myristate-13-acetate (PMA) was able to activate NF-κB (Fig. 7A). Surprisingly, however, addition of the Ca^{2+} ionophore inonomycin at concentrations ranging from 0.01 to 10 μg/ml was unable to further activate NF-κB (data not shown). Nevertheless, CaN seems to be required for the induction of NF-κB by PMA because this activation was sensitive to both W13 and the CaN inhibitor CsA. And as expected, these agents had no effect on the induction of NF-κB after LPS treatment of W231.Bcl-X_L cells. To determine, then, whether CaN plays a role in the constitutive maintenance of NF-κB we incubated cells with either W13 or CsA and measured NF-κB activity over 3 h. Although W13 was effective, we were unable to detect a reduction of NF-κB DNA binding activity in cells treated with CsA (Fig. 7B). We conclude from these studies that a CaN requiring event is unlikely to be the Ca^{2+}/CaM-dependent step for maintaining constitutively active NF-κB.

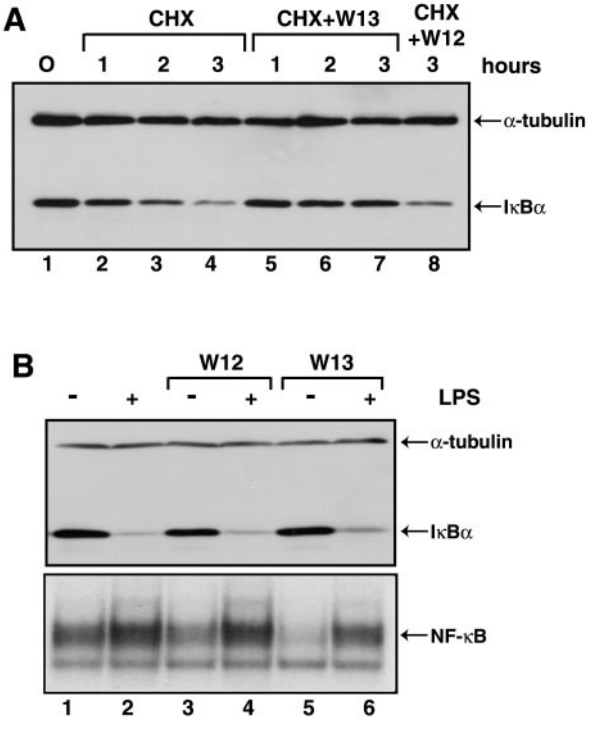

Fig. 6. W13 prevents the constitutive but not the inducible degradation of IκBα in W231.Bcl-X_L cells. A, W231.Bcl-X_L cells were incubated with 25 μg/ml cycloheximide (CHX) alone (lanes 1–4), or in addition to either W13 (lanes 5–7) or W12 (lane 8), each at 20 μM concentration. The cells were incubated for approximately 15 min to allow for drug action before terminating the reference sample (lane 1). Other samples were terminated at the indicated times. Whole cell extract (20 μg) was used for Western blotting against the IκBα and α-tubulin proteins. B, W231.Bcl-X_L cells were left untreated or were incubated over a period of 3 h with 20 μM W12 or 20 μM W13. After this pretreatment the cells were either left unstimulated or were stimulated with 20 μg/ml LPS for 20 min. Top, Western blot performed on 20 μg of total protein and probed for IκBα and α-tubulin. Bottom, EMSA demonstrating κB DNA binding activity that was performed on the same whole cell extracts described for the top. The p50/c-Rel homodimeric complex is indicated as NF-κB.

Discussion

We have identified previously a novel proteolytic inactivation of the NF-κB inhibitory protein IκBα. Whereas IκBα is typically degraded by the ubiquitin-dependent 26S proteasome, in WEHI-231 cells the ongoing turnover of IκBα is not blocked by excess concentrations of proteasome inhibitors. However, these inhibitors effectively block the LPS-induced degradation of IκBα in these cells. Conversely, the intra- and extracellular Ca^{2+} chelators BAPTA-AM and EGTA, respectively, are able to block the constitutive turnover of IκBα but not that of the LPS-induced turnover of IκBα (Miyamoto et al., 1998; Fields et al., 2000). We further provided evidence that this calcium-dependent degradation of IκBα is preceded by a proteasome-dependent mechanism during differentiation of B cells in vitro (Fields et al., 2000). However, both upstream and downstream components required for rapid IκBα degradation and the maintenance of constitutive NF-κB activity in B cells remain undefined. In this study, we show that the activity of the calcium-sensing protein CaM is necessary for these processes both in the WEHI-231 B cell line and in primary splenocytes.

The WEHI-231 line is an immature B-cell lymphoma (Ig-

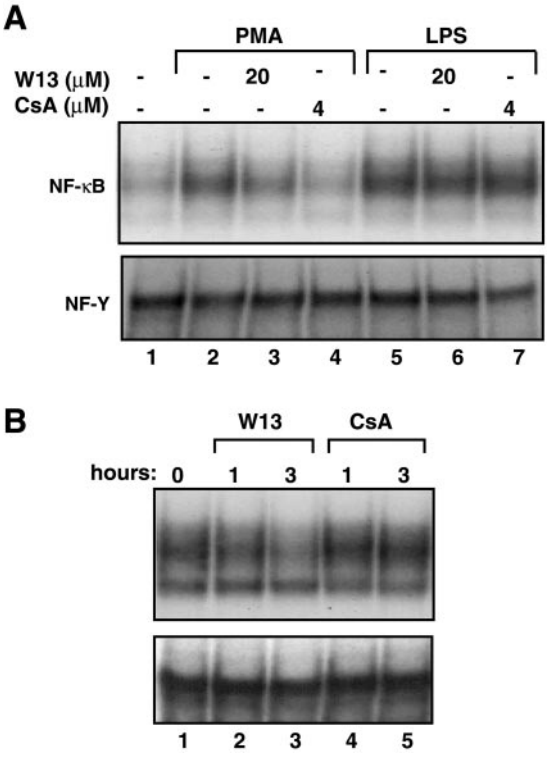

Fig. 7. CaN inhibition is unable to block constitutive NF-κB. A, W231.Bcl-X_L cells were either left untreated (lanes 1, 2, and 5) or were incubated for 30 min with 20 μM W13 (lanes 3 and 6) or 4 μM CsA (lanes 4 and 7). After this pretreatment, cells were stimulated with either 10 nM PMA (lanes 2–4) or 20 μg/ml LPS (lanes 5–7) for an additional 30 min. Whole cell extracts were analyzed by EMSA for κB binding activity (top) or NF-Y binding activity (bottom). B, W231.Bcl-X_L cells were treated with 20 μM W13 (lanes 2 and 3) or 4 μM CsA (lanes 4 and 5) and were terminated at the times indicated. Whole cell extracts (untreated cells are included in lane 1) were analyzed as in A.

M^{hi}/IgD^{lo}) that undergoes apoptosis after cross-linking of surface IgM antigen receptors, and therefore has been widely studied as a model for the clonal deletion of self-reactive B lymphocytes. It has been shown using WEHI-231 cells that, in the absence of costimulation, surface IgM cross-linking results in a transient increase of NF-κB activity followed by a complete loss of the constitutive NF-κB activity at approximately 4 to 6 h after treatment (Lee et al., 1995). Detectable signs of apoptosis under these conditions have been detected at 18 to 24 h after treatment (Benhamou et al., 1990; Gottschalk et al., 1994). Furthermore, when an inducible dominant negative form of IκBα was used to inhibit the constitutive NF-κB activity in an Epstein-Barr virus-transformed cell line, appreciable apoptosis was observed at 24 h after a reduction in NF-κB activity (Cahir-McFarland et al., 2000). This lapse in time between a reduction in NF-κB activity and the onset of apoptosis is not seen when TPCK, PDTC, or BAPTA-AM is used to inhibit NF-κB (Fig. 1; Wu et al., 1996). Perhaps, then, a drop in NF-κB function alone may not be sufficient to cause a rapid onset of apoptosis in these cells. Additional events influenced by these agents are probably causing rapid apoptotic cell death. Thus, our data suggest that the use of these pharmacological agents to study NF-κB–dependent antiapoptotic processes in B cells should be viewed with caution. Moreover, our data suggest that any antiapoptotic gene product(s) produced in a NF-κB–dependent manner probably has a relatively long half-life, making a sustained drop in NF-κB activity a requirement for the onset of apoptosis.

How long is a drop in NF-κB activity required for WEHI231 B cells to commit to apoptotic cell death? We have failed to detect either apoptosis or loss of cell viability in WEHI-231 cells after treatment with W13 for indefinite periods. Surprisingly, time course experiments revealed that the NF-κB inhibitory effect of W13 was completely lost at approximately 8 to 10 h after its addition to the culture medium (Fig. 3D). Incubating W13 in media alone for 6 h abrogated its ability to inhibit NF-κB, suggesting that the loss of a W13 effect was due to instability of W13 in the medium. Therefore, the cytotoxic consequences of W13-mediated inhibition of NF-κB function could not be directly assessed at time points greater than 8 h after treatment. Nevertheless, because W13 is able to inhibit NF-κB activity with similar kinetics as for TPCK, PDTC, and BAPTA-AM, yet does not induce apoptosis up to 8 h, we propose that NF-κB activity needs to be down-regulated for a time greater than 8 h for proapoptotic events to be able to overcome the antiapoptotic effects of NF-κB. There are a number of antiapoptotic genes implicated as direct targets of NF-κB, such as Bfl-1/Al, IAP1/2, and Bcl-X_L. Thus, it is likely that one or a combination of these antiapoptotic genes is regulated by NF-κB in WEHI-231 cells and their in vivo half-lives are such that at least 8 h or more is required to lose their antiapoptotic effects. A role for NF-κB–dependent expression of A1 in WEHI-231 cells is supported by our finding that A1 mRNA levels drop in the presence of W13 but return after its removal (Fig. 5C).

Treatment of T lymphocytes with PMA in conjunction with ionomycin mimics cross-linking of surface TCRs. One result of this is the activation of diverse transcription factors such as nuclear factor of activated T cells, AP-1, and NF-κB. Using PMA plus ionomycin to treat T cells, it has been shown that the Ca^{2+}/CaM-dependent phosphatase calcineurin is required for activation of NF-κB (Mattila et al., 1990; Frantz et al., 1994; Steffan et al., 1995). And although a requirement for CaM activity has been implicated in this model, it was not until recently that a necessary role for Ca^{2+}/CaM activity upstream of IκBα phosphorylation and degradation was more directly supported (Hughes et al., 1998). Hughes et al. (1998) conducted studies that demonstrate that in T cells NF-κB activation, as well as IκBα phosphorylation and degradation, are sensitive to an array of CaM antagonists after treatment with either PMA alone or PMA plus ionomycin. Because CsA could not inhibit NF-κB activation by PMA alone in T cells, Hughes et al. (1998) conclude that CaM is involved in CaN-independent as well as CaN-dependent NF-κB activation pathways. In support of this, our results show that although CaM antagonists block the constitutive activity of NF-κB in the W231.Bcl-X_L cell line, inhibition of CaN does not (Fig. 7). Furthermore, a slower migrating phosphorylated form of IκBα was not detectable in W231.Bcl-X_L cells in which IκBα degradation was blocked by W13 (Fig. 6A; data not shown). Therefore, the step in the constitutive IκBα proteolytic pathway in W231.Bcl-X_L cells at which CaM inhibitors intercede seems to be distinct from that in which CaM inhibitors block the inducible IκBα proteolytic pathway in T cells. The nature of the Ca^{2+}/CaM-dependent step in the constitutive, signal-independent IκBα degradative pathway is currently under investigation.

Although we investigated potential downstream effectors of Ca^{2+} that are critical for the regulation of constitutive NF-κB in B cells, we did not address potential upstream regulators of the Ca^{2+} levels required for constitutive NF-κB activity in B cells. In our recent study, we have obtained several lines of evidence that 1,4-dihydropyridine-sensitive calcium channels may be involved in the maintenance of Ca^{2+} levels in B cells (C. M. Berchtold, S. D. Shumway, S. Miyamoto, M. N. Gould, manuscript in preparation). Together, these studies suggest that there is a signaling system in B cells that begins with 1,4-dihydropyridine-sensitive calcium channels to maintain Ca^{2+}/CaM activity that then leads to downstream events, ultimately causing the degradation of IκBα in a proteasome-independent manner followed by the release of NF-κB into the nucleus. Because NF-κB (p50/cRel) can stimulate the synthesis of p50, c-Rel, and IκBα in this system (Miyamoto et al., 1994b), these events lead to the maintenance of constitutive NF-κB activity to continue their viability in vitro and in vivo. Thus, important future investigations include the identification of an IκBα protease responsible for its Ca^{2+}/CaM-dependent degradation and an "endogenous signal" that maintains this process independent of an added exogenous stimulus.

Acknowledgments

We thank Dr. G. Nuñez for generously providing flag-Bcl-X_L, A. Moser for C57BL/6 mice, and members of the Miyamoto lab for many helpful suggestions throughout the course of this work.

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