Suppression of Calcium Release from Inositol 1,4,5-Trisphosphate-sensitive Stores Mediates the Anti-apoptotic Function of Nuclear Factor-κB*

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The activation of the transcription factor nuclear factor-κB (NF-κB) by growth factors, cytokines, and cellular stress can prevent apoptosis, but the underlying mechanism is unknown. Here we provide evidence for an action of NF-κB on calcium signaling that accounts for its anti-apoptotic function. Embryonic fibroblasts lacking the transactivating subunit of NF-κB RelA (p65) exhibit enhanced inositol 1,4,5-trisphosphate (IP₃) receptor-mediated calcium release and increased sensitivity to apoptosis, which are restored upon re-expression of RelA. The size of the endoplasmic reticulum (ER) calcium pool and the number of IP₃ receptors per cell are decreased in response to stimuli that activate NF-κB and are increased when NF-κB activity is suppressed. The selective antagonism of IP₃ receptors blocks apoptosis in RelA-deficient cells, whereas activation of NF-κB in normal cells leads to decreased levels of the type 1 IP₃ receptor and decreased calcium release. Overexpression of Bcl-2 normalizes ER calcium homeostasis and prevents calcium-mediated apoptosis in RelA-deficient cells. These findings establish an ER calcium channel as a pivotal target for NF-κB-mediated cell survival signaling.

Activation of the transcription factor NF-κB¹ by a variety of signaling pathways provides protection against apoptotic insults both in vitro and in vivo (1). The kinds of death signals that NF-κB counteracts include cytokines such as tumor necrosis factor-α (TNFα) and Fas ligand (2), trophic factor deprivation (3), overactivation of ionotropic glutamate receptors (4), and various types of oxidative stress (5). The gene targets of NF-κB that mediate its anti-apoptotic function have not been established, although several candidates have been identified including Bcl-2 family members (6), manganese superoxide dismutase (5), and members of the inhibitor of apoptosis protein family (7). These proteins may block the apoptotic process by stabilizing mitochondrial membranes, decreasing oxyradical levels, and inhibiting caspases. Recent findings suggest that NF-κB can also repress pro-apoptotic proteins such as GADD153 (8) and JNK (9, 10).

Calcium is a second messenger that mediates cellular responses to various stimuli; examples include cell proliferation, motility, secretion, and neurotransmission (11–13). Calcium is also a trigger of apoptosis in physiological and pathophysiological processes. For example, increases of intracellular calcium levels mediate Fas ligand induction and killing by cytotoxic T lymphocytes (14), ischemic death of neurons (15), and the deaths of cells induced by cytotoxic chemicals (16). Recently, interest has focused on the role of the endoplasmic reticulum (ER) in cell deaths that occur in a variety of physiological and pathological conditions (17, 18). Calcium release from the ER can trigger apoptosis in many different types of cells including fibroblasts, neurons, and tumor cells (18–20). Moreover, data suggest that pro-apoptotic proteins such as presenilins (21), Bax (22), and caspase-12 (23), and anti-apoptotic proteins such as Bcl-2 (24) may exert their actions by modulating ER calcium release. It was recently reported that activation of inositol 1,4,5-trisphosphate (IP₃) receptors regulates NF-κB activity (25), suggesting a possible role for NF-κB in modulation of cell survival/death decisions under conditions in which ER calcium release plays a role. Here we identify suppression of calcium release through IP₃ receptors as a key mechanism whereby NF-κB prevents apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Bradykinin, ATP, caffeine, ryanodine, 2-aminoethoxydiphenyl borate (APB), ISP-1, and dantrolene were purchased from Sigma. Xestospongin C was purchased from Calbiochem. TNFα was purchased from Pepro-Tech Inc. (Rocky Hill, NJ). Fura-2/AM, Fura-2FF/AM, and thapsigargin were purchased from Molecular Probes. The p55EGFP expression plasmid was a generous gift from E. De Smaele and G. Franzoso. The Bcl-2 expression plasmid was a gift from A. Cheng.

Decoy DNA and Antisense Treatments—NF-κB decoy DNA was synthesized and prepared as described previously (5). IκBα antisense and scrambled control DNA oligodeoxynucleotides were synthesized and prepared as described previously (26). Decoy and antisense were added to neuronal cultures in Neurobasal medium. The cell uptake was evaluated by using fluorescence-tagged decoy DNA and confocal microscopy analysis. The efficacy of the decoy DNA and antisense approaches to decrease and increase NF-κB binding activity, respectively, was assessed by electrophoretic mobility shift assay.

Cell Cultures and Experimental Treatments—RelA⁻/⁻ and wild-type...
**Fig. 1. Deficiency of RelA^−/− results in increased ER calcium release.**

*Panel A:* Immunoblot analysis showing RelA protein levels in WT (lane 1) and RelA^−/− (lane 2) MEFs. 

*Panel B:* Typical traces showing calcium signals induced by 1 μM bradykinin (BK). The inset shows quantitative data for the peak [Ca^{2+}]_c (2 separate experiments, 6–8 cultures, 59–78 cells). *, p = 0.003 compared with WT. 

*Panel C:* Representative traces showing the effects of 100 μM ATP on [Ca^{2+}]_c in WT cells, RelA^−/− MEFs, and WT cells pretreated for 24 h with 20 μM NF-κB decoy DNA. 

*Panel D:* Pseudocolor images of WT and RelA^−/− MEFs before (Basal) and after (ATP) addition of 100 μM ATP. 

*Panel E:* Effect of the membrane-permeable IP₃R antagonist APB on response to 100 μM ATP in the presence of extracellular calcium. WT and RelA^−/− MEFs loaded with Fura-2 were incubated with 100 μM APB for 10 min before starting the recording. ATP was added in the presence of APB. The inset shows values of the peak [Ca^{2+}]_c response (measurements made in 3–18 cultures and 43–360 cells). *, p < 0.001 compared with WT MEFs; #, p < 0.001 compared with RelA^−/− MEFs. 

*Panel F:* Comparison of ATP-evoked peak [Ca^{2+}]_c responses in untransfected WT and RelA^−/− cells and in RelA^−/− cells stably transfected with green fluorescent protein (pEGFP) or a fusion protein of green fluorescent protein and RelA (p65EGFP). The inset shows the expression of p65EGFP. Values are mean ± S.D. of 3–4 separate experiments (WT, 20 dishes, 208 cells; RelA^−/−, 19 dishes, 360 cells) and three clones for each transfected line (pEGFP, 7 cultures, 34 cells; p65EGFP, 8 cultures, 18 cells). *, p = 0.001 compared with WT MEFs; #, p < 0.001 compared to RelA^−/− MEFs transfected with pEGFP.
WT immortalized mouse embryonic fibroblasts were a gift from H. Nakshatri, D. Baltimore, and A. Hoffmann. Primary MEFs were prepared from E16 WT and p50 knock-out mouse embryos according to standard procedures (27). Fibroblasts were maintained at 37 °C (5% CO2 atmosphere) in Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin mixture. Stable cell lines expressing p65EGFP and overexpressing Bcl-2 were generated by transfecting RelA−/− MEFs with 1–2 μg of cDNAs using CLONfectin according to the manufacturer’s instructions (Clontech). Selection was carried out for 4 weeks in Dulbecco's modified Eagle's medium containing either G418 (pEGFP and p65EGFP) or puromycin (pBabe and pBabe-Bcl2). Dissociated cell cultures of primary rat cerebral cortical neurons were prepared using methods similar to those described previously (28).

FIG. 2. NF-κB regulates calcium stores. A, typical traces show [Ca2+]i responses to 100 μM ATP in the absence of external Ca2+ in WT and RelA−/− cells. The inset reports values for the peak [Ca2+]i (measurements made in 15 cultures of WT cells (168 cells) and 16 cultures of RelA−/− cells (264 cells)). *, p < 0.001 compared with WT MEFs. B, to measure calcium release from ER stores, 1 μM thapsigargin (Tg) was added to WT and RelA−/− cells in the absence of extracellular calcium. After return of the [Ca2+]i to the basal level, Ca2+ was reintroduced to the incubation buffer to a final concentration of 3 mM. The inset shows the peak [Ca2+]i responses to thapsigargin and to addition of extracellular calcium (6-8 cultures, 43–131 cells). *, p < 0.001 compared with WT MEFs. Average [Ca2+]i (C) and pseudocolor images (D) in permeabilized fibroblasts of 3 cultures. *, p < 0.001 compared with WT MEFs. E, SERCA activity was measured in microsome preparations from WT and RelA−/− cells as a decrease in absorption at 340 nm indicative of NADH consumption. Treatment with thapsigargin was used as negative control (data not shown). The values are the average Δ/min of three separate preparations assayed in duplicate. The inset shows a representative determination. #, p < 0.01 compared with WT cells. F, immunoblot analysis showing levels of the indicated proteins in whole cell lysates and microsomal fractions from WT (lane 1) and RelA−/− (lane 2) MEFs. G, RelA−/− MEFs were transfected with an expression vector encoding Bcl-2 (pBcl2) or the empty vector (pBabe). Cells were selected for resistance to puromycin, and stable lines were established. Overexpression of Bcl-2 was confirmed by Western blot, and three clones for each type were used for measurements of cytosolic calcium responses to 100 μM ATP in the presence of extracellular calcium. The graph shows values of the peak [Ca2+]i responses in RelA−/− pBabe, RelA−/− pBcl-2 (9 cultures, 113–261 cells); the response of WT cells was added for comparison. *, p = 0.004 compared with pBabe-transfected cells.
FIG. 3. Blockade of IP₃-sensitive calcium release abolishes the cell death-promoting effect of RelA deficiency. A, WT and RelA⁻/⁻ MEFs were treated with 50 ng/ml (TNF50) or 100 ng/ml (TNF100) hTNFα in the absence or presence of either the IP₃R antagonist xestospongin C (X, 1 μM) or the ryanodine receptor antagonist dantrolene (Dant, 50 μM). Cell survival was assessed using the MTS assay after 24 h of treatment (n = 16 cultures in 2 separate experiments). *, p < 0.0001 compared with control cells; #, p < 0.0001 compared with TNF-treated cells. B, images showing nuclear DNA morphology (Hoechst staining) in cells treated with 100 ng/ml hTNFα in the presence or absence of 1 μM xestospongin C (X). C, hTNFα exacerbated the calcium response to ATP. Cells were treated for 8 h with 100 ng/ml hTNFα, and [Ca²⁺]c responses to 100 μM ATP were measured. The inset shows quantification of the peak [Ca²⁺]c responses (measurements made in 3 cultures, 14–46 cells). *, p < 0.05 compared with WT MEFs; #, p < 0.05 compared with RelA⁻/⁻ MEFs. D, peak [Ca²⁺]c responses to 100 μM ATP in WT and RelA⁻/⁻ cells pretreated with xestospongin C (X, 1 μM), or dantrolene (Dant, 50 μM). Values are the mean ± S.D. of two separate experiments (6–8 cultures). *, p < 0.01 compared.
Cerebral cortices were removed from embryonic day 18 Harlan Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN). Cells were dissociated by mild trypsinization and triturated and seeded on 22-mm coverslips or 60-mm polyethyleneimine-coated culture dishes containing Eagle’s minimal essential medium supplemented with 26 mM NaHCO₃, 40 mM glucose, 20 mM KCl, 1 mM sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum, and 0.001% gentamycin sulfate. After a 3-5-h incubation period to allow cell attachment, the medium was replaced with Neurobasal medium with B27 supplements (Invitrogen). Experimental treatments were performed on 7-9-day-old neuronal cultures in which ~95% of the cells were neurons, and the remaining cells were astrocytes.

**Fluorometric Measurements of [Ca²⁺]**—Cytosolic Ca²⁺ was measured by ratiometric (F₉₀/F₅₀) imaging of Fura-2 fluorescence as described previously (39). Briefly, MEFs were loaded for 45 min with 2 μM Fura-2/AM in the presence of 0.3% pluronic acid in HBCS (120 mM NaCl, 5.4 KCl, 0.8 mM MgCl₂, 2 mM CaCl₂, 5 mM Hepes, 0.5% phenol red, and 20 mM Hepes, pH 7.3). [Ca²⁺], measurements were obtained using a Zeiss AttoFluor system with a ×40 oil objective. The system was calibrated using stock solutions containing either no calcium or a saturating concentration of calcium (1 mM) using the formula [Ca²⁺] = Kₛ[R - R₉₀]/(R₉₀ - R₅₀) (F₅₀/F₉₀). The experiments in the absence of calcium were performed using HBCS lacking CaCl₂ and supplemented with 1 mM EGTA; the ATP solution was also prepared in Ca²⁺-free medium. Analysis of [Ca²⁺], was performed as described previously (30). Briefly, Fura-2/FF/AM-loaded fibroblasts were washed with Ca²⁺-free buffer and permeabilized using a protocol that preserves the functional integrity of the ER Ca²⁺ stores by incubation for 3 min with 15 ng/ml digitonin in intracellular medium (120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM Tris-Hepes, pH 7.2, and 2 mM ATP). The specificity of calcineurin was assessed by the null effect of IP₃ on Fura-2 FF and ER was assessed in pilot studies by addition of 10 μM IP₃, to the permeabilized cells and detection of a decrement of the Fura-2/FF/AM ratio.

**Preparation of Microsomal Fractions**—Microsomes from WT and RelA−/− cells were prepared as described previously (25). Briefly, cells on 150-mm plates were rinsed twice with phosphate-buffered saline, detached, and pelleted by centrifugation at 500 × g for 10 min. The pellet was resuspended in ice-cold buffer containing 1 mM EDTA, 0.32 M sucrose, 0.1 mM dithiothreitol, 1 mM Hepes, pH 7.4, and protease inhibitors. Cells were homogenized with 40 strokes in a Dounce homogenizer with type A pestle. The cellular debris were removed by centrifugation at 500 × g for 10 min, and supernatants were centrifuged at 20,000 × g for 20 min to pellet intact mitochondria and nuclei. The supernatant was then transferred to a polycarbonate tube and centrifuged at 100,000 × g for 1 h to obtain the microsomal fraction. Microsomes were resuspended in ice-cold buffer, and protein concentration was measured by Bradford assay using bovine serum albumin as the standard. Protein concentration was adjusted to 2 μg/μl, and aliquots were prepared for Western blot analysis or snap-frozen in liquid nitrogen and stored at −80 °C for sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) activity determination.

**Electrophoretic Mobility Shift Assay and Western Blots**—Preparation of cell extracts and electrophoretic mobility shift assays were performed as reported previously (31). For Western blot analysis, solubilized proteins were separated by electrophoresis on a 10% polyacrylamide gel for whole cell extracts (50 μg) and on a 4% Tris-glycine gel for microsomes (20 μg). After transfer to a nitrocellulose sheet, the membrane was blocked at room temperature for 1 h in 5% non-fat milk in Tris-buffered saline containing Tween 20 and immunoreacted overnight at 4 °C with the different primary antibodies. The immunoreacted bands were visualized by incubation with a horseradish peroxidase-conjugated secondary antibody and a chemiluminescence detection kit (Amer sham Biosciences). The following antibodies were used: mouse monoclonal anti-NF-κB p65 (Sigma), mouse monoclonal anti-Bcl-2 (Transduction Laboratories), rabbit polyclonal anti-SERCA2b (Santa Cruz Biotechnology, Santa Cruz, CA).

**Measurements of SERCA Activity**—SERCA activity was measured using an enzyme-coupled spectrophotometric assay in which hydrolysis of ATP is coupled with the oxidation of NADH (32). The depletion of NADH was detected by a decrease in absorbance at 340 nm using a Beckman DU7500 spectrophotometer. The assay buffer contained 120 mM KCl, 2 mM MgCl₂, 1 mM ATP, 1.5 mM phenylphosphorylvurate, 1 mM dithiothreitol, 0.45 mM CaCl₂, 0.5 mM EGTA, 25 mM MOPS/KOH, pH 7.0, 0.32 mM NADH, 10 units/ml pyruvate kinase, 11 units/ml lactate dehydrogenase, and 2 μM of the calcium ionophore 4-bromo-A23187. The reaction was started with the addition of 10 μg of microsomal membranes and monitored at 30-s intervals for 10 min. The rate measured with the SERCA inhibitor thapsigargin was used as negative control.

**Analysis of mRNA Encoding IP₃R1 and GAPDH by Reverse Transcription-PCR**—Total RNA was isolated from cells using TRIzol reagent (Invitrogen). Two μg of RNA was reverse-transcribed using random primers with SuperScript First-Strand Synthesis System according to the manufacturer’s protocol (Invitrogen). PCR amplification of cDNA was performed using the following primer sets, encoding for IP₃R1 and GAPDH, respectively, of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C. Gel electrophoresis with PCR products was performed using ethidium bromide-stained 1.2% agarose gels, and PCR products were identified via UV transillumination at their predicted size by using a 100-bp DNA ladder as a molecular weight marker. Images of UV-transilluminated gels were acquired with a FLA 3000 (Fujifilm, Tokyo, Japan). The band intensities were assessed using the NIH Image software, and densitometric measurements were performed using the NIH Image software.

**Quantification of Cell Survival**—Fibroblasts were seeded in 96-well plates, allowed to adhere for 24 h, and then treated with 50 or 100 ng/ml hTNFα at 37 °C for 24 h. Experiments were also performed in the presence of 1 μM xestospongin C (a specific inhibitor of IP₃ receptor (IP₃R) activation) (12) and 1 μM diphenylketone-4-thiosemicarbazone (dantrolene, 50 μM) and serine-palmitoyltransferase (IP₃R, 1 μM). The inhibitors were added 1 h prior to the addition of hTNFα. At the end of the incubation period, cell survival was assessed using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) following the manufacturer’s instructions. The assay measures the conversion of a tetrazolium compound (MTS) by dehydrogenase enzymes found in metabolically active cells. The quantity of soluble formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture. Results were expressed as a percentage compared with control.

**Assessment of apoptotic cell death was performed by staining fixed MEFs or neurons with the fluorescent DNA binding dye bisbenzamidile (Hoechst stain). Nuclei were visualized and photographed under epifluorescence illumination (340 nm excitation and 510-nm barrier filter) using a ×20 oil immersion objective. Cells in which nuclear staining was diffuse were considered viable, and cells in which nuclear staining was condensed and fragmented were considered apoptotic.

**Immunocytochemistry**—Following experimental treatments, cells were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline and then permeabilized by incubation in 0.2% Triton X-100 in phosphate-buffered saline. Cultures were then incubated for 1 h in 5% normal goat serum and stained for indirect immunofluorescence using a rabbit polyclonal anti-IP₃R1 antibody (Affinity BioReagents) and a biotinylated goat-anti-mouse secondary antibody (Molecular Probes). Nonspecific labeling was determined by omission of the primary antibody. Confocal images were acquired using a Zeiss 510 CSLM microscope.
FIG. 4. NF-κB activation decreases the levels of IP₃R1. A, neurons were treated with vehicle (water), 20 μM NF-κB decoy DNA, or 20 μM IκBα antisense oligodeoxynucleotide. At designated time points, cell extracts were prepared, and electrophoretic mobility shift assays were performed using specific oligonucleotides for NF-κB. The autoradiogram shows NF-κB binding activity in extracts from cells that had been exposed...
Lipid Extraction and Ceramic Measurements—Lipid extraction from WT and RelA−/− cells and electrospray-tandem mass spectrometry analyses were performed using methods described previously (33). Briefly, equal amounts of cell pellets were homogenized in deionized water, and lipids were extracted by sequential addition of methanol: ammonium acetate and chloroform. After centrifugation, the chloroform phase was collected and used for the lipid analysis. Samples were injected using a Harvard Apparatus pump at 15 μl/min into an electrospray ionization Sciex API 3,000 triple stage quadrupole tandem mass spectrometer from Sciex Inc. (Thorntown, Ontario, Canada) operated in a positive mode. Precursor ion scanning or neutral loss scanning of purified standards of C16:0, C18:0, C20:0, C24:0, C24:1 ceramides and C18:0-C18:1 phosphatidylethanolamine were used to identify each species. Quantification of ceramides in the samples was accomplished by summing the total mass counts accumulated under each peak after a 3-min injection period.

Statistical Analyses—Data are presented as means ± S.D. (or S.E., where indicated). One-way analysis of variance combined with Fisher’s protected least significant difference test was used for pairwise comparisons.

RESULTS
Lack of RelA Results in Increased Endoplasmic Reticulum Calcium Release—To investigate the role of NF-κB in calcium homeostasis, we monitored cytosolic free calcium levels ([Ca2+]c) in WT MEFs and in MEFs lacking the transactivating subunit of NF-κB RelA/p65 (RelA−/−) during exposure to different agonists that alter [Ca2+]c (Fig. 1A). To activate the IP3 calcium signaling pathway, cells were stimulated with the surface receptor agonists bradykinin (34) (Fig. 1B) and ATP (35) (Fig. 1, C–E). A dramatic enhancement of calcium release from IP3-sensitive stores was documented in RelA−/− MEFs compared with WT cells. In both WT and RelA−/− MEFs we were unable to detect responses elicited by caffeine or ryanodine (data not shown), suggesting a lack of functional ryanodine receptors in these cells. The calcium response to ATP was also significantly enhanced in WT MEFs in which NF-κB activity was suppressed by a 24-h pretreatment of the cells with xB decoy DNA (Fig. 1C). Treatment with APB, a specific inhibitor of IP3 receptor-mediated calcium release from the ER (36), completely abolished the [Ca2+]c response to ATP in both WT and RelA−/− cells (Fig. 1E). Stable transfection of RelA−/− MEFs with a fusion protein of green fluorescent protein and RelA(p65EGFP) significantly attenuated the calcium response to ATP, whereas the expression of EGFP alone did not (Fig. 1F), confirming a specific effect of NF-κB on calcium signaling.

NF-κB Modulates IP3-gated, Thapsigargin-sensitive Calcium Stores—To determine whether the sustained elevation in [Ca2+]c resulted from calcium influx across the plasma membrane or release from intracellular calcium stores, we stimulated the cells with ATP in calcium-deficient medium. Removal of extracellular calcium resulted in a transient calcium response to ATP, a response that was dramatically enhanced in RelA−/− MEFs (Fig. 2A). Therefore, the enhanced calcium response in cells lacking RelA can be attributed to an effect of NF-κB on calcium release from intracellular stores rather than calcium influx. In most cell types IP3-gated Ca2+ stores overlap with thapsigargin-sensitive Ca2+ stores (37). We sought to determine whether an increased ER Ca2+ pool storage capacity was responsible for the enhanced [Ca2+]c responses, of RelA-deficient cells to agonists that trigger calcium release from IP3-sensitive stores. We monitored [Ca2+]c, in WT and RelA−/− cells before and during exposure to thapsigargin, an inhibitor of ER Ca2+-ATPases, in Ca2+-free medium. Cells lacking RelA displayed a significantly greater rise in [Ca2+]c than did WT MEFs (Fig. 2B), suggesting that constitutive NF-κB activity reduces the total pool of ER calcium available for release. Notably, the magnitude of the capacitative Ca2+ entry, analyzed by the addition of 3 mM Ca2+ following the return of [Ca2+]c to basal levels, was essentially indistinguishable in WT and RelA−/− cells (Fig. 2B). This experiment demonstrated that the activity of Ca2+ release-activated Ca2+ channels is not altered in RelA−/− cells. The Ca2+ content of the intracellular stores, as assessed by equilibrium loading experiments with 45Ca2+, also demonstrated an increased ER calcium pool in cells lacking RelA (data not shown). We further assessed the ER luminal Ca2+ concentration ([Ca2+]lm) by monitoring the fluorescence of compartmentalized Fura-2FF/AM in permeabilized cells (Fig. 2, C and D). In agreement with a previous report (30), the basal ER Ca2+ concentration was estimated to be ~790 nm in normal cells. In contrast, RelA−/− fibroblasts exhibited a higher [Ca2+]lm of ~1700 nm (Fig. 2, C and D). Moreover, when 10 μM IP3 was added to the Fura-2FF/AM-permeabilized cells, a greater decrease in [Ca2+]lm was observed in the RelA−/− cells (data not shown).

Considering the significant [Ca2+]lm difference observed in WT as compared with RelA−/− MEFs, we measured the SERCA activity in isolated microsomes. SERCA activity was higher in RelA−/− cells compared with WT cells (Fig. 2E), suggesting that the constitutive ER calcium overload observed in RelA−/− cells is linked to increased active uptake. We then analyzed the

to vehicle (lane 1), NF-κB decoy DNA (lane 2), and IxBα antisense (lane 3) for 24 h. B, confocal laser scanning microscope images of IP3, R1 immunoreactivity in rat cortical neurons treated for 24 h with either vehicle (Control), 20 μM NF-κB decoy DNA (xB decoy), or 20 μM IxBα antisense oligonucleotide (IxBα ant). The graph shows values for immunoreactivity per neuron (average pixel intensity in the cell body; mean ± S.E. of determinations made in 3–6 separate cultures with 19–30 cells evaluated per culture). *, p < 0.05 compared with Control. C, total RNA was isolated from cortical neurons that had been exposed for 16 h to the indicated treatments, and it was subjected to reverse transcription-PCR analysis to determine relative levels of IP3, R1 mRNA. The two neuronal splice variants of IP3, R1 were detected as 535- and 410-bp PCR products. The relative band intensities were normalized using GAPDH as an internal control. *, p < 0.05 compared with Control. D, representative confocal laser scanning microscope images showing IP3, R1 immunoreactivity in a control culture and in a culture that had been exposed to lTNFa for 24 h. The graph shows values for immunoreactivity per neuron (average pixel intensity in the cell body) as the mean ± S.E. of determinations made in five separate neuronal cultures treated for 24 h with either vehicle (Control), 100 ng/ml hTNFa (TNFa), 20 μM NF-κB decoy DNA and 100 ng/ml hTNFa (Decoy+TNFα), or 20 μM NF-κB decoy DNA (Decoy). *, p < 0.05 compared with Control; #, p = 0.0004 compared with Control. E, cortical neurons were treated for 24 h with either vehicle, 20 μM NF-κB decoy DNA, or 20 μM IxBα antisense oligonucleotide and then exposed for 24 h to 100 ng/ml hTNFa as indicated. Relative levels of IP3, R1 mRNA were determined by reverse transcript-PCR. F, neuronal cultures were treated with either vehicle, 100 ng/ml hTNFa (TNFa), 20 μM NF-κB decoy DNA (xB decoy), 20 μM NF-κB decoy DNA (xB decoy), 20 μM IxBα antisense (IxBα ant) or 100 ng/ml hTNFa in the presence of NF-κB decoy DNA (xB decoy + TNFa) or IxBα antisense (IxBα ant + TNFa) for 24 h. The medium was then replaced with Neurobasal medium without B27, and 15 μM glutamate (Glut) was added for an additional 24 h in the presence or absence of the various compounds. Apoptotic cell death was assessed by Hoechst staining. Values are the mean ± S.E. of determinations made in four to nine separate cultures. *, p < 0.001 compared with Control. #, p = 0.001 compared with hTNFa. G, hippocampal fibroblast cultures were established from p50−/− and WT mice. Cultures were exposed to either vehicle or 100 ng/ml hTNFa for 24 h, and levels of IP3, R1 immunoreactivity were determined. Values are the mean ± S.E. of determinations made in six separate cultures (108–600 cells). *, p = 0.006 compared with WT MEFs; #, p < 0.001 compared with WT MEFs.
levels of proteins that reside in the ER that are involved in SERCA type 2b, as well as the expression of the calcium-binding protein calreticulin and of IP$_3$R type 2, were comparable in the two cell types (Fig. 2F). However, IP$_3$R1 and IP$_3$R3 levels were 3.2 and 2 times higher, respectively, in RelA$^{-/-}$ MEFs compared with WT cells (Fig. 2F). Because Bcl-2 has been reported to be a gene controlled by NF-$\kappa$B (38), and because it was previously shown that overexpression of Bcl-2 reduces the [Ca$^{2+}$]$_{er}$ and that this action of Bcl-2 is part of its anti-apoptotic mechanism (39), we also measured the levels of Bcl-2. Levels of Bcl-2 were increased compared with RelA$^{-/-}$ MEF clones overexpressing Bcl-2 exhibited a markedly decreased [Ca$^{2+}$]$_{er}$ response to ATP compared with cells transfected with the empty vector, such that the magnitude of the calcium response in RelA$^{-/-}$ MEFs overexpressing Bcl-2 was comparable to the response observed in WT MEFs (Fig. 2F). These data indicate that the lack of NF-$\kappa$B activity as the result of RelA deletion causes a profound alteration of ER calcium signaling that involves increased SERCA activity, ER calcium uptake, and IP$_3$-mediated calcium release and that this alteration can be counteracted by Bcl-2.

**Inhibition of IP$_3$-induced Calcium Release Protects RelA$^{-/-}$ Cells from TNF$\alpha$-induced Cytotoxicity**—In order to link the abnormal ER calcium homeostasis in cells lacking RelA to the cell survival-promoting function of NF-$\kappa$B, we quantified cell death induced by TNF$\alpha$. As expected, RelA$^{-/-}$ MEFs exhibited increased vulnerability to killing by TNF$\alpha$ compared with WT cells (Fig. 3A and B). When RelA$^{-/-}$ MEFs were pretreated for 8 h with TNF$\alpha$, their [Ca$^{2+}$]$_{er}$ response to ATP was greatly enhanced compared with RelA$^{-/-}$ MEFs not treated with TNF$\alpha$ (Fig. 3C). In contrast, pretreatment of WT cells with TNF$\alpha$ did not result in an increased [Ca$^{2+}$]$_{er}$ response to ATP; rather, an opposite trend was observed. We next determined the role of ER calcium release in the enhanced sensitivity of RelA$^{-/-}$ MEFs to apoptosis by blocking calcium release from IP$_3$ and ryanodine receptors with xestospongin C (40) and dantrolene (41), respectively. Because these experiments required a 24-h exposure time, we opted for the use of xestospongin C because it has a similar ability to decrease IP$_3$-induced calcium release (compare Figs. 1E and 3D), without the cellular toxicity observed with long-term exposures to ABP (data not shown). Xestospongin C completely prevented killing of RelA$^{-/-}$ cells by TNF$\alpha$ (Fig. 3A and B), whereas dantrolene did not modify the calcium response (Fig. 3D) or the survival of RelA$^{-/-}$ cells (Fig. 3A). Thus, opening of IP$_3$R channels is essential for the cell death-promoting effect of RelA deficiency.

In our studies we used human recombinant TNF$\alpha$, which in rodent cells activates the p55 TNF$\alpha$ receptor and signaling pathways that generate ceramide (42, 43); ceramide has been shown to trigger intracellular calcium release through IP$_3$ receptors (44). It appears that one factor controlling the cell susceptibility to ceramide-induced apoptosis is the [Ca$^{2+}$]$_{er}$ (39). Generation of ceramide requires the production of the precursor sphinganine arising from the condensation of serine and active palmitate through the catalysis of the enzyme serine-palmitoyltransferase. We studied the involvement of ceramide generation in the sensitivity of RelA$^{-/-}$ MEFs to TNF$\alpha$-induced apoptosis by using the inhibitor of serine-palmitoyltransferase ISP-1 (32). Blockage of de novo ceramide synthesis was very effective in preventing apoptosis (Fig. 3E). Notably, we also found that the activity of neutral sphingomyelinase was about two times higher in RelA$^{-/-}$ MEFs compared with WT cells under basal culture conditions (Fig. 3F). In addition, tandem mass spectrometry analysis of lipid extracts from WT and RelA$^{-/-}$ cells demonstrated that under basal conditions, RelA$^{-/-}$ cells contain a 2-fold higher level of the long chain ceramides C16 and C24 (Fig. 3G). When we analyzed the effect of ISP-1 treatment on the calcium response to ATP, we found a reduced average fold increase in RelA$^{-/-}$ cells (Fig. 3H). Szalai et al. (45) recently demonstrated that the Ca$^{2+}$ sensitivity of the permeability transition pore in mitochondria increases in cells exposed to ceramide. In such “ceramide-primed” cells, IP$_3$-induced calcium spikes (which are normally buffered by mitochondrial uptake) cause the opening of permeability transition pores, cytochrome c release, and apoptosis. Our data suggest that inhibition of NF-$\kappa$B activity places cells in a similar apoptosis-prone state with regard to sensitivity to IP$_3$-induced calcium release.

**Activation of NF-$\kappa$B Leads to Decreased Expression of IP$_3$R Type 1**—It was recently reported that activation of TNF$\alpha$ receptors can decrease levels of IP$_3$ receptors as well as intracellular calcium mobilization in lymphoma cells (46). Because our data pointed to a specific effect of NF-$\kappa$B activation on IP$_3$ receptor channels, we determined whether IP$_3$R levels are regulated by NF-$\kappa$B. For these experiments, we employed primary embryonic rat cortical neurons, a cell type with a relatively high level of constitutive NF-$\kappa$B activity (47). NF-$\kappa$B activity was either induced by introducing NF-$\kappa$B decoy DNA into the cells or enhanced by antisense-mediated reduction in the levels of IxB$a$, the inhibitory subunit of NF-$\kappa$B (Fig. 4A). We determined the effects of reducing or increasing NF-$\kappa$B activity on the expression of IP$_3$R$_1$, the major IP$_3$R isoform in neurons (48) and the one primarily affected by RelA deficiency (Fig. 2F). We found that NF-$\kappa$B decoy DNA increased the amount of IP$_3$R$_1$ protein (Fig. 4B) and IP$_3$R$_1$ messenger RNA (Fig. 4C) in the neurons, whereas IxB$a$ antisense had the opposite effect. Correspondingly, exposure of neurons to TNF$\alpha$ also resulted in a decrease in the levels of IP$_3$R$_1$ protein and mRNA (Fig. 4, D and E); these effects of TNF$\alpha$ were prevented in neurons co-treated with NF-$\kappa$B decoy DNA (Fig. 4, D and E), demonstrating a requirement of NF-$\kappa$B activation in modulation of IP$_3$R$_1$ by TNF$\alpha$. Increases in intracellular calcium levels are closely linked to excitotoxicity (49). We therefore analyzed the effect of NF-$\kappa$B activity on calcium mobilization and sensitivity to apoptosis in response to glutamate. Pretreatment with TNF$\alpha$ significantly protected neurons from glutamate-induced apoptosis (Fig. 4F), an effect mimicked by IxB$a$ antisense treatment and completely prevented by co-treatment with NF-$\kappa$B decoy DNA (Fig. 4F). In agreement with a previous report (46), treatment with TNF$\alpha$ resulted in diminished levels of calcium release, an effect abolished by co-treatment with NF-$\kappa$B decoy DNA (Fig. 4G). Notably, neurons treated with NF-$\kappa$B decoy DNA exhibited an enhanced calcium response to glutamate (Fig. 4G), thus substantiating our findings in RelA$^{-/-}$ cells. Previous studies have shown that neurons lacking the p50 subunit of NF-$\kappa$B exhibit enhanced elevations of intracellular calcium levels and increased sensitivity to death after exposure to glutamate (4). We found that cultured primary fibroblasts (Fig. 4H), as well as cortical and hippocampal neurons (data not shown), established from p50-deficient mice did not exhibit a decrease in IP$_3$R$_1$ levels following treatment with TNF$\alpha$, in contrast to cells from WT mice. Moreover, cells lacking p50 exhibited an increased basal level of IP$_3$R$_1$ protein, supporting a role for NF-$\kappa$B in determining the basal level of IP$_3$R$_1$ (Fig. 4H).
Increasing evidence points to events occurring in the ER as pivotal determinants of cell death and life decisions. For example, ER stress responses such as the unfolded protein response can trigger apoptosis (50), and ER protein chaperones can prevent apoptosis (51). Our data identify the ER (and specifically, ER calcium signaling) as a pivotal subcellular target that mediates the cell survival-promoting function of NF-κB. Our analysis of cells lacking RelA and of cells in which NF-κB activity was increased or decreased suggests that NF-κB prevents cell death by reducing calcium release from IP3-sensitive ER stores. The down-regulation of IP3R1 expression by NF-κB appears to be a pivotal action of NF-κB that promotes cell survival by preventing excessive elevations of cytoplasmic calcium levels.

Abnormalities in ER calcium release have been implicated in diseases that involve aberrant apoptosis. For example, mutations in presenilin 1 that cause early-onset Alzheimer disease may act, in part, by enhancing calcium release from ER stores (21). Perturbations in NF-κB activity have been documented in studies of Alzheimer disease (52), but a possible link between the calcium signaling defect and altered NF-κB activation has not been explored. Beyond establishing a key role for modulation of calcium release from IP3-sensitive stores in the cell survival-promoting function of NF-κB, our findings suggest that NF-κB may modify calcium signaling events involved in various other physiological processes. Intracellular calcium release channels play important roles in regulating cell proliferation (53) and differentiation during embryogenesis (54), in lymphocyte signaling (55), and in modulation of synaptic plasticity in the nervous system (56). It was recently reported that calcium release from IP3-sensitive stores could trigger activation of NF-κB in cultured neurons (25). NF-κB may therefore serve in a dynamic transcription-dependent feedback mechanism to down-regulate ER calcium release responses.

Signaling pathways that affect transcription of the genes encoding IP3 receptors are being identified. The promoter of IP3R1 contains regulatory elements responsive to several different transcription factors including AP-1, estrogen receptors, and NF-κB (57). In addition, it has been shown that calcium-responsive signaling pathways can control IP3R1 expression. For example, the calcium/calmodulin-dependent protein phosphatase calcineurin enhances IP3R1 expression in neurons (58). Our data show that NF-κB down-regulates expression of IP3R1 in fibroblasts and neurons and further suggest that this action of NF-κB on IP3R1 expression plays an important role in the anti-apoptotic action of NF-κB. Interestingly, another link between mechanisms regulating apoptosis and IP3Rs was identified in a study showing that the anti-apoptotic protein Bcl-xL affects calcium homeostasis in lymphoid cells by altering the expression of IP3Rs (59). Interactions between calcium signaling pathways and proteins that regulate apoptosis are therefore likely to play important roles in various physiological and pathological conditions that involve apoptosis. Our findings reveal a novel cell survival-promoting feedback pathway in which activation of IP3R in the ER stimulates NF-κB (25), which, in turn, negatively regulates expression of IP3R1.

NF-κB is often activated in cells in response to potentially lethal stimuli including exposure to TNFα, Fas ligand, oxidative stress, and calcium influx (60, 61). Although it was initially thought that NF-κB plays a role in the cell death process, subsequent studies showed that selective inhibition of NF-κB activity exacerbated cell death, whereas enhanced activation of NF-κB prevented cell death (5, 26, 62, 63). Indeed, RelA-null mice die during embryonic development as the result of massive hepatocyte apoptosis related to the deregulated TNFα/

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