Endoplasmic Reticulum \(d\text{-}myo\text{-}\)Inositol 1,4,5-Trisphosphate-sensitive Stores Regulate Nuclear Factor-\(\kappa\)B Binding Activity in a Calcium-independent Manner*

Gordon W. Glazner‡§¶, Simonetta Camandola‡, Jonathan D. Geiger§, and Mark P. Mattson‡**

From the ‡Laboratory of Neurosciences, NIA Gerontology Research Center, National Institutes of Health, Baltimore, Maryland 21224, the §Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Manitoba R3E 0T6, Canada, and the ¶Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received for publication, February 12, 2001
Published, JBC Papers in Press, April 17, 2001, DOI 10.1074/jbc.M101315200

The transcription factor nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) plays critical roles in neuronal survival and plasticity in activation of immune responses. The activation of NF-\(\kappa\)B has been closely associated with changes in intracellular calcium levels, but the relationship between the two remains unclear. Here we report that inhibition of endoplasmic reticulum (ER) \(d\text{-}myo\text{-}\)inositol 1,4,5-trisphosphate (IP\(_3\))-gated calcium release decreased basal NF-\(\kappa\)B DNA-binding activity in cultured rat cortical neurons. Activation of NF-\(\kappa\)B in response to tumor necrosis factor-\(\alpha\) and glutamate was completely abolished when IP\(_3\) receptors were blocked, and NF-\(\kappa\)B activation in response to depletion of ER calcium by thapsigargin treatment was also decreased by IP\(_3\) receptor blockade. We further investigated the relationship between IP\(_3\) receptor activation and NF-\(\kappa\)B activity using a cell-free system. Microsomes enriched in the ER were isolated from adult rat cerebral cortex, resuspended, and treated with agents that induce or inhibit ER calcium release. They were then recentrifuged, and the supernatant was added to cytoplasmic extract isolated from the same source tissue. We found that microsomes released an NF-\(\kappa\)B-stimulating signal in response to activation of IP\(_3\) receptors or inhibition of the ER Ca\(_{\text{2+}}\)-ATPase, but not in response to ryanodine. Studies of intact cells and cell-free preparations indicated that the signal released from the ER was not calcium and was heat- and trypsin-sensitive. Our data suggest that activation of IP\(_3\) receptors is required for a major component of both constitutive and inducible NF-\(\kappa\)B binding activity in neurons and that decreasing ER intraluminal calcium levels triggers release of a diffusible NF-\(\kappa\)B-activating signal from the ER.

The transcription factor NF-\(\kappa\)B\(^1\) is a member of the NF-\(\kappa\)B/Rel family, which includes p50, p52, p65 (RelA), c-Rel, and RelB proteins (1, 2). Prototypical NF-\(\kappa\)B is a p50-p65 heterodimer that is retained in the cytoplasm of unstimulated cells in an inactive form by the inhibitory protein I\(\kappa\)B. In response to various stimuli, I\(\kappa\)B is phosphorylated, rapidly ubiquitinated, and subsequently proteolyzed by a 26 S proteasome complex. The degradation of I\(\kappa\)B unmaskes the nuclear localization signal of the NF-\(\kappa\)B heterodimer, which then translocates into the nucleus, where it binds to its cognate sequence on DNA and thereby regulates gene transcription. In the brain, NF-\(\kappa\)B is constitutively active in many neurons (3), wherein its activity may be further increased by excitation (4, 5) and ischemia (6, 7) and in neurodegenerative disorders (8–10). Because activation of NF-\(\kappa\)B is associated with cell injury and death, it has been proposed that this transcription factor contributes to the neuronal death process (6, 11). However, results from cell culture and in vivo studies have demonstrated that activation of NF-\(\kappa\)B in neurons represents a highly protective response that promotes cell survival and plasticity (12–16).

Stimuli that can activate NF-\(\kappa\)B include TNF-\(\alpha\) (12, 17, 18), interleukin-1\(\beta\) (19), glutamate (20), nerve growth factor (21, 22), and secreted amyloid precursor protein (23). Increased NF-\(\kappa\)B activity may also occur in response to oxidative stress, perturbed calcium homeostasis, and DNA damage (2, 24–26). However, the mechanisms that regulate NF-\(\kappa\)B activity in neurons under basal conditions and in response to various physiological and pathological conditions are not known. Changes in the concentration of intracellular free calcium (\(\text{[Ca}^{\text{2+}}\)) regulate numerous functions in neurons, including synaptic plasticity and cell survival (27, 28). Influx through the plasma membrane has long been regarded as the main source of Ca\(_{\text{2+}}\)-mediated intracellular signals. However, the significance of Ca\(_{\text{2+}}\) released from internal stores such as the ER has become increasingly apparent. In neurons, the ER is distributed throughout the cytoplasm and represents a large and releasable pool of calcium. Increased \(\text{[Ca}^{\text{2+}}\)) can stimulate release of Ca\(_{\text{2+}}\) stored in the ER, a phenomenon known as calcium-dependent calcium release that is regulated by the ER-resident IP\(_3\) and ryanodine-sensitive receptor calcium channels (29). The intracellular signaling molecule IP\(_3\), the ligand for ER IP\(_3\) receptors, is generated by activation of phospholipase C and cleavage of phosphoinositide bisphosphate into diacylglycerol and IP\(_3\). Calcium regulates the sensitivity of these channels to IP\(_3\), increasing the calcium current (28). Therefore, alterations in \(\text{[Ca}^{\text{2+}}\)) will affect the activity of the ER IP\(_3\) receptor and thus affect cellular events regulated by this receptor.

Recent studies have shown that impairment of ER function tetraacetic acid; EMSA, electrophoretic mobility shift assay; CREB, cAMP-responsive element-binding protein; MSE, microsomal extract.
with accumulation of proteins in the ER and the consequent release of Ca\(^{2+}\) from this organelle stimulates NF-κB DNA-binding activity and NF-κB-dependent gene expression (30, 31). Because IP\(_{3}\) receptors are highly sensitive to many of the same stimuli that activate NF-κB, and alterations in ER function are associated with increased NF-κB binding and transcription, we examined the relationship between ER IP\(_{3}\) receptors and NF-κB activation.

**MATERIALS AND METHODS**

Cortical Cell Cultures and Experimental Treatments—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 trituration as described previously (32) and were seeded into 60-mm polyethyleneimine-coated culture dishes containing Eagle’s minimum essential medium supplemented with 26 mM NaHCO\(_3\), 40 mM glucose, 20 mM KCl, 1 mM sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum (Sigma), and 0.001% gentamycin sulfate. After a 3–5-h incubation period to allow for cell attachment, the medium was replaced with 2 ml of neurobasal medium with B27 supplements (Life Technologies, Inc.). 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. IP\(_{3}\), glutamate, and ryanodine were purchased from Sigma; xestospongin C (XeC) was purchased from Calbiochem; TNF-α was purchased from Pepro-Tech Inc. (Rocky Hill, NJ); and thapsigargin and BAPTA free acid were purchased from Molecular Probes, Inc. (Eugene, OR). Each reagent was prepared as a 500× or 1000× stock in an appropriate solvent (saline or dimethyl sulfoxide).

Total Cell Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Total cell extracts were prepared as described (33). Briefly, cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed for 30 min at 4°C in ToX buffer (350 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl\(_2\), 0.5 mM EDTA, 0.1 mM EGTA, 5 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, 1% aprotinin, 0.05% Nonidet P-40, 2 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 100 mM HEPES, pH 7.9; and 0.5 mM EDTA, 0.25% Nonidet P-40, 2 m M dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 20 mM HEPES, pH 7.9). Samples were centrifuged at 14,000 × g for 10 min, and aliquots of the supernatant were collected and stored at −70°C until taken for assay. The protein content of the extract (supernatant) was measured by the Bradford method (Bio-Rad). For EMSA, equal amounts of protein were incubated in a 20-μl reaction mixture containing 20 μg of bovine serum albumin; 1 μg of poly(dI-dC); 2 μl of buffer containing 20% glycerol, 100 mM KCl, 0.5 mM MgCl\(_2\), 0.25% Nonidet P-40, 2 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 20 mM HEPES, pH 7.9; 5 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 20 mM HEPES, pH 7.9; and 0.001% gentamycin sulfate. Total cell extracts were prepared, and EMSAs were performed using oligonucleotides specific for NF-κB or CREB-binding sites. b, films from four separate gel-shift assays were scanned, and densitometric analyses were performed. Data are the means ± S.E. (n = 4), *p < 0.05 versus control (Con) (analysis of variance with Scheffe’s post-hoc test).

**RESULTS**

**Inhibition of IP\(_{3}\) Receptor-mediated Calcium Release Decreases and Thapsigargin Increases NF-κB Activity in Cortical Neurons**—The possible involvement of ER pools of calcium regulated by IP\(_{3}\) receptors in controlling basal levels of NF-κB activation was investigated using XeC, a membrane-permeable blocker of IP\(_{3}\)-induced Ca\(^{2+}\) release (36–38). Conversely, to release the total pool of ER luminal calcium, we used thapsigargin, a specific inhibitor of the ER Ca\(^{2+}\)-ATPase (39, 40). Cultured primary cortical neurons were treated with 1 μM XeC, 100 nm thapsigargin, or vehicle for 6 h, and protein extracts were analyzed for NF-κB DNA-binding activity. XeC caused a significant decrease in the basal levels of activated NF-κB relative to control levels (Fig. 1, a and b). The decrease in NF-κB activation was not due to interference of XeC with the assay used to detect NF-κB activity as determined by addition of XeC to the reaction (data not shown). As previously reported (41), treatment with thapsigargin produced a large and significant increase in NF-κB activity (Fig. 1, a and b). To determine if the effects of XeC and thapsigargin were specific for NF-κB, we measured the levels of CREB DNA-binding activity. CREB

![FIG. 1. Effects of xestospongin C and thapsigargin on basal levels of NF-κB and CREB DNA-binding activities in cerebral cortical cell cultures.](http://www.jbc.org/)
is a transcription factor thought to play a role in synaptic plasticity and neuronal survival (42, 43), and activation of CREB is thought to be primarily regulated by calcium (44). In contrast to their effects on NF-κB activity, XeC and thapsigargin each produced a small increase in CREB binding activity (Fig. 1a). We next determined whether IP$_3$ receptor blockade would alter the thapsigargin-induced activation of NF-κB. XeC was added to the cortical cultures 30 min before thapsigargin, and binding activity was analyzed after 6 h; IP$_3$ receptor blockade inhibited the ability of thapsigargin treatment to activate NF-κB (Fig. 2a).

**Inhibition of IP$_3$ Receptor-mediated Calcium Release Inhibits Inducible NF-κB Activity in Cortical Neurons**—NF-κB is not only a constitutively active transcription factor, but is also highly inducible in neurons by several stimuli, including the excitatory neurotransmitter glutamate (20, 45, 46) and the pro-inflammatory cytokine TNF-α (12). We therefore determined whether activation of IP$_3$ receptor-mediated calcium channels is necessary for NF-κB DNA-binding activity induced by glutamate and TNF-α. Cells were pretreated for 30 min with vehicle or XeC and were then exposed to 100 ng/ml TNF-α or 20 μM glutamate for 6 h. TNF-α produced a significant increase in NF-κB activity that was completely abolished by pretreatment with XeC (Fig. 2b). This effect was specific for NF-κB because neither TNF-α nor XeC affected the binding activity of CREB (Fig. 2b). Induction of NF-κB DNA-binding activity was observed with glutamate treatment, and pretreatment with XeC completely abolished the activation of NF-κB by this excitatory neurotransmitter (Fig. 2d). Cells pretreated with XeC prior to exposure to glutamate demonstrated a level of NF-κB activity lower than that of control neurons exposed to glutamate. Measurement of [Ca$^{2+}$] in neurons by imaging of the calcium indicator dye fura-2 showed that XeC treatment resulted in only a small attenuation of the glutamate-induced increase in [Ca$^{2+}$]. Values were as follows: control, 86 ± 14 nM; 1 μM XeC (5-min exposure), 96 ± 24 nM; 20 μM glutamate (peak response), 618 ± 87 nM; and 1 μM XeC plus 20 μM glutamate (peak response), 410 ± 49 nM (values are the means ± S.D. of determinations made in four cultures with measurements made in at least 12 neurons/culture).

**Microsomal Extract Regulates Cytoplasmic NF-κB Activity in an ER Calcium Channel-dependent Manner**—Our results to this point indicated that ER IP$_3$-sensitive calcium pools can regulate NF-κB activity in neurons. To gain further insight into the mechanism behind this, we used an in vitro cell-free system to separate cytoplasmic pools of inactive NF-κB from the ER, therefore allowing us to study ER-derived signals that might modulate NF-κB activity. Microsomal preparations were isolated as illustrated (Fig. 3a), and tests were conducted to establish their content of ER. Immunoblot analysis (Fig. 3b) of grp78, an ER-resident chaperone protein, performed on the various fractions demonstrated that the microsomal fraction was highly enriched in this ER marker. Moreover, measurements of the concentration of intramicrosomal calcium and the subsequent reduction in this value with exposure of the microsomes to IP$_3$ demonstrated high levels of functional IP$_3$ receptors in this preparation (Fig. 3c).

To determine the identity of the major NF-κB-binding complexes found in the cytoplasmic fraction, supernatants from the 100,000 × g centrifugation were incubated with anti-p50 antibody, anti-p65 antibody, or a combination of anti-p50 and anti-p65 antibodies for 45 min. There were two major bands identified in these experiments, bands A and B (Fig. 3d). Addition of anti-p50 antibody shifted band A in its entirety to a much higher position, called supershift 1, and shifted the lower band to a slightly higher position, called supershift 2. Band A was also greatly diminished in intensity by anti-p50 antibody treatment. Incubation with anti-p65 antibody caused a disappearance of band A, but had no apparent effect on band B. Incubation with both anti-p50 and anti-p65 antibodies caused a complete shift upward of band A to the supershift 1 position and shifted band B slightly upwards and reduced its intensity. Thus, band A is the p50-p65 heterodimer, and band B contains p50-p50 homodimers. A binding pattern identical to that seen in the cytoplasmic extracts of this study was previously seen in whole cell extracts of cultured rat cortical neurons (47).

Microsomal preparations were treated with thapsigargin and calcium in the absence or presence of XeC for 1 h and centrifuged, and 3 μl of supernatant (MSE) was added to 10 μl of the cytoplasmic fraction. After 1 h at room temperature, samples were examined for NF-κB DNA-binding activity. Significant increases in NF-κB activity were observed following addition of MSE, thus indicating the presence of a microsome-derived NF-κB-stimulating signal (Fig. 4, a and b). MSE from thapsigargin-treated microsomes caused a significant enhancement of this binding activity (Fig. 4, a and b). MSE from XeC-treated microsomes did not elevate NF-κB activity, and treatment with XeC reduced by ~50% the thapsigargin-stimulated NF-κB activity (Fig. 4, a and b).

A recent report suggested that NF-κB may localize to the ER (48), raising the possibility that the NF-κB-stimulating agent released by microsomes was the activated transcription factor itself. To examine this possibility, EMSAs were performed on cytoplasmic fractions, MSEs, and microsomal pellets following treatment with vehicle, thapsigargin, or XeC. Although there was binding activity found in MSE and microsomes, this binding was >10-fold less than that seen in the cytoplasm. Neither thapsigargin nor XeC had any effect on NF-κB DNA-binding activity levels in any of the three fractions tested (Fig. 4c). We
then assessed the necessity of the ER in the microsomal preparation for NF-κB activation by treating isolated cytoplasmic extracts from whole adult rat cortices with 100 nM thapsigargin or 1 μM XeC (X) and then incubated for 1 h with vehicle (control (C)), 100 nM thapsigargin (Tg), or 10 μM Ca²⁺. The microsome suspension was centrifuged at 100,000 × g, and 3 μl of MSE was added to 10 μl of cytoplasmic fraction. After incubation for 1 h at 37 °C, NF-κB DNA-binding activity was determined. As a control, the cytoplasmic fraction without addition of microsomes (Cyt) was also analyzed. b, exposures from four different experiments were scanned, and densitometry was performed. Data are the means ± S.E. (n = 4). *, p < 0.05, and **, p < 0.01 compared with the cytoplasm alone; †, p < 0.05 compared with thapsigargin alone; ‡, p < 0.05 compared with cytoplasmic extract/MSE (Cyt/MSE) (analysis of variance with Scheffe’s post-hoc test). c, the cytoplasmic fraction, MSE, or pelleted microsomes (Pellet) were treated with vehicle, 1 μM XeC, or 100 nM thapsigargin for 1 h prior to being analyzed for NF-κB DNA-binding activity.

A Signal Other than Calcium Mediates NF-κB Activation in Response to ER Calcium Depletion—We next asked whether NF-κB could be induced by calcium directly. Aliquots of the cytoplasmic fraction were treated with calcium at concentrations ranging from 1 μM to 10 mM for 1 h, followed by analysis of NF-κB binding by EMSA. As shown in Fig. 5b, calcium did not affect NF-κB DNA-binding activity at any concentration tested. These same extracts were analyzed for CREB binding activity; calcium greatly enhanced CREB binding, which likely results from stimulation of calcium-activated kinases (49). These data suggest that although the elevation of calcium in the cytoplasm by itself is able to induce CREB, it is not sufficient to cause NF-κB activation. Conversely, chelation of cal-

Fig. 3. Subcellular fractionation protocol and characterization of microsomes and cytosolic NF-κB proteins. a, shown are the microsomal, MSE, and cytoplasmic extract isolation protocols. XeC, xestospongion C; Thaps, thapsigargin. b, equal amounts of protein from the subcellular fractions were separated by SDS-polyacrylamide gel electrophoresis and probed with an antibody against the ER-resident protein grp78. Similar results were obtained in a separate experiment. Super, supernatant. c, the intraluminal calcium concentration was measured prior to and after exposure to 10 μM IP3. Values (nanomolar) are the average of measurements made in two separate experiments. d, supernatant fractions from the cortex following a 100,000 × g centrifugation for 1 h (cytoplasmic fraction (Cyt)) were incubated for 45 min with anti-p50 antibody (Ab), anti-p65 antibody, or a combination of anti-p50 and anti-p65 antibodies. Binding activity and band position were then analyzed by EMSA. The four sites of NF-κB binding identified are referred to as band A (A), band B (B), supershifted band 1 (ss 1), and supershifted band 2 (ss 2).

Fig. 4. Evidence that an NF-κB-activating factor is released from the ER in response to calcium release. a, shown are the effects of the microsomal extract on NF-κB binding activity. Microsomes were pretreated for 30 min with vehicle or 1 μM XeC (X) and then incubated for 1 h with vehicle (control (C)), 100 nM thapsigargin (Tg), or 10 μM Ca²⁺. The microsome suspension was centrifuged at 100,000 × g, and 3 μl of MSE was added to 10 μl of cytoplasmic fraction. After incubation for 1 h at 37 °C, NF-κB DNA-binding activity was determined. As a control, the cytoplasmic fraction without addition of microsomes (Cyt) was also analyzed. b, exposures from four different experiments were scanned, and densitometry was performed. Data are the means ± S.E. (n = 4). *, p < 0.05, and **, p < 0.01 compared with the cytoplasm alone; †, p < 0.05 compared with thapsigargin alone; ‡, p < 0.05 compared with cytoplasmic extract/MSE (Cyt/MSE) (analysis of variance with Scheffe’s post-hoc test). c, the cytoplasmic fraction, MSE, or pelleted microsomes (Pellet) were treated with vehicle, 1 μM XeC, or 100 nM thapsigargin for 1 h prior to being analyzed for NF-κB DNA-binding activity.
NF-κB activation was determined by EMSA. b, aliquots of cytoplasmic fractions were left untreated or were treated for 1 h with 10 μM Ca²⁺, 10 μM BAPTA (B), or Ca²⁺ plus BAPTA, and NF-κB DNA-binding activity was determined.

Microsomes were inactivated by heating MSE to 100 °C (Fig. 6a-c) and was also inactivated by trypsin treatment (Fig. 6a). Collectively, these results indicate that a diffusible factor that is heat- and trypsin-sensitive is released from the ER, in response to stimuli that induce calcium release from IP₃-susceptible stores, and is capable of activating NF-κB.

**DISCUSSION**

NF-κB is believed to play important roles in brain development and in neurodegenerative disorders (2, 51). In unstimulated cells, NF-κB subunits are present in the cytoplasm, including neurites and synapses, in an inactive form complexed with IκBα (45, 52). Increases in cytosolic calcium levels had previously been associated with activation of NF-κB (41, 53–57); but it was not established if and how calcium itself activates NF-κB, and increased [Ca²⁺], is an event common to several different NF-κB-activating pathways (24). Studies of other transcription factors such as CREB have shown that the source of calcium entry may affect which genes are transcribed through distinct DNA regulatory elements (44). To help elucidate the relationship between ER calcium release through IP₃ receptors specifically and NF-κB activation, we conducted a series of experiments in cortical neurons and found that 1) inhibition of IP₃ receptor channels reduces both basal and thapsigargin-induced NF-κB DNA-binding activity; 2) the decrease in NF-κB activity is independent of changes in intracel-
ular calcium levels; 3) extracts from microsomes release a diffusible NF-κB-stimulating signal that is augmented by IP_3 and thapsigargin and diminished by XeC; and 4) the ER-derived NF-κB-activating signal is not calcium. This factor appears to be regulated by IP_3 receptor activity and/or the filling state of the IP_3-sensitive pools of intracellular calcium. Thus, our data indicate that it is not the calcium release from ER stores that activates cytoplasmic NF-κB, but rather the decrease in intraluminal calcium that signals release of a diffusible NF-κB-activating factor from the ER.

We found that a concentration of XeC reported to selectively block IP_3 calcium channels in a noncompetitive manner (36–38) can decrease the basal level of NF-κB activation. It has been shown that the constitutively active form of NF-κB in neurons is transcriptionally active and may drive basal expression of several different genes (58). Although the basis of this constitutive activity has been unclear, our data suggest that it is dependent upon IP_3 receptor activation. It has been previously shown that there is a spontaneous release of calcium from IP_3-sensitive calcium stores (59) resulting from a basal level of IP_3 production and the ER luminal content of calcium. It should be emphasized that IP_3 receptors can be activated in response to a broad range of stimuli present in neuronal cultures. IP_3 is endogenously produced in cultures as a result of the actions of growth factors, neuropeptides, and other bio-molecules coupled to phospholipase C-linked GTP-binding proteins. Activation of purinergic receptors and glutamate receptors, depolarization of the membranes, and synaptic activity will all activate the IP_3 receptors present in the ER. According to our data, if the constitutive activity of IP_3 receptors is blocked, the basal activation of NF-κB in neurons is largely abolished, strongly suggesting that the two events are functionally related.

Activation of TNF receptors results in recruitment of TNF receptor-associated death domain, which acts as an adaptor for TNF receptor-associated factor-2, which in turn mediates NF-κB activation (60–62). In our model, XeC significantly inhibited the ability of TNF-α to induce NF-κB. TNF-α does not evoke calcium transients over short time periods (63); however, it does activate phospholipase C (64) and can thereby activate ER IP_3 receptors even in the absence of elevated intracellular calcium levels (65). Supporting this is the recent finding of Liu et al. (66) that activation of NF-κB in astrocytes by TNF-α is dependent upon metabolic P2Y receptors. Glutamate, which causes a large influx of calcium in neurons, also greatly induces NF-κB DNA-binding activity (20, 67). XeC was able to totally abolish this induction by glutamate, although XeC treatment only partially attenuated the increase in intracellular calcium levels caused by glutamate. Glutamate induces ER calcium release by increasing intracellular calcium (68–70) and by stimulating IP_3 production through phospholipase C activation (71, 72). Therefore, each of the inducers of NF-κB examined in this study (glutamate, TNF-α, and thapsigargin) have in common the ability to initiate calcium release from ER IP_3-sensitive stores.

The cell-free system that was used as a model in our studies consisted of a membrane-free cytoplasmic fraction and an extract taken from ER-rich microsomes after treatment with agents that affect ER calcium pools. We manipulated the ER calcium regulatory proteins with specific agents: thapsigargin to irreversibly inhibit Ca^{2+}-ATPases and to decrease intramicrosomal calcium maximally, XeC to specifically block calcium release from IP_3 stores, IP_3 to mimic the in vitro system of IP_3 receptor activation, and ryanodine to examine the other major pool of ER calcium. Treatment of microsomes with thapsigargin resulted in release into MSE of a factor(s) that activates cytoplasmic NF-κB to a level 3-fold that of the control level. This level of activation is comparable to that seen in intact cultured cells exposed to agents that release ER calcium (Refs. 30 and 73 and this study). Conversely, treatment of microsomes with XeC totally abolished the stimulatory effect of MSE, resulting in NF-κB binding levels that were no different from the cytoplasm alone.

Addition of thapsigargin and XeC directly to the cytoplasmic extract did not alter NF-κB DNA-binding activity, indicating that these factors must work through microsomes to stimulate NF-κB. Furthermore, the fact that neither calcium nor BAPTA was able to directly affect NF-κB DNA-binding activity shows that the calcium released from the microsomes is not the factor that regulates NF-κB. Indeed, the role calcium plays in this paradigm seems to be as an intraluminal signal, such that the depletion of calcium from the IP_3 pool is a signal for release of the NF-κB-activating factor. This is supported by the observation that direct activation of IP_3 receptors on microsomes using IP_3 itself led to MSE that had NF-κB-stimulating properties similar to those seen with thapsigargin. In addition, pretreatment of microsomes with XeC completely abolished this effect. Release of calcium by ER IP_3 receptors results in a decrease in the pool of calcium in those ER stores, and it is this calcium depletion that apparently leads to release of the diffusible factor that stimulates NF-κB activity. Microsomes pretreated with XeC followed by calcium produced no NF-κB-stimulating activity, indicating that although calcium can directly modulate ER calcium release, it has no effect when IP_3 receptors are blocked. Addition of ryanodine to microsomes did not result in an increase in NF-κB-stimulating activity in MSE, indicating that it is the ER IP_3 pool specifically that regulates release of this factor. These findings reveal a heretofore unknown mechanism for modulation of NF-κB activity by the ER and suggest the presence of a novel factor released by microsomes that activates NF-κB. The identity of this factor remains to be determined, but it could conceivably be a protein because it is heat- and trypsin-sensitive and because previous studies have shown that luminal proteins are released from the ER in response to calcium depletion (74).

Increasing data implicate cell calcium-regulating mechanisms as having a central role in governing NF-κB activity. We have shown in our in vitro studies that calcium per se does not change NF-κB binding. However, our cell-free system examined only the NF-κB-signaling pathway from the ER to NF-κB activation. Indeed, in the whole cell, the hypothesis that it is the filling state of the ER IP_3 pool that signals NF-κB activation would place calcium as a major upstream effector. Both IP_3 and ryanodine receptors are sensitive to Ca^{2+}, as is the ER Ca^{2+}-ATPase. In addition, IP_3 production is sensitive to calcium levels (75, 76). In fact, in the absence of calcium, phospholipase C-mediated production of IP_3 drops precipitously (77). Our data suggest that an array of signals ranging from neurotransmitters to neurotrophic factors and cytokines may modulate NF-κB activity, in part, by affecting ER calcium release. Both ER calcium release (78–80) and NF-κB activation (13, 16, 52, 81) play pivotal roles in regulating synaptic plasticity and survival of neurons during development of injury and disease conditions. In addition, an ER overload (unfolded protein) response occurs in cells exposed to a variety of stressors (82) and results in NF-κB activation by a mechanism involving release of calcium from the ER (41). Our findings therefore identify a novel signaling pathway whereby activation of IP_3 receptors results in generation of a diffusible signal that activates a transcription factor known to play important roles in neuronal plasticity and survival.
Endoplasmic Reticulum d-myoinositol 1,4,5-Trisphosphate-sensitive Stores Regulate Nuclear Factor-κB Binding Activity in a Calcium-independent Manner

Gordon W. Glazner, Simonetta Camandola, Jonathan D. Geiger and Mark P. Mattson

J. Biol. Chem. 2001, 276:22461-22467.
doi: 10.1074/jbc.M101315200 originally published online April 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101315200

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 82 references, 30 of which can be accessed free at http://www.jbc.org/content/276/25/22461.full.html#ref-list-1