Elevated Calcium in Preneoplastic Cells Activates NF-κB and Confers Resistance to Apoptosis*

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Early preneoplastic cells (sup+) exhibit increased susceptibility to apoptosis, which is lost in late stage preneoplastic cells (sup−). Sup+ cells, which undergo apoptosis when cultured in low serum, show little or no DNA binding activity to nuclear factor (NF)-κB either in 10% or 0.2% serum. In contrast sup− cells, which are resistant to apoptosis in low serum, show a sustained constitutive activation of NF-κB. The constitutive activation of NF-κB observed in sup− cells is not due to loss of IkBa. We considered that the activation of NF-κB in sup+ cells might be secondary to an increase in cytosolic Ca2+, since sup− cells have a cytosolic Ca2+ level that is double that in sup+ cells. In support of a role for Ca2+, lowering cytosolic Ca2+ in sup− cells by addition of the cell-permeable Ca2+ chelator 1,2 bis(O-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) reduced cytosolic Ca2+ by ~31% relative to untreated sup− cells, concomitant with a 65% reduction in NF-κB DNA binding activity and a reduction in IkB kinase (IKK) activity. In sup− cells in low serum, addition of BAPTA-AM also resulted in a significant (~50%) increase in caspase-3 activity. Raising extracellular Ca2+ in sup+ cells resulted in a slight activation of IkB kinase and in enhanced NF-κB DNA binding activity. Using proteasome and calpain inhibitors, we determined that the basal activity of NF-κB in sup− cells is largely proteasome-independent, but sensitive to calpain inhibitors. Taken together these data suggest that the elevated Ca2+ in sup− cells causes a modest activation of IKK, which likely contributes to the enhanced basal activation of NF-κB in sup− cells; however, the predominant effect of Ca2+ appears to be mediated by Ca2+-enhanced degradation by calpain.

It has been shown that signals that lead to proliferation frequently stimulate apoptosis unless they are counteracted by specific survival signals (1–3). Thus it is not surprising that a common feature of early preneoplastic cells is a susceptibility to apoptosis, which is overcome in later stage neoplastic cells by either stimulating survival signals or by inhibiting apoptotic signals (2, 4). To better understand how cells overcome apoptosis during neoplastic progression, we have examined variants of Syrian hamster embryo cells that have been mutagenized to yield two immortalized cell lines representing different stages of neoplastic progression. It has been shown previously that early preneoplastic cells (sup+) are more susceptible to apoptosis than late preneoplastic cells (sup−) (4). The mechanism involved in this altered susceptibility to apoptosis could have important implications for carcinogenesis.

Many apoptotic stimuli activate NF-κB1 (8, 9), and it was originally assumed that the increase in NF-κB activity might be involved in stimulating apoptosis. However, recent studies have shown that if NF-κB activation is inhibited, apoptosis is enhanced (5–7). Because of the importance of NF-κB in apoptosis we were interested in investigating whether there were differences in NF-κB activity in early versus late preneoplastic cells, as these cells have differing sensitivity to apoptosis.

NF-κB is a transcription factor that is activated by numerous cytokines and stresses. NF-κB is normally maintained in an inactive state in the cytoplasm, because it is bound to inhibitors of the IkB family. Phosphorylation of IkB targets it for degradation, thereby leading to activation of NF-κB. We found a sustained basal activation of NF-κB in late (sup−), but not early stage (sup+) preneoplastic cells. The sustained activation of NF-κB in the sup− cells correlates with their resistance to apoptosis following treatment with medium containing low serum, a condition that leads to apoptosis in the sup+ cells. We examined possible mechanisms for the sustained activation of NF-κB in the sup− cells and noted a correlation with elevated cytosolic calcium. The late preneoplastic sup− cells have a basal cytosolic free Ca2+ level that is more than double that observed in the early preneoplastic sup+ cells. An elevation in cytosolic Ca2+ is commonly noted in transformed cells and has been proposed to be important in signaling cell proliferation (10, 11). In this study, we present data showing that an elevation in cytosolic Ca2+ can also provide survival signals by contributing to a sustained activation of NF-κB.

**Experimental Procedures**

Cell Culture—Two cell lines (sup+ and sup−), originally immortalized via asbestos mutagenesis of Syrian hamster embryo cells, were used in these studies (12). Cells were maintained in Dulbeco’s modified IBR medium containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 37°C incubator with 10% CO2/90% air. Low serum conditions were either 0.2 or 0.8% fetal calf serum, depending upon the age of the serum. As the serum aged, later experiments required the higher percentage of fetal calf serum.

Measurement of NF-κB DNA Binding Activity—Extracts of nuclear proteins were prepared using a modification of the method of Dignam et al. (13). Syrian hamster embryo cells were plated at a density of 1.1 × 10^4 per dish and were allowed to attach for 24 h before treatment. The cells were then washed and incubated with...
10^6 cells/150-mm diameter plate in 20 ml of IBR medium with 10% FCS for 24 h, then switched to various treatments for 16–18 h. Cells were scraped into medium plus 20 ml of cold calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), centrifuged at 480 × g, and then washed in 10 ml of cold CMF-PBS. Following centrifugation, cell pellets were resuspended in 500 µl of lysis buffer (10 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 0.1% IGEPA, 0.5 mM dithiothreitol, 1.0 mM orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each of aprotinin, leupeptin, and pepstatin), vortexed, and the nuclei pelleted by centrifugation at 10,000 × g for 5 min. Nuclear pellets were resuspended in 30 µl of extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 10% glycerol, 0.5 mM dithiothreitol, 1.0 mM orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each of aprotinin, leupeptin, and pepstatin) and centrifuged at 16,000 × g for 15 min. Supernatants were collected and protein content quantified using the Pierce BCA assay.

Binding reactions for NF-κB/DNA electrophoretic mobility shift assays were performed for 20 min at room temperature in binding buffer (15 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM PMSF) using 10 µg of nuclear protein, 169 µg of 32P-labeled oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGG-C'-3'), and 1.5 µg of poly(dI-dC)/poly(dI-dC) in a total volume of 18 µl. Bound complexes were resolved on 6% polyacrylamide nondenaturing gels, and densitometry of autoradiographs was performed using the software package ImageJ.

**Calcium Measurements**—Cytosolic calcium was measured as described previously (14). Briefly, cells were plated at a density of 6 × 10^4 cells/plate in IBR medium with 10% serum in 30-mm plates containing 22-mm diameter round glass coverslips. Fura-2 AM (2 µg/ml, Invitrogen) was added. After 16–18 h, cells (adherent and floating cells) were harvested by scraping into treatment medium followed by centrifugation at 480 × g for 5 min at 4 °C. The cells were resuspended in 5 ml of cold CMF-PBS, centrifuged as above, and the resulting pellets lysed in 40 µl of hypotonic lysis buffer (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 5 mM EDTA, 5 mM dithiothreitol, 2 mM PMSF, 10 µg/ml pepstatin A, 10 µg/ml leupeptin) followed by four cycles of freezing and thawing in liquid nitrogen. The lysate was centrifuged in a 4 °C microcentrifuge for 20 min at 16,000 × g. Supernatants were collected and protein content quantified using the Pierce BCA assay. The caspase-3 activity assay was performed according to the protocol in the CaspACE Assay System (Promega). Data are expressed as caspase activity (change in arbitrary fluorescence units) corrected for background fluorescence measured from a field not containing cells, which was subtracted.

**Caspase-3 Activity Assay**—Cells were seeded in 100-mm plates at a density of 5 × 10^4 cells/plate and grown for 24 h. Cells were washed with CMF-PBS, and 10 ml of the appropriate treatment medium was added. After 16–18 h, cells (adherent and floating cells) were harvested by scraping into treatment medium followed by centrifugation at 480 × g for 5 min at 4 °C. The cells were resuspended in 5 ml of cold CMF-PBS, centrifuged as above, and the resulting pellets lysed in 40 µl of hypotonic lysis buffer (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 5 mM EDTA, 5 mM dithiothreitol, 2 mM PMSF, 10 µg/ml pepstatin A, 10 µg/ml leupeptin) followed by four cycles of freezing and thawing in liquid nitrogen. The lysate was centrifuged in a 4 °C microcentrifuge for 20 min at 16,000 × g. Supernatants were collected and protein content quantified using the Pierce BCA assay. The caspase-3 activity assay was performed according to the protocol in the CaspACE Assay System (Promega). Data are expressed as caspase activity (change in arbitrary fluorescence units) corrected for background caspase activity.

**IKK Activity Assays**—Activity of IκB kinase (IKK) was determined by immunoprecipitation followed by a phosphorylation assay. Cells were seeded in 100-mm diameter plates at a density of 5 × 10^5 cells/plate and grown for 24–40 h before treatments. Whole cell lysates were prepared by scraping the cells into medium, centrifuging at 480 × g, and washing the pellets twice with 5 ml of cold CMF-PBS. Pellets were lysed in 100 µl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 1% Nonidet P-40, 10 mM p-nitrophenyl phosphate, 10 mM β-glycerophosphate, 1 mM benzamidine, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml pepstatin). Lysates were then centrifuged at 14,000 rpm in a microcentrifuge for 30 min at 4 °C and the supernatants taken for immunoprecipitation, which was performed in 500 µl of lysis buffer for 2–3 h at 4 °C using 200 µg of cell lysate and an agarose-conjugated antibody to IκB-α (Santa Cruz Biotechnology catalog number sc-7182AC). Immunoprecipitates were washed four times with lysis buffer and once with kinase buffer (Cell Signaling Technology catalog number 9802), supplemented with 10 mM p-nitrophenyl phosphate, 2 mM MnCl₂, 1 mM benzamidine, 10 mM PMSF, 10 µg/ml aprotinin, and 1 µg/ml leupeptin. Kinase reactions were carried out for 30 min at 30 °C in 200 µl of kinase buffer containing 2 µg of GST-IκB-α substrate (Santa Cruz Biotechnology catalog number 4094), 10 µg of [γ-32P]ATP, and 10 µM ATP. Reactions were terminated with 5x Laemmli sample buffer and boiled 5 min at 95 °C before performing SDS-polyacrylamide gel electrophoresis using 8% Tris-glycine gels. Densitometry of autoradiographs was performed using NIH image.

**Western Blots**—Cells were plated at a density of 1.1 × 10^6 cells/150-mm diameter plate in 20 ml of IBR medium with 10% serum for 24 h, then switched to various treatments for 16–18 h. Whole cell lysates were prepared by scraping the cells into medium plus 20 ml of cold CMF-PBS, centrifuging at 480 × g, and then washing twice in 5 ml of cold CMF-PBS. Pellets were then lysed in 500 µl of lysis buffer (1% Nonidet P-40, 0.5% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4) supplemented with protease and phosphatase inhibitors (2 µg/ml leupeptin, 2 µg/ml pepstatin A, 2 µg/ml aprotinin, 400 µM PMSF, 1 mM sodium orthovanadate). Protein concentrations were determined using the Pierce BCA assay, and 25 µg of total protein per lane was loaded on a 10% acrylamide SDS-polyacrylamide gel electrophoresis gel. Following Western blotting to a nitrocellulose membrane, 1B-α and 1B-β were detected using Santa Cruz antibodies sc-371 and sc-945, respectively, and Bcl-2 was detected using Transduction Laboratories antibody number B46620.

**MATERIALS**—The NF-κB oligonucleotide probe was purchased from Promega, proteasome K from Boehringer-Mannheim, and RNase A from Sigma-Aldrich. IBR medium was obtained from Life Technologies Inc., and fetal calf serum was obtained from Summit Technologies.

**Statistics**—Results are expressed as mean ± S.E. For comparison between two groups, statistical significance was determined by Student's t test. For multiparameter comparisons, statistical significance was determined by analysis of variance, adjusting for multiple comparisons using Tukey's post hoc test. A value of p < 0.05 was considered to be significant.

**RESULTS**

Previous studies have shown that early stage preneoplastic sup+ cells have enhanced susceptibility to apoptosis under antiproliferative conditions such as treatment with low serum, whereas later stage preneoplastic sup− cells do not (4). We were interested in determining the basis for this difference in susceptibility to apoptosis. We examined whether there were differences in Bcl-2 expression levels between sup+ and sup− cells. As shown in Fig. 1, there is no significant difference in Bcl-2 levels between sup+ and sup− cells. Because NF-κB activation has been shown to be anti-apoptotic, we next investigated whether differences in NF-κB activation between the two cell lines might explain the difference in apoptotic susceptibility. To determine whether NF-κB activation occurs in sup+ versus sup− cells, we cultured each cell line in medium supplemented with 10% FCS for 24 h. The medium containing 10% FCS was then replaced with medium containing either 10% or low serum for an additional 18 h prior to cell harvest. Extracts enriched for nuclear proteins were prepared and analyzed by electrophoretic mobility shift assay (EMSA). Sup+ cells showed relatively little DNA binding activity of NF-κB, either in 10% or low serum (Fig. 2, lanes 1 and 3). In contrast, sup− cells showed constitutive NF-κB activation in 10% FCS (3.5 times sup+ levels in 10% serum) and

![Fig. 1. Western blot showing Bcl-2 levels in sup+ and sup- cells.](image-url)
of IκB and decreased susceptibility to apoptosis in low serum, we tested whether pharmacological lowering of NF-κB in sup–cells would render them more susceptible to apoptosis. BAY 11-7082 has been reported to inhibit phosphorylation of IκB and thus to decrease NF-κB activation (15, 16). Sup–cells were cultured for 24 h and then treated for 18 h with 1 or 2 μM BAY 11-7082. NF-κB activation was decreased with BAY 11-7082 treatment (Fig. 3A). BAY 11-7082 treatment also inhibited IKK activity (Fig. 3B). Similarly treated sup–cells were assayed for apoptotic induction using caspase-3 activity as a measure of apoptosis after 18 h treatment in low FCS, with and without BAY 11-7082. As shown in Fig. 3C, caspase-3 activity was significantly higher in low serum–treated sup–cells in the presence of BAY 11-7082, compared with sup–cells in low serum alone. Fig. 3C further shows that caspase-3 activity in sup–cells in low serum plus BAY 11-7082 was similar to that in sup+ cells in low serum.

We were interested in the mechanism responsible for the constitutive activation of NF-κB in the sup–cells. Constitutive activation of NF-κB is observed in many tumor and viral-infected cells. Some tumor cells have constitutive activation of NF-κB due to mutation or loss of IκBα (17, 18). However, loss of IκBα does not account for the constitutive activation of NF-κB in sup–cells, because we found slightly increased levels of IκBα in sup–versus sup+ cells (see Fig. 4A). An increase in IκBα in sup–cells is consistent with constitutive activation of NF-κB in these cells, because expression of IκBα has been shown to be up-regulated by NF-κB (19, 20). Constitutive activation of NF-κB can also occur because of hypophosphorylation of IκBβ (21). However, as shown in Fig. 4B, IκBβ has a similar electrophoretic mobility in sup+ and sup–cells, suggesting a similar level of phosphorylation.

Interestingly, as shown in Fig. 5, sup–cells, which have constitutive activation of NF-κB, have a 2-fold increase in cytosolic Ca2+ compared with sup+ cells, which lack basal NF-κB activation. Cytosolic free Ca2+ in sup–cells was 220 ± 26 nM, a value double that measured in the sup+ cells (97 ± 18 nM). An elevation in Ca2+ has been suggested to activate NF-κB (22–26). To determine whether the constitutive activation of NF-κB in sup–cells is related to the observed increase in cytosolic free Ca2+, we treated sup–cells in medium plus 10% serum with the cell-permeable Ca2+ chelator BAPTA-AM. Cells were grown for 24 h and then treated with 10 μM BAPTA-AM for 1 or 2 h. Base-line NF-κB binding activity decreased to ~34% of control at 1 and 2 h after treatment with BAPTA-AM (Fig. 6). To determine whether BAPTA-AM treatment in fact lowered cytosolic Ca2+ levels, intracellular Ca2+ was measured spectrophotometrically in fura-2-loaded cells. Cells were treated with BAPTA-AM for 2 h, loaded with fura-2 AM for 30 min, washed in PBS, and fluorescence was measured. Cytosolic Ca2+ levels of BAPTA-AM-treated sup–cells decreased by ~31% compared with untreated sup–cells (Fig. 7). Thus, lowering cytosolic Ca2+ levels with BAPTA-AM also lowers NF-κB binding activity. Furthermore, as shown in Fig. 8, lowering cytosolic Ca2+ with BAPTA-AM resulted in a significant increase in caspase-3 activity measured at 4 h after treatment of sup–cells in low serum. Sup–cells placed in low serum showed only minimal activation of caspase-3. However,
lowering cytosolic Ca\textsuperscript{2+} with BAPTA-AM resulted in a 48% increase in caspase-3 activity (Fig. 8). Caspase-3 activity was measured at 4 h to ensure that measurements were made at a time point comparable with the NF-\kappa B and cytosolic Ca\textsuperscript{2+} measurements, as well as to ensure that BAPTA-AM was still present in the cells. These data suggest that the lowering cytosolic Ca\textsuperscript{2+} in the sup\textsuperscript{+} cells causes a decrease in NF-\kappa B activation and an increased susceptibility to apoptosis. Thus, reducing NF-\kappa B in sup\textsuperscript{+} cells increases apoptosis, as would be expected if activation of NF-\kappa B in sup\textsuperscript{+} cells is responsible for their observed resistance to apoptosis.

We also tested whether elevating calcium in sup\textsuperscript{−} cells by raising extracellular calcium would alter NF-\kappa B. As shown in Fig. 9, raising extracellular Ca\textsuperscript{2+} to 3 mM also resulted in a slight but significant activation of NF-\kappa B. We also investigated whether altering cell calcium altered the activity of IKK. As shown in Fig. 10, raising extracellular calcium also caused a significant, but modest, 39% increase in IKK activity in sup\textsuperscript{+} cells. In contrast, lowering cytosolic calcium with addition of BAPTA-AM significantly decreased (by 66%) IKK activity in sup\textsuperscript{−} cells.

Raising extracellular Ca\textsuperscript{2+} in sup\textsuperscript{+} has a very modest effect of NF-\kappa B activation and IKK activity. These data contrast with the robust Ca\textsuperscript{2+} dependence of IKK and NF-\kappa B activity in sup\textsuperscript{−} cells. Fig. 11 shows the relationship of cytosolic Ca\textsuperscript{2+} to NF-\kappa B activation and to IKK activity, standardized to their activities in sup\textsuperscript{+} cells in 10% FCS. In sup\textsuperscript{+} cells, increased cytosolic Ca\textsuperscript{2+} results in a proportionately similar increase in IKK and NF-\kappa B. Sup\textsuperscript{−} cells show a Ca\textsuperscript{2+}-related increase in IKK, which is similar to that observed in sup\textsuperscript{+} cells; however, in sup\textsuperscript{−} cells...
increased Ca\textsuperscript{2+} correlates with a much larger percentage increase in NF-\kappaB activity versus IKK activity. These data suggest that a large proportion of the enhancement of NF-\kappaB activation attributable to Ca\textsuperscript{2+} is not mediated by IKK.

To better understand the mechanism involved in the Ca\textsuperscript{2+}-induced activation of NF-\kappaB, we examined the effect of proteasome and calpain inhibition on the basal activity of NF-\kappaB in sup\textsuperscript{+} cells. Fig. 12A shows that neither the proteasome inhibitor lactacystin nor proteasome inhibitor-I reduced the level of basal NF-\kappaB activity. Fig. 12A further demonstrates that the inability of proteasome inhibitors to block basal NF-\kappaB activation is not due to lack of an effective concentration, because both inhibitors significantly inhibited the LPS-induced activation of NF-\kappaB. Since the basal NF-\kappaB activity in sup\textsuperscript{−} cells is Ca\textsuperscript{2+}-dependent, but proteasome-independent, we examined whether calpain inhibition would affect basal NF-\kappaB activity. As shown in Fig. 12B, the calpain inhibitors EST and calpain inhibitor-V significantly reduced basal NF-\kappaB activity. Taken together these data suggest that the elevated Ca\textsuperscript{2+} in sup\textsuperscript{−} cells causes a modest activation of IKK, which likely contributes to the enhanced basal activation of NF-\kappaB in sup\textsuperscript{−} cells; however, the predominant effect of Ca\textsuperscript{2+} appears to be mediated by Ca\textsuperscript{2+}-enhanced degradation by calpain.

DISCUSSION

Two preneoplastic Syrian hamster embryonic cell lines representing different stages of neoplastic progression have been used for these studies. Early stage preneoplastic cells (sup\textsuperscript{+}) have been shown to suppress tumorigenicity when hybridized with tumor cells, whereas sup\textsuperscript{−} cells do not suppress tumorigenicity in cell hybrids. The RB and p53 genes are wild type in both cell types. Previous studies have shown that the sup\textsuperscript{−} cells are more susceptible to apoptosis than the late stage preneoplastic sup\textsuperscript{−} cells (4, 27).

In this paper we report that late stage preneoplastic cells that are resistant to apoptosis have a high basal level of NF-\kappaB activation. As NF-\kappaB has been reported to be anti-apoptotic, we examined whether pharmacologically reducing NF-\kappaB would enhance apoptosis. As shown in Figs. 3 and 8,
preneoplastic cells often exhibit enhanced apoptosis (28). It has been shown that activation of cell proliferation can lead to apoptosis unless cell survival pathways such as NF-κB are also activated (1–3). This study suggests that increased activation of NF-κB is involved in the reduced apoptotic susceptibility of the late stage preneoplastic sup cells. To gain insight into how cells activate NF-κB as part of the neoplastic progression, we investigated the mechanism by which NF-κB is activated under basal conditions in sup cells. Loss or mutation of IκBα has been reported to activate NF-κB in some tumor cells (17, 18). However, loss of IκBα does not account for the enhanced NF-κB levels in sup cells. In fact we find enhanced levels of IκBα in sup cells, consistent with NF-κB regulation of the promoter for IκBα (19, 20). Hypophosphorylated IκBα has also been reported to lead to constitutive activation of NF-κB (21). However, we find no evidence for hypophosphorylation of IκBα.

There are recent data suggesting that Ca2+ can modulate NF-κB activity (22–26, 29–34). Since we find a greater than 2-fold higher basal cytosolic Ca2+ concentration in sup cells compared with sup cells, we considered the possibility that the elevation in cytosolic Ca2+ might be involved in the increased NF-κB activation observed in the sup cells. In support of this hypothesis, we report that lowering cytosolic Ca2+ by addition of the cell-permeant Ca2+ chelator BAPTA-AM to sup cells reduced NF-κB activation and significantly reduced IKK activity. Furthermore, the BAPTA-AM-dependent lowering of NF-κB binding also rendered the sup cells susceptible to apoptosis in low serum. These data suggest that the increased NF-κB binding in the sup cells is secondary to the increase in Ca2+ and is responsible for the reduced apoptosis observed in the sup cells.

An elevation in cytosolic Ca2+ is a common observation in transformed cells (10). It has been shown that lowering extracellular Ca2+ results in a reversible block of the cell cycle in normal cells, whereas SV40-transformed cells continue to proliferate in low Ca2+ media (11). The data in this manuscript are consistent with the hypothesis that high cytosolic Ca2+ in transformed cells may provide the cell with a reduced susceptibility to undergo apoptosis by activating NF-κB, an anti-apoptotic factor.

The mechanism by which Ca2+ regulates NF-κB is unclear, and there are data to suggest that multiple pathways may be involved (22–26, 31–34). Ca2+ is reported to activate IKK (via calcineurin, see Refs. 23 and 32), and Ca2+ is also reported to activate calpain-dependent degradation of IκB (31, 33). Lowering Ca2+ in the sup cells causes a large reduction in NF-κB, consistent with the hypothesis that the higher Ca2+ in sup cells is necessary for the higher basal NF-κB. However, raising Ca2+ in the sup cells only slightly elevates NF-κB and is not sufficient to elevate NF-κB to the same level as found in sup cells. Thus the data suggest that high Ca2+ in the sup cells is required for activation of NF-κB, but raising Ca2+ in the sup cells is not sufficient. Furthermore, the data in Fig. 11 suggest that the constitutive activation of NF-κB in sup cells is Ca2+-dependent, but largely IKK-independent. These data are consistent with Ca2+ activation of NF-κB in sup cells being primarily mediated by calpain. Calpain is a Ca2+-activated protease, which has been reported to play a role in the degradation of IκBα (31), and calpain 3 deficiency is associated with increased apoptosis in limb-girdle muscular dystrophy type 2A (33). Also, these data are similar to those observed in primary B cells that have constitutive activation of NF-κB. Fields et al. (30) have reported that the constitutive activation of NF-κB in B lymphocytes is calcium-dependent, but proteasome-independent.

In summary, many transformed cells exhibit an increase in cytosolic Ca2+. We demonstrate that lowering cytosolic Ca2+ reduces the activation of NF-κB and confers susceptibility to apoptosis.

![Graph showing relationship between NF-κB binding activity and IKK activity in sup cells.](image)

**Fig. 11.** Relationship of NF-κB binding activity and IKK activity to cytosolic Ca2+ levels. NF-κB binding activity, IKK activity, and calcium are expressed as a percentage of the respective sup 10% FCS controls. Calcium levels for sup cells were measured in 10% FCS or in 10% FCS supplemented with 3 mM Ca2+. Calcium levels for sup cells were measured in 10% FCS or in 10% FCS plus 10 μM BAPTA-AM (4-h treatment).

![Graph showing effects of proteasome inhibitors and calpain inhibitors on basal NF-κB binding activity in sup cells.](image)

**Fig. 12.** Effects of proteasome inhibitors and calpain inhibitors on basal NF-κB binding activity in sup cells. A, extracts for EMSA’s were prepared from sup cells treated for 4 h with either 20 μM lactacystin, 10 μM proteasome inhibitor-I, 1 μg/ml LPS, or a 30 min pretreatment with proteasome inhibitors followed by LPS treatment. B, extracts for EMSA’s were prepared from sup cells treated for 4 h with the calpain inhibitors EST (20 μM) or calpain inhibitor-V (20 μM). *, indicates significantly different from control; **, indicates significantly different from LPS (p < 0.05).
apoptosis in sup− cells. We further show that the constitutive activation of NF-κB observed in sup− cells is proteasome-independent. Taken together these data suggest that an elevation in Ca2+, which is common in tumor cells, may provide these cells with resistance to apoptosis via activation of NF-κB.

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