Characterization of Calcium Release-activated Apoptosis of LNCaP Prostate Cancer Cells*

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Apoptosis inhibition rather than enhanced cellular proliferation occurs in prostate cancer (CaP), the most commonly diagnosed malignancy in American men. Therefore, it is important to characterize residual apoptotic pathways in CaP cells. When intracellular Ca$^{2+}$ stores are released and plasma membrane "store-operated" Ca$^{2+}$ entry channels subsequently open, cytosolic [Ca$^{2+}$] increases and is thought to induce apoptosis. However, cells incapable of releasing Ca$^{2+}$ stores are resistant to apoptotic stimuli, indicating that Ca$^{2+}$ store release is also important. We investigated whether release of intracellular Ca$^{2+}$ stores is sufficient to induce apoptosis of the CaP cell line LNCaP. We developed a method to release stored Ca$^{2+}$ without elevating cytosolic [Ca$^{2+}$]; this stimulus induced LNCaP cell apoptosis. We compared the apoptotic pathways activated by intracellular Ca$^{2+}$ store release with the dual insults of store release and cytosolic [Ca$^{2+}$] elevation. Earlier processing of caspases-3 and -7 occurred when intracellular store release was the sole Ca$^{2+}$ perturbation. Apoptosis was attenuated in both conditions in stable transfectected cells expressing antiapoptotic proteins BclxL and catalytically inactive caspase-9, and in both scenarios inactive caspase-9 became complexed with caspase-7. Thus, intracellular Ca$^{2+}$ store release initiates an apoptotic pathway similar to that elicited by the dual stimuli of cytosolic [Ca$^{2+}$] elevation and intracellular store release.

Apoptosis is the physiological counterbalance to mitosis and a fundamental mechanism for achieving tissue homeostasis (1). Accordingly, disorders of apoptosis contribute to a number of disease states. Degenerative conditions, such as Alzheimer’s disease and AIDS, occur in the setting of excess apoptosis, whereas hyperplastic diseases, including cancer, often occur when apoptosis is attenuated (2). Specifically, inhibition of apoptosis rather than enhanced cellular proliferation is the most critical pathophysiological insult that contributes to the development of prostatic adenocarcinoma (CaP) (3, 4). CaP is the most commonly diagnosed malignancy in American men and is the second most common cause of death due to cancer, with estimates that 37,000 men died from CaP in 1999 (5).

Successful CaP therapies have been shown to induce tumor regression via apoptosis; however, therapy-resistant cells fail to die in response to treatment (6). It is therefore important to characterize the residual functional apoptotic pathways in therapy-resistant CaP cells. Perturbation of calcium ion (Ca$^{2+}$) homeostasis is a common occurrence in CaP cells induced to undergo apoptosis by a variety of agents, including chemotherapeutics (7) and androgen ablation (8, 9).

Although the influx of Ca$^{2+}$ across the cell membrane results in a dramatic increase in cytosolic [Ca$^{2+}$], this influx is often invoked by a less readily detectable cytosolic [Ca$^{2+}$] elevation as a consequence of organelar Ca$^{2+}$ store release (10). For example, the endoplasmic reticulum (ER) serves as an intracellular Ca$^{2+}$ store that is released into the cytosol in response to a variety of stimuli, including binding of inositol 1,4,5-trisphosphate (IP$_3$) to its cognate ER-localized receptor (IP$_3$R) (11). The release of ER Ca$^{2+}$ stores in turn induces a Ca$^{2+}$ influx across the plasma membrane, also known as the Ca$^{2+}$ release-activated Ca$^{2+}$ current (I$_{URAC}$) (12). Thus, the release of ER Ca$^{2+}$ stores serves to trigger a larger perturbation in cytosolic [Ca$^{2+}$].

Studies with an IP$_3$R-deficient Jurkat T-lymphocyte cell line underscore the importance of ER Ca$^{2+}$ stores in cellular signaling. In contrast to the parental cell line, these cells are defective in antigen-specific T-cell signaling and are resistant to multiple inducers of apoptosis, including Fas ligand, CD3 antibody, glucocorticoids, and irradiation (13). These results are of interest because Jurkat cells have been used to study the role of caspases in apoptosis. Caspases are aspartate-specific cysteine proteases that constitute the effector arm of cell death (14). Upon receipt of an apoptotic stimulus, large pro-domain initiator caspases (such as caspase-8 and -9) are activated by autoproteolysis, which converts the single polypeptide zymogen to form the active dimeric protease. Caspase-8 is activated when it is recruited by the adapter molecule FADD (Fas/APO-1-associated death domain protein) to death receptors upon extracellular ligand binding (15, 16). Caspase-9 is activated when cells are subjected to intracellular insults that result in the release of cytochrome c from damaged mitochondria. In the presence of cytochrome c the ATPase adapter molecule Apaf-1

ER, endoplasmic reticulum; IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, inositol 1,4,5-trisphosphate receptor; I$_{URAC}$, calcium release-activated calcium current; Apaf-1, apoptotic protease activating factor-1; TG, thapsigargin; SERCA, sarco/endo/sarcoplasmic/endoplasmic reticulum calcium ATPase; Me$_2$SO, dimethyl sulfoxide; CPA, cyclopiazonic acid; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks’ balanced salt solution; AM, acetoxymethyl ester; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; caspase-9 DN-FLAG, FLAG-tagged dominant negative caspase-9; PCNA, proliferating cell nuclear antigen.
binds to and activates caspase-9 in a reaction requiring ATP or dATP hydrolysis (17, 18). Initiator caspases amplify the death signal by proteolytically processing and activating downstream small pro-domain effector caspases (such as caspase-3 and -7). Activated effector caspases subsequently cleave a variety of apoptotic substrates, resulting in the biochemical and morphological changes that characterize apoptosis (19).

Because IP₃-R-deficient Jurkat cells are incapable of mobilizing Ca²⁺ stores and are resistant to apoptosis, the question has arisen as to whether release of ER Ca²⁺ is by itself sufficient to induce apoptosis. To address this we chose to study thapsigargin (TG)-induced [Ca²⁺] perturbations in the human Ca²⁺-sensitive Ca²⁺ store release-independent of IP₃-R-mediated cytosolic [Ca²⁺] elevation, we developed a technique to hold cytosolic [Ca²⁺] constant after TG-mediated ER store release. This release of ER [Ca²⁺] was found to be sufficient to induce apoptosis of LNCAp cells. To determine whether the apoptotic pathway elicited by ER Ca²⁺ store release differed from that elicited by the dual perturbations of cytosolic [Ca²⁺] increase and ER Ca²⁺ store release, we compared the caspase activation profiles evoked by each stimulus as well as the effects of antiapoptotic proteins.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagent Treatment Conditions—LNCAp human prostatic carcinoma cells were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% l-glutamine. For dose-response studies, 1 × 10⁵ cells were seeded in duplicate 6-well plates. Indicated concentrations of the drugs were achieved by adding 1000× stock solutions dissolved in Me₂SO. Untreated cell culture medium and medium with 0.1% Me₂SO were used as controls. Cells were trypsinized with 10 μg/ml trypsin/EDTA (Invitrogen, San Diego, CA) with a 5 min incubation at 37 °C and washed. Final concentrations of drug stock (Molecular Probes, Eugene, OR) were prepared in 20% Pluronic F-127 (Molecular Probes) in anhydrous Me₂SO and was diluted in HBSS for a final concentration of 3 μM Fura-2/AM. Cells were loaded (30 min, 37 °C) in 2.75 ml of this solution and washed (2 × 2 ml of HBSS). Fluorescence ratios (340/380-nm excitation) were measured using a spectrophotometer equipped with an xenon lamp (model F-2000, Hitachi, San Jose, CA) with a 510-nm filter and stored at 4 °C. Because IP₃-R-deficient Jurkat cells are incapable of mobilizing Ca²⁺ stores, Jurkat cells were transfected with antiapoptotic proteins, multiple time points for each condition were assessed and results reported are for those time points that gave the most significant results. This occurred at 24 h of TG treatment for cells in control [Ca²⁺] conditions and at 18 h of TG treatment for cells under the [Ca²⁺] clamp conditions.

Ca²⁺ Imaging—The [Ca²⁺] Imaging protocol was performed as previously described (27). In brief, 1 × 10⁶ cells were plated onto 9 × 22-mm poly-L-lysine-coated glass coverslips in 35-mm plates and washed with 2 ml of HBSS (Life Technologies, Inc.). HBSS was warmed to 37 °C for preparation of TG. Fura-2/AM stock solutions (Molecular Probes) were prepared in 20% Pluronic F-127 (Molecular Probes) in anhydrous Me₂SO and was diluted in HBSS for a final concentration of 3 μM Fura-2/AM. Cells were loaded (30 min, 37 °C) in 2.75 ml of this solution and washed (2 × 2 ml of HBSS). Fluorescence ratios (340/380-nm excitation) were measured using a spectrophotometer equipped with an xenon lamp (model F-2000, Hitachi, San Jose, CA) with a 510-nm filter and stored at 4 °C.

Intracellular [Ca²⁺] Analysis—For intracellular [Ca²⁺] analysis of cytosolic [Ca²⁺] was measured using Indo-1/AM or Indo-1/AM loading. All [Ca²⁺] measurements were performed in HBSS having [Ca²⁺] matched to the cell culture medium [Ca²⁺], because [Ca²⁺] measurements in the culture medium, even without phenol red, produced lower signals (data not shown).

Expression Vectors—The expression construct encoding FLAG-tagged dominant negative caspase-9 (caspase-9 DN-FLAG) has been previously described (28). Full length BclxL was cloned in-frame into the pcDNA3.1(-) myc-his vector (Invitrogen, San Diego, CA).

Generation of Stable Cell Lines—Stable LNCAp cell lines were generated using CaPO₄ precipitation-mediated transfection. Following transfection, cells were cultured in supplemented RPMI 1640 culture medium with 380 μg/ml Geneticin (Life Technologies, Inc.).

Immunoprecipitation and Western Blot Analysis—For caspase analysis, we used the method of the manufacturer (40). BclxL cells treated with 10 μM PMA and 1%L-glutamine. Full length BclxL was cloned in-frame into the pcDNA3.1(-) myc-his vector (Invitrogen, San Diego, CA).

Ca²⁺ Release-activated Apoptosis of LNCaP Prostate Cancer Cells

Electron Microscopy—Cells were seeded onto 12-well plates, treated as indicated, and fixed with Karnovsky’s fixative following by postfixation in osmium tetroxide in 0.1 M Sorenson’s buffer. After fixation, cells were dehydrated in a graded series of acetone and embedded in epoxy resin. Sections were cut en face and stained with both uranyl acetate and lead citrate. Electron micrographs were photographed using a Philips EM-400 electron microscope.

Flow Cytometric Analysis of Hypodiploid DNA—Flow cytometric analysis was performed as previously described (25). For analysis of LNCAp cells transfected with antiapoptotic proteins, multiple time points for each condition were assessed and results reported are for those time points that gave the most significant results. This occurred at 24 h of TG treatment for cells in control [Ca²⁺] conditions and at 18 h of TG treatment for cells under the [Ca²⁺] clamp conditions.

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Measurements of Solutions—[Ca²⁺] Measurement of Solutions was performed using a Ca²⁺ pump inhibition, ER Ca²⁺ stores are rapidly released into the cytosol via ER Ca²⁺ leak channels, thereby activating an IC₃R-mediated sustained elevation of cytosolic [Ca²⁺] (22). To study the effects of ER Ca²⁺ store release independent of IC₃R-mediated cytosolic [Ca²⁺] elevation, we developed a technique to hold cytosolic [Ca²⁺] constant after TG-mediated ER store release. This release of ER [Ca²⁺] was found to be sufficient to induce apoptosis of LNCAp cells. To determine whether the apoptotic pathway elicited by ER Ca²⁺ store release differed from that elicited by the dual perturbations of cytosolic [Ca²⁺] increase and ER Ca²⁺ store release, we compared the caspase activation profiles evoked by each stimulus as well as the effects of antiapoptotic proteins.

DNA Extraction and Analysis—Internucleosomal DNA fragmentation as a consequence of TG treatment was evaluated using a modification of the original method (23). LNCAp cells treated with 10 μM PMA served as the positive control for inducing internucleosomal DNA fragmentation (24). LNCAp cells cultured in 0.1% Me₂SO and untreated cells served as the negative control. In brief, 2 × 10⁶ cells were seeded in duplicate 100-mm plates, treated as indicated, and collected at the appropriate time point. Cells were lysed (10 volumes of 25 mM sodium acetate buffer, pH 6.6) and centrifuged (12,400 × g, 45 min) to remove insoluble DNA. The supernatant containing soluble DNA was precipitated (1 volume of 0.13 M NaCl in 70% ethanol) and recovered by vacuum centrifugation. The DNA was then dissolved (50 μl of 10 mM Tris buffer with 1 mM EDTA and 1% SDS, pH 7.4), extracted (4 × 50 μl, 24:1 chloroform/isomyl alcohol) and resolved by electrophoresis on an agarose gel (1.8% for 4 h at 75 V). Fragment size was calibrated using a 1 Kb ladder (Life Technologies, Inc.).

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samples were rotated for an additional 2 h. The beads were recovered by centrifugation, washed three times in lysis buffer, boiled in sample buffer to elute bound proteins, and the divided samples were recombined and resolved on 4–20% (caspase 7) or 10–20% (caspase-9) acrylamide gels. Immunoprecipitated caspase-7 was detected by immunoblotting using 1 μg/ml anti-caspase-7 antibody (GNE clone 2347) and caspase-9 using a monoclonal antibody mixture (gift from Yuri Lazebnik, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY plus 4 μg/ml PharMingen clone B40).

For expression analysis of transfected cell lines, standard Western blot protocols were followed as above using 40 μg of cellular lysates and a monoclonal antibody to BclxL (2 μg/ml, Pharmingen), the anti-caspase-9 antibody mixture described above, or a polyclonal antibody to neomycin phosphotransferase II for control pcDNA3-transfected cells (15 μg/ml, 5 Prime → 3 Prime, Inc., Boulder, CO). Positive controls included BclxL/D or caspase-9 recombinant protein and lysate from a caspase-9 DN stable transfected LNCaP cell for neomycin phosphotransferase II analysis. Wild-type LNCaP cell lysate served as the negative control in all cases.

RESULTS AND DISCUSSION

TG Induces LNCaP Cell Apoptosis—To determine the optimal TG concentration for inducing LNCaP cell death, lethality curves as functions of both dose and time were established (Fig. 1A). Most cell death occurred between 24 and 48 h using 100 nM TG. Based on these results and on phase-contrast microscopic evaluation of cellular morphology, treatment with 100 nM TG was chosen as the standard concentration for inducing LNCaP cell death. This is consistent with the concentration needed for complete SERCA pump blockade (29). We used the structurally unrelated SERCA pump inhibitor cyclopiazonic acid (CPA) to confirm that SERCA pump blockade was indeed the mechanism responsible for TG-induced LNCaP cell death. At 100 μM, a concentration also consistent with complete SERCA pump blockade (29), CPA induced LNCaP cell death following a similar time course as TG-induced lethality (data not shown). Because the only similarity shared between TG and CPA is the ability to inhibit SERCA pumps (29), we concluded that the [Ca^{2+}] perturbation induced by SERCA pump inhibition was likely responsible for inducing apoptosis in LNCaP cells.

Because cell death can occur via apoptosis or necrosis, it was important to determine which mechanism was deployed in TG-treated LNCaP cells. Apoptosis is characterized by a num-
number of morphological and biochemical events that distinguish it from necrosis. Whether a cell undergoes apoptotic or necrotic demise is cell type- and stimulus-specific (30). TG-induced cell death was therefore assessed by several measures. These included: 1) electron microscopic analysis, which revealed morphological changes characteristic of apoptosis, including nuclear fragmentation and plasma membrane blebbing (Fig. 1B); 2) internucleosomal DNA fragmentation, the biochemical hallmark of apoptosis (Fig. 1C); and 3) flow cytometric analysis of DNA, demonstrating the progressive accumulation of hypodiploid DNA (Fig. 1D). Together, these observations confirmed that TG treatment of LNCaP cells induced apoptosis.

Establishment of Cytosolic [Ca\(^{2+}\)] Clamp Conditions—TG irreversibly inhibits the SERCA-type Ca\(^{2+}\)-pumps, which lead to two distinct intracellular [Ca\(^{2+}\)] perturbations: release of ER Ca\(^{2+}\) stores and a sustained increase in cytosolic [Ca\(^{2+}\)] via I\(_{\text{CRAC}}\) (22). The Ca\(^{2+}\) indicator dyes Indo-1 and Fura-2 were used to characterize these TG-induced intracellular [Ca\(^{2+}\)] fluctuations. Indo-1 was used to measure and compare the intracellular [Ca\(^{2+}\)] of individual cells, and Fura-2 was used to measure the intracellular [Ca\(^{2+}\)] of cell populations. Both Ca\(^{2+}\) dyes shared similar profiles, and Indo-1 analysis revealed that intercellular variation in cytosolic [Ca\(^{2+}\)] was minimal (data not shown). Following exposure to 100 nM TG, a sustained plateau in cytosolic [Ca\(^{2+}\)] was induced in LNCaP cells (Fig. 2A). To determine the relative contribution of each TG-induced [Ca\(^{2+}\)] perturbation to the induction of LNCaP apoptosis, conditions were established to permit the release of intracellular Ca\(^{2+}\) stores while maintaining a constant (“clamped”) cytosolic [Ca\(^{2+}\)]. We reasoned that, if the [Ca\(^{2+}\)] clamp conditions protected LNCaP cells from TG-induced apoptosis, this would suggest that elevation of cytosolic [Ca\(^{2+}\)] rather than release of intracellular Ca\(^{2+}\) stores was critical for the induction of apoptosis. However, if apoptosis nevertheless proceeded under these conditions, then release of intracellular Ca\(^{2+}\) stores was likely a critical factor.

We first attempted to clamp cytosolic [Ca\(^{2+}\)] using the fast-acting Ca\(^{2+}\) chelator BAPTA. When LNCaP cells were loaded in 5–30 \(\mu\)M extracellular BAPTA, the TG-induced rise in cytosolic [Ca\(^{2+}\)] was only transiently attenuated (Fig. 2B). This was consistent with previous reports that the buffering capacity of BAPTA is overwhelmed with time (31). Extracellular BAPTA concentrations greater than 30 \(\mu\)M were unsatisfactory because they induced LNCaP cell death (data not shown). Because it was also noted that the transient buffering capacity of BAPTA diminished after 6–8 h, possibly due to intracellular compartmentalization, export, or hydrolysis (26) (data not shown), we decided to replenish BAPTA every 6 h, which maintained an effective cytosolic BAPTA concentration (Fig. 2).

Because BAPTA loading alone was ineffective in preventing TG-induced elevation of cytosolic [Ca\(^{2+}\)], we decided to reduce I\(_{\text{CRAC}}\)-mediated Ca\(^{2+}\) influx using econazole and NiCl\(_2\), reagents that block depletion-activated Ca\(^{2+}\) channels (32). Econazole and NiCl\(_2\) were unsatisfactory, because concentrations inhibiting TG-induced Ca\(^{2+}\) influx were lethal over 12–24 h (50 \(\mu\)M and 5 mM, respectively) and lower concentrations were ineffective (data not shown). However, these studies indicated that Ca\(^{2+}\) store release-activated Ca\(^{2+}\) channels are present and functional on LNCaP cell membranes (33, 34).

Because econazole and NiCl\(_2\) were unsatisfactory for attenuating TG-induced Ca\(^{2+}\) influx, EGTA was added to the extracellular medium to reduce the [Ca\(^{2+}\)] gradient across the plasma membrane. To determine if LNCaP cells could be grown in reduced extracellular [Ca\(^{2+}\)], cells were cultured in medium having [Ca\(^{2+}\)] ranging from 6 to 165 \(\mu\)M, and viability was assessed after 60 h. The TG-induced I\(_{\text{CRAC}}\) was negligible in extracellular [Ca\(^{2+}\)] up to 120 \(\mu\)M (Fig. 2C), and decreased viability was detected in medium having 80 \(\mu\)M [Ca\(^{2+}\)] or less (data not shown). Thus, the range of extracellular [Ca\(^{2+}\)] that permitted cell viability without contributing to a TG-induced cytosolic [Ca\(^{2+}\)] increase was 90–120 \(\mu\)M. However, as expected, the approximately 0.1-\(\mu\)M increase in cytosolic [Ca\(^{2+}\)] resulting from intracellular store release was not attenuated by extracellular EGTA (Fig. 2C). Given this, we decided to use

**Fig. 2.** Fura-2 analysis of intracellular [Ca\(^{2+}\)] of LNCaP cells to establish [Ca\(^{2+}\)] clamp conditions. In A–D: TG indicates the addition of 100 nM TG, *iono* indicates the addition of 5.0 \(\mu\)M of the Ca\(^{2+}\) ionophore ionomycin, and *CaCl*\(_2\) indicates the addition of 5.0 mM CaCl\(_2\). Ionomycin and CaCl\(_2\) were added to *iono* to perturb functioning of Fura-2. A, control cell culture conditions. TG induces an increase in cytosolic [Ca\(^{2+}\)], which plateaus at approximately 1.8 \(\mu\)M. B, LNCaP cells loaded in 7 \(\mu\)M BAPTA/AM. The TG-induced rise in cytosolic [Ca\(^{2+}\)] is transiently attenuated. C, LNCaP cells cultured in medium with 110 \(\mu\)M extracellular Ca\(^{2+}\). The TG-induced release of intracellular Ca\(^{2+}\) stores is denoted by an increase of approximately 0.1 \(\mu\)M 100 s after TG addition. The larger elevation of cytosolic [Ca\(^{2+}\)] due to Ca\(^{2+}\) influx across the plasma membrane, depicted in A and B, is inhibited. D, LNCaP cells cultured for 6 h under the [Ca\(^{2+}\)] clamp conditions (110 \(\mu\)M extracellular Ca\(^{2+}\) and loading in 7 \(\mu\)M BAPTA/AM). The TG-induced elevation of cytosolic [Ca\(^{2+}\)] due to the release of intracellular Ca\(^{2+}\) stores and the Ca\(^{2+}\) influx across the cell membrane are inhibited, although intracellular Ca\(^{2+}\) store release presumably occurs.
Calcium Clamp Conditions Do Not Deplete Intracellular Calcium Stores—Because high concentrations of Ca\(^{2+}\) chelators have been shown to deplete intracellular Ca\(^{2+}\) stores (34), it was important to determine whether the [Ca\(^{2+}\)] clamp conditions led to Ca\(^{2+}\) store release. This was accomplished by exploiting the properties of reagents that specifically block depletion-activated Ca\(^{2+}\) channels to result in I\(_{\text{CRAC}}\) such as econazole and NiCl\(_2\) (32). Following treatment with 5 mM NiCl\(_2\), the TG-induced elevation of cytosolic [Ca\(^{2+}\)] due to I\(_{\text{CRAC}}\) is diminished (Fig. 3A). Therefore, we reasoned that the attenuation of I\(_{\text{CRAC}}\)-mediated Ca\(^{2+}\) influx with NiCl\(_2\) can be used to detect the presence of intracellular Ca\(^{2+}\) stores, because by definition I\(_{\text{CRAC}}\) only occurs after intracellular Ca\(^{2+}\) stores have been released. When cells were cultured under the [Ca\(^{2+}\)] clamp conditions for 24 h and treated with TG, as expected no fluctuation in cytosolic [Ca\(^{2+}\)] occurred, although the depletion-activated Ca\(^{2+}\) channels likely opened in response to TG-induced Ca\(^{2+}\) store release. However, when extracellular Ca\(^{2+}\) was added, the cytosolic [Ca\(^{2+}\)] rapidly increased presumably via Ca\(^{2+}\) influx through the already opened depletion-activated Ca\(^{2+}\) channels, which overwhelmed the chelating ability of the cytosolic BAPTA (as in Fig. 2D). That the Ca\(^{2+}\) influx occurred via depletion-activated Ca\(^{2+}\) channels was confirmed by Ca\(^{2+}\) influx attenuation with NiCl\(_2\). As an additional control, cells were cultured under the clamp conditions for 24 h, and extracellular Ca\(^{2+}\) was added prior to adding TG (Fig. 3C). Before TG addition, no elevation of cytosolic [Ca\(^{2+}\)] was detected even though the [Ca\(^{2+}\)] gradient was increased. This was likely because the Ca\(^{2+}\) channels on the cell membrane were closed, which would not be the case if the [Ca\(^{2+}\)] clamp conditions had even partially led to Ca\(^{2+}\) store release. Thus, culturing cells under the [Ca\(^{2+}\)] clamp conditions did not deplete intracellular Ca\(^{2+}\) stores. For the above experiments, similar results were obtained using Indo-1 as the Ca\(^{2+}\) indicator dye and 50 \(\mu\)M econazole as the depletion-activated Ca\(^{2+}\) channel blocker (data not shown).

ER Ca\(^{2+}\) Store Depletion Induces LNCaP Cell Apoptosis—Having established conditions that sustained a cytosolic [Ca\(^{2+}\)] clamp yet did not deplete intracellular Ca\(^{2+}\) stores or induce apoptosis, it was possible to determine whether Ca\(^{2+}\) release from intracellular stores was critical for TG-induced LNCaP cell death. No protection against TG-induced apoptosis was conferred by clamping cytosolic [Ca\(^{2+}\)], indicating that LNCaP cell death was likely induced by the release of intracellular Ca\(^{2+}\) stores (Fig. 4). Bearing this in mind, it is noteworthy to recall that loading LNCaP cells with high (>30 \(\mu\)M) extracellular concentrations of BAPTA, which has been reported to cause the release of ER Ca\(^{2+}\) stores in the absence of cytosolic

FIG. 4. Evaluation of LNCaP cell hypodiploid DNA. LNCaP cells are not protected from TG-induced apoptosis by clamping cytosolic [Ca\(^{2+}\)], indicating that the release of intracellular Ca\(^{2+}\) stores is critical for inducing LNCaP cell apoptosis. Note that the amount of cell death detected when cells are cultured under control conditions and under [Ca\(^{2+}\)] clamp conditions is comparable.
TG under the [Ca\(^{2+}\)] clamp conditions.

To permit better visualization of the processed fragments of caspases-9 and -3, the film was exposed for a longer duration than that for the corresponding zymogen. A, caspase-9 proteolytic fragments are detected in LNCaP cells after 24 h of TG treatment under both control and [Ca\(^{2+}\)] clamp conditions. B, caspase-7 processing. Proteolytic fragments are detected at 36 h of TG treatment when cultured under control [Ca\(^{2+}\)] conditions but are detected by 12 h of treatment when cultured under the [Ca\(^{2+}\)] clamp conditions. C, caspase-3 processing is also detected after 36 h of TG treatment with control [Ca\(^{2+}\)] conditions and after 12 h of treatment with the [Ca\(^{2+}\)] clamp conditions.

Because TG-induced caspase activation was earlier than the dual perturbations of [Ca\(^{2+}\)] elevation and [Ca\(^{2+}\)] store depletion (35), this discrepancy could be due to the different properties of Dunning rat AT-3 androgen-independent prostate cells and androgen-sensitive LNCaP human prostate cancer cell lines used in the study or to the different criteria used for assessing apoptosis.

**Caspase Processing in LNCaP Cells**—Because caspases are the enzymes responsible for the execution phase of apoptosis, it was of interest to determine which caspases were activated, the relative timing of their activation, and whether differences occurred in caspase processing when apoptosis was induced by TG under the [Ca\(^{2+}\)] clamp conditions versus control [Ca\(^{2+}\)] conditions. Processing of caspase-9, the initiating caspase associated with intracellular apoptotic stimuli, occurred by 24 h under both the control [Ca\(^{2+}\)] and the [Ca\(^{2+}\)] clamp conditions (Fig. 5A). The effector caspases-3 and -7 were also processed by 12 h under the [Ca\(^{2+}\)] clamp conditions and by 36 h with control [Ca\(^{2+}\)] (Fig. 5, B and C). That processing of the effector caspases is detected earlier than the initiator caspase-9 under the [Ca\(^{2+}\)] clamp conditions is possibly a reflection of relative antibody sensitivity but could also imply the existence of a mechanism that directly activates the small pro-domain effector caspases. Regardless, the release of intracellular Ca\(^{2+}\) stores in the absence of cytosolic [Ca\(^{2+}\)] elevation results in earlier caspase activation than the dual perturbations of sustained cytosolic [Ca\(^{2+}\)] increase and intracellular Ca\(^{2+}\) store release. It is important to note that no caspase activation was detected when cells were cultured for 36 h under the [Ca\(^{2+}\)] clamp conditions (Fig. 5).

**Inhibition of TG-induced Apoptosis by Antia apoptotic Proteins**—Because TG-induced caspase activation was earlier than when cells were cultured under the [Ca\(^{2+}\)] clamp conditions versus under control [Ca\(^{2+}\)] conditions, we further characterized the death pathways activated by these stimuli to determine if they were distinct. We generated stable transfected LNCaP cell lines expressing the specific inhibitor of apoptosis Bcl\(_x\), a potent inhibitor of apoptosis that is thought to function by directly inhibiting Apaf-1-mediated activation of caspase-9 or by attenuating release of mitochondrial cytochrome c (36, 37). These stably transfected cell lines, as well as a pcDNA3 vector-transfected LNCaP cell line control, were treated with TG under control [Ca\(^{2+}\)] conditions or under the [Ca\(^{2+}\)] clamp conditions, and apoptosis was assessed by quantitation of hypodiploid DNA.

The protection afforded by the antia apoptotic proteins was consistent with the profiles of caspase activation (Fig. 6). Caspase-9 DN reduced apoptosis in cells treated with TG under control [Ca\(^{2+}\)] and [Ca\(^{2+}\)] clamp conditions. However, based on our previous studies indicating that caspase-9 DN potently blocks apoptosis (36), we would have predicted a greater degree of protection than that observed in these studies. This is, however, consistent with the possibility of direct activation of effector downstream caspases, a situation where caspase-9 DN would be of limited effectiveness given that it is thought to work by blocking the ability of endogenous caspase-9 from interacting with downstream pro-domain caspases. Bcl\(_x\), a potent inhibitor of apoptosis that is thought to function at many levels, provided roughly equal levels of protection in cells treated under both [Ca\(^{2+}\)] conditions.

**Caspase-9 DN Interacts with Caspase-7**—We questioned whether the ability of caspase-9 DN to inhibit apoptosis, albeit partially, was related to its forming an activation-dependent complex with downstream effector caspases. Such complexes formed from the binding of catalytically inert caspase-9 DN would potentially be more stable than those formed with the
wild-type protease, which would facilitate “trapping” of an interacting substrate. Indeed, caspase-7 was found complexed to caspase-9 DN under both control cell culture conditions and under \([\text{Ca}^{2+}]\) clamp conditions. The relative expression level of each transfected gene compared with its expression in wild-type LNCaP cells is indicated.

Fig. 6. Evaluation of hypodiploid DNA in TG-treated stable transfected LNCaP cell lines. Bcl-x\(_L\) is a more potent inhibitor of TG-induced LNCaP apoptosis than caspase-9 DN under both control cell culture conditions and under \([\text{Ca}^{2+}]\) clamp conditions. The relative expression level of each transfected gene compared with its expression in wild-type LNCaP cells is indicated.

A
control calcium conditions
untreated
wt LNCaP Casp-9 DN FLAG
IP: -- FLAG -- FLAG
32.5 caspase-7
WB: caspase-7

36h TG
wt LNCaP Casp-9 DN FLAG
IP: -- FLAG -- FLAG
32.5 caspase-7
WB: caspase-7

B
calcium clamp conditions
untreated
wt LNCaP Casp-9 DN FLAG
IP: -- FLAG -- FLAG
32.5 caspase-7
WB: caspase-7

18h TG
wt LNCaP Casp-9 DN FLAG
IP: -- FLAG -- FLAG
32.5 caspase-7
WB: caspase-7

has been elucidated in hippocampal neurons involving calcineurin-mediated dephosphorylation of the Bcl2 family member BAD (Bcl2-associated death promoter), which displaces Bcl-x\(_L\) from Apaf-1 to permit caspase-9 activation (38). Because \(\text{Ca}^{2+}\) is a second messenger involved in multiple signaling pathways, it is likely that induction of apoptosis due to TG-induced \([\text{Ca}^{2+}]\) perturbation is also multifactorial.

Our studies also show that caspase-9 is one initiator protease activated during TG-induced cell death. Caspase-9 is required for the execution of intracellular apoptotic stimuli, which is clearly demonstrated by caspase-9 knockout mice. Embryonic stem cells and fibroblasts derived from these mice are resistant to ultraviolet and gamma irradiation and their thymocytes are resistant to dexamethasone and gamma irradiation (39). How these intracellular stimuli, including \([\text{Ca}^{2+}]\) perturbation, activate caspases remains unclear.
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However, it has been shown that caspase-9 and Apaf-1 are essential downstream components of p53-induced apoptosis (40). The p53 tumor suppressor protein has been implicated as a sensor of intracellular perturbations and is involved in regulating cell cycle arrest and apoptosis (41). The cyclin-dependent kinase inhibitor p21 is a downstream effector of p53 and causes an arrest in the G1 phase of the cell cycle by at least two mechanisms: dephosphorylation of the retinoblastoma protein (Rb) (42) and complexing to proliferating cell nuclear antigen (PCNA) (43). Our previous studies have shown that TG treatment of LNCaP cells results in p21 induction, progressive Rb dephosphorylation, and p21-PCNA complex formation (44, 45). Flow cytometric analyses performed in our current studies are consistent with these results, showing an accumulation of cells in G1-phase and a reduction of S-phase cells (data not shown). It is therefore possible that p53 is a sensor of TG-induced [Ca$^{2+}$] perturbations that induces cell cycle arrest and caspase-9 activation.

That p53 is induced in TG-treated cells is also suggested by the finding that treating LNCaP cells with TG under the [Ca$^{2+}$] clamp conditions leads to earlier caspase activation than treatment under control [Ca$^{2+}$] conditions. It has been shown that the TG-induced elevation of cytosolic [Ca$^{2+}$] transiently suppresses p53-induced apoptosis, possibly by mimicking mitogenic stimuli (46). Indeed, our data also indicate that lower concentrations of TG result in LNCaP cell proliferation and that under the [Ca$^{2+}$] clamp conditions, the TG-induced cytosolic [Ca$^{2+}$] elevation would be inhibited and result in earlier caspase activation.

In summary, we have shown that TG-induced [Ca$^{2+}$] perturbations can induce apoptosis of LNCaP prostate cancer cells. By developing a technique to isolate the TG-induced release of ER Ca$^{2+}$ stores from the elevation of cytosolic [Ca$^{2+}$], we found intracellular Ca$^{2+}$ store release is by itself sufficient to induce apoptosis and that this stimulus results in earlier caspase processing than the dual stimuli of ER Ca$^{2+}$ store release and cytosolic [Ca$^{2+}$] elevation. However, once activated, the apoptotic pathways induced by intracellular Ca$^{2+}$ store release or by Ca$^{2+}$ store release coupled with elevated cytosolic [Ca$^{2+}$] are similar, as indicated by a similar degree of protection via antiapoptotic proteins and the formation of a complex of caspase-9 DN and caspase-7 in both scenarios.

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