The Role of Calcium, pH, and Cell Proliferation in the Programmed (Apoptotic) Death of Androgen-independent Prostatic Cancer Cells Induced by Thapsigargin

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ABSTRACT

Calcium (Ca^{2+}) accumulates within the endoplasmic reticulum of cells through function of the sarcoplasmic reticulum and endoplasmic reticulum Ca^{2+}-dependent ATPase family of intracellular Ca^{2+}-pumping ATPases. The resulting pools have important signaling functions. Thapsigargin (TG) is a sesquiterpene \(-\)-lactone which selectively inhibits the sarcoplasmic reticulum and endoplasmic reticulum Ca^{2+}-dependent ATPase pumps with a 50\% inhibitory concentration of approximately 30 nM. Treatment of androgen-independent prostate cancer cells of both rat and human origin with TG inhibits their endoplasmic reticulum Ca^{2+}-dependent ATPase activity, resulting in a 3–4-fold elevation in the level of intracellular free Ca^{2+} (Ca_{i}) within minutes of exposure. Due to a secondary influx of extracellular Ca^{2+}, this increase in Ca_{i} is sustained, resulting in morphological (cell rounding) and biochemical changes within 6–12 h (enhanced calmodulin, glucose regulated protein, and tissue transglutaminase expression, and decreased expression of the G_{1} cyclins). Within 24 h of exposure, androgen-independent prostate cancer cells stop progression through the cell cycle, arrest out of cycle in G_{0} and irreversibly lose their ability to proliferate with a median effective concentration value of 31 nM TG. During the next 24–48 h, the genomic DNA of the G_{0}-arrested cells undergoes double-strand fragmentation. This is followed by the loss of plasma membrane integrity and fragmentation of the cell into apoptotic bodies. During this process, there is no acidification in the intracellular pH. Using cells transfected with the avian Mr 28,000 calbindin D Ca^{2+}-buffering protein, it was demonstrated that the programmed death initiated by TG is critically dependent upon an adequate (i.e., 3–4-fold) sustained (>1 h) elevation in Ca_{i}, and not depletion of the endoplasmic reticulum pools of Ca^{2+}. These results demonstrate that TG induces programmed cell death in androgen-independent prostatic cancer cells in a dose-dependent manner and that this death does not require proliferation or intracellular acidification but is critically dependent upon an adequate, sustained (i.e., >1 h) elevation in Ca_{i}.

INTRODUCTION

Androgen ablation therapy is initially beneficial to nearly all men with metastatic prostatic cancer. Unfortunately, relapse to an androgen-insensitive state is almost inevitable for essentially all patients, and additional forms of anti-androgen therapy are ineffective, no matter how aggressively given, due to the continued growth of androgen-independent prostatic cancer cells (1, 2). Typically, chemo-therapeutic agents for the treatment of cancer are toxic for dividing cells only. However, the low rate of proliferation of androgen-independent prostatic cancer cells renders such agents ineffective (3). Thus, a novel cytotoxic therapy not requiring cell proliferation is urgently required for these devastating cells.

Previously, we have demonstrated that both androgen-dependent normal prostatic glandular cells and androgen-dependent prostatic cancer cells can be induced to undergo apoptosis following androgen ablation and that this programmed cell death does not require entry into or progression through the cell cycle (4–6). Associated with this apoptosis is the enhanced expression of a series of genes (7, 8), resulting in a sustained elevation of Ca_{i} (9, 10). This results in the activation of Ca^{2+}-/Mg^{2+}-dependent endonuclease present within the cell's nucleus which double strand fragments the genomic DNA, thus committing the cell to death (9–11). Androgen ablation does not induce the changes in Ca_{i} required to induce programmed cell death in androgen-independent prostatic cancer cells (12); however, these cells can be induced to undergo programmed cell death if a modest (3–4-fold) elevation in Ca_{i} is sustained for a sufficient time (minutes to hours) (12).

TG, a sesquiterpene \(-\)-lactone isolated from the root of the umbelliferous plant, Thapsia garganica, is highly lipophilic and irreversibly inhibits the SERCA enzymes with an IC_{50} value of \(\sim 30 \text{nM}\) (13). TG is highly selective since PMCA are not inhibited by TG, even at \(\mu \text{M}\) concentrations (13). Cytoplasmic Ca^{2+} levels are maintained in the order of 20–40 nM by both classes of Ca^{2+}-pumping ATPases which either transport Ca^{2+} out of the cell across the plasma membrane (PMCA) or sequester Ca^{2+} within internal pools such as those located in the ER (SERCA). While the absolute concentration of Ca^{2+} within the ER is not known, it is likely to exceed 100 \(\mu \text{M}\), and it is apparent that the SERCA family of Ca^{2+}/ATPases constantly compensate for the release of Ca^{2+} across the ER membrane, either by passive leak or by second messenger-activated Ca^{2+} channels (13). Interestingly, depletion of the ER Ca^{2+} pool results in the generation of a signal, possibly an ER-derived diffusible messenger, which induces an increased permeability of the plasma membrane to divalent cations and a sustained elevation of Ca_{i} (14, 15). Previous studies have demonstrated that TG treatment rapidly stops cells from entering the S phase of the cell cycle (16). Given that TG can cause sustained elevation in Ca_{i} and stop entrance into S phase, we tested whether TG treatment alone may be sufficient to induce apoptosis of androgen-independent prostatic cancer cells without entrance into the S phase. These studies demonstrated that TG blocks entrance of the androgen-independent prostatic cancer cells into the S phase and induces their programmed death (17, 18). These results raised three major questions: (a) does the elevation of Ca_{i} induced by TG result in a secondary decrease in cellular pH? This possibility is based upon the demonstration that, in certain cell types induced by certain agents, such a secondary acidification, not the rise in Ca_{i}, activates the endonuclease involved in the double strand DNA frag-
vention characteristic of apoptotic cell death (19); (b) is the deple-
tion of the Ca2+ pools, and not the elevation of Ca2+ induced by TG, the
critical initiator of programmed cell death in prostatic cancer
cells? This possibility is based upon the demonstration that depletion of
the ER pools of Ca2+ can up-regulate the expression of a series of
genes encoding ER proteins without a requirement for a elevation in
Ca2+ (20, 21); (c) is the inhibition of entrance of the cells into the S
phase of the cell cycle induced by TG due to a block in progression of
the cells in G1 of the cycle, or do prostatic cancer cells leave the
cycle and enter a G0 state from which the cells are recruited to undergo programmed cell death? This possibility is based upon the
demonstration that androgen ablation recruits androgen-dependent
protein cells from G0 into the programmed death pathway (6, 8). The
present studies were performed to evaluate these three questions using a
series of four distinct androgen-independent prostatic cancer cell
lines of both rat (Dunning AT-3) and human (TSU-Pr1, DU-145, and
PC-3) origin.

MATERIALS AND METHODS

Cell Lines. The rat AT-3, and human TSU-Pr1, DU-145, and PC-3 andro-
gen-independent prostatic cancer cell lines were all cultured with RPMI 1640
media containing 10% fetal calf serum (Hyclone, Logan, UT) with 25 nm
examined in total cell pools by flow cytometry using the detergent-trypsin
viable cells was determined by trypan blue exclusion analysis of total cell
conjugated mouse monoclonal antibody to bromodeoxyuridine; Accurate
were similarly incubated with an irrelevant monoclonal antibody (FITC
tomycin sulfate (antibiotics from M. A. Bioproducts, Walkerville, MD) as
pared from —5 x 107 cells, were isolated as described previously (27).

G0-G1 when they had a diploid DNA content with a small nucleus, in S when
and by nuclear size forward angle light scatter. Cells were judged to be in
stained nuclei was performed on an Epics 752 flow cytometer (Coulter Corp.,
ability to 50% was extrapolated to determine the EC50. Cell cycle progression
media containing 10% fetal calf serum (Hyclone, Logan, UT) with 250 nM
microsomes were suspended to a protein concentration of 1 mg/ml, and
pared from —5 x 107 cells, were isolated as described previously (27).

Determination of Microsomal Ca2+—ATPase Activity. Microsomes, pre-
pared from ∼5 x 106 cells, were isolated as described previously (27).
Microsomes were suspended to a protein concentration of 1 mg/ml, and
aliquots (50 μg protein) were assayed for their Ca2+—ATPase activity using the
coupled enzyme assay of Seidler et al. (25). These experiments were reported
three independent times using microsomes isolated from three different
batches of cells.

DNA Fragmentation Analysis. Double-strand DNA fragmentation was
quantitated according to a modification of the pulse field gel electrophoresis
method of Stamato and Denko (29) as described in detail previously (18). This
method involves uniformly labeling the genomic DNA with [3H]thymidine,
sodium dodecyl sulfate/1% β-mercaptoethanol. DNA was sheared by repeated pipetting, and 50-μl aliquots were applied to glass microfiber filters (Whatman, Maidstone, United Kingdom). The filters were washed three times with 10% trichloroacetic acid and once with 70% ethanol, dried, and finally counted. The results were initially expressed as dpm incorporated into protein per 10⁶ cells. These values were then normalized to the value for TG untreated control cells (i.e., 32,300 ± 1,480 dpm/h/10⁶ AT-3 cells = 100%).

CAM Radioimmunooassay. Aliquots of total cell pools (i.e., attached plus unattached) in triplicate were washed with PBS. Ten volumes of 50 mM Tris-HCl (pH 7.8), 3 mM MgSO₄, 1 mM dithiothreitol, and 1 mM EDTA were added, and the tissue was homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1,000 × g for 15 min, and the resulting supernatant was further centrifuged at 100,000 × g for 1 h. The final supernatant was used for determination of CAM using a radioimmunoassay kit (NEN Research Products, Boston, MA) according to the manufacturer's recommendation without heating.

Statistical Analysis. Values are expressed as the mean ± SE. Statistical analyses of significance were made by one-way analysis of variance with the Newman-Keuls test for multiple comparison. Unless otherwise stated, each experiment was performed at least three independent times in triplicate for each data point.

RESULTS

Inhibition of the ER Ca²⁺ ATPase from Prostatic Cancer Cells by TG. Microsomes from both the rat AT-3 and human TSU-Prl cell lines were assayed for their ER Ca²⁺-dependent ATPase activity. These results (Table 1) demonstrate that, while there is a 10-fold difference in the amount of Ca²⁺-independent ATPase activity in these two prostatic cancer cell lines, there is only an ~3-fold difference in the amount of Ca²⁺-dependent ATPase activity. Previous studies of Thastrup et al. (13) reported that TO inhibits the microsomal Ca²⁺-ATPase with an IC₅₀ of ~30 nM. Based upon these data, the ability of TG to inhibit the microsomal Ca²⁺-dependent ATPase from AT-3 and TSU-Prl cells was tested. Both lines were treated during exponential growth with 500 nM TO for ~18 h, and microsomal preparations were made and assayed for their Ca²⁺-dependent and independent ATPase activity as described in "Materials and Methods." As shown in Table 1, even after 18 h, TG inhibited the Ca²⁺-dependent ATPase activity by greater than 80% in both lines. Additionally, microsomes isolated from untreated AT-3 and TSU-Prl cells were assayed in the presence or absence of TG (500 nM). Within 1 min of addition of TG to the assay tube, the Ca²⁺-dependent ATPase was completely (i.e., <99%) inhibited (data not shown).

Effect of TG on Ca$_4$ and pH. The Ca$_4$ concentration in exponentially growing androgen-independent prostatic cancer cells of rat and human origin range between 16–40 nM (Table 2). Following exposure to TG (500 nM), Ca$_4$ rose rapidly (i.e., within 1 min) to values exceeding 100 nM, regardless of the cell line. During the next 5–10 min, the Ca$_4$ decreased to values in the range of 80–160 nM, which were maintained for hours, if extracellular free Ca$_4$ was maintained at a value of at least 400 μM (Table 2 presents data at 4 h of TG exposure). If extracellular free Ca$_4$ was lowered to less than 50 nM by including EDTA (5 mM) in the media, the initial TG-induced rise in Ca$_4$ remained unchanged. However, prolonged elevation of Ca$_4$ was not observed, and Ca$_4$ returned to ~10–30 nM. These results are consistent with TG inducing an initial elevation in Ca$_4$ as pools of Ca$_4$²⁺ stored in the ER were released, followed by a sustained elevation due to the influx of extracellular Ca$_4$²⁺ across the plasma membrane.

As pointed out by Barry et al. (19), under certain conditions, an increase in Ca$_4$ can result in a secondary acidification of the cell to a pH of below 6.5. To test if this occurs following TG treatment of AT-3 cells, the pH$_4$ was determined over the first 48 h of chronic exposure to 500 nM TG. The starting pH$_4$ in AT-3 cells is 7.30 ± 0.05. At no time point did the pH$_4$ of the AT-3 cells ever decrease to below 7.00 (i.e., pH$_4$ was 7.20 ± 0.06, 7.18 ± 0.05, 7.21 ± 0.07, 7.27 ± 0.15, 7.10 ± 0.15, and 7.05 ± 0.10 at 1, 2, 4, 24, 36, and 48 h of TG treatment, respectively). These results demonstrate that a secondary acidification to a pH of below 7.0 following TG treatment of the AT-3 cells does not occur.

Effect of TG on the Morphology of Prostatic Cancer Cells. Time-lapse videomicroscopy demonstrated that no morphological changes occur during the first several hours of TG treatment. By 3–6 h of initial exposure to TG (500 nM), however, morphological changes began to occur in all of the androgen-independent prostatic cancer lines tested (AT-3, TSU-Prl, DU-145, and PC-3). By 12–24 h of treatment, all cells are smaller and more rounded (compare Fig. 1, a and b; note that all six of the panels of Fig. 1 are from the exact same field over a 68-h observation period). After 24 h of treatment, the cells undergo periods of plasma membrane hyperactivity characterized by plasma membrane blebbing (Fig. 1, c and f, arrows). These surface blebs are highly dynamic, giving the appearance of membrane boiling, which is characteristic of programmed cell death as reported previously (12). Episodes of such blebbing continued until the cell lysed into membrane-bounded apoptotic bodies (Fig. 1, d, e, and f, arrowheads), which eventually detach from the plate. The time required before apoptotic lysis began varied between 36 and 72 h after initiation of TG treatment, depending on the different cell lines used. Times for morphological lysis of 50% of the rodent AT-3 cells was ~60 h, while ~90 h was required for morphological lysis of 50% of the human cells into apoptotic bodies.

Effect of TG on Cell Cycle Progression. Bivariate flow cytometric analysis was used to determine whether TG treatment affects the progression of androgen-independent prostatic cancer cells through the cell cycle. These analyses demonstrated that, within 24 h of exposure to TG (500 nM), prostatic cancer cells arrested in the G$_2$-M compartment of the cell cycle, (Fig. 2 presents data for AT-3 cells; TSU-Prl, PC-3, and DU-145 cells gave essentially identical results). This conclusion is based upon the observation that, by 24 h of exposure, the percentage of cells in S-phase decreases (P < 0.05) by more than 85% from values of ~30% to less than 5% for all four of the cell lines. During this first day of TG exposure, the percentage of cells in G$_2$-M likewise decreases by one-half from values of ~15 to ~7% (Fig. 2). By 48 h of TG treatment, the percentage of cells in the

<table>
<thead>
<tr>
<th>Table 1 Response of the microsomal ATPase to TG treatment</th>
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<tr>
<td>Microsomal ATPase activity (nmol of ATP hydrolyzed/mg protein)</td>
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<tr>
<td>Cell line</td>
</tr>
<tr>
<td>Ca²⁺-independent TG</td>
</tr>
<tr>
<td>AT-3</td>
</tr>
<tr>
<td>TSU-Prl</td>
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<td>Ca²⁺-dependent TG</td>
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| * Cells treated overnight with either control media (i.e., 0 nM) or 500 nM TG before microsomes were isolated and assayed for ATPase activity. |

<table>
<thead>
<tr>
<th>Table 2 Ability of TG treatment to raise the Ca$_4$ level in prostatic cancer cells</th>
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<tr>
<td>Cell line</td>
</tr>
<tr>
<td>AT-3</td>
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<tr>
<td>TSU-Prl</td>
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<td>DU-145</td>
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<td>PC-3</td>
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* Extracellular Ca$_4$ in 0.4 mM for all cell lines. |

* P < 0.05 difference when compared to no treatment value. |
S and G2-M phases combined was less than 2% of the total, regardless of the cell line examined. After this 48-h period, there was an increased incidence of apoptotic bodies detectable as small-sized particles with a less than G0 DNA content (Fig. 2). Enhanced detection of these apoptotic bodies occurred after differing times of TG exposure for each of the four cell lines (i.e., >48 h for AT-3 cells and >72 h for TSU-Pr1, PC-3, and DU-145 cells). These results demonstrate that, within 24 h of TG treatment, androgen-independent prostatic cancer cells are no longer entering the S phase of the proliferative cell cycle.

To determine whether this inhibition of S phase entrance was due to arrest in G1 or to the cells leaving the cell cycle completely (i.e., cells in G0), two approaches were undertaken. The first was to use flow cytometric immunocytochemical staining analysis using the Ki67 monoclonal antibody to determine the percentage of cells which were negative for the antigen detected by this antibody. Schwarting et al. (26) have demonstrated that this Ki67 monoclonal antibody reacts with a nuclear antigen present in all human cells in the G1, S, G2, or M phase of the cycle but not with cells in G0. Unfortunately, this antibody is human specific and does not stain rodent cells consistently (26). Thus, the human TSU-Pr1, DU-145, and PC-3 prostatic cancer cells were analyzed for the percentage of cells Ki67 negative (i.e., in G0) following exposure to 500 nM TG. (Note: cells were defined as Ki67 negative if their fluorescence value was below 20. This cutoff value was determined by staining cells with irrelevant FITC-labeled anti-bromodeoxyuridine monoclonal antibody). When the human prostatic cancer cells in exponential growth are analyzed, greater than 98% are positive for Ki67 expression (Fig. 3 presents data for TSU-Pr1 cells; PC-3 and DU-145 cells gave essentially identical results). Within 24 h of TG exposure, the percentage of Ki67 negative cells increases from values <2% to values >80%, and by 36 h of exposure, >90% of the cells are Ki67 negative (Fig. 3). These results demonstrate that TG treatment does not arrest the human prostatic cancer cells in G1 but instead induces them to leave the proliferation cell cycle within 24 h of TG exposure and enter G0.

Similar flow cytometric analysis could not be performed with the rat AT-3 cells due to the human-specific nature of the Ki67 antibody. To determine whether a similar G0 arrest occurs within 24 h of 500 nM TG treatment of rat prostatic AT-3 cancer cells, the expression of a series of G1-specific genes was used. The genes chosen were the G1-cyclins (i.e., cyclin C, D1, and E) and thymidine kinase (33). As controls, expression of the CAM, GRP, and TTG genes were also analyzed, since we have demonstrated previously that these are unregulated within the first 24 h of exposure of AT-3 cells to 500 nM TG (18). These studies (Fig. 4) demonstrated that, within 6–12 h, there was a greater than 80% decrease in the expression of all of G1 cyclin (i.e., cyclin E and thymidine kinase data are not shown in Fig. 4). This decrease is not due to generalized RNA degradation, as demonstrated when blots were stripped and rehybridized with probes to CAM, GRP, TTG, or γ-actin. These results demonstrated that CAM mRNA is up-regulated within 1 h and decreases at 12 h of TG treatment. In contrast, GRP mRNA induction occurs at 6 h and decreases after 12 h, while TTG mRNA is constant until there is an ~3-fold induction per cell between 24 and 36 h after initiation of TG treatment. γ-Actin mRNA remains constant per cell, at least up to 36 h of TG treatment.
50-kilobase size pieces become detectable and eventually, even small-sized (i.e., <10-kilobase) pieces become detectable. Qualitative analysis of the small (i.e., <10-kilobase) size DNA fragments using standard DNA isolation and gel electrophoresis demonstrated a nucleosomal size-repeating ladder characteristic of programmed cell death (Fig. 5, right side).

After an additional 10–24 h period, the cells begin to undergo changes in their plasma membrane integrity, and by 58 h for the AT-3 cells and 84 h for the TSU-Pr1 cells, 50% of the cells take up trypan blue. These kinetics are consistent with morphological data which demonstrated that 50% of the AT-3 and TSU-Pr1 cells lyse into apoptotic bodies by 60 and 90 h, respectively. Likewise, changes are consistent in nuclear morphology, detectable by staining cells at various times of TG exposure with the fluorescent DNA binding dye, Hoescht 33342. These studies demonstrated that nuclear chromatin initially becomes condensed and marginalized, and the nuclei become smaller. Eventually, the nuclei themselves become fragmented within the cell. A comparison of the frequency of such nuclear change demonstrated that these changes are coincident with the kinetics of DNA fragmentation.

The data in Fig. 5 demonstrated that exposure to 500 nM TO for up to 12 h results in no loss of clonogenic ability or viability. This confirms that TO effects are reversible up to a certain time point. After 24 h of TO exposure (the time when DNA fragmentation is initiated), however, AT-3 cells become irreversibly committed to eventual death, although during this period, there is no decrease in cell viability, as judged either by videomicroscopy or trypan blue exclusion. These data demonstrate that TO treatment initially arrests androgen-independent rodent and human prostatic cancer cells in G0, and then
THAPSIGARGIN AND PROGRAMMED CELL DEATH

Fig. 5. Temporal changes in the clonogenic ability, degree of double-stranded DNA fragmentation, and viability of cells during exposure to 500 nM TG. Values represent the mean (n = 3–5/point); bars, SEM. For some points, the SEM is too small to be presented graphically. Inset on right of figure is the qualitative pattern of double-stranded DNA fragments isolated at 24 h of TG treatment. A, data for rodent AT-3 cells. B, data for TSU-Prl cells.

TG induced the initiation of DNA fragmentation. Once DNA fragmentation is initiated, cellular fragmentation into apoptotic bodies occurs 12 h later for rodent cells and 24 h later for human cells. Thus, DNA fragmentation is not the result of cellular fragmentation and death. These data are consistent with double-strand DNA fragmentation being the irreversible commitment step in TG-induced death of nonproliferating cancer cells. To further test this possibility, AT-3 cells were exposed to varying concentrations of TG for 48 h and then tested for the clonogenic survival in TG-free media. These dose-response studies demonstrated that the concentration of TG required to irreversibly commit 50% (i.e., EC_{50}) of AT-3 cells to programmed death within 48 h of chronic exposure is 31 nM, a value essentially identical to the IC_{50} value for TG inhibition of the ER Ca^{2+}-ATPase.

Role of Elevated Ca_{i} versus Ca^{2+} Depletion. The 3–4-fold elevation in Ca_{i} in AT-3 cells induced by treatment with 500 nM TG (Table 2) is rather modest. This raises the issue of whether it is this modest elevation in Ca_{i}, or the depletion of the ER pools of Ca^{2+}, which is the actual initiator of programmed death of the AT-3 cells. One way to differentiate between these two possibilities, initially developed by Dowd et al. (32), is to transfected cells with an expression vector for the calbindin D-28Kd calcium binding protein and test the effect that high calbindin protein expression has upon the toxic response to TG (32). Since one molecule of calbindin D-28Kd protein binds six molecules of Ca^{2+} (32), overexpression of this protein should be able to buffer elevations in Ca_{i} induced by TG treatment without preventing the depletion of the ER pool of sequestered Ca^{2+}. Thus, if elevation in Ca_{i} and not simply depletion of the stored ER Ca^{2+} pool are critically required to activate programmed death of AT-3 cells induced by TG, then such calbindin D-28Kd overexpression should protect these cells from such activation.

To test these possibilities, AT-3 cells were transfected with either a neomycin resistance expression vector containing the full-length complementary DNA for the calbindin D-28Kd protein or a control vector containing only the neomycin resistance gene. Multiple calbindin D-28Kd neomycin resistance clones (i.e., CaBP clones) and neo-only control (i.e., neo) clones were isolated following both types of
transfection and screened for expression of calbindin D-28Kd by Western blot analysis. Such analysis identified two CaBP clones expressing large amounts of the calbindin D-28Kd protein (i.e., clones 23 and 15) and one CaBP clone expressing low amounts (i.e., clone 9; see Fig. 6, upper panel, lower right-hand inset, for Western blot analysis of calbindin D-28Kd protein expression by these CaBP doses). No expression of the calbindin D-28Kd protein was detected in either the parental or neo-only transfectants (Fig. 6, upper panel, right-hand inset). The Ca\textsubscript{i} response of the differing types of AT-3 cells to treatment with 500 nM TG demonstrated that only in high calbindin D-28Kd protein-expressing clones (e.g., CaBP clone 15; Table 3) is the elevation in Ca\textsubscript{i} induced by TG treatment buffered (i.e., only a ~2-fold not a 3–4-fold increase).

To test the effects that such buffering of the TG-induced elevation in Ca\textsubscript{i} has on programmed death of the AT-3 cells, these clones were separately exposed to 500 nm TG for either 36 h (Fig. 6, upper panel) or 48 h (Fig. 6, lower panel), and then the clonogenic survival was determined. These results demonstrated that, in both of the high calbindin D-28Kd protein-expressing clones (i.e., CaBP clones 23 and 15), there is a greater than 50% inhibition in the loss of clonogenic ability induced by 36 h of 500 nm TG treatment as compared to either the parental AT-3 cells, neo-only transfectants, or CaBP clone 9 cells expressing only low levels of calbindin D-28Kd protein (Fig. 6, upper panel). This inhibition is not observed, however, if the chronic TG treatment is extended to 48 h (Fig. 6, lower panel). These results suggest that, by 48 h of continuous TG treatment, the influx of extracellular Ca\textsuperscript{2+} saturates the binding capacity of the intracellular calbindin D-28Kd protein, even in the high-expressing CaBP clones 15 and 23 cells.

To further clarify the role of elevation in Ca\textsubscript{i} in the cytotoxic response of TG, the effect of varying the extracellular Ca\textsuperscript{2+} on the TG-induced loss of clonogenic ability of control AT-3 cells and high-expressing CaBP clones 15 and 23 were tested. The standard media (i.e., RPMI 1640 containing 10% fetal calf serum) has rather low levels (0.4 mM) of free Ca\textsubscript{i}. Sufficient Ca\textsuperscript{2+} was added to this standard media to raise the concentration of free Ca\textsuperscript{2+} from 0.4 mM to 1.0 mM. Using both medias containing 0.4 or 1.0 mM free Ca\textsuperscript{2+}, the extent of loss of clonogenic ability induced by 36 h of exposure to 500 nm TG was determined (Table 4). These results demonstrated that, by increasing the extracellular Ca\textsuperscript{2+}, the extent of loss of clonogenic ability induced by 36 h if exposure to 500 nm TG was increased (P < 0.05) for all cell types. The extent of loss of clonogenic ability in the high calbindin-expressing CaBP 15 and 23 clones were still, however, lower (P < 0.05) than for the AT-3 parental or neo control transfectants. These increases in loss of clonogenic ability in the higher Ca\textsuperscript{2+}-containing media were consistent with an enhanced ability of 500 nm to TG to elevate the Ca\textsubscript{i} in these cells. For example, the Ca\textsubscript{i} after 20 min of exposure to 500 nm TG in the 1.0 mM as compared to 0.4 mM Ca\textsuperscript{2+}-containing media was ~2.2-fold higher in the AT-3 parental and neo control transfectant (i.e., 150 ± 20 versus 67 ± 4 nm) and 1.8-fold higher in the high calbindin-expressing CaBP clones (i.e., 77 ± 9 versus 44 ± 3 nm). Again, even in the higher Ca\textsuperscript{2+}-containing media, TG treatment did not induce as high an increase in Ca\textsubscript{i} in high calbindin-expression CaBP clones as parental or neo-only control transfectants.

**Effect of TG on Protein Synthesis.** Programmed cell death often involves transcriptional induction of specific genes and enhanced translational expression of the corresponding encoded protein product. When a 3–4-fold elevation in Ca\textsubscript{i} is maintained in AT-3 cells by exposure to TG (500 nm), a series of such epigenetic changes are induced (Fig. 4). One of the genes induced encodes for the multifunctional calcium-binding protein, CAM, which is involved in calcium-dependent regulation of more than a dozen known enzymes (34). The level of CAM protein per AT-3 cell doubles between 6 and 12 h of exposure to 500 nm TG (Fig. 7). In contrast, total protein synthesis

Table 4: Effect of raising the extracellular Ca\textsuperscript{2+} concentration in culture media on the loss of clonogenic ability induced by 36 h of exposure of 500 nm TG

<table>
<thead>
<tr>
<th>Cell type</th>
<th>0.4 mM Ca\textsuperscript{2+}</th>
<th>1.0 mM Ca\textsuperscript{2+}</th>
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<tbody>
<tr>
<td>AT-3 parental</td>
<td>55 ± 8</td>
<td>81 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AT-3 neo</td>
<td>51 ± 10</td>
<td>85 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AT-3 CaBP clone 15</td>
<td>21 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AT-3 CaBP clone 23</td>
<td>24 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 difference when compared to parental cells in 0.4 mM Ca\textsuperscript{2+} media.
<sup>b</sup> P < 0.05 difference when compared to cell type in 0.4 mM Ca\textsuperscript{2+} media.
<sup>c</sup> P < 0.05 difference when compared to parental cells in 1.0 mM Ca\textsuperscript{2+} media.
concentrations (13). Based upon the stoichiometric and quantitative
The intracellular targets for TO are the Ca\(^{2+}\)-dependent ATPases
proliferate during a given day (35, 36). In contrast, high numbers of
there is no effective systemic therapy for the control of androgen
general protein synthesis is decreased while the synthesis of specific
decreases by 60% within 12 h of TG treatment (Fig. 7). These results
designated as a percentage of untreated control AT-3 cells (i.e., 100% = 34 ± 2 µg
CAM protein/10\(^6\) cells and 32,300 ± 1,480 dpm of methionine incorporated into protein/
h!10\(^6\) cells).

DISCUSSION
Metastatic prostatic cancer is a fatal disease due to the fact that
there is no effective systemic therapy for the control of androgen-
independent prostatic cancer cells (3). A major reason why androgen-
independent prostatic cancer cells are resistant to present therapies is
their extremely low rate of proliferation. Less than 10% of such cells
proliferate during a given day (35, 36). In contrast, high numbers of
normal host cells proliferate daily (e.g., 10—20% per day for gut, skin,
and blood cells). Thus, there is an extremely small therapeutic index
for anti-proliferation drugs targeted at the limited number of proliferating
androgen-independent prostatic cancer cells.

Androgen-independent prostatic cancer cells can be induced to
undergo proliferation-independent programmed cell death if their Ca\(_i\)
can be elevated for a sustained period (12). As demonstrated in the
present study, one method to support an elevated Ca\(_i\) in these cells is
to selectively inhibit the ER Ca\(^{2+}\)-dependent ATPase by treatment
with TG. TG, a sesquiterpene \(\gamma\)-lactone, is the active principle of
the root of the umbelliferous plant, *Thapsia garganica* (37). Resin from
this plant was used as early as 300 B.C. as a medicine for rheumatic
pains by the Greeks and has also been used in traditional Arabic
medicine for centuries (38). However, TG is also a skin irritant
inducing mast cells to release histamine in an extracellular Ca\(^{2+}\)-
dependent manner similar to the action of calcium ionophores (38).
The intracellular targets for TG are the Ca\(^{2+}\)-dependent ATPases
present in the ER. TG potently [IC\(_{50}\) of \(\approx 30\) nM; (13)] and selectively
inhibits the SERCA family of ATPases.

In contrast, PMCA are not inhibited by TG, even at micromolar
concentrations (13). Based upon the stoichiometric and quantitative
effects of TG on the ER Ca\(^{2+}\)-ATPase, the inhibition appears to be
irreversible (39—40). In order for the ER Ca\(^{2+}\)-ATPase to function
cyclically as a pump, it must form an aspartylphosphoenzyme inter-
mediate (the phosphate being derived from ATP) which must, in time,
be dephosphorylated for the protein to recycle (41). Mechanistic
studies have demonstrated that the basis for the action of TG is its
ability to inhibit the production of the aspartylphosphoenzyme inter-
mediate required for the ER Ca\(^{2+}\)-ATPase protein to function as
a transport pump (42). TG does not interfere with the dephosphorylation
step in the enzymatic cycling (41).

In the present study, treatment of both human and rat androgen-
independent prostatic cancer cells with TG results in sustained Ca\(_i\)
elevations. The mechanism for this sustained Ca\(_i\) elevation is likely to
be as follows. TG inhibits the ER Ca\(^{2+}\)-ATPase, which prevents
resequestration of Ca\(_i\) as it leaks out of the ER. The resultant depletion
of ER Ca\(^{2+}\) pools results in capacitative entrance of extracellular
Ca\(^{2+}\) into the cell, which is sustained (14, 15). In addition, the
depletion of Ca\(^{2+}\) from the ER has significant effects on ER function,
which includes changes in protein synthesis, assembly, and processing
(16, 43). In the current study, within the first 12 h of TG exposure,
there are a series of epigenetic (i.e., mRNA profile) events which are
coupled with morphological changes in the cells (i.e., cell rounding
and shrinkage). Concurrent with these changes is an enhanced syn-
thesis of specific protein (e.g., camcalmodulin), although total protein
synthesis is greatly decreased (i.e., >50%). By 24 h of TG treatment,
the prostatic cancer cells ceased progression through the cell cycle
and arrested in G\(_0\), losing their clonogenic ability. At this time, the cells
begin to undergo DNA fragmentation, initially into 300 then 50-
kilobase-sized pieces. These larger DNA fragments subsequently are
degraded further into smaller nucleosomal-sized (i.e., \(\geq 1\) kilobase)
pieces before being eventually reduced to individual nucleotides.
Between 24 and 48 h of TG treatment, the majority of cells undergo
dNA fragmentation. Following G\(_0\) arrest and subsequent DNA frag-
mentation, the cells lose their plasma membrane viability, as assayed
by trypan blue exclusion and flow cytometric assays, and undergo
cellular fragmentation into apoptotic bodies, as documented by time
lapse videomicroscopy.

Based upon the temporal sequence of events following initiation of
TG treatment, initiation of DNA fragmentation is not an epiphem-
enon occurring as a result of the cell losing its viability but is a causal
event in committing the cell to complete programmed death. Inhibi-
tion of ER Ca\(^{2+}\)-dependent ATPase appears to be the major pathway
for the induction of programmed cell death by TG, since the IC\(_{50}\) for
both TG inhibition of the ER Ca\(^{2+}\)-ATPase and EC\(_{50}\) for TG-
duced loss of clonogenic ability and programmed death are \(\approx 30\) nM.
No changes in pH\(_i\) occurred during TG treatment, documenting that
intracellular acidification is not the mechanism for activation of the
genomic DNA and cellular fragmentation. The inhibition of ER Ca\(^{2+}\-
ATPase by TG results in both a depletion of the ER pool of seques-
trated Ca\(^{2+}\) and to a secondary rise in Ca\(_i\). In high calbindin trans-
flectants, both the rise in Ca\(_i\) and cytotoxicity induced by TG treatment
are inhibited without prevention of the depletion of the ER pool of
Ca\(^{2+}\). These results demonstrate that it is the elevation in Ca\(_i\) to a
critical level which is the initiator of the programmed death of
androgen-independent prostatic cancer cells. Thus, these studies have
identified the ER Ca\(^{2+}\)-ATPase pump as a new therapeutic target for
activating programmed cell death of nonproliferating, androgen-inde-
pendent prostatic cancer cells.

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THAPSIGARGIN AND PROGRAMMED CELL DEATH


The Role of Calcium, pH, and Cell Proliferation in the Programmed (Apoptotic) Death of Androgen-independent Prostatic Cancer Cells Induced by Thapsigargin

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