Cell Death and Differentiation (2002) 9, 561–573

www.nature.com/cdd

A supramicromolar elevation of intracellular free calcium ($[Ca^{2+}]_i$) is consistently required to induce the execution phase of apoptosis

B Tombal*[,1,2], SR Denmeade[1], J-M Gillis[2] and JT Isaacs[1,3]

[1] Johns Hopkins Oncology Center, Johns Hopkins School of Medicine, Baltimore, MD 21231, USA
[2] Department of Physiology, Université catholique de Louvain, Brussels B-1200, Belgium
[3] Department of Urology, James Buchanan Brady Urological Institute, Johns Hopkins School of Medicine, Baltimore, MD 21231, USA
* Corresponding author: B Tombal, Department of Physiology, UCL St-Luc, UCL/Harvey/FYMU 5540, Avenue Hippocrate, 55, B-1200 Brussels, Belgium. Tel: 32 2 764 5540; Fax: 32 2 764 5580; E-mail bertrand.tombal@fymu.ucl.ac.be

Received 28.9.01; revised 30.11.01; accepted 5.12.01
Edited by J Cidlowsky

Abstract

Many agents, such as the endoplasmic reticulum Ca^{2+} ATPase inhibitor, thapsigargin, or the ionophore, ionomycin, induce apoptosis by transiently elevating $[Ca^{2+}]_i$. The role of $[Ca^{2+}]_i$ in apoptosis induced by agents that do not immediately increase $[Ca^{2+}]_i$, such as 5-FdUr, TGFβ-1, doxorubicin, or radiation, is far more controversial. In the present paper, $[Ca^{2+}]_i$ was measured continuously for 120 h. in prostate and bladder cancer cell lines exposed to these four agents: 5-FdUR, TGFβ-1, doxorubicin, or radiation. Each of them consistently induced a delayed $[Ca^{2+}]_i$ rise associated with the morphological changes that characterize the execution phase of apoptosis (i.e. rounding, blebbing). This $[Ca^{2+}]_i$ rise occurred in two consecutive steps ($\leqslant 10$ μM and >10 μM) and resulted from a Ca^{2+} influx from the extracellular medium. This delayed supramicromolar $[Ca^{2+}]_i$ rise was also observed previously in breast, prostate and bladder cancer cell lines exposed to thapsigargin. This influx regulated transcriptional reprogramming of Gadd153 and is required to activate cytochrome c release, caspase-3 activation, loss of clonal survival and DNA fragmentation. When cells were maintained in low extracellular Ca^{2+} media, these phenomena were temporarily delayed but occurred on return to normal Ca^{2+} medium. Similarly, apoptosis could be delayed by overexpressing the Ca^{2+}-binding proteins, Calbindin-D_{28K} and parvalbumin. As this delayed $\geqslant 10$ μM $[Ca^{2+}]_i$ elevation was observed in a number of cell lines exposed to a variety of different agents, we conclude that such elevation constitutes a key and general event of apoptosis in these malignant cells.
Cell Death and Differentiation (2002) **9**, 561–573. DOI: 10.1038/sj/cdd/4400999

Keywords: apoptosis; calcium; Fura Dextran; Prostate; DNA fragmentation

Abbreviations: 5-FdUr, 5-fluorodeoxyuridine; TGFβ-1, transforming growth factor β1

Introduction

Apoptosis is a widespread phenomenon, occurring normally at different stages of morphogenesis, growth and development and in normal turnover in adult tissue.[1] The correct completion of apoptosis requires a highly diversified machinery to detect and decode death signals and transform them into a lethal cascade. The first steps of apoptosis are usually reversible and define the 'decision to dye' or signaling phase.[2] This phase varies between cells types, and can be activated either by injury to the cell by various exogenous agents (e.g., radiation, chemicals, virus), or by changes in the levels of a series of endogenous signals (e.g., hormones and growth/survival factors).[3] The final step or execution phase of apoptosis is usually irreversible and comprises a series of morphological changes that characteristically occur during apoptosis: blebbing and deformation of the plasma membrane, fragmentation of the nucleus, and fragmentation of the cell into apoptotic bodies.[4] These changes mostly result from the activation of a proteolytic cascade organized upstream of a special set of cysteine proteases: the caspases.[5–8] In contrast to the signaling phase, the execution phase occurs asynchronously in prostate cancer cells over a period of 12–96 h.[2]

Since it was recognized more than twenty years ago that a rise in $[Ca^{2+}]_i$ precedes apoptosis induced by glucocorticoids in thymocytes, and that $Ca^{+}-Mg^{2+}$-dependent endonucleases were involved in the fragmentation of genomic DNA nucleus, intracellular free calcium ($[Ca^{2+}]_i$) has been considered as one of the key elements in the cell death pathway.[9–12] To date, $[Ca^{2+}]_i$ measurements in individual cells undergoing apoptosis have been performed by passively loading fluorescent Ca^{2+} indicators such as Fura-2AM. These types of dyes were initially designed to monitor transient $[Ca^{2+}]_i$ changes occurring shortly after exposure to the inducing agent.[13] When used in cells undergoing apoptosis, the use of dyes like Fura-2AM limits the analysis of $[Ca^{2+}]_i$ changes to the period of time ($\leqslant$120 min) immediately following the application of the apoptotic stimulus.[14] Using these types of dyes, a series of agent that mobilize intracellular or extracellular Ca^{2+} stores and trigger apoptosis by transiently elevating $[Ca^{2+}]_i$ have been identified, and include the glucocorticoids, the Ca^{2+} ionophore ionomycin, or the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (TG).[15–17]

In contrast, there are many agents that activate apoptosis without inducing an early measurable rise of $[Ca^{2+}]_i$ such as TGFβ-1, 5-FdUr, doxorubicin or radiation.

The precise involvement of $[Ca^{2+}]_i$ changes in apoptosis induced by these agents is still therefore largely unexplored. Experimental evidence suggest, however, that several key enzymes involved in the biochemical and enzymatic cascade that characterize the execution phase of apoptosis are tightly regulated by $[Ca^{2+}]_i$. These proteins include calpain,[18] Ca^{2+}-dependent nuclear scaffold proteases involved in the degradation of the cellular and nuclear matrix,[19] Ca^{2+}-dependent transglutaminases,[20] and calcineurin.[21] Moreover, recent reports suggest that apoptosis is also associated with the overexpression of genes encoding plasma membrane or intracellular ligand-gated calcium channels such as $P2X_1$ and type I and III $InsP_3$ receptors.[22–24]

The standard method for monitoring changes in $[Ca^{2+}]_i$ in cells treated with apoptosis inducing agents has been to treat cells with the agent and at various time points load these cells with fluorescent Ca^{2+} probes (i.e. post-loading). The existence of any elevation in $[Ca^{2+}]_i$ that is directly related to these events described above has been extremely difficult to demonstrate using these AM fluorescent Ca^{2+} probes because of severe limitations in dye uptake, metabolism and retention in post-loaded cells that have already been injured by the toxic agent.[13]

In the present report we have studied apoptotic pathways in four different cancer cell lines treated with cytotoxic agents that do not produce an early and transient rise of $[Ca^{2+}]_i$. In individual cells, continuous $[Ca^{2+}]_i$ measurements and detection of morphological changes were made up to 120 h after application of the apoptotic agent, thus overlapping the signaling phase and the execution phase of apoptosis, using a previously validated procedure.[13] This longitudinal analysis of $[Ca^{2+}]_i$ was complemented by longitudinal measurements of key features of the execution phase of apoptosis: DNA fragmentation, loss of clonogenic survival, release of cytochrome *c*, caspases-3 activation, and transcriptional up-regulation of death related protein Gadd153. These additional analyses allowed us to establish the temporal relationships between some of these features and the changes of $[Ca^{2+}]_i$.

Results

Temporal characteristics of the execution phase of apoptosis in a population of normal and malignant epithelial cells

There are a variety of agents that induce apoptotic death of normal and malignant cells through highly variable signaling pathways that do not produce an initial rise in $[Ca^{2+}]_i$: the thymidylate synthase inhibitor, 5-FdUR;[25] the alkylating/topoisomerase inhibitor, doxorubicin;[26] ionizing γ-irradiation;[27,28] and growth factor TGFβ-1.[29,30] We followed the kinetics of apoptosis for each of these agents (see Table 1 for concentrations) in a series of normal and malignant cell lines by measuring the loss of clonogenic survival and the DNA fragmentation at 0, 12, 24, 48, 72 and 96 h post-exposure to the inducing agent. An example of the kinetics of DNA fragmentation (expressed as the % of Tunnel-positive cells) is illustrated at the Figure 1 where a sigmoid curve was fitted through the data to define the time required to induce DNA fragmentation in 50% of the cells (i.e. it is well known that some cells escape apoptosis, so that DNA fragmentation never reaches 100%; the time for 50% DNA fragmentation was calculated on the whole population and not on the number of cells that eventually died). The same type of analysis was made for the loss of clonogenic survival (for technical limitations, clonogenic survival was not measurable in the NRP152 cell line). Table 1 summarizes the average time required for the various agents, at their optimal dosage, to induce a 50% reduction of clonogenic survival and DNA fragmentation in 50% of the cells. This demonstrates that the execution phase of apoptosis requires at least 24 h exposure for the most sensitive cell line, while in most cases, 40–48 h are needed, regardless of the inducing agent.

Longitudinal measurement of $[Ca^{2+}]_i$ in individual cells undergoing apoptosis with Fura Dextran

TSU, PC3, AT3.1 and NRP154 cells were individually microinjected with Fura Dextran and exposed to the same inducing agents as above, in the same conditions. Fluorescent radiometric measurement of $[Ca^{2+}]_i$ and analysis of morphological changes were performed continuously on individual cells starting shortly before exposure to the inducing agent until completion of the apoptotic program as defined by the fragmentation of the cells into apoptotic bodies (usually up to 96 h, sometimes up to 120 h).[13,31] No apoptosis-related morphological changes were observed by videomicroscopy before 12 h of exposure of any of the agents. Within this 12 h initial period, no elevation from baseline $[Ca^{2+}]_i$ was observed. This confirms previous data demonstrating that, in contrast to TG or ionomycin, these agents trigger apoptosis independently of an initial $[Ca^{2+}]_i$ rise.[13,31]

On the contrary, during the execution phase of apoptosis, a delayed rise of $[Ca^{2+}]_i$ was observed in all cell lines, regardless of the nature of the inducing agent. This $[Ca^{2+}]_i$ rise occurred only in cells presenting the early morphological changes characteristic of the execution phase of apoptosis such as cell rounding, reduction in overall cell size and plasma membrane blebbing (Figure 2). The delayed $[Ca^{2+}]_i$ elevation was detected asynchronously in the cell populations, starting not earlier than after 12 h of exposure to the inducing agent and occurring thereafter stochastically within the cell population, mostly between 24 and 96 h post-exposure.

Based upon temporal analysis of $[Ca^{2+}]_i$ values, morphological changes and total content of fluorescent dye, the apoptotic process could be divided into four separate phases as illustrated in Figure 2.

Phase 1, or baseline phase, showed neither an elevation of $[Ca^{2+}]_i$ (which remained within the 50–70 nM range) nor any detectable morphological change. Its duration is very variable from cell to cell; in most cases, it lasted 28–48 h after exposure to the inducing agent, but infrequent extreme values ranging from 12–96 h were also observed.

Phase 2, or submicromolar phase, occurred stochastically within the cell population and is characterized by a sustained

Table 1 Time required to induce 50% of DNA fragmentation and loss of clonogenic survival in prostate cancer cells

Agent (dose)	Prostatic cell line	Time (h) required to induce a 50% reduction in clonogenic survival			Time (h) required to induce DNA fragmentation in 50% of the cells		
		n	mean	S.E.M	n	mean	S.E.M
5-FdUr	TSU	5	47.5	4.9	7	48.8	2.5
	PC3	3	44.0	8.1	5	46.6	3.4
	AT3.1	3	35.8	4.9	3	35.8	4.9
Doxorubicin	TSU	3	42.8	2.6	3	45.5	4.9
(100 nM)	AT3.1				3	35.6	5
TGFβ-1	NRP152				5	26.2	5
Radiation	TSU	3	42.7	6.6	5	46.5	4.7
(800 cGy)	PC3	3	61.5	4.3	5	69.6	3.8

n, number of experiments; S.E.M.: standard error of the mean

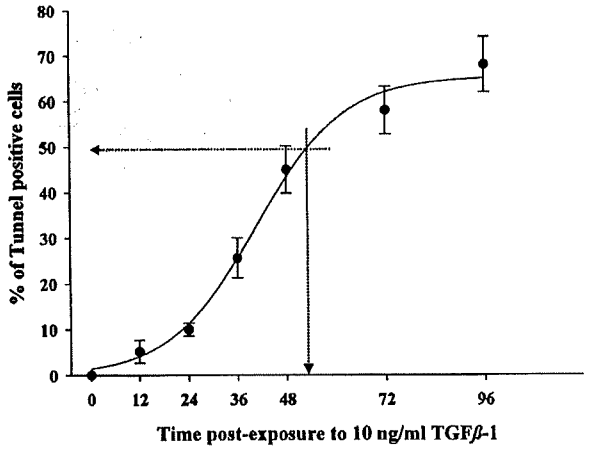


Figure 1 Kinetics of induction of DNA fragmentation in TSU cells exposed for increasing periods of time to 10 μM 5-FdUr. At each time point, cells were trypsinized and percentage of DNA fragmentation was measured using Tunel assay. Each experiment was performed in triplicate. Mean $\pm$ S.D. A sigmoid curve (line) was mathematically fitted through the data to calculate the time required to induce DNA fragmentation in 50% of the cells

(30 min to 2 h) but moderate $[Ca^{2+}]_i$ elevation from baseline to 250–750 nM. This second phase is systematically associated with the earliest onset of apoptosis-related morphological changes (i.e. cell rounding and reduction of cell size).

Phase 3, or supramicromolar phase, was characterized by an abrupt rise of $[Ca^{2+}]_i$ to > 1.5 μM, the Fura Dextran saturating values. This third > μM phase is characteristically associated with periods of plasma membrane hyperactivity (blebbing) and occurs without reduction of the total Fura Dextran dye content or increased permeability to Trypan blue, thus before major alterations of plasma membrane permeability. Once initiated, this $[Ca^{2+}]_i$ elevation was quantitatively equal in the cytoplasm and the nucleus and the duration (typically 4–8 h) was similar in the different cells types treated with the different inducing agents.

Phase 4 eventually followed phase 3 and was associated with major alterations of the plasma membrane permeability resulting in a rapid leakage of the dye from the cell. Further $[Ca^{2+}]_i$ measurements are at that point possible.

Determination of maximal $[Ca^{2+}]_i$ value with Mag-Indo Dextran

Because of its high affinity for Ca^{2+}, Fura Dextran is inappropriate to measure $[Ca^{2+}]_i$ in the micromolar range. As shown in Figure 2, $[Ca^{2+}]_i$ rose systematically during phase 3 to concentrations which saturate the probe, making accurate measurement impossible. To overcome this limitation, cells were injected with Mag-Indo Dextran which has lower affinity for Ca^{2+} and is appropriate for $[Ca^{2+}]_i$ measurements in the micromolar range. In these cells, we observed that the amplitude of the $[Ca^{2+}]_i$ rise in phase 3 ranged from 6.7 to 50 μM, with a mean value of 18.2 $\pm$ 5.7 μM (SD) (Figure 3). This average value was independent of the type of cell line and the nature of the inducing agent. Previously we have demonstrated that this magnitude of $[Ca^{2+}]_i$ rise was also observed in prostate, bladder and breast cancer cells following exposure to thapsigargin.[31,32]

The time-course of $[Ca^{2+}]_i$ changes consistently precedes the time-course of DNA fragmentation regardless of the inducing agent

Determination of DNA fragmentation and of clonogenic survival was made on different cell populations analyzed after predefined time points (i.e. at 0, 12, 24, 48, 72 and 96 h after exposure to the inducing agent), as explained previously (Table 1 and Figure 1). In contrast, measurements of $[Ca^{2+}]_i$ and analysis of morphological changes were made continuously on individual cells, observed up to 96 h (in some cases up to 120 h). To compare the time course of $[Ca^{2+}]_i$ changes with that of DNA fragmentation and clonogenic survival, we measured the time needed for 50% of the microinjected cells of each cell line to present a submicromolar elevation of $[Ca^{2+}]_i$ (phase 2 defined above, Table 2). These results are summarized in Table 1 and 2 and reveal that for each cell line and each agent, the time required to observe a delayed submicromolar $[Ca^{2+}]_i$ rise in 50% of the cells was consistently shorter than for attaining 50% decrease of clonogenic survival or DNA fragmentation in 50% of the cells. Figure 4 shows the strong correlation between the time for 50% of cells to enter phase 2 of $[Ca^{2+}]_i$ changes vs onset of DNA fragmentation. Furthermore, a weighted regression was calculated which yielded the following equation:

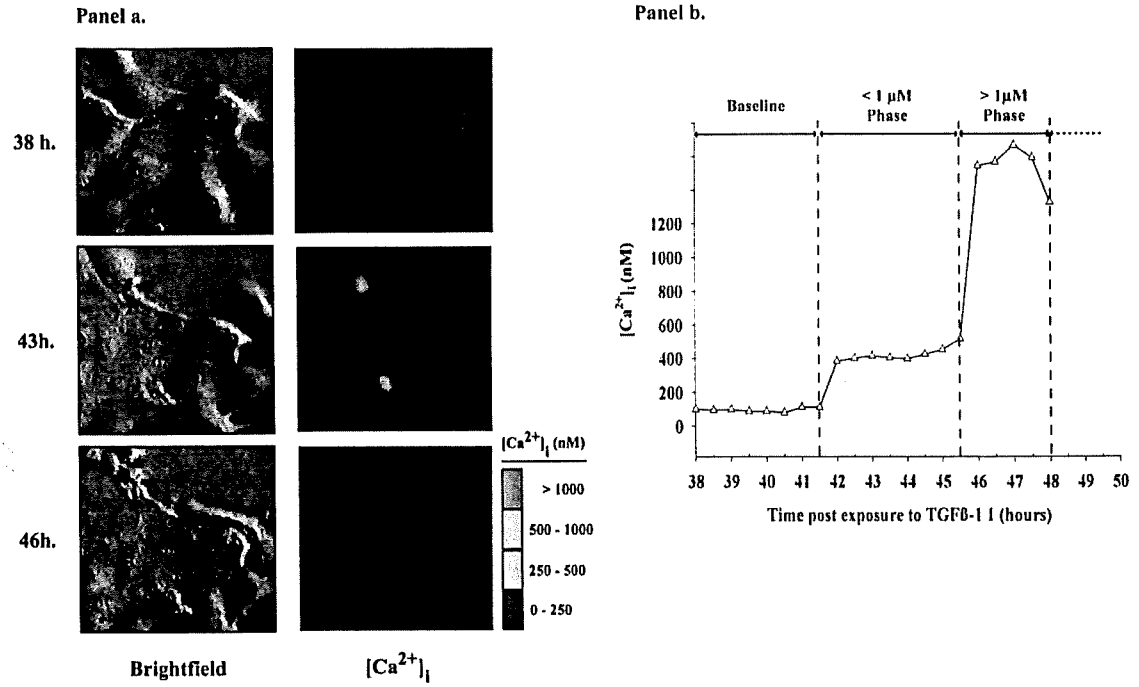


Figure 2 (a) NRP152 cells exposed for 38, 43 and 46 h to 10 ng/ml TGFβ-1. These pictures provide an example of the asynchronous induction of morphological changes and delayed $[Ca^{2+}]_i$ rise in two of the three NRP152 cells microinjected with Fura Dextran (indicated by the color stars). Phase contrast images are presented in the left columns and computerized ratiometric measurement of $[Ca^{2+}]_i$ in the right column. At 41 and 46 h, two of the cells demonstrate apoptosis-related morphological changes: ie, detaching, cell rounding and blebbing. These two cells also present an elevation of $[Ca^{2+}]_i$ which is color-coded (see insert in the bottom right corner). In (b) the average $[Ca^{2+}]_i$ of the NRP152 cell with the green star has been plotted every 30 min starting 39 h after exposure to 10 ng/ml TGFβ-1. This longitudinal analysis defines the four phases of the observed $[Ca^{2+}]_i$ changes during the execution of apoptosis: phase 1 or baseline phase, phase 2 or submicromolar phase, phase 3 or supra-micromolar phase, and phase 4 or leakage phase (see text for details). As demonstrated in the present experiment, Fura Dextran does not allow for precise measurement of $[Ca^{2+}]_i$ changes occurring in the μM range. $[Ca^{2+}]_i$ values obtained during phase 3 and 4 are therefore largely underestimated using this technique

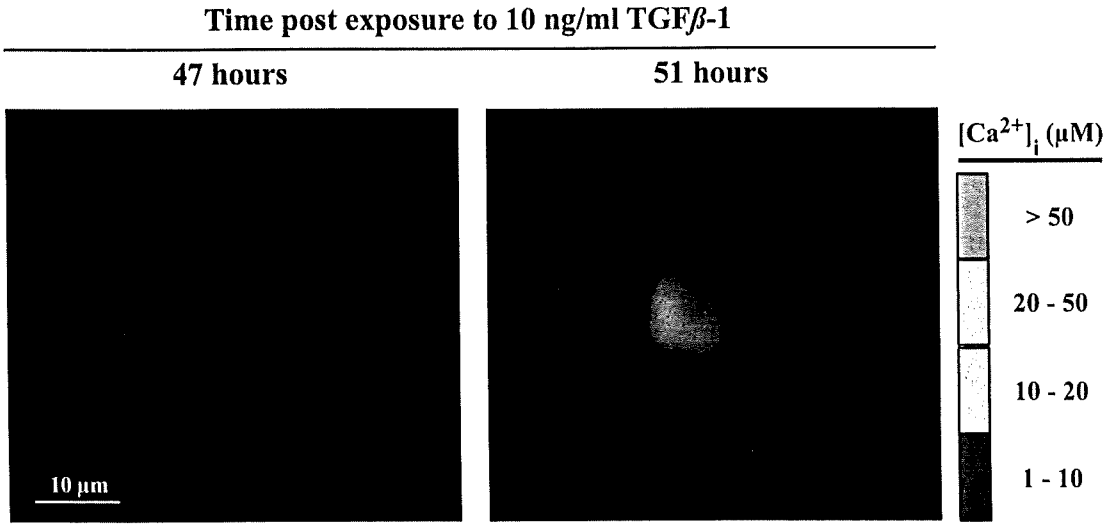


Figure 3 A typical example of the induction of a supramicromolar elevation of $[Ca^{2+}]_i$ in one TSU cells microinjected with Mag-Fura Dextran. Dark and light blue colors were computerized from the ratio between fluorescent images emitted at 405 and 495 nm. Using the color code inserted on the right, $[Ca^{2+}]_i$ can be estimated to rise to between 10–20 μM at 51 h

Table 2 Time required to induce a rise of $[Ca^{2+}]_i$ in 50% of prostate cancer cells microinjected with Fura Dextran

Agent (dose)	Prostatic cell line	Number of microinjected cells	Time (h) required to induce a submicromolar $[Ca^{2+}]_i$ rise in 50% of the cells
5-FdUr	TSU	57	39.6
	PC3	46	40.5
	AT3.1	31	28.9
Doxorubicin	TSU	25	37.2
(100 nM)	AT3.1	33	25.9
TGFβ-1	NRP152	78	21.5
Radiation	TSU	32	43.0
(800 cGy)	PC3	25	65.8

n, number of experiments; SEM: standard error of the mean

t_{50} for DNA = 10.4 h ($\pm$2.6) + 0.91 ($\pm$0.065)
t_{50} phase 2 ($r = 0.98$, $P < 0.001$).

Interestingly, the extrapolated ordinate, 10.4, is significantly different from zero ($P<0.05$). This indicates that DNA fragmentation lags behind the submicromolar rise of $[Ca^{2+}]_i$ by an average delay of 10 h, confirming that the latter is not a consequence of the former. The fact that the slope of the regression is not significantly different from 1.0 ($P>0.2$) suggests that the spread of the induction of the $[Ca^{2+}]_i$ changes and of DNA fragmentation among the cell population, though delayed in time, evolved in direct proportion. Moreover, results plotted in Figure 4 show that two essential features of the execution phase of apoptosis are mechanistically linked by a unique relationship for each of the four different cell lines studied here and treated with apoptosis inducing agents as diverse as a pyrimidine analog, an alkylating agent, a cytokine or radiation.

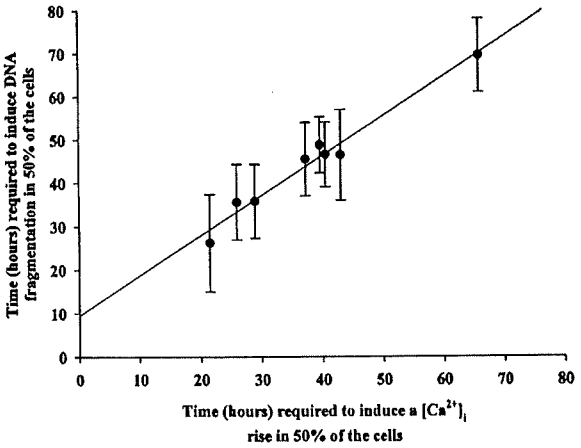


Figure 4 The time required to induce DNA fragmentation in 50% of the cells (Data from Table 1 and 2; bars: standard deviation) was plotted against the time required to induce a delayed submicromolar $[Ca^{2+}]_i$ rise in 50% of the cells. A weighted linear regression was calculated on these individual points which crossed the ordinate at 10.4 h

Involvement of extracellular Ca^{2+} in the delayed $[Ca^{2+}]_i$ elevation initiated during the execution phase of apoptosis

To clarify the mechanism involved in the delayed $[Ca^{2+}]_i$ elevation, TSU cells and NRP154 cells were maintained in normal medium (RPMI1640/10%FBS containing 400 μM Ca^{2+}) and exposed for 12 h to the inducing agent to allow the completion of the signaling phase of apoptosis. After this initial incubation period of 12 h, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) was decreased from 400 μM to 100$\pm$25 nM by addition of 800 μM EGTA to the standard medium. $[Ca^{2+}]_e$ was monitored every 6 h on a sample aliquot. Decreasing $[Ca^{2+}]_e$ down to undetectable levels by adding EGTA in large excess (>1 mM) resulted in cells rounding and detaching from the support and cell deaths. In contrast, TSU, PC-3 and NRP152 cells could be maintained alive for up to 120 h when carefully controlling $[Ca^{2+}]_e$ at 100 nM.

In 100 nM $[Ca^{2+}]_e$, no significant delayed $[Ca^{2+}]_i$ elevation was detected regardless of the cell type and the nature of the stimulus, during the period of observation when it normally occurs; thus the delayed $[Ca^{2+}]_i$ elevation resulted from an influx of extracellular Ca^{2+} (Figure 5). Importantly, morphological changes did not occur as long as the cells were maintained in 100 nM $[Ca^{2+}]_e$, suggesting

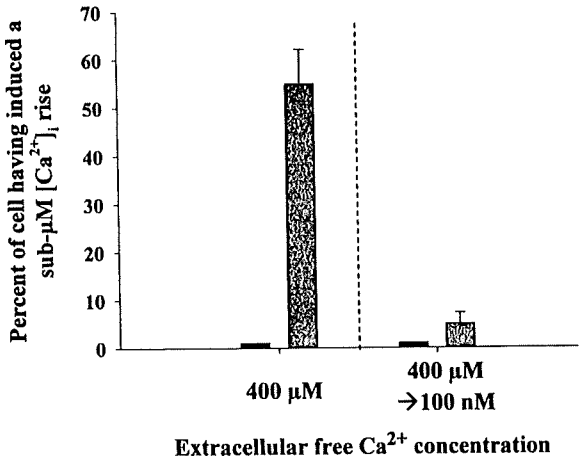


Figure 5 Percentage of TSU cells microinjected with Fura-Dextran that display a >10 μM $[Ca^{2+}]_i$ rise, just before (black columns) and 48 h after exposure to 10 μM 5-FdUr (grey columns). Cells were incubated either in a 400 μM Ca^{2+} medium for 48 h (left, labeled 400 μM) or after an initial 12 h period in 400 μM Ca^{2+}, for the remaining 36 h into a 100 nM Ca^{2+} medium (right, labeled 400 μM→100 nM). Results were corrected by subtraction of the percentage observed in control untreated cells (usually 3–5%)

that the $[Ca^{2+}]_i$ elevation was required for cells to enter into the execution phase of apoptosis. However, replacing the 100 nM $[Ca^{2+}]_e$ medium by standard medium containing 400 μM $[Ca^{2+}]_e$ at any time during the next 72 h period, resulted in a rapid (i.e. < 1 h) $[Ca^{2+}]_i$ elevation to values > 1 μM in the micro-injected cells. Once this occurs, the cells subsequently undergo apoptosis within less than 6 h. This suggests that during the baseline phase (phase 1) following exposure to the agent the cells were already programmed to die but required an influx of extracellular Ca^{2+} to proceed to the final execution steps. Maintaining the cells after the initial signaling phase in 100 nM $[Ca^{2+}]_e$ did not suppress apoptosis but rather prevented cells from proceeding to the execution phase.

Overexpression of Ca^{2+} binding proteins, Calbindin-D_{28k} and Parvalbumin, delays DNA fragmentation and clonogenic survival

It has been suggested that the ability of Ca^{2+} to interact with a family of high affinity Ca^{2+}-binding proteins, known as EF-Hand proteins, plays a pivotal role in the transduction of the calcium signal into a biological response.[33,34] Thus, we next investigated whether or not these Ca^{2+} binding proteins could influence apoptosis.[34] To answer this question we examined the role of two different calcium binding proteins. Calbindin-D_{28k} is normally expressed in restricted neuronal populations of the mammalian brain where it plays a role in protecting neurons against excitotoxic insults.[35] Parvalbumin is implicated in the relaxation of muscle after contraction where it helps to terminate contraction by soaking up Ca^{2+} from the cytosol.[36,37] It may also play a role in protecting non-muscle cells, particularly neurons, from Ca^{2+}-induced cell damage.[38]

On this basis, TSU and PC3 cells were transfected with expression vectors encoding for these two 'E-F hand' Ca^{2+}-binding proteins. The transfected cell lines TSU–CaBP (for Calbindin) and PC3-Parva (for Parvalbumin) were obtained. The intracellular concentration of either protein was not measured, but in both cases, it was high enough to prevent any significant rise of $[Ca^{2+}]_i$ when cells were challenged with 1 μM thapsigargin. TSU–Neo and PC3–Neo cell lines were also obtained after transfection with a plasmid encoding for a neomycin resistance gene. Two distinct clones of TSU–CaBP, PC3–Parva, TSU–Neo and PC3–Neo were separately exposed to 10 μM 5-FdUr or irradiated up to 800 cGy. Clonogenic survival and DNA fragmentation were measured at different time-points for 120 h after exposure. Two examples (Figure 6) illustrate how overexpression of Calbindin-D_{28k} or of Parvalbumin effectively reduced by half the effects of 5-FdUr and irradiation on clonogenic survival and on DNA fragmentation, respectively, during the first 48 h of exposure. A similar protective effect was obtained for the first 48 h following exposure to other inducing agents (data not shown). However this protective effect was lost after longer exposure (i.e. 96 h) as shown in Figure 6). This suggests that the micromolar Ca^{2+} influx eventually overwhelms the buffering capacity of the Ca^{2+}-binding proteins. Interestingly, a similar protective effect was also observed when cells were incubated with the cell permeant calcium buffer BAPTA–AM (data not shown). Therefore, the protective effect of Parvalbumin and Calbindin-$D28_k$ most likely resulted from their Ca^{2+}-buffer capacity.

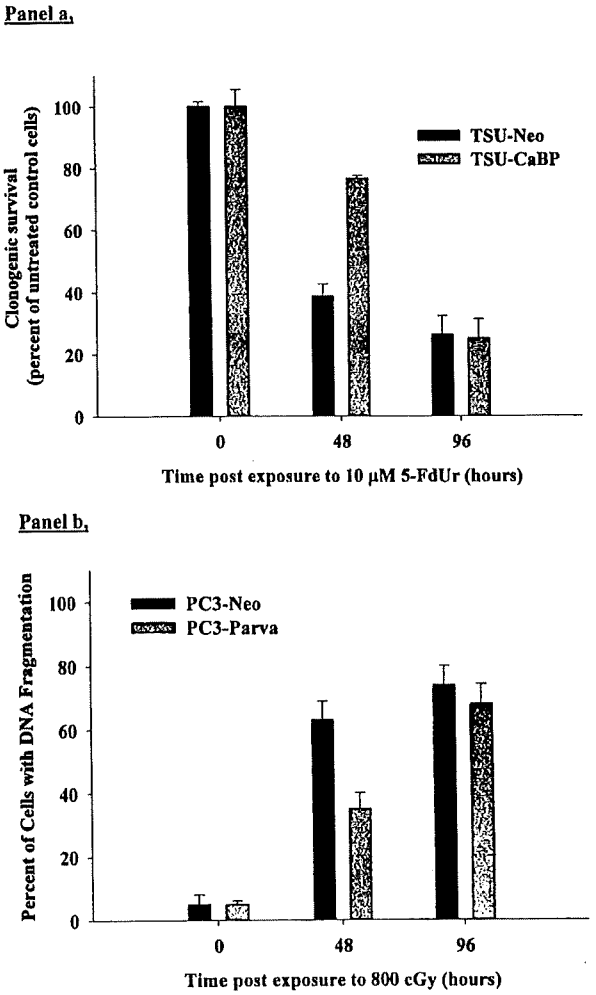


Figure 6 (a) Changes in clonogenic survival of TSU–Neo or TSU–CaBP cells exposed to 10 μM 5-FdUr. For each transfection, two different overexpressing clones have been separately exposed to 5-FdUr and analyzed. Results are expressed in per cent of untreated cells (see methods). Means and S.D. (b) Changes of DNA fragmentation of PC3–Neo or PC3–Parva cells exposed to 800 cGy. For each transfection, two different overexpressing clones have been separately exposed to radiation and analyzed. Results are expressed in per cent of Tunel positive cells. Means and S.D.

The delayed $\geqslant$10 μM elevation of $[Ca^{2+}]_i$ is associated with transcriptional reprogramming of death associated proteins

Gadd153 is a ubiquitously expressed member of the CCATT/enhancer-binding protein (C/EBP) family of transcription factors.[39] The expression of Gadd153 is minimal during normal growth but is highly induced in response to a variety of cellular stresses, including after an elevation of $[Ca^{2+}]_i$.[31,40,41] Such elevation can be observed in TSU cells exposed to 1 μM

TG, in which Gadd153 expression is detected by Western blot analysis as soon as 3 h after exposure and maximally at 24 h of exposure (Figure 7). In contrast, when TSU cells are exposed to 10 μM 5-FdUr, a more delayed increase in Gadd153 expression occurs after 24 h of exposure and peaked after 48–72 h (Figure 7). A similar delayed elevation was also observed when NRP154 cells were exposed to TGFβ-1 (data not shown). We therefore postulated that this delayed up-regulation of Gadd153 resulted from the induction of the delayed $[Ca^{2+}]_i$ rise.

To verify this hypothesis, we cloned into an EGFP reporter plasmid the promoter sequence of *Gadd153* (*pGadd153*–EGFP-1), which consists of the 900 first base pairs of the entire *Gadd153* cDNA. This promoter sequence has previously been used to monitor gadd153 up-regulation, in particular after stimulation by agents that increase $[Ca^{2+}]_i$ such as ionomycin.[42,43] A series of experiments with agents that directly elevate $[Ca^{2+}]_i$ demonstrated that, in TSU and NRP152 stably transfected with *pGadd153*–EGFP-1, *gadd153* RNA expression could be monitored in cells by measuring the amount of EGFP fluorescence. In individual cells, green fluorescence and morphological changes were monitored using the longitudinal spectrofluorometric method previously validated for $[Ca^{2+}]_i$ measurements and adjusted for GFP wavelength. In TSU and NRP152 cell transfected with *pGadd153*–EGFP, a 1.5 to 3.5 increase in green fluorescence was observed asynchronously in the cell population 12 to 96 h after exposure to 10 μM 5-FdUr or 10 ng/ml TGFβ-1 respectively (Figure 8). This increase expression of green fluorescence occurs simultaneously with the induction of the apoptosis related changes. When TSU or NRP 152 transfected *pGadd153*–EGFP-1 were first pre-incubated respectively with 5-FdUr or TGFβ-1 for 12 h in normal RPMI and then maintained in 100 nM $[Ca^{2+}]_e$, the increase in gadd153–EGFP fluorescence was dramatically reduced (data not shown). These data strongly suggest that the delayed elevation of $[Ca^{2+}]_i$ observed during the execution phase of apoptosis is involved in RNA transcription of gadd153.

Relationship between the delayed $\geqslant$10 μM $[Ca^{2+}]_i$ elevation, release of cytochrome *c*, caspase 3 activation, and DNA fragmentation

It has become widely accepted that, in most cell types, the ultimate decision to proceed to the final lethal steps of apoptosis occurs in mitochondria which release into the cytosol a cocktail of pro-apoptotic proteins that consistently includes cytochrome *c*.[6,8] Once in the cytosol, cytochrome *c* forms a complex with apaf-1 and caspase-9 that ultimately activates the downstream caspase cascade.[8] Thus far, no consensus has been reached on the exact nature of the intracellular signal that stimulates mitochondria to release their apoptogenic cocktail except that it seems to include opening of the mitochondrial transition pore and translocation and homo/hetero dimerization of several members of the BCL-2 family.[6,31]

In TSU cells exposed to 5-FdUr or doxorubicin, cytosolic translocation of cytochrome *c*, enzymatic activation of caspase-3, and DNA fragmentation was detectable in the cytosol as soon as 12 h after exposure to the inducing agent and was usually maximum at 48 h after exposure (Figure 9). When TSU cells were first exposed to the inducing agent for 12 h in normal medium and then maintained in 100 nM $[Ca^{2+}]_e$ to prevent the induction of the delayed $[Ca^{2+}]_i$ rise, cytosolic translocation of cytochrome *c*, enzymatic activation of caspases-3 and DNA fragmentation were decreased to close to background level. These results demonstrated that the delayed secondary rise was required for any of these events to be activated. Interestingly enough, as shown in Figure 9, restoring extracellular calcium to normal values (400 μM) for 12 h restored the apoptotic potential, enzymatic activation of caspases-3 and DNA fragmentation. Similar results were observed when incubating the AT3.1 cells for 48 h with 5-FdUr or when using NRP152 induced by TGFβ-1 (data not shown).

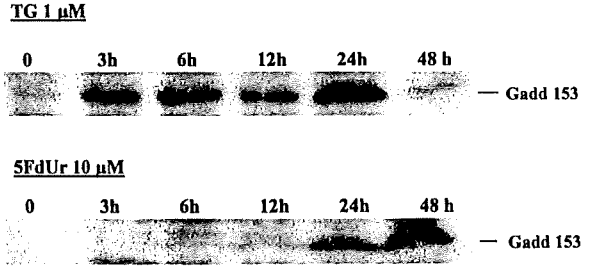
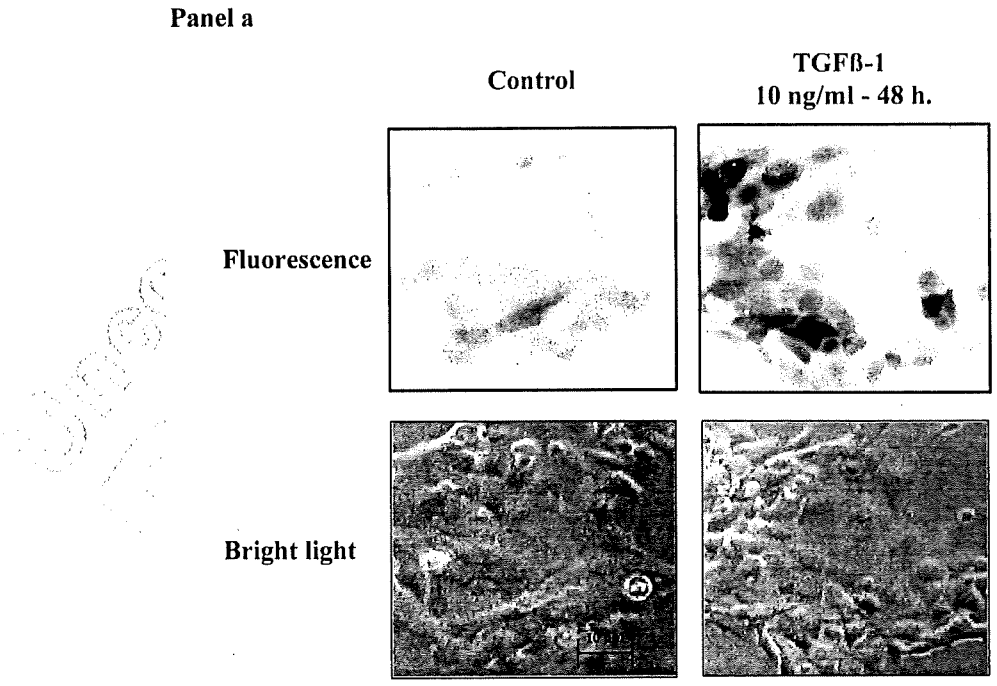


Figure 7 Western blot analysis of the time course of GADD153 protein expression in 1.5 10^6 TSU cells exposed to either 1 μM TG or 10 μM 5-FdUr

Discussion

Programmed cell death is a widespread phenomenon induced by both endogenous tissue-specific agents and exogenous cell-damaging agents. Since the initial observation of Kaiser *et al* in 1977, it is known that several of agents such as ionomycin or thapsigargin activate apoptosis by an early and transient elevation of $[Ca^{2+}]_i$. Several attempts were made to link this transient elevation of $[Ca^{2+}]_i$ with biochemical and enzymatic events occurring during the execution phase of apoptosis.[12,15,17] However, in these studies $[Ca^{2+}]_i$ measurements during apoptosis were usually performed with Ca^{2+} fluorescent probes that were inappropriate to measure $[Ca^{2+}]_i$ changes over the long period of time (12–96 h) needed to activate the complete apoptotic cascade.[13]

In this study we used a recently developed procedure to perform longitudinal kinetic analysis of $[Ca^{2+}]_i$ in individual cells, microinjected with high-molecular weight Fura-Dextran. This analysis was coupled with videomicroscopic monitoring of the morphological changes characteristics of the execution phase of apoptosis. This method allowed us to monitor $[Ca^{2+}]_i$ for 120 h after TG exposure and to observe a typical early rise of $[Ca^{2+}]_i$ followed by the late rise described here.[31] In the present study, this technique was used to study individual prostate cells after exposure to apoptotic agents (5-FdUr, Doxorubicin, radiation or TGF-β1) that, unlike TG or ionomycin, do not induce an immediate elevation in $[Ca^{2+}]_i$. We demonstrated that the execution



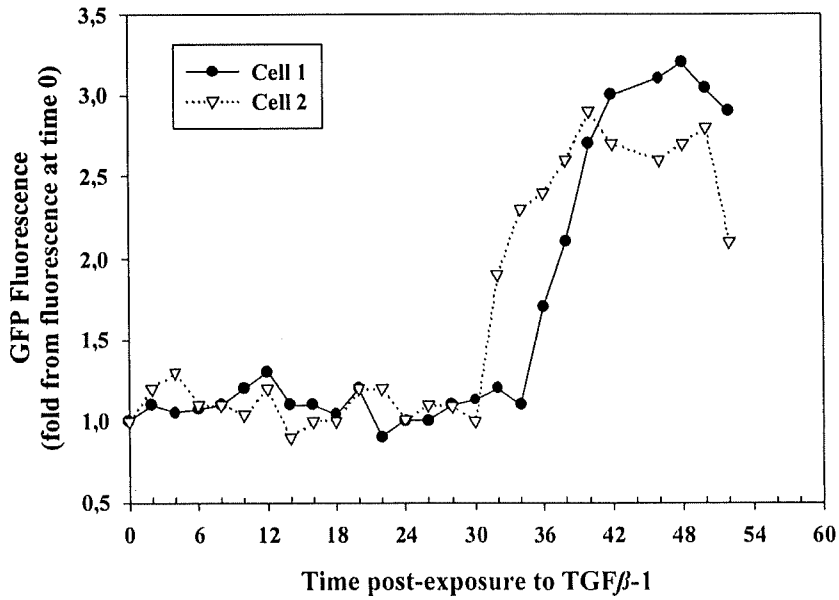


Figure 8 (**a**) Simultaneous analysis of *gadd153* transcription driven green fluorescence and morphological changes in NRP153 prostate cancer cells transfected with *gadd153*-EGFP-1 reporter gene. Left column: untreated cells; right column: cells exposed for 48 h to 10 ng/ml TGFβ-1. Notes that the cells with the highest green content are also those in which morphological signs of apoptosis such as cell detaching, rounding or blebbing can be identified. (**b**) Kinetic analysis of green fluorescence in two typical NRP152 cells. EGFP fluorescence is measured continuously from exposure to inducing agent until induction of morphological changes and reported in fold increase from fluorescence at time 0

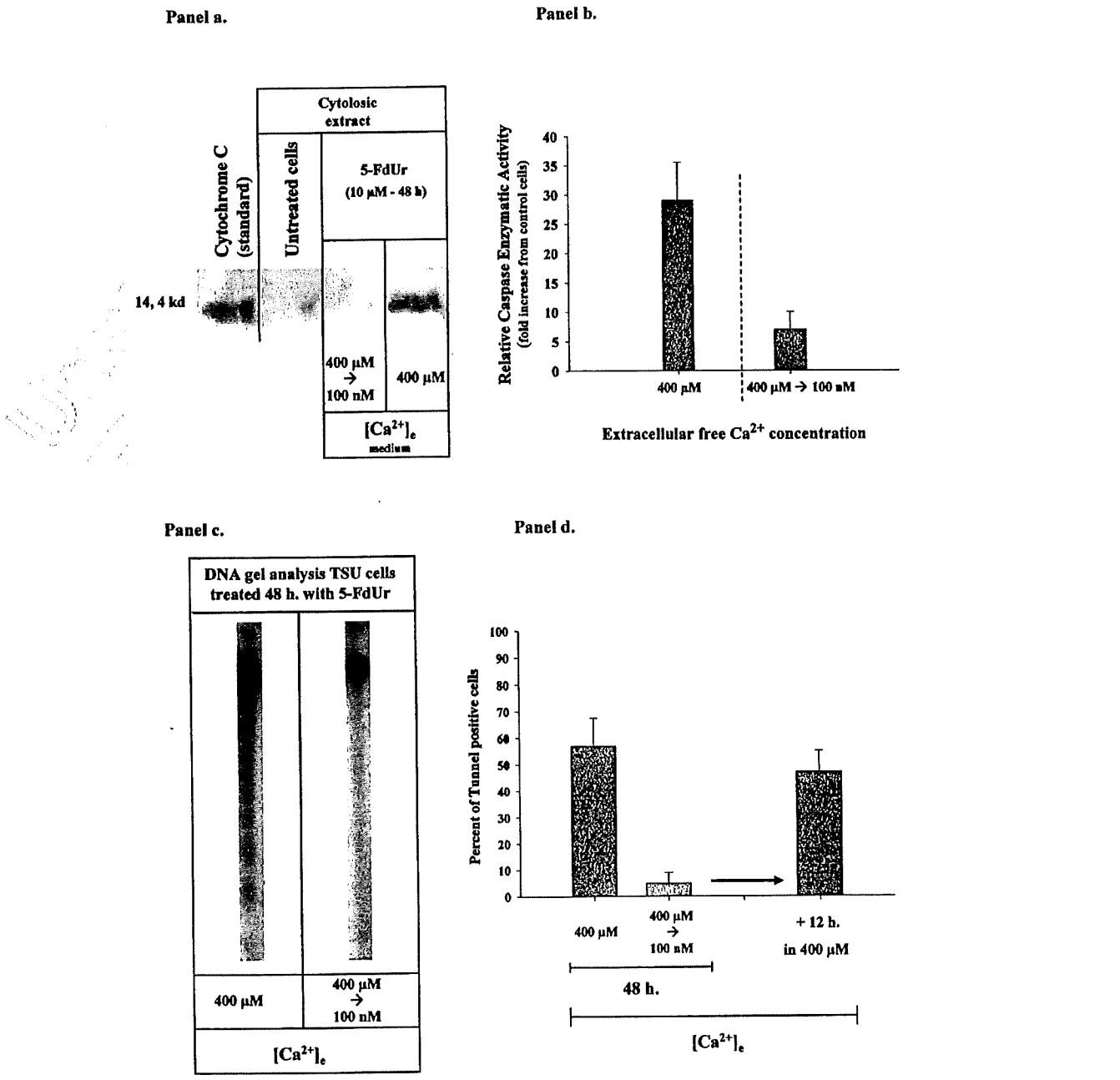


Figure 9 Influence of the concentration of the extracellular Ca^{2+} on four parameters of the execution phase of apoptosis in TSU cells exposed for 48 h to 10 μM 5-FdUr. In each case (**a–d**) left-hand side results (labeled 400 μM) were obtained from cells maintained for 48 h in RPMI medium containing 400 μM Ca^{2+}; right-hand side results (labeled 400 μM→100 nM) were obtained on cells maintained in normal medium for the first 12 h and then incubated 36 h in medium containing 100 nM Ca^{2+}. (**a**) Cytochrome *c* release in cytosolic fraction; (**b**) increase in caspase 3 activity; (**c**) gel electrophoresis of DNA fragmentation; (**d**) percentage of Tunel-positive cells, here the 400 μM→100 nM protocol was followed by the return to 400 μM Ca^{2+} for an additional period of 12 h

phase of apoptosis, that occurred from 12 to 96 h after exposure to the inducing agent, was consistently associated with an increase in intracellular $[Ca^{2+}]_i$ which occurred in two phases in sequence: an initial submicromolar rise followed by a supramicromolar one. The sequence occurred asynchronously among the cultured cells.

Rather than being a consequence of a non-specific increase in membrane leakiness, we present here experimental evidence suggesting that, at least in these models, the $[Ca^{2+}]_i$ elevation plays a pivotal role in the induction of the biochemical cascade that characterizes the execution phase of apoptosis. First, the submicromolar $[Ca^{2+}]_i$ rise

always preceded DNA fragmentation and the loss of clonogenic survival (Tables 1 and 2 and Figure 4). Indeed, the average delay of about 10 h between the submicromolar rise of $[Ca^{2+}]_i$ and DNA fragmentation is not much larger than the sum of the duration of the $[Ca^{2+}]_i$ rise observed in phase 2 (0.5–2 h) and phase 3 (4–8 h). The process terminates in phase 4 when large increases of permeability causes presumably $[Ca^{2+}]$ to equilibrate with the external medium, i.e. to reach millimolar values. Second, when the cells were maintained, after the signaling phase (12 h), in a low extracellular Ca^{2+} medium (100 nM), the delayed $[Ca^{2+}]_i$ rise did not occur as it depended on an influx of Ca^{2+}. Maintaining cells in low extracellular Ca^{2+} medium also greatly decreased transcriptional up-regulation of apoptosis related gene *gadd*153, release of cytochrome *c*, activation of caspase 3, DNA fragmentation and loss of clonogenic survival. Conversely, when extracellular Ca^{2+} concentration was re-increased to normal values (400 μM), caspase 3 activation and DNA fragmentation were restored. Third, to a lesser extent, a similar preventative effect could be obtained by overexpressing intracellular, high affinity Ca^{2+}-binding proteins, Calbindin-D_{28k} and Parvalbumin, or by loading the cells with a permeant Ca^{2+} buffer. In these cells, the execution phase of apoptosis was not suppressed but delayed up to 48 h; presumably the execution phase proceeded when the buffering capacity for Ca^{2+} was overwhelmed by the influx of extracellular Ca^{2+}. The last two sets of results suggest that during the signaling phase, cells are programmed to undergo apoptosis but that the entry into the execution phase requires the sub- and supramicromolar sequence of changes in $[Ca^{2+}]_i$. Although the existence of such a delayed, secondary rise in $[Ca^{2+}]_i$ had been previously suggested in model systems where inducing agents produce an early, transitory rise of $[Ca^{2+}]_i$,[44,45] this work provides the first experimental demonstration that (1) a large and sustained supramicromolar $[Ca^{2+}]_i$ rise occurs during the execution phase of apoptosis, independently of the nature of the inducing agent, and (2) that this supramicromolar rise is a prerequisite for activation of the execution phase.

It is clear that this work is mostly observational and that what remains is to elucidate the mechanism(s) underlying the delayed rise of $[Ca^{2+}]_i$ that heralds the execution phase. However, this observation is critical to demonstrate that the biochemical conditions (ie, high $[Ca^{2+}]_i$) necessary to activate a series of enzymes do exist for sustained period of time during the late phase of apoptosis. This $[Ca^{2+}]_i$ rise is definitely produced by an increased influx of Ca^{2+} from the medium, but it seems unlikely that it results from a non specific increase of membrane permeability. On the contrary, we have demonstrated that the $[Ca^{2+}]_i$ rise follows a well-defined temporal sequence: an initial submicromolar phase that lasts for some time (0.5–2 h), followed by a supramicromolar one that lasts even longer (4–8 h). However, even during this last phase, $[Ca^{2+}]_i$ remained 10–100 times below the external $[Ca^{2+}]$. While our observation that Ca^{2+} influx from the external medium is essential for the execution phase of apoptosis, it does not preclude the possibility that apoptotic agents first produce a Ca^{2+} release from the internal stores which, subsequently, induces the large influx of Ca^{2+} needed to bring $[Ca^{2+}]$ up to the 10 μM level, through capacitative calcium entry mechanisms.

Altogether, these results indicate that coordinated mechanisms do control the influx of Ca^{2+} that triggers execution of apoptosis. Previously we have observed a delayed supramicromolar elevation in breast, bladder and prostate cancer lines exposed to thapsigargin. In the present study we again observe a delayed $\geqslant 10$ μM $[Ca^{2+}]_i$ elevation following treatment of human bladder and prostate cancer cells with a number of mechanistically distinct cytotoxic agents. These combined results support the conclusion that this delayed supramicromolar $[Ca^{2+}]_i$ elevation constitutes a key and general event in the induction of the execution phase of apoptosis.

Materials and Methods

Materials

Thapsigargin, 5-Fluorodeoxyuridine, equine cytochrome *c*, and ionomycin were purchased from Sigma Chemical Co, St. Louis, MO, USA. Doxorubicin was purchased from Pharmacia Inc., Kalamazoo, MI, USA. TGF-β1 was purchased from Austral Biologicals, San Ramon, CA, USA.

Cell lines

The androgen-independent rat AT 3.1, human PC3 epithelial prostate cancer and the human TSU bladder cancer cell lines were cultured in RPMI1640 media supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 250 nM dexamethasone (AT3.1 cells only), 100 units/ml Penicillin G and 100 units streptomycin in 5% CO2/95% Air at 37°C. The origins of AT3.1, TSU, and PC3 cell lines have been described previously.[46–48] Tumorigenic NRP152 normal rat epithelial cell lines were kindly provided by D Danielpour, Case Western Reserve University, Cleveland, OH, USA. NRP152 were serially passaged in GM2 medium as described by Hsing *et al* and were maintained in DMEM/F12, 15 mM HEPES and 1% calf serum for apoptosis assays.[29] For ionizing radiation exposure, cells were exposed to a one time high dose rate 800 cGy radiation using a Gammacell 40 ^{137}Cs irradiator (Atomic Energy of Canada, Ltd).

$[Ca^{2+}]_i$ measurements

Simultaneous longitudinal analysis of $[Ca^{2+}]_i$ and morphological changes was performed using image analysis of cells microinjected with high molecular weight Fura Dextran according to a previously validated method develop in our laboratory.[13] Briefly, individual cells were grown on 4.5 cm cover slipped incubation chambers and microinjected directly in the cytosol with either 10 k or 70 k Fura Dextran, 10 k Mag-Indo Dextran (Molecular probes, Eugene, OR). Simultaneous analysis of $[Ca^{2+}]_i$ and morphological changes were performed in a controlled 37°C, 5% CO_2/95% air-humidified chamber mounted on a Zeiss Axiovert T-100 microscope (Zeiss, Oberkochen, Germany). Images were acquired every 30 min for 5 days with a Princeton CCD1300-v Camera (Princeton Instruments, Trenton, NJ, USA) and analyzed with MetaFluor software (Universal Imaging Corporation, West Chester, PA, USA). This frequency of measure-

ments was not detrimental to cell survival.[13] $[Ca^{2+}]_i$ measurements and calibration procedures of Fura Dextran signals have been described in detail previously.[13] Because Fura-2AM cannot be used to accurately measure $[Ca^{2+}]_i$ above 1 μM, measurement of $[Ca^{2+}]_i$ values between 1 and 100 μM was performed by microinjecting the cells with the low-affinity Ca^{2+} indicator 10 k Mag-Indo Dextran.[49] Fluorescent images of cells excited at 355 nm were alternatively recorded through a 405 and a 495 nm filter. $[Ca^{2+}]_i$ was calculated from the emission ratio using a titration curve prepared from three different concentrations of Mag-Indo Dextran (5, 10, 50 μM) in 1.37 μM to 1 mM extracellular Ca^{2+}, at 37°C, using the Calcium Calibration Buffer Kit from Molecular Probes, Eugene, OR, USA.

Apoptosis assays

The kinetics of the morphological changes of both microinjected and surrounding control population during $[Ca^{2+}]_i$ measurements was followed by serial acquisition of phase contrast images of cells within the microscopic field. The characteristics of these morphological changes associated with apoptosis in prostate cancer cell lines have been reported in detail elsewhere.[50] Measurement of loss of clonogenic ability using total cell pool was performed as described previously.[50] Briefly, both floating and attached cells were collected by trypsinization at defined time-points after exposure to the inducing agent. Cells were washed, counted and diluted in large volumes of HBSS solution (Sigma Chemical Co, St. Louis, MO, USA) to the concentration of 500 cells per ml. One ml of the suspension was then plated in culture flask (6 flasks in parallel) and grown in normal medium. After 1 week, cells were stained with crystal violet and colonies counted. The clonogenic survival is expressed as the ratio, x 100, between the number of colonies produced by the cells treated with the inducing agent and the number of colonies produced by untreated cells cultured in identical conditions.

Qualitative DNA fragmentation analysis was performed with the Genzyme TACS Apoptotic DNA Laddering kit according to the manufacturer's instructions (Genzyme, Cambridge, MA, USA). Quantitative detection of individual cells undergoing apoptosis was achieved by terminal transferase end-labeling apoptotic nuclei with the commercially available *In Situ* Cell Death Detection kit from Boerhringer, Indianapolis, IN, USA. The results were expressed as the per cent of cells undergoing apoptosis (i.e. the number of labeled divided by total number of cell x 100). For each experiment, the results were corrected for the background percentage of cells end-labeled in an untreated control.

Overexpression of 'EF-Hand' Ca^{2+}-binding proteins Calbindin-$D28_K$ and Parvalbumin

TSU and PC3 cells were respectively transfected with either a neomycin resistance vector containing the full-length Calbindin-$D28_k$ cDNA (i.e. pRCaBP neo plasmid generously supplied by Dr. D Dowd, St. Louis University, St. Louis, USA) or a plasmid carrying the entire cDNA of rat muscle parvalbumin under control of a SV40 promoter (i.e. SV40-cPV neo plasmid generously supplied by Dr. MW Berchtold, Institute of Molecular Biology, University of Copenhagen, Copenhagen K, Denmark). Controls cells were obtained by transfecting the same cell lines with a neomycin-resistance pZipNeo control vector. Clones expressing high levels of either Calbindin-D28k or Parvalbumin protein were selected by Western-Blot analysis and measurement for their ability to inhibit a rapid and transient rise in $[Ca^{2+}]_i$ induced by exposure to 10 μM TG or 1 μM ionomycin (as reported previously).[31]

Cytochrome *c* experiments

Extraction and Western-Blotting analysis of cytochrome *c* was performed as previously described.[31]

Gadd153 experiments

Detection of Gadd153 For protein analysis, Gadd153 was immunoprecipitated from whole cell extract obtained at various time after exposure to inducing agents with a polyclonal anti-Gadd153 IgG (Santa Cruz, Santa Cruz, CA, USA), resolved on a 18% SDS-acrylamide gel and detected by Western-blot detection using a performed monoclonal anti-Gadd153 antibody (Santa Cruz).

Analysis of transcriptional up-regulation of Gadd153 by EGFP Green Fluorescence reporter assay *pGadd153*-EGFP-1 vector was constructed by inserting the *gadd153* promoter (kindly provided by Dr. Holbrook, Laboratory of Biological Chemistry, NIA, National Institutes of Health, Baltimore, MD, USA) in the multiple cloning site of pEGFP-1 promoterless mammalian Expression Vectors (Clontech, Palo Alto, CA, USA). The promoter of *gadd153* has been previously used in other mammalian reporter systems and has been reported to be sensitive to $[Ca^{2+}]_i$ elevation.[40] TSU and NRP152 cells were transfected with Gadd153-EGFP-1 vector using Lipofectamine Plus reagent, according to the manufacturer's instructions. Transfected cells were grown in coverslipped micro-incubation chambers and mount on the setup used to monitor $[Ca^{2+}]_i$. Fluorescence was measured using an EGFP enhanced FITC filter (excitation 485 nm, emission 510 nm) set from Chroma Technologies. Individual cells were followed longitudinally. GFP fluorescence was measured continuously and expressed as *n* fold from the resting fluorescence measured before exposure to the inducing agent and integrated over the surface of the cells. Control cells consisted of TSU or NRP152 cells transfected with EGFP-N1 vector containing a constitutive CMV promoter or Neomycine resistant pZipNeo vector.

Stable transfectants of *pGadd153*-EGFP-1 transfected TSU and NRP152 cells were selected based on their ability to increase green fluorescence in response to a rise in $[Ca^{2+}]_i$ induced by 10 μM ionomycin or 1 μM TG. In both transfected cell lines, a 4-6 h exposure to 1 μM TG induced a 2.5-3-fold increase in green fluorescence. This increase in fluorescence occurs fairly synchronously in the cells population (data not shown). No increase in green fluorescence was detected in control cells exposed to the same agent. When cells are pre-incubated with Ca^{2+}-chelator BAPTA-AM to buffer $[Ca^{2+}]_i$ or maintained in low extracellular free Ca^{2+}, no increase in fluorescence was detected, demonstrating that the activation of the Gadd153 promoter is, at least in this system, calcium dependent (data not shown).

Measurements of caspase enzymatic activity

Crude cell extract of pooled floating and attached TSU cells were prepared at different time point. Caspase 3 enzymatic activity was measured using FluorAce Apopain Assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Acknowledgements

This work was generously supported by an award from the Foundation for the Cure of Prostate Cancer (CaPCURE) and a Special Project on Research Excellence (Prostate Cancer 2P50 CA58236-07) to Dr. J

Isaacs, by a Department of Defense Award to Dr. S Denmeade (DAMD17-00-1-0028) and by a grant from the FNRS and by a 'Fond Joseph Maisin' grant to Dr. B Tombal. Irradiation of prostate cancer cell lines was performed with the expert assistance of Dr. TL Deweese, Johns Hopkins Oncology Center, Baltimore, whose help is greatly appreciated.

References

1. Bowen ID and Lockshin RA (1981) Cell Death in Biology and Pathology. London: Chapman and Hall
2. Denmeade SR and Isaacs JT (1996) Activation of programmed (apoptotic) cell death for the treatment of prostate cancer. Adv. Pharmacol. 35: 281–306
(1) 3. Denmeade SR, Tombal B and Isaacs JT (2001) Apoptotic pathways in Prostate Cancer. In: Programmed Cell Death. Vol. II. Mattson MP, Estus S, Rangnekar VM (eds) Amsterdam Publisher pp. 23–54
4. Kerr JF and Searle J (1973) Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Archiv., B. Cell Pathology 13: 87–102
5. Zhivotovsky B, Gahm A, Ankarcrona M, Nicotera P and Orrenius S (1995) Multiple proteases are involved in thymocyte apoptosis. Exp. Cell Res. 221: 404–412
6. Hengartner MO (2000) The biochemistry of apoptosis. Nature 407: 770–776
7. Thornberry NA and Lazebnik Y (1998) Caspases: enemies within. Science 281: 1312–1316
8. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479–489
9. Kyprianou N and Isaacs JT (1988) Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology 122: 552–562
10. Nakamura M, Sakaki Y, Watanabe N and Takagi Y (1981) Purification and characterization of the Ca2+ plus Mg2+-dependent endodeoxyribonuclease from calf thymus chromatin. J. Biochem. (Tokyo) 89: 143–152
11. Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555–556
12. Kaiser N and Edelman IS (1977) Calcium dependence of glucocorticoid-induced lymphocytolysis. Proc. Natl. Acad. Sci. USA 74: 638–642
13. Tombal B, Denmeade SR and Isaacs JT (1999) Assessment and validation of a microinjection method for kinetic analysis of [Ca2+]i in individual cells undergoing apoptosis. Cell Calcium 25: 19–28
(2) 14. Skryma R, Mariot P, Bourhis XL, Coppenolle FV, Shuba Y, Abeele FV and *et al* (2000) Store depletion and store-operated Ca2+ current in human prostate cancer LNCaP cells: involvement in apoptosis. J. Physiol. 527: 71–83
15. Jiang S, Chow SC, Nicotera P and Orrenius S (1994) Intracellular Ca2+ signals activate apoptosis in thymocytes: studies using the Ca(2+)-ATPase inhibitor thapsigargin. Exp. Cell Res. 212: 84–92
16. Martikainen P and Isaacs J (1990) Role of calcium in the programmed death of rat prostatic glandular cells. Prostate 17: 175–187
17. Furuya Y, Lundmo P, Short AD, Gill DL and Isaacs JT (1994) The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. Cancer Res. 54: 6167–6175
18. Squier MK, Miller AC, Malkinson AM and Cohen JJ (1994) Calpain activation in apoptosis. J. Cell Physiol. 159: 229–237
19. Madsen KR, Fiskum G and Clawson GA (1990) Regulation of nuclear scaffold protease activity by calcium. Exp. Cell Res. 187: 343–345
20. Fesus L, Thomazy V and Falus A (1987) Induction and activation of tissue transglutaminase during programmed cell death. FEBS Lett. 224: 104–108
21. Shibasaki F and McKeon F (1995) Calcineurin functions in Ca(2+)-activated cell death in mammalian cells. J. Cell Biol. 131: 735–743
22. Distelhorst CW and Dubyak G (1998) Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings. Blood 91: 731–734
23. Jayaraman T and Marks AR (1997) T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. Mol. Cell. Biol. 17: 3005–3012
24. Khan AA, Soloski MJ, Sharp AH, Schilling G, Sabatini DM, Li SH, Ross CA and Snyder SH (1996) Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor. Science 273: 503–507
25. Kyprianou N and Isaacs JT (1989) "Thymineless" death in androgen-independent prostatic cancer cells. Biochem. Biophys. Res. Commun. 165: 73–81
26. Denmeade SR, Nagy A, Gao J, Lilja H, Schally AV and Isaacs JT (1998) Enzymatic activation of a doxorubicin-peptide prodrug by prostate-specific antigen. Cancer Res. 58: 25537–2540
27. Kyprianou N and Rock S (1998) Radiation-induced apoptosis of human prostate cancer cells is independent of mutant p53 overexpression. Anticancer Res. 18: 897–905
28. DeWeese TL, Shipman JM, Dillehay LE and Nelson WG (1998) Sensitivity of human prostatic carcinoma cell lines to low dose rate radiation exposure. J. Urol. 159: 591–598
29. Hsing AY, Kadomatsu K, Bonham MJ and Danielpour D (1996) Regulation of apoptosis induced by transforming growth factor-beta1 in nontumorigenic rat prostatic epithelial cell lines. Cancer Res. 56: 5146–5149
30. Martikainen P, Kyprianou N and Isaacs JT (1990) Effect of transforming growth factor-beta 1 on proliferation and death of rat prostatic cells. Endocrinology 127: 2963–2968
31. Tombal B, Weeraratna AT, Denmeade SR and Isaacs JT (2000) Thapsigargin induces a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells. Prostate 43: 303–317
32. Jackisch C, Hahm HA, Tombal B, McCloskey D, Butash K, Davidson NE and Denmeade SR (2000) Delayed micromolar elevation in intracellular calcium precedes induction of apoptosis in thapsigargin-treated breast cancer cells. Clin. Cancer Res. 6: 2844–2850
33. Christakos S, Barletta F, Huening M, Kohut J and Raval-Pandya M (2000) Activation of Programmed Cell Death by Calcium: Protection against Cell Death by the Calcium Binding Protein Calbindin-D_{28k}. In: Calcium: The molecular Basis of Calcium Action in Biology and Medicine Pochet R, Donato R, Haiech J, Heizmann C, Gerke V (eds) Amsterdam: Kluwer Academic Publisher pp 259–275
34. Maki M and Penta-EF-Hand (2000) Proteins and Calsenilin/DREAM: Involvement of the New EF-Hand Calcium-Binding Proteins in Apoptosis and Signal Transduction. In: Calcium: The molecular Basis of Calcium Action in Biology and Medicine Pochet R, Donato R, Haiech J, Heizmann C, Gerke V, editors. Amsterdam: Kluwer Academic Publisher pp 245–248
35. Mattson MP, Rychlik B, Chu C and Christakos S (1991) Evidence for calcium-reducing and excito-protective roles for the calcium-binding protein calbindin-D28k in cultured hippocampal neurons. Neuron 6: 41–51
36. Gillis JM, Thomason D, Lefevre J and Kretsinger RH (1982) Parvalbumins and muscle relaxation: a computer simulation study. J. Muscle Res. Cell Motil. 3: 377–398
37. Gillis JM (1985) Relaxation of vertebrate skeletal muscle. A synthesis of the biochemical and physiological approaches. Biochimica Et Biophysica Acta 811: 97–145
38. Pauls TL, Cox JA and Berchtold MW (1996) The Ca2+(-)binding proteins parvalbumin and oncomodulin and their genes: new structural and functional findings. Biochimica Et Biophysica Acta. 1306: 39–54
39. Choi AM, Tucker RW, Carlson SG, Weigand G and Holbrook NJ (1994) Calcium mediates expression of stress-response genes in prostaglandin A2-induced growth arrest. FASEB J. 8: 1048–1054
40. Bartlett JD, Luethy JD, Carlson SG, Sollott SJ and Holbrook NJ (1992) Calcium ionophore A23187 induces expression of the growth arrest and DNA damage inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153. Ca2+ increases transcriptional activity and mRNA stability. J. Biol. Chem. 267: 20465–20470
41. Lin XS, Denmeade SR, Cisek L and Isaacs JT (1997) Mechanism and role of growth arrest in programmed (apoptotic) death of prostatic cancer cells induced by thapsigargin. Prostate 33: 201–207
42. Luethy JD, Fargnoli J, Park JS, Fornace AJ and Holbrook NJ (1990) Isolation and characterization of the hamster gadd153 gene. Activation of promoter activity by agents that damage DNA. J. Biol. Chem. 265: 16521–16526
43. Park JS, Luethy JD, Wang MG, Fargnoli J, Fornace AJJ, McBride OW and Holbrook NJ (1992) Isolation, characterization and chromosomal localization of the human GADD153 gene. Gene 116: 259–267
44. Oshimi Y and Miyazaki S (1995) Fas antigen-mediated DNA fragmentation and apoptotic morphologic changes are regulated by elevated cytosolic Ca2+ level. J. Immunol. 154: 599–609

45. D'Herde K and Leybaert L (1997) Intracellular Free Calcium Related to Apoptotic Cell Death in Quali Granulosa Cell Kept in Serum-Free Culture. Cell Death Differ. 4: 59–65
46. Isaacs JT, Isaacs WB, Feitz WF and Scheres J (1986) Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. Prostate 9: 261–281
47. Iizumi T, Yazaki T, Kanoh S, Kondo I and Koiso K (1987) Establishment of a new prostatic carcinoma cell line (TSU-Pr1). J. Urol. 137: 1304–1306
48. van Bokhoven A, Varella-Garcia M, Korch C and Miller GJ (2001) TSU-Pr1 and JCA-1 cells are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. Cancer Res. 61: 6340–6344
49. Tse FW, Tse A and Hille B (1994) Cyclic Ca2+ changes in intracellular stores of gonadotropes during gonadotropin-releasing hormone-stimulated Ca2+ oscillations. Proc. Natl. Acad. Sci. USA 91: 9750–9754
50. Martikainen P, Kyprianou N, Tucker RW and Isaacs JT (1991) Programmed death of nonproliferating androgen-independent prostatic cancer cells. Cancer Res. 51: 4693–4700