Research Article

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Calcium signaling in prostate cancer cells of increasing malignancy

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Abstract: Calcium signaling controls a large variety of cell functions, including proliferation and apoptosis, and plays a major role in neoplastic transformation. Prostate cancer (PCa) is one of the most common malignancies in men. The transition to castration-resistant prostate cancer (CRPC), a lethal form that is still lacking an effective cure, could be influenced by fine tuning intracellular calcium ($[Ca^{2+}]_i$) homeostasis. This study investigates $[Ca^{2+}]_i$ dynamics in metastatic PCa cell lines that mimic the progression of PCa to CRPC: (i) well differentiated LNCaP cells that require androgen for survival, and (ii) poorly differentiated, highly aggressive androgen-insensitive prostate cancer (AIPC) PC3 and DU145 cells. In AIPC cells, ATP induces a fast rise in $[Ca^{2+}]_i$, due to release from intracellular stores and sensitive to phospholipase C inhibitors, while LNCaP cells do not respond to ATP challenge. Moreover, AIPC cells showed a reduced capacity to store Ca^{2+} in thapsigargin-sensitive stores and limited store-operated calcium entry, with respect to androgen-dependent LNCaP cells. Finally, green tea extract causes $[Ca^{2+}]_i$ elevation and inhibits proliferation in PC3 and DU145 cells, but is ineffective in LNCaP cells. The consequences of these differences are discussed and interpreted in this study with reference to previously proposed models for Ca^{2+} dependence of prostate carcinogenesis.

Keywords: PC3 cells, DU145 cells, LNCaP cells, store-operated calcium entry, ATP, green tea

Introduction

Calcium signaling controls a large variety of cell functions, including fertilization, learning and memory, contraction, secretion and gene expression, as well as proliferation and apoptosis [1]. It plays a key role in several pathologies and is correlated to abnormal tissue growth and invasion in neoplastic transformation [2,3].

Due to its multifaceted action, intracellular Ca^{2+} level ($[Ca^{2+}]_i$) is regarded as the leading factor of major cellular functions, but also a difficult-to-control parameter [4]. In malignant transformation, $[Ca^{2+}]_i$ signaling can induce cell survival or trigger apoptosis, but it is generally believed that $[Ca^{2+}]_i$ oscillations promote cell proliferation and survival, whereas sustained cytosolic $[Ca^{2+}]_i$ induces cell death by apoptosis [5,6]. So, in principle, it might be possible to antagonize the malignant transformation and proliferation of cancer cells with disturbing cell $[Ca^{2+}]_i$ dynamics, targeting the altered Ca-modulating proteins, and channels during cancer development.

Prostate cancer (PCa) is the most common malignancy in men in the industrialized countries [7,8]. Androgen deprivation, the mainstay of treatment for advanced PCa, frequently but not always, results in tumor remission, while in many cases, the disease progresses to castration-resistant prostate cancer (CRPC), a lethal form lacking an effective cure [9,10]. Recent studies have provided evidence that in malignant transformation, PCa cells do not simply become androgen-independent, but rather become more sensitive to androgen and androgen precursors or bypass the androgen receptor [11–13].

In any case, the transition to CRPC involves a significant modification of the Ca^{2+}-dependent elements that mediate cell apoptotic pathways [14,15]. In previous studies, transition to CRPC phenotype was found to be associated with a significantly lowered filling of intracellular Ca^{2+} stores (endoplasmic reticulum (ER)), which was due to under expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, combined with enhanced Ca^{2+} leak and decreased store-operated Ca^{2+} entry store-operated calcium entry (SOCE) [16–19].

For a long time, three established metastatic prostate carcinoma cell lines (DU145, PC3, and LNCaP) were the system of choice for many experimental approaches [20]. PC3 cells are a characteristic of small cell neuroendocrine carcinoma, an aggressive variant of PCa, which is

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harmful for androgen-receptor and prostate-specific antigen and does not respond to hormonal therapy [21]. In contrast, LNCaP cells share common features with adenocarcinoma cells [22] and require a concentration of four orders of higher magnitude 5α-dihydrotestosterone for survival with respect to PC3 and DU145 cells [23].

These three PCa cell lines are suitable models to investigate the Ca^{2+} signaling machinery in the progression of cancer and how to enhance the proapoptotic potential of malignant cells in the treatment of advanced PCa [14,17]. In this respect, there is a growing interest in the natural compounds that can provide chemical agents to improve PCa cure [13]. For example, we have recently described how green tea extract and green tea catechins may stimulate entry of very small amounts of Ca^{2+} from outside and initiate Ca^{2+}-induced Ca^{2+} release (CICR) [24,25], a mechanism that was also described for different substances and can be a characteristic of hormone-refractory PCa cells [26,27] and could antagonize proliferation of these cells.

This study investigates some functional differences that may be significant for increasing malignant behavior and identifying specific targets to stimulate apoptosis in CRPC cells.

Methods

Chemicals

Analytical grade chemicals were purchased from Merck Life Science (Sigma-Aldrich, Milano, Italy), unless otherwise specified. Food/cosmetic grade ethanol 70/water 30 extract of *Camellia sinensis* (L.) Kuntze (green tea, CAS 84650-60-2), in the form of dry powder, was purchased from Farmalabor Srl, Canosa di Puglia, Italy. Dry extract was titrated in 50% polyphenols and contained approx. 20% (−)-Epigallocatechin-3-gallate (EGCG) w/w according to the manufacturer's specifications, as well as total catechins ≥30% w/w, epicatechin gallate ≥4% w/w and caffeine ≤3% w/w.

Cell models and viability assay

Human LNCaP, DU145 and PC3 prostate carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS and 2 mM glutamine, at 37°C, in 5% CO_2, fully humidified atmosphere. LNCaP cell-medium was also supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate and 4.5 mg/mL glucose.

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Cells were settled in 96-well plates for 24 h and exposed to various agents for 48 h as specified. After the stipulated time, cells were incubated with 100 µL/mL MTT; 5 mg/mL phosphate buffered saline was added to the cell culture medium without serum for 3 h at 37°C; the cells were treated with a solution of 1 N HCl–isopropanol (1:24, v/v) and mixed to dissolve the dark-blue formazan crystals, formed as a result. After a few minutes at room temperature, the plates were read at 550 nm in a V_{Max} microplate reader (Molecular Devices, Sunyvale, CA).

Intracellular calcium measurements

Cytosolic Ca^{2+} concentration was measured by loading cells with the intracellular fluorescent Ca^{2+} chelator Fura-2-acetoxymethyl ester (Fura-2, AM) (Thermo Fisher Scientific) and using a microspectrophotometry fluorescence-ratio setup, equipped with a perfusion system [24,28]. Cells were plated on poly-L-lysine-coated glass coverslips and incubated with 5 µM Fura-2, AM in a physiological saline (see below) at 37°C for 40 min. After that time, the coverslips were washed and mounted on the stage of an inverted microscope (Axiovert Zeiss, Germany), where they were continually superfused with different solutions. Cells were illuminated using a xenon lamp through a wavelength selector monochromator; emission was observed through an X40 quartz objective and recorded by a photomultiplier. The ratio R = E340/E380 was calculated every 40 ms to acquire a time-dependent internal Ca^{2+} sensitive signal. At the end of each experiment, cells were incubated with 2 µM ionomycin in 1 mM Ca^{2+} until the ratio reached a maximum value of R_{max}, then 10 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was applied until the ratio reached a minimum value (R_{min}). Finally, $MnCl_2$ (5 mM) was added to the bath to quench the Fura-2 fluorescence and determine the background fluorescence values. The fluorescence emissions relative to each excitation wavelength (E 340 and E 380 respectively) were corrected for this background signal before ratio R determination. Internal Ca^{2+} was calculated according to the Grynkiewicz equation [29].

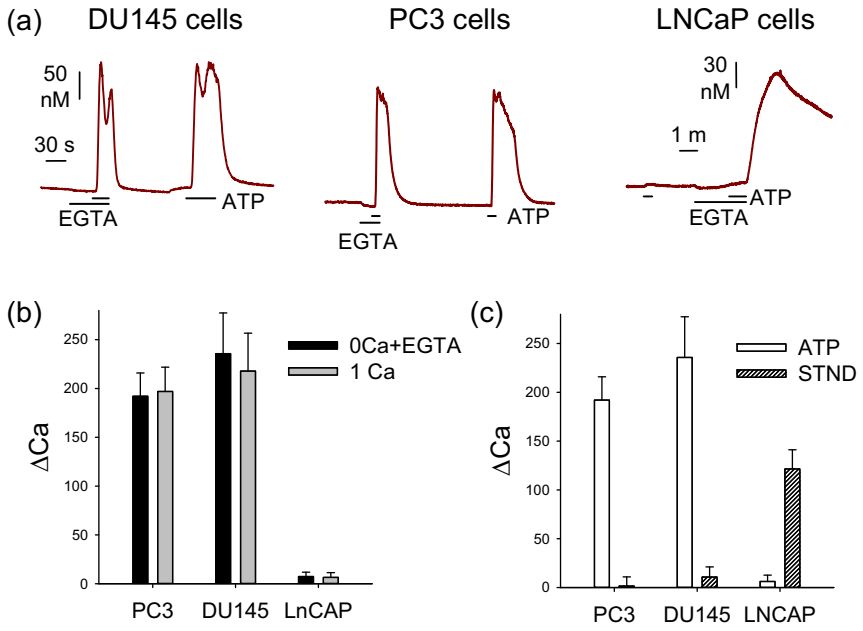

Figure 1: The effect of ATP application on $[Ca^{2+}]_i$ in PCa cells. (a) Internal $[Ca^{2+}]_i$ spikes in Fura-2 loaded DU145 and PC3 cells exposed to 10 μM ATP for 20 s in 0Ca-EGTA external solution (EGTA) and in basal (1 mM) external Ca. The same protocol did not cause any $[Ca^{2+}]_i$ rise in LNCaP cells, but $[Ca^{2+}]_i$ increased when external Ca^{2+} returned to the control value (1 mM). (b) Internal Ca^{2+} level following 10 μM ATP application in the presence and absence of external Ca in the three cell lines, expressed as $\Delta Ca = [Ca^{2+}]_{i(ATP)} - [Ca^{2+}]_{i(basal)}$. Lines represent SEM over the number of experiments, which were LNCaP $n = 9$, PC3 $n = 7$, DU145 $n = 4$. (c) $[Ca^{2+}]_i$ level following ATP application in 0 Ca EGTA external solution in the three cell lines, as in B (ATP), and after external Ca^{2+} 1 mM was restored (STND). Despite the low response to ATP, a SOCE response was always present in LNCaP cells, but not in the other two, ATP-respondent cell types. Number of experiments were LNCaP $n = 7$, PC3 $n = 9$, DU145 $n = 4$. Lines represent SEM.

The physiological standard bath solution contained (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10, and glucose 10. The pH was adjusted at 7.4 with NaOH. The Ca^{2+}-free solution (indicated as EGTA in the figures) had the same composition with 0 $CaCl_2$ added, 4 $MgCl_2$ and 2 EGTA.

Statistical analysis

Data were analyzed using Sigma Plot software (Systat Software Inc.) and shown as mean ± SD for cytotoxicity assays and mean ± SEM in all other experiments. Statistical significance was evaluated using Student's *t*-test or Mann–Whitney Rank Sum test, as indicated by the software.

Results

The first functional difference between the different cell types was the response to stimulation by ATP. In both PC3 and DU145 cells, application of ATP (10 μM) induced a rapid and elevated $[Ca^{2+}]_i$ rise, which was not dependent on external Ca^{2+}, but it was entirely due to the release of Ca^{2+} from intracellular stores (Figure 1). As reported in PC3 cells [24], the aminosteroid U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione), a potent inhibitor of phospholipase C (PLC) enzymes and inositol trisphosphate (IP_3)-mediated Ca^{2+} release, completely abolished the Ca^{2+} peak following ATP application in PC3 and this observation was confirmed in DU145 cells, with undetectable $[Ca^{2+}]_i$ rise with 5 μM U73122 in four experiments. Therefore the ATP-driven internal Ca^{2+} rise is dependent on IP_3 production in CRPC cells and mediated through P2Y receptors. In contrast, in androgen-sensitive LNCaP cells, ATP never determined a sizeable $[Ca^{2+}]_i$ rise, either in the presence or in the absence of Ca^{2+} in the external bath. In addition, when external Ca^{2+} was restored (1 mM), after ATP treatment in EGTA, an increase in $[Ca^{2+}]_i$ was always observed in LNCaP cells, but not in PC3 and DU145 cells (Figure 1).

This observation is in agreement with differences in Ca^{2+} storing and releasing capacity between two different cell types. In LNCaP cells, in a Ca^{2+}-free EGTA-containing external solution, the SERCA blocker thapsigargin caused

a transient $[Ca^{2+}]_i$ rise and emptying of sensitive stores. When extracellular Ca^{2+} was restored, $[Ca^{2+}]_i$ increased rapidly and sizably due to SOCE. These effects where blunted in PC3 and DU145 cells, which showed a reduced capacity to store Ca^{2+} in thapsigargin-sensitive stores and limited SOCE, with respect to androgen-dependent LNCaP cells (Figure 2).

In LNCaP, SOCE was antagonized by gadolinium (Gd, 1 µM) and by 4-methyl-'-[3,5-bis[trifluoromethyl]-1*H*-pyrazol-1-yl]-1,2,3-thiadiazole-5-carbox-anilide (YM-58483, 5 µM), an highly specific SOCE inhibitor [30] (Figure 3).

Previous studies have shown that both (−)-epigallocatechin-3-gallate (EGCG), the main green tea catechin, and green tea extract (GTE) cause $[Ca^{2+}]_i$ elevation and inhibit proliferation in PC3 and DU145 cells [24,25]. The first step of GTE-induced toxicity was mediated by a $[Ca^{2+}]_i$ increase, through release from thapsigargin-sensitive and insensitive stores. Viability experiments indicated that in these cells, GTE was able to inhibit proliferation with IC_{50} close to 62 µg/mL, which corresponds to a concentration of approximately 30 µM EGCG in the extract. In contrast, in LNCaP cells, EGCG and GTE hardly induced any Ca rise in five experiments

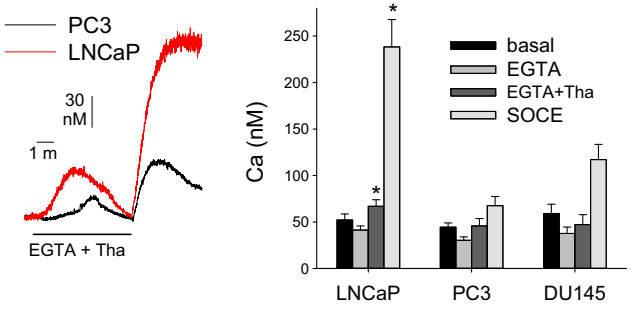

Figure 2: $[Ca^{2+}]_i$ level in the three cell lines following treatment with thapsigargin (1 µM, Tha) in 0 Ca EGTA (EGTA) and in control Ca^{2+} (1 mM). Traces on the left show two representative experiments, in PC3 (black trace) and LNCaP (red trace) cells, respectively. The $[Ca^{2+}]_i$ level decreased slightly, but not significantly, when switching from basal external Ca^{2+} (1 mM) to 0 Ca-EGTA in all cell types. Following thapsigargin application in 0 Ca-EGTA bath, $[Ca^{2+}]_i$ rise was larger in LNCaP than PC3 and DU145 and $[Ca^{2+}]_i$ level was significantly different from basal level only in these cell type. When external Ca^2 was restored to 1 mM, $[Ca^{2+}]_i$ increased as a consequence of SOCE and this response was more pronounced in LNCaP cells than in the other cell lines. Lines represent SEM over the number of experiments, which were LNCaP and PC3 n = 16, DU145 n = 10. (*) indicates significantly different from basal Ca level $p < 0.001$ by paired t-test.

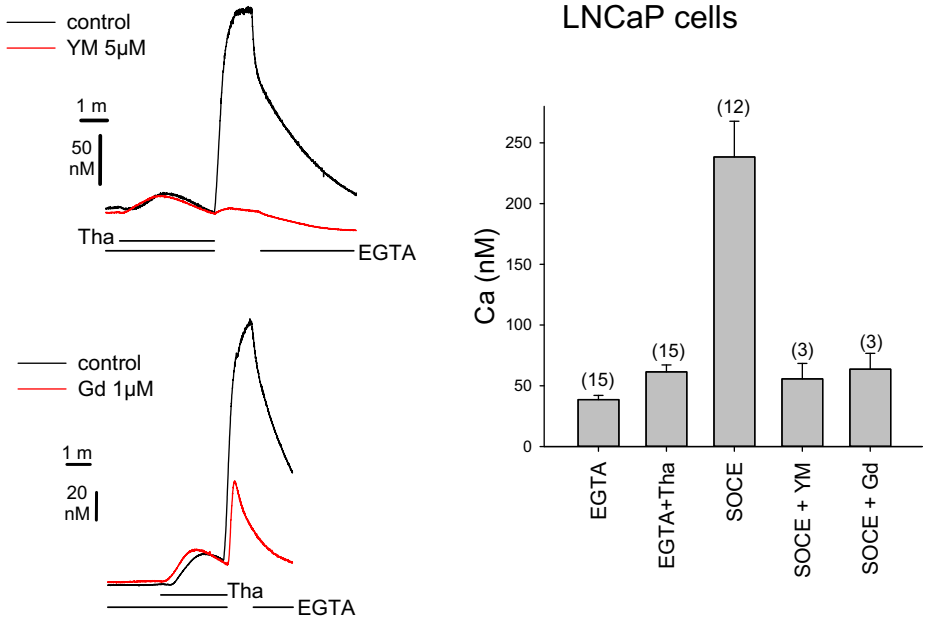

Figure 3: Effect of modifiers on the SOCE $[Ca^{2+}]_i$ response in LNCaP cells. On the left, traces show the effect of YM-58483 (above) and 1 µM Gd (below) on LNCaP SOCE response. The graph on the right shows the summary of the effects on SOCE $[Ca^{2+}]_i$ response of the two modifiers in LNCaP cells. Lines represent SEM over the indicated number of experiments.

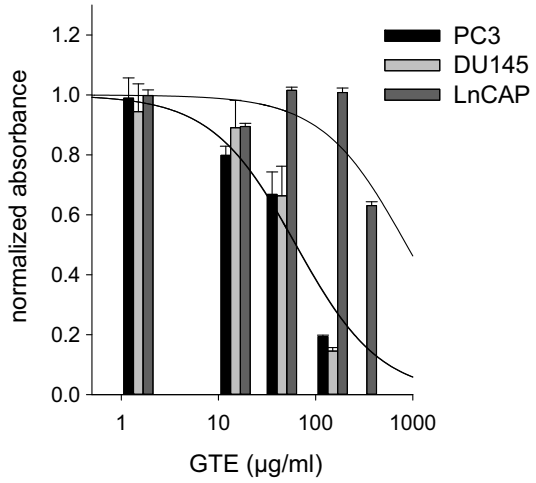

Figure 4: Dose–response data of cell viability obtained with the MTT assay after exposure of PCa cells to different concentrations of green tea extract (1.5, 15, 45, and 150 µg/mL GTE) for 48 h. Bars represent mean ± SD of percent MTT-formazan absorbance (see Materials and Methods section), normalized to the control absorbance (proliferation in the absence of GTE). In PC3 and DU145 cells, the IC_{50} was 62 and 63 µg/mL, respectively. In LNCaP cells, the additional dose of 300 µg/mL GTE was tested and of IC_{50} was estimated 862 µg/mL.

(not shown) and viability measured by MTT assay was much less sensitive to GTE, with an estimated IC_{50} close to 850 µg/mL (Figure 4).

The main differences in Ca^{2+} signaling described in this study are summarized in Figure 5.

Discussion

The malignant, hormone-refractory, or more precisely, castration-resistant stage of PCa is characterized by inhibition

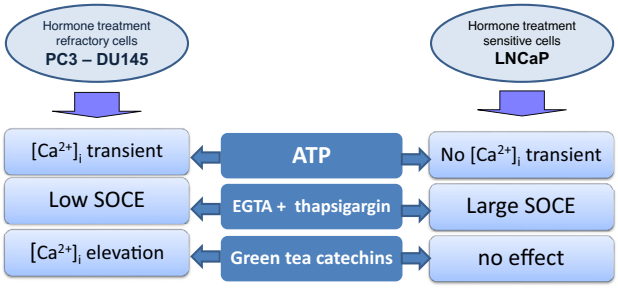

Figure 5: The three main differences in Ca^{2+} signaling between hormone treatment refractory and sensitive cells: response to purinergic stimulation, $[Ca^{2+}]_i$ storing and releasing capacity and sensitivity to GTE catechins.

of apoptosis and abnormal cell proliferation. Several models have established the central role of Ca^{2+} signaling in this progression [14,15,31,32]. This study outlined some aspects of cytosolic Ca^{2+} imbalance that define the most aggressive stage of PCa and characterize the different behavior of hormone-refractory cells.

Purinergic receptors are overexpressed in some types of cancer cells and tissues and specific subtypes are implicated in numerous oncogenic processes [33,34]. A role of P2Y receptors was postulated in PC3 and DU145 cells [35,36]. The expression of $P2Y_1$ receptors was confirmed by subsequent studies in these cells [37,38] and experiments suggested that their activation may inhibit growth and induce cell death [37]. In LNCaP cells, ATP did not stimulate any cytosolic Ca^{2+} peak [36] and no effect of nucleotides on either IP_3 or cyclic AMP was detectable, despite a significant expression of $P2Y_2$ and $P2Y_{11}$ transcripts [39].

This study is in agreement with previous observations and confirms that in PC3 and DU145 cells, ATP mediates the activation of PLC and IP_3-driven transient cytosolic release of Ca^{2+}, without involvement of Ca^{2+} influx from the external medium. In contrast, in LNCaP cells, ATP did not induce any transient Ca^{2+} peak, either in the presence or in the absence of external Ca^{2+}. The possibility to use $P2Y_1$ receptor agonists as a therapeutic drug for treating refractory PCa is linked to the ability of these activated receptors to promote apoptosis which was recently investigated [38]. So it appears that if these receptors can be considered to modulate $[Ca^{2+}]_i$ in PCa cell, they would represent a more specific target for CRPC cells.

Previous studies investigated the mechanism of SOCE during PCa progression to malignity [18]. It was proposed that the enhanced expression of ORAI3 subunit, which forms heteromultimeric ORAI1/ORAI3 store-independent channels [19], promotes PCa cell proliferation and confers apoptosis resistance [31].

Our data confirmed that the hormone-sensitive LNCaP cells are well equipped to activate SOCE, while hormone-refractory PCa cells are less capable to accumulate and release less Ca^{2+} from ER stores and have reduced SOCE. Another study has suggested that lowering the ER Ca^{2+}-store content is a mechanism by which hormone-refractory cells escape death in the absence of androgenic stimulation [40].

Interestingly, in LNCaP cells, external Ca^{2+} withdrawal and ATP application appeared to be effective stimulators of SOCE even in the absence of a net $[Ca^{2+}]_i$ transient. This observation may be explained by a close connection between ER and mitochondria [41,42]. The

contact between these two compartments is significantly favored by the overexpression of the voltage-dependent anion channels (VDACs), located on the outer mitochondrial membrane [43]. VDACs, also called mitochondria porins, enhance Ca^{2+} signal propagation into the mitochondria, increasing the extent of mitochondrial Ca^{2+} uptake [44]. In LNCaP cells, various evidences suggested that VDACs are involved in the extrinsic apoptotic pathway [45] and control LNCaP cells that appear to overexpress the mitochondria porins with respect to similar cells that had already started the transition to CRPC status [46]. ER depletion and Ca^{2+} overloading of the mitochondria has been found to play an important role in triggering apoptotic cell death [47].

As hormone-refractory PCa cells have lost the normal control on apoptotic stimuli, targeting the key players determining Ca^{2+} homeostasis to enhance the pro-apoptotic potential of malignant cells may prove to be a useful strategy in the treatment of advanced PCa [17]. For this, great attention is devoted to bioactive natural compounds that, through oxidation-induced alterations in Ca^{2+} signaling, may induce apoptosis [13]. It has been recently seen that the major green tea catechin, EGCG, induces a $[Ca^{2+}]_i$ increase in PC3 and DU145 cells through intracellular Ca^{2+} release triggered by a moderate Ca^{2+} influx from the outside [24]. This mechanism is dependent on CICR through ryanodine receptors (RyRs), with an important contribution of IP_3-driven Ca^{2+} release from ER.

Both EGCG and total GTE have a similar way of action in these cells and reduce cell viability following a 48 h exposure [24,25]. In contrast, the results presented in this study show that GTE is much less effective in diminishing viability in LNCaP cells and this may be linked to the presence of different specific RyRs. While little is known about expression and function of RyRs in PC3 cells, both LNCaP and DU145 cells have been reported to express functional RyRs [48,49]. Different RyR isoforms are expressed differently and a comparison with nontumorogenic cell lines suggested that the expression of the RyRs and tumor aggression is not related to specific RyR isoforms, but may be linked to the activity and number of receptors [49].

In summary (Figure 5), ATP-driven Ca^{2+} transients are a peculiar response of hormone-refractory cells, which appear to have lost the full SOCE capacity. While SOCE, with presumptive ensuing mitochondrial overload, would drive LNCaP cells toward the apoptotic demise, in PC3 and DU145 cells, an external stimulus such as natural compounds from green tea is required for triggering a consistent $[Ca^{2+}]_i$ elevation and switching the cell fate from proliferation to apoptosis.

The differences in Ca^{2+} signaling between hormone-sensitive and hormone-refractory cancer cells are complex and hardly described by as simple a scheme as that depicted in Figure 5. However, a better comprehension of the individual players in the intricate malfunctioning of malignant PCa cells is essential to define a more specific, less invasive and hopefully, effective therapy.

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Data availability statement: The datasets generated during and/or analyzed during this study are available from the corresponding author on reasonable request.

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