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Research article

Green tea catechins and intracellular calcium dynamics in prostate cancer cells

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Abstract: Perturbations of internal Ca^{2+} ($[Ca^{2+}]_i$) homeostasis play a key role in several pathologies and in neoplastic transformation, where deregulated cell proliferation, together with the suppression of apoptosis, provides the condition for abnormal tissue growth and invasion. Green tea catechins have been shown to affect cancer development by interference with basic cellular functions, most of which are mediated by $[Ca^{2+}]_i$. Prostate cancer (PCa) is one of the most common malignancy in men in Western countries and the androgen-independent carcinoma is a lethal form for which there is still no effective therapy. Different evidences suggested that consumption of green tea may have beneficial effects against PCa. We have previously described how the main green tea flavonoid, (−)-epigallocatechin-3-gallate (EGCG), inhibited proliferation and induced dose-dependent peaks of $[Ca^{2+}]_i$ in metastatic androgen-insensitive DU145 and PC3 PCa cells, by a mechanism that combined Ca^{2+} entry and Ca^{2+}-induced Ca^{2+} release. In the present study, we studied the effect of green tea extract (GTE) on the same cell lines. Proliferation, measured by MTT assay, was inhibited by GTE with IC_{50} close to 60 μg/ml, a value that is higher than that expected by EGCG effect alone. $[Ca^{2+}]_i$, measured in real time by the fluorescent dye Fura-2, was transiently increased by GTE by a mechanism that resembled that described for EGCG, but was largely independent of external Ca^{2+}. These observations suggested that other components, acting in synergy with EGCG, were involved in GTE effect, and confirmed the view that the alleged health benefits of green tea for PCa prevention may be related to $[Ca^{2+}]_i$ deregulation in malignant cells. These results may be significant to understand the functional mechanisms by which flavonoids exert their beneficial or toxic actions.

Keywords: androgen-resistant prostate cancer PC3 cells; DU145 cells; green tea extract; internal Ca2+ release

1. Introduction

Calcium signaling controls a large variety of physiological cell functions, including proliferation and apoptosis [1]. In order to control such a variety of different, and sometimes even mutually exclusive, physiological cell functions, internal calcium ($[Ca^{2+}]_i$) signals are modulated by an array of diverse mechanisms and specific interactions [1,2]. $[Ca^{2+}]_i$ transients trigger all patterns of cellular demises. While a high and prolonged surge of cytosolic $[Ca^{2+}]_i$, with subsequent $[Ca^{2+}]_i$ accumulation in mitochondria, promote cell death by necrosis, programmed cell or apoptosis is driven by increased $[Ca^{2+}]_i$, through activation of Ca^{2+}-dependent enzymes and Ca^{2+}-activated proteases [3,4]. Deregulation of cell proliferation, together with the suppression of apoptosis, provides the condition for abnormal tissue growth and cancer development and invasion [1–4]. Therefore, specific apoptosis induction in malignant cells is regarded as a useful strategy in the treatment of advanced cancer [5].

Prostate cancer (PCa) remains one of the most common malignancy in men [6,7]. PCa is initially androgen dependent and androgen deprivation leads to a remission of the tumor. However, after a few years of treatment, most patients develop an androgen-independent carcinoma, a lethal form for which there is still no effective therapy [8,9]. In androgen-sensitive cells, it is believed that androgen withdrawal causes apoptosis by sustained elevation of $[Ca^{2+}]_i$ [5]. On the contrary, androgen-independent PCa cells escape androgen withdrawal dependent cell death with altered $[Ca^{2+}]_i$ homeostasis, for example through lowered endoplasmic reticulum (ER) Ca^{2+} content by increased IP3R1 levels and IP3R1 phosphorylation by PKA [10], or through downregulation of ORAI1 and consequently store-operated calcium entry (SOCE) in favour of a pro-proliferative Ca^{2+} influx pathway involving ORAI1/3 heteromeric channels [11,12], a down regulation that was confirmed in *in vivo* models [13]. The hormone-refractory PCa cell is thus protected against diverse apoptosis-inducing pathways [4,11] and it could be possible to induce apoptosis in androgen-independent PCa cells by triggering a surge of $[Ca^{2+}]_i$.

Different plant polyphenols have been shown to affect cancer cell growth not only by modifications of the redox status, but also by interference with basic cellular functions, most of which are mediated by $[Ca^{2+}]_i$ [14].

Several evidences have suggested a negative correlation between PCa incidence and green tea consumption in Asian countries [15]. Data from case-control studies [15–17] and meta-analysis [19–21] indicate green tea polyphenols may be effective for preventing PCa and dose-dependently decrease the risk of advanced PCa [22]. Although recent analysis of epidemiological studies reported conflicting results [18,23], the interest for the alleged anti-cancer effects of green tea polyphenols has not diminished [24,25].

Most polyphenols in green tea are catechins (30–40% by dry weight), including (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC), and (−)-

epigallocatechin-3-gallate (EGCG), which the most abundant and active. Water-extractable material of green tea leaves also contain 2–5% caffeine [26]. A great number of *in vitro* and animal studies investigated and validated the activity of green tea constituents against PCa [27], with special focus on EGCG [28–30], while the effect of the other components is less documented [25]. Different mechanisms have been proposed, the *in vivo* relevance of which remains to be demonstrated [31,32]. These include antioxidation and proxidation activity [33], induction of apoptosis [30,34], inhibition of enzyme activities [32], influences on key enzymes in the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway [24,35], effect on microRNA and direct antagonism of androgen action [36]. Synergistic interactions were also reported for green tea catechins in different cancer models, where single catechins were inactive when tested alone [37].

We have recently described the mechanism by which EGCG mediates $[Ca^{2+}]_i$ increase in androgen-independent PCa cells [38]. Focusing on the role of $[Ca^{2+}]_i$ dynamics, we have studied how the model we have described also fits to green tea extract (GTE) effect in the same androgen-independent DU145 and PC3 PCa cells [39,40] and found out obvious similarities, but also notable differences that argue for the synergic intervention of the other components.

2. Material and methods

2.1. 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% EGCG w/w according to manufacturer specifications, as well as total catechins ≥ 30% w/w, epicatechin gallate ≥ 4% w/w and caffeine ≤ 3% w/w.

2.2. In vitro cell culture and viability assay

In vitro experiments were carried out on androgen-independent PC3 and DU145 PCa cells [39,40]. Cells were grown in RPMI medium, supplemented with 10% foetal bovine serum at 37 ℃, in a 5% CO_2, fully humidified atmosphere.

Cell viability was determined by the MTT assay. Cells were settled in 96-well plates for 24 h and exposed to various agents for 48 h. After that time, cells were incubated with 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt (MTT, 0.5 mg/ml) added to the cell culture medium without serum for 3 h at 37 ℃, treated with a solution of 1 N HCl–isopropanol (1:24, v/v) and mixed to dissolve the dark-blue formazan crystals formed. After a few minutes at 37 ℃, the plates were read at 570 nm in a VMax microplate reader (Molecular Devices, Sunyvale, CA).

2.3. Intracellular Ca^{2+} measurements

$[Ca^{2+}]_i$ was measured using a microspectrophotometry fluorescence ratio setup equipped with a perfusion system, as previously described [38]. Cells were incubated with 5 μM Fura-2-acetoxymethyl ester (Fura-2-AM, Thermo Fisher Scientific) in physiological saline (see below), at 37 ℃ for 40 min, washed and mounted on the stage of an inverted microscope (Axiovert Zeiss, Germany), where they were continually superfused with different solutions. Cells were illuminated by a xenon lamp through a wavelength selector monochromator; emission was observed through an X40 quartz objective and recorded by a photomultiplier. The ratio E340/E380 was calculated every 40 msec to acquire a time-dependent $[Ca^{2+}]_i$ 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 (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 = E340/E380 determination. $[Ca^{2+}]_i$ was calculated according to the Grynkiewicz equation [41].

The physiological standard bath solution contained (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, and glucose 10. The pH was adjusted at 7.4 with NaOH. The Ca^{2+}-free solution had the same composition with 0 $CaCl_2$ added, 4 Mg Cl_2 and 2 EGTA.

2.4. Statistical analysis

Data were analyzed using Sigma Plot software (Systat Software Inc.). Data are shown as mean ± sd for cytotoxicity assays and mean ± sem in all other experiments. Statistical significance was evaluated by Student's t-test or Man-Whitney Rank Sum test, as indicated by the software. The difference between two conditions was considered significant if $p < 0.05$.

3. Results and discussion

3.1. Cell viability assay

The green tea extract (GTE) used for experiments is titrated at 30% (w/w) in catechins, and (−)-epigallocatechin-3-gallate (EGCG) represents at least 20% of the total extract weight, according to the provider. GTE inhibited proliferation of both PC3 and DU145 cells with IC_{50} = 60 and 72 μg/ml, respectively (Figure 1). These figures are in agreement with previous results with similar ethanol/water tea extracts [42] and correspond approximately to a concentration of 27 and 32 μM EGCG in the extract. Our previous experiments with purified EGCG yielded the values of IC_{50} = 56 μM for PC3 and 46 μM for DU145 cells [38], indicating that the potency of EGCG is approximately doubled in synergy with the other components.

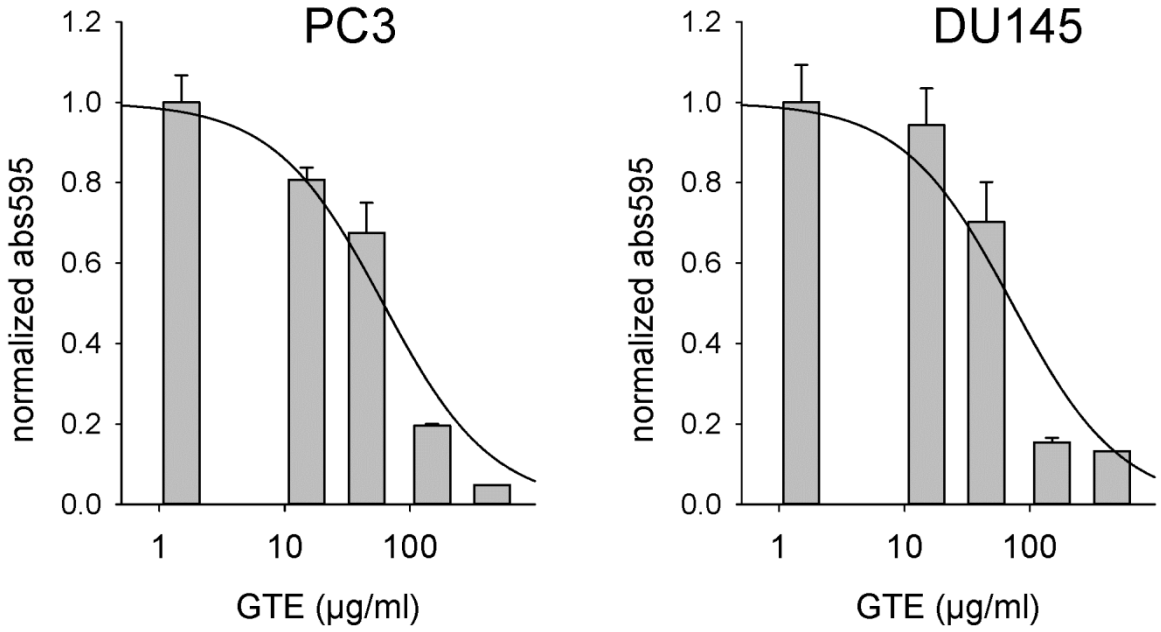

Figure 1. Dose-response data of cell viability obtained with the MTT assay after exposure of PCa cells to different concentrations of green tea extract (GTE) for 48 h. Bars represent means ± s.d. of percent MTT-formazan absorbance (see Materials and Methods). In PC3 cells (left), the IC_{50} was 60 µg/ml and in DU145 cells (right), the IC_{50} was 72 µg/ml.

3.2. Effects on intracellular Ca^{2+} level

$[Ca^{2+}]_i$ elevations were observed upon application of increasing doses of GTE to Fura-2-loaded PC3 and DU145 cells (Figure 2A). GTE showed an intrinsic fluorescence signal partially overlapping the Fura-2 Ca^{2+}-dependent signal. The increase in fluorescence at 510 nm following 380 nm excitation was higher than that following 340 nm excitation, causing a transient decrease in the E340/E380 ratio when the extract was applied, and a parallel transient increase in the ratio when the extract was washed out. This artifact is reflected in the Ca^{2+}-dependent signal time course (see * in Figure 2A), but did not hindered to quantify the response to the GTE, which resulted similar to that of purified EGCG [38]. The effect was clearly dose dependent (Figure 2B), but $[Ca^{2+}]_i$ elevations are not the result of a single site interaction and are likely to be due to a multi-step mechanism, as we have already described for EGCG effect [38].

GTE mediated $[Ca^{2+}]_i$ rise was weakly dependent on the presence of external Ca^{2+} in the medium, and could be triggered also in Ca-free EGTA-containing external bath (Figure 3A). As the second response to GTE was frequently lower than the first (see graph on the left in Figure 3A), only the first response to GTE was included in statistics in the graph of Figure 3B. The response in 0 Ca-EGTA was not significantly different from that in 1 mM Ca. This observation is in partial contrast with the results obtained with purified EGCG [38], where the response in 0 Ca-EGTA was always significantly lower than that in 1mM external Ca. Therefore it is possible that other components in GTE play a role in generating the $[Ca^{2+}]_i$ peak. First of all, caffeine, a well-known Ca mobilizer, may

contribute the trigger for Ca^{2+} release, without the need of Ca^{2+} influx. As the concentration of caffeine in the extract is ≤ 3% w/w, 150 µg/ml GTE would contain around 20 µM caffeine, a dose which is too low to mediate a sizeable effect, but can induce a first $[Ca^{2+}]_i$ surge, followed by EGCG-mediated Ca^{2+}-induced Ca^{2+} release. In addition, synergic intervention of other GTE catechins [26] is also possible [37,43]. In our previous work, we proposed a model in which EGCG response is initiated by a limited entry of Ca^{2+} from the outside, through a mechanism involving the interaction with redox-sensitive sulfhydryls [38]. Another GTE catechin, epigallocatechin gallate (EGC), was previously shown to decrease tumor cell viability and interact with protein sulfhydryls [44], thus enhancing GTE effect on $[Ca^{2+}]_i$ with respect to that of purified EGCG.

To confirm the role of Ca release from intracellular stores, we treated the cells with the non-competitive inhibitor of the ER Ca^{2+} ATPase (SERCA) thapsigargin. It was applied in 0 Ca EGTA solution and caused a transient $[Ca^{2+}]_i$ increase of moderate amplitude (18 ± 5 nM in 20 cells). When external Ca was shortly reintroduced, it was observed a small $[Ca^{2+}]_i$ rise that hardly exceeded the level of basal $[Ca^{2+}]_i$, indicating that the contribution of store-operated calcium entry (SOCE) is not very significant in androgen-independent PCa cells [11]. When GTE was applied shortly after, it elicited a much smaller response than in control (Figure 3B). As already mentioned, sometimes the second response to GTE was smaller than the first. Therefore we applied a different protocol and incubated the cells with thapsigargin before the first application of GTE. Responses were compared to that obtained in control conditions in the same day and on the same culture and found that thapsigargin-treated cells exhibited a significantly smaller response to GTE, which was on average 40% of that in the absence of thapsigargin (Figure 3D). This observation confirms the role of ER in mediating the $[Ca^{2+}]_i$ elevation driven by GTE, although a thapsigargin-insensitive component was also present.

We verified that GTE-induced $[Ca^{2+}]_i$, increase was not affected by several Ca channel modifiers (Figure 4). The GTE response was not modified by lanthanum (La^{3+}, 10 µM), suggesting that Ca channels of the TRPC family were not involved [45]. Other modifiers of these channels were equally ineffective; these included SK&F 96365 and 2-aminoethoxydiphenyl borate (2-APB) [45,46] (not shown) and ML204 a potent, and selective inhibitor of TRPC4 channel [47], whose expression was revealed in PC3 cells [38] (Figure 4B,C). $[Ca^{2+}]_i$ increase triggered by GTE was not modified by nifedipine, a specific blocker of a L-type voltage-dependent Ca channels, which have been reported to induce an intracellular calcium increase in PCa cells [48,49]. On the other hand, similar to EGCG [38], the GTE-induced increase in internal Ca was totally abolished by 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 IP_3-mediated Ca^{2+} release. All these observations indicate that the source of $[Ca^{2+}]_i$ rise triggered by GTE is mainly intracellular and confirm a role of IP_3 receptors in thapsigargin-sensitive and thapsigargin-insensitive stores.

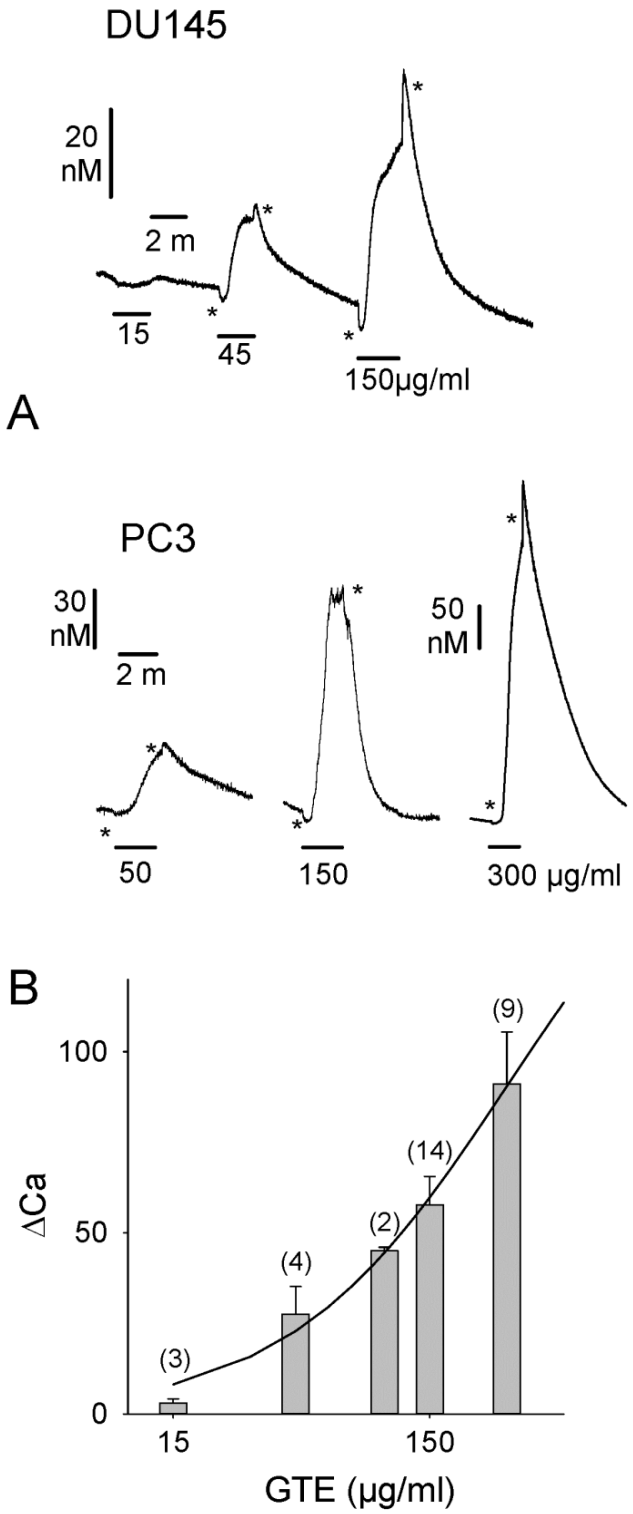

Figure 2. Effect of GTE on $[Ca^{2+}]_i$ in PCa cells. (A) $[Ca^{2+}]_i$ spikes in Fura-2 loaded DU145 cells and PC3 following transient exposure to different doses of GTE. Artifacts caused by GTE autofluorescence are marked by * and were much faster than cellular response. (B) Dose dependent $[Ca^{2+}]_i$ elevation cause by GTE in PCa cells. Data of DU145 and PC3 cells are pooled. The line represent best fit with the function: $Ca = ([Ca^{2+}]_{i(GTE)} - [Ca^{2+}]_{i(basal)}) = Ca_{max}/(1 + (K/[GTE]))$, with $Ca_{max} = 180$ nM and $K = 300$ µg/ml.

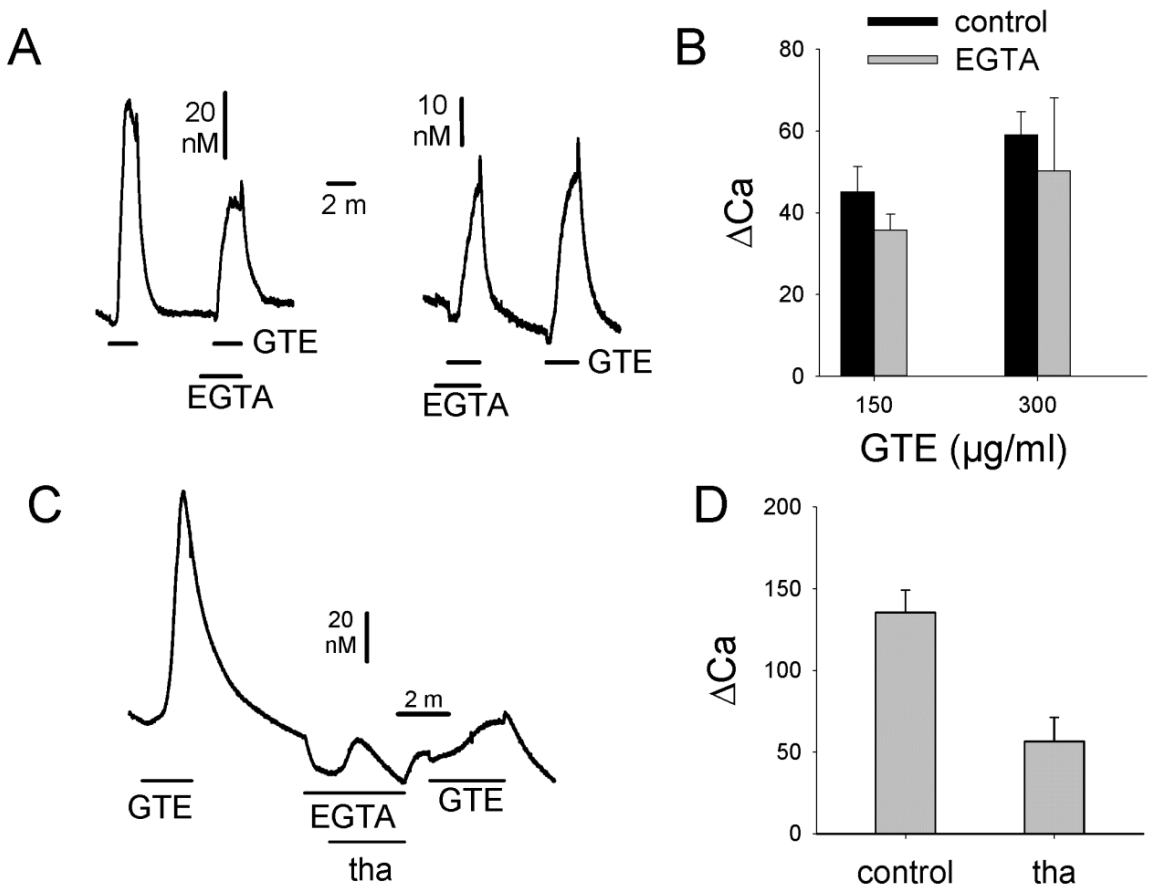

Figure 3. Effect of Ca^{2+} and thapsigargin (tha) on GTE-induced $[Ca^{2+}]_i$ peak in PC3 cells. (A) $[Ca^{2+}]_i$ spikes in Fura-2 loaded PC3 cells following transient exposure to 150 μg/ml GTE in 1 mM external Ca^{2+} and in 0 external Ca^{2+}, in the presence of 1 mM EGTA. (B) Summary of the effects of 2 different concentrations of GTE in 1 mM external Ca^{2+} (control) and in 0 external Ca^{2+} with 1 mM EGTA (EGTA) in PC3 cells (n = 6). Bars represent sem. (C) $[Ca^{2+}]_i$ response to 150 μg/ml GTE before and after incubation with 1 μM thapsigargin. See text for further details. (D) Summary of the effects of 1 μM thapsigargin (tha) on $[Ca^{2+}]_i$ response to 150 μg/mL GTE (n = 4). Bars represent sem. The responses after thapsigargin treatment were significantly different from control with $p < 0.05$.

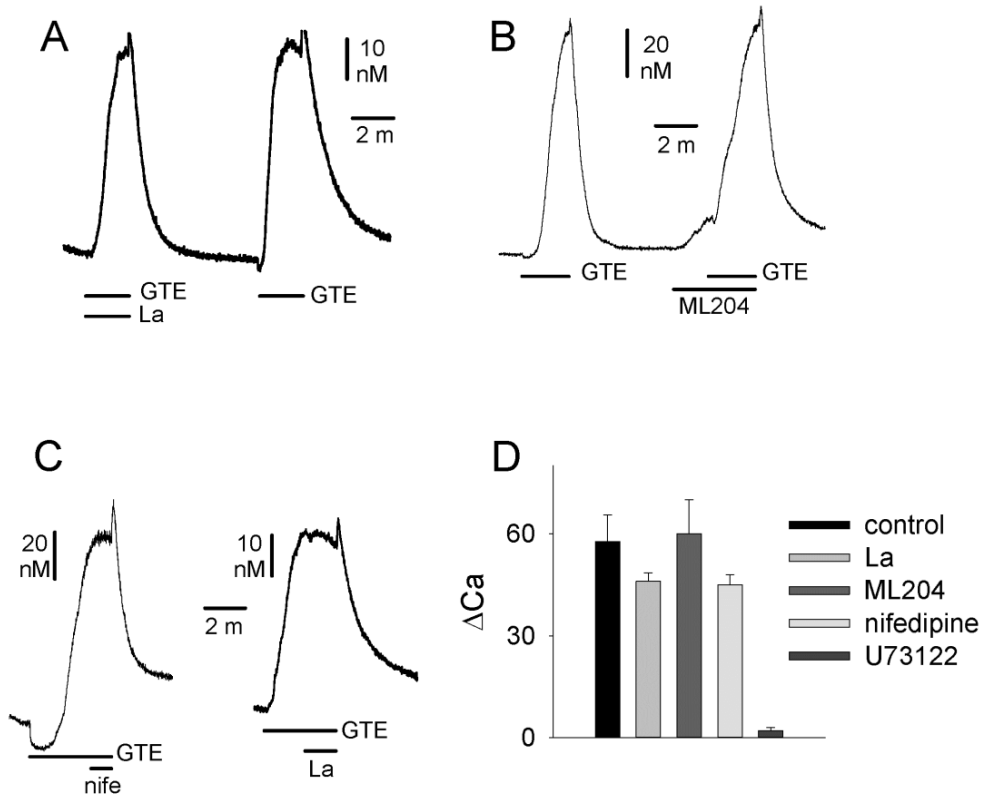

Figure 4. Effect of different modifiers on the $[Ca^{2+}]_i$ response to GTE (150 μg/mL) in PC3 cells. (A) Lack of effect of 10 μM La on $[Ca^{2+}]_i$ response to GTE. (B) Lack of effect of 5 μM ML204 on $[Ca^{2+}]_i$ response to GTE. (C) Lack of effect of 10 μM nifedipine (nife, left) and of 10 μM La on the plateau phase of $[Ca^{2+}]_i$ response to GTE. (D) Summary of the effects of different agents on $[Ca^{2+}]_i$ response to GTE (n = 3). Bars represent sem.

4. Conclusions

The present results indicate that in androgen-independent PCa cells the first step of GTE induced-toxicity is mediated by a $[Ca^{2+}]_i$ increase, occurring through release from thapsigargin-sensitive and insensitive stores. This mechanism is qualitatively similar to that described for the purified EGCG [38], which appears the main effector of the $[Ca^{2+}]_i$ surge. However, while EGCG alone was not able to trigger $[Ca^{2+}]_i$ release without Ca^{2+} influx from the outside, the effect of GTE was largely independent on external Ca^{2+} and this suggested that other GTE components would act in synergy with EGCG. In this respect a major contribution is expected to derive from the effect of caffeine, a well-known Ca mobilizer, that would act as a booster for EGCG activity, but synergic intervention of other green tea catechins is also possible [37,43,44].

These data support the view that the alleged health benefits of green tea for PCa prevention may be related to $[Ca^{2+}]_i$ deregulation in malignant cells and androgen-resistance transformation and proliferation of PCa cells can be antagonized by disturbing cell Ca dynamics. Moreover these results may be significant to understand the mechanisms by which flavonoids exert their beneficial or toxic actions.

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Conflict of interest

The author declares no conflicts of interest in this paper.

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