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Casein phosphopeptides modulate proliferation and apoptosis in HT-29 cell line through their interaction with voltage-operated L-type calcium channels

Silvia Perego[a], Stefania Cosentino[a], Amelia Fiorilli[a], Guido Tettamanti[a,b], Anita Ferraretto[a,*]

[a] *Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina, Università degli Studi di Milano, Milano, Italy*
[b] *IRCCS Policlinico San Donato, San Donato Milanese, Milano, Italy*

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Abstract

At the intestinal level, proliferation and apoptosis are modulated by the extracellular calcium concentration; thus, dietary calcium may exert a chemoprotective role on normal differentiated intestinal cells, while it may behave as a carcinogenesis promoter in transformed cells. Calcium in milk is associated with casein and casein phosphopeptides (CPPs), hence is preserved from precipitation. CPPs were demonstrated to induce uptake of extracellular calcium ions by *in vitro* intestinal tumor HT-29 cells but only upon differentiation. Here, the hypothesis that CPPs could differently affect proliferation and apoptosis in undifferentiated and differentiated HT-29 cells through their binding with calcium ions was investigated. Results showed that CPPs protect differentiated intestinal cells from calcium overload toxicity and prevent their apoptosis favoring proliferation while inducing apoptosis in undifferentiated tumor cells. The CPP effect on undifferentiated HT-29 cells, similar to that exerted by ethyleneglycol-O, O′-bis(2-aminoethyl)-N, N, N′, N′-tetraacetic acid (EGTA), is presumably due to the ability in binding the extracellular calcium. The effect on differentiated HT-29 cells is coupled to the interaction of CPPs with the voltage-operated L-type calcium channels, known to activate calcium entry into the cells under depolarization and to exert a mitogenic effect: the use of an agonist potentiates the cell response to CPPs, while the antagonists abolish the response to CPPs (36% of examined cells) or reduce both the percentage of responsive cells and the increase of intracellular calcium concentration. Taken together, these results confirm the potentialities of CPPs as nutraceuticals/functional food and also as modulators of cellular processes connected to the expression of a cancer phenotype.

Keywords: Casein phosphopeptides; Intestinal cells; Calcium; Depolarization; Calcium channels; Cell cycle

1. Introduction

A new promising field in nutrition is the identification of bioactive compounds, that is to say molecules naturally occurring in food, which can exert physiologically relevant actions either directly, or upon enzymatic hydrolysis, when inactive within the parent molecules, carried on by digestive enzymes or by proteolytic microorganisms. All these proteolytic processes can be achieved *in vitro*, for instance, using proteolytic enzymes extracted by plants or microorganisms, but also *in vivo*, during the gastrointestinal digestion and the fermentation of food by the gut bacteria (for a review, see Korhonen and Pihlanto [1]). A lot of effects exerted by these bioactive compounds are now recognized at multiple levels in the body, such as at cardiovascular, nervous, gastrointestinal and immune level. The goal of all the studies devoted to discover new bioactive molecules in food is to improve human health simply by consuming a "normal diet" that naturally contains a consistent amount of these bioactive molecules or by using them as additives in food.

Milk and dairy products are well-known sources of bioactive peptides originating during milk fermentation with the starter cultures by the dairy industry or by cheese ripening [1]. A family of bioactive peptides derived from the digestion of casein, the milk main protein fraction, is named casein phosphopeptides (CPPs), due to their high content of phosphorylated regions [2]. In contrast to casein, which does not possess any bioactivity and serves as a source of amino acids for human nutrition, CPPs are characterized by the ability to bind and solubilize cations, especially calcium [3]. Numerous studies arose in the last decades trying to address to CPPs also the aptitude to enhance calcium absorption in the intestine, but the difficulties of the both human and animal studies have provided conflicting results, as reviewed in Ferraretto and Fiorilli [4].

Instead, studies using *in vitro* cell cultures have revealed new and interesting potentialities for CPPs, such as the modulation of the intestinal immune system by triggering cytokine secretion and stimulating immunoglobulin A production [5,6], the release of interleukin-6 cytokine in human epithelial intestinal cell lines [7], the protective antiproliferative effects on cultured mammalian intestinal cells [8] and the modulation of cell viability, that is, proliferation and apoptosis, in different human cell cultures [9,10]. In this context, the recent findings that CPPs can induce intracellular calcium increases in intestinal human tumor cell lines HT-29 and Caco2 but only upon cell differentiation [11] can be considered not

* Corresponding author. Tel.: +39 0250330341; fax: +39 0250230365.
E-mail address: anita.ferraretto@unimi.it (A. Ferraretto).

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only from the calcium absorption process point of view, since the calcium ion influx induced by some mitogenic factors can play a role as messengers of cellular events linked to control of the proliferation, thus cell viability [12]. In fact, abnormal cell proliferation is often associated to pathological conditions such as inflammation and tumor [13,14]. The calcium ion entry mechanisms in cells can be more than one — voltage-operated calcium channels, receptor-operated channels and store-operated calcium entry — and all of them have been associated with cell growth in tumor and normal tissues [12]. More recently, it was demonstrated that in HT-29 cells, as well as in a primary human colon cancer cell line (AZ-97), the activation of voltage-activated L-type calcium channels, which mediate the calcium influx according to the depolarization state of the cell, is correlated to apoptosis, and their blockade may promote the growth of colon cancer cells [15].

The human HT-29 cell line, derived from an intestinal adenocarcinoma, is frequently used as an intestinal *in vitro* cell model due to its ability to undergo different patterns of cell differentiation, in dependent of the modifications in their growth medium [16]. When cultivated in Dulbecco's modified Eagle's medium (DMEM), HT-29 cells do not display any signs of differentiation, apart from rare apical microvilli and desmosomes; on the contrary, when cultivated in Roswell Park Memorial Institute 1640 (RPMI) medium, HT-29 cells display a differentiated morphological phenotype characterized by a complete junctional apparatus (tight junctions, adherens junctions, desmosomes) and very abundant microvilli [11,17]. Despite these morphological differences, no significative variation in the activities of alkaline phosphatase and sucrase–isomaltase, known enzymatic markers of intestinal cell differentiation, between HT-29 DMEM and RPMI cells was observed, as already reported [11,17], and HT-29 RPMI cells are considered a heterogeneous cell population.

The aims of the present work are therefore to explore the possibility that the CPP-mediated calcium influx in differentiated HT-29 cells could involve the L-type calcium channels known to be expressed in this cell line [15,18] and to study the possible correlated modulation of cell proliferation and apoptosis. Moreover, the same studies were carried on in undifferentiated tumor HT-29 cells, in order to verify the possibility that CPPs, due to their property to complex calcium ions, may behave as ethyleneglycol-O, O′-bis(2-aminoethyl)-N, N, N′, N′-tetraacetic acid (EGTA), the known calcium-chelating agents, affecting the extracellular medium and influencing cell functions [19,20].

All the experiments were done at physiological condition, the calcium concentration present in the growing medium, and at calcium overload. The supraphysiological concentrations of calcium used in this work — 6 mM for undifferentiated DMEM cells, 2 and 6 mM for differentiated RPMI cells — were chosen in order to mimic the situation at intestinal lumen after a meal, since it is reported in literature that in this particular condition, the extracellular calcium concentration can reach values as high as 10 mM [21-23]. Since tumor DMEM cells grow in 2 mM calcium concentration, while differentiated RPMI cells require small amounts of calcium ions to survive [24,25] (0.4 mM calcium in RPMI medium), the experiments with RPMI cells were carried on both at 2 and 6 mM calcium, in order to compare results with DMEM cells and to reproduce more than one step in the increasing calcium concentration after a meal.

2. Materials and methods

2.1. Materials and reagents

Cell culture media, Bay-K8644, Nifedipine, Nimodipine and all other reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was from EuroClone Ltd. (West Yorkshire, UK). Fura-2 acetoxymethyl ester was from Calbiochem (La Jolla, CA, USA). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, $DiBAC_4(3)$, was obtained from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

The human colon carcinoma cell line HT-29 (BS TCL 132) was obtained from Istituto Zooprofilattico Sperimentale di Brescia (Brescia, Italy). Cell growth (75-cm^2 plastic flasks; Costar, Concorezzo, Italy) in high D-glucose (4.5 g/L) DMEM medium, HT-29 DMEM cells, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mg/L streptomycin, 100,000 U/L penicillin and 0.25 mg/L amphotericin-B, provides an undifferentiated tumor cell population [16]. The replacement of DMEM medium with low D-glucose (2 g/L) RPMI 1640 medium supplemented as above reported, after cell subcultivation for at least five to six passages, switched the cell phenotype toward a population of differentiated and polarized HT-29 cells, HT-29 RPMI cells, although sometimes exhibiting morphofunctional heterogeneity (features of absorptive and mucous-like cells) [26]. Cultures, kept at 37°C in a 5% CO_2–95% air atmosphere, were periodically checked for the presence of mycoplasma and were found to be free of contamination. The culture medium was changed every 2 days.

2.3. CPPs

The CPP preparation (CE 90 CPP III) was a gift by DMV International, Veghel, The Netherlands. As a casein-derived hydrolysate, this CPP preparation is a peptide mixture containing the characteristic CPP "cluster sequence," Ser(P)-Ser(P)-Ser(P)-Glu-Glu, whose average molecular weight is 2500. CPP powder was dissolved in doubly distilled water in stock solutions (1000× concentrated, with respect to the final concentration) and stored at −20°C.

2.4. Measurement of intracellular free calcium concentration, $[Ca^{2+}]_i$, at a single-cell level

Cytoplasmic calcium, $[Ca^{2+}]_i$, was measured on differentiated HT-29 RPMI cells seeded on a glass coverslips (24-mm diameter, thickness 0.13–0.17 mm) and loaded with 2.5 μM fura-2 acetoxymethyl ester and 2.5 μM Pluronic F-127 in a Krebs-Ringer HEPES solution (KRH) containing the following (in mM): NaCl 125.0, KCl 5.0, KH_2PO_4 1.2, $CaCl_2$ 2.0, $MgSO_4$ 1.2, glucose 6.0 and HEPES 25.0, and were adjusted to pH 7.4, according to the procedure already described [11,27]. The experiments were carried out by means of a thermostated (TC-202 A; Medical System Corporation, Harvard Apparatus, Holliston, MA, USA) perfusion chamber (PDMI-2, same commercial source) and a microscope (TE 200; Nikon, Tokyo, Japan) connected to a CCD intensified camera (Extended Isis; Photonic Science, Millham, UK). The fluorescence image acquisition and data analysis system (Applied Imaging; High Speed Dynamic Video Imaging Systems, Quanticell 700, Sunderland, UK) allowed to determine the $[Ca^{2+}]_i$ on single cells. The amount of intracellular free calcium, $[Ca^{2+}]_i$, within the cells was calculated from the 340/380-nm images by means of a calibration performed with external standards of calcium and Fura-2, according to the equation of Grynkiewicz et al. [28].

2.5. Use of activators/inhibitors of the L-type Ca^{2+} channels on CPP bioactivity evaluation

Bay-K8644, a known agonist of the L-type Ca^{2+} channels, previously solubilized in DMSO, was administered to HT-29 cells at 400 nM concentration. Both the dihydropyridine derivatives Nimodipine and Nifedipine, known L-type Ca^{2+} channels antagonists, were solubilized in dimethyl sulfoxide (DMSO) and administered to HT-29 RPMI cells at 30 μM concentration. In videoimaging experiments, the responsiveness to CPPs was primarily evaluated, and then after a preincubation with the agonist or the antagonists, cells were stimulated with a second CPP dose. La_2Cl_3, known to inhibit Ca^{2+} entry and cause an irreversible decrease in $[Ca^{2+}]_i$ [29], was administered to cells at 250 μM concentration after treatment with CPPs.

2.6. Cell proliferation assay

Cells (1×10^4 cells/well), 24 h after plating in a Microtiter plate (96 well, Greinerbio-one; Cellstar, Frickenhausen, Germany), were treated for 24 h with either 1280 μM CPP or 500 μM EGTA in fetal bovine serum free media, in the presence of 0.4–2–6 mM $CaCl_2$. The effect of 400 nM Bay-K8644, 30 μM Nimodipine and 30 μM Nifedipine in the presence of 0.4 and 2 mM $CaCl_2$ was determined in HT-29 RPMI cells following the same procedure. At the end of the incubation, cells were submitted to a 2-h pulse with bromodeoxyuridine and bromodeoxyuridine incorporation into DNA was quantified by the chemiluminescent immunoassay (Roche Applied Science, Milan, Italy) following the manufacturer's instructions. The results are expressed as percentage referred to control cells.

2.7. Cell apoptosis, Apo-ONE assay

The Apo-ONE assay (Promega, Madison, WI, USA) is a practical tool to measure the activities of caspases 3 and 7, which are known to act as key effectors in the apoptosis of mammalian cells [30].

Cells (1×10^4 cells/well), plated in a Microtiter plate (96 well, Greinerbio-one; Cellstar), were treated for 24 h with either 1280 μM CPP or 500 μM EGTA in fetal bovine serum free media, in the presence of 0.4–2–6 mM $CaCl_2$. At the end of the incubation,

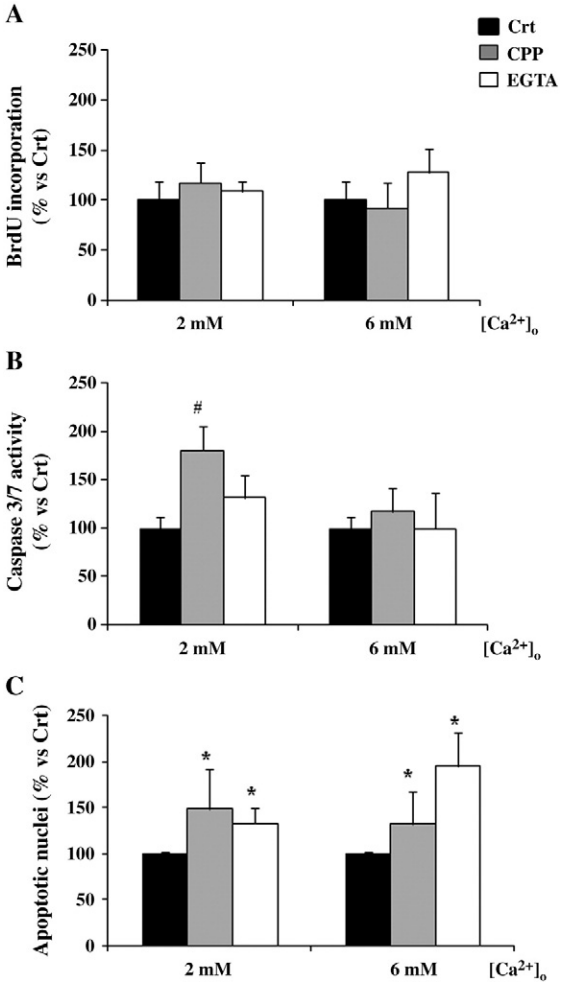

Fig. 1. Effect of CPPs on the proliferation rate and apoptosis activity in undifferentiated HT-29 DMEM cells. Proliferation rate (A) and apoptotic activity determined by caspase 3/7 assay (B) and by DAPI staining (C) were evaluated in CPP (1280 μM)- and EGTA (500 μM)-treated cells versus control cells (Crt; 100%) in the presence of 2 and 6 mM $[Ca^{2+}]_o$. Each bar represents the mean±S.D. of three analogous experiments. Symbols indicate a statistically different value from control (*P<.05, #P<.01).

the samples were processed as indicated by the manufacturer. The fluorescence emission at 530 nm, with excitation at 485 nm, was monitored between 1 and 7 h by VICTOR^2 Wallac 1420 Multilabel Counter (Perkin Elmer, Beaconsfield, UK). Data reported in the graph refer to the values registered after 5 h and are expressed as percentage with respect to control value.

2.8. Cell apoptosis, DAPI staining

Cells (5×10^4 cells) seeded on glass coverslips were treated for 24 h with either 1280 μM CPP or 500 μM EGTA in fetal bovine serum free media, in the presence of 0.4–2–6 mM $CaCl_2$. The same procedure was adopted to study the effect of 400 nM Bay-K8644, 30 μM Nimodipine and 30 μM Nifedipine in the presence of 0.4 and 2 mM $CaCl_2$ in HT-29 RPMI cells. After the incubation, cells were fixed in 70% ethanol for 15 min at –20°C. Then they were stained with 0.4 mg/μl 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and the morphology of the nuclei was examined by Olympus microscope BX50 (Olympus, Tokyo, Japan) at excitation and emission wavelengths of 358 and 460 nm, respectively. The results are expressed as percentage of apoptotic nuclei with respect to the total nuclei present in each examined cellular fields, for a total of 12 cellular fields analyzed for each treatment in a single experiment.

2.9. Measurement of membrane potential

$DiBAC_4(3)$ belongs to a class of anionic slow potential-sensitive dyes [31] and has been shown to respond to membrane depolarization with an increase in fluorescence resulting from the increased intracellular concentration and accumulation in intracellular lipid-rich compartments [32]. HT-29 RPMI cells (2.5×10^4 cells/well), plated in a Microtiter plate (96-well, Greinerbio-one; Cellstar), were loaded with 500 nM $DiBAC_4(3)$ in KRH solution. After 15 min of incubation at 37°C and 300 rpm in a Thermomixer (Eppendorf, Hamburg, Germany), fluorescence was recorded (λ_{ex} 490 nm, λ_{ex} 510 nm) and here reported as "before stimulus." Then, 1280 μM CPP, or 1 μM Gramicidin or a hyperpolarizing KRH solution (5 mM KCl, 0 mM NaCl) was added, and fluorescence immediately was recorded and here reported as "after stimulus."

2.10. Statistical analysis

The data reported in Figs. 1, 2, 5 and 8 are expressed as percentage versus control cells (100%) due to the variability in the absolute values between the different cell lines and the different cell treatments. Each treatment was applied to at least 8–10 cell wells in the single experiment (8–10 replicates), and every experiment was repeated at least three to four times. The correspondent bar in the graphs represents the mean calculated among all experiments±standard deviation. Statistically significant differences between two mean values were established by the Student's t test, independent of two population t test, performed with Origin 6.0 (a P value <.05 was considered significant).

3. Results

3.1. CPP effects on proliferation rate and apoptosis in undifferentiated/differentiated HT-29 cells

In HT-29 DMEM cells, the proliferation rate both at physiological $[Ca^{2+}]_o$ (2 mM) in the growth medium and in calcium overload

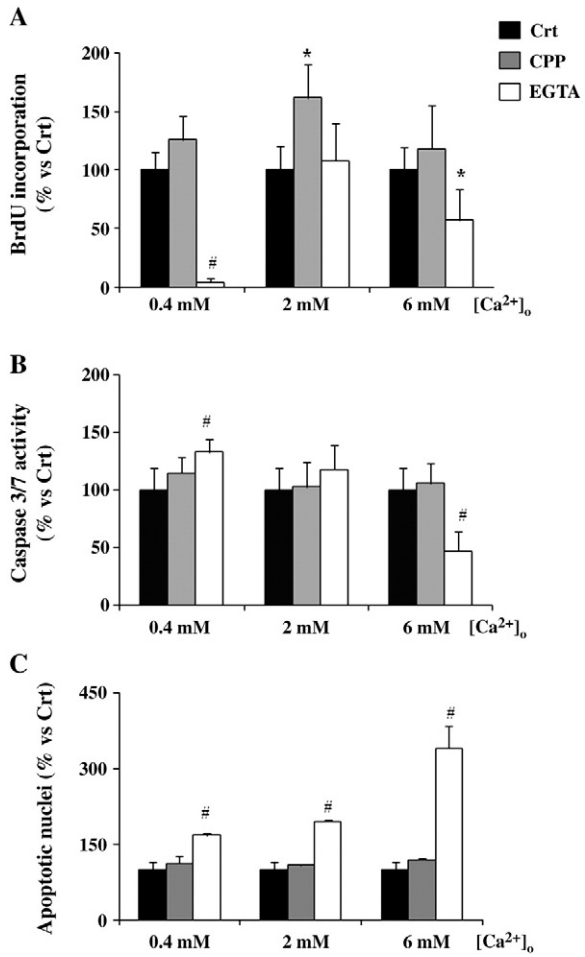

Fig. 2. Effect of CPP administration on the proliferation rate and apoptosis activity in differentiated HT-29 RPMI cells. Proliferation rate (A) and apoptotic activity determined by caspase 3/7 assay (B) and by DAPI staining (C) were evaluated in CPP (1280 μM)- and EGTA (500 μM)-treated cells versus control cells (Crt; 100%) in the presence of 0.4, 2 and 6 mM $[Ca^{2+}]_o$. Each bar represents the mean±S.D. of three analogous experiments. Symbols indicate a statistically different value from control (*P<.05, #P<.01).

(6 mM) was not affected by the cell incubation with 1280 μM CPPs, a dose at which they are demonstrated to exert the maximal bioactivity [11,17], or 500 μM EGTA (Fig. 1A). On the contrary, the apoptotic activity was increased by CPP administration both at 2 mM $[Ca^{2+}]_o$ (caspase 3/7 activity and apoptotic nuclei staining; Fig. 1B, C) and at 6 mM $[Ca^{2+}]_o$ (apoptotic nuclei staining; Fig. 1C). The administration of EGTA similarly induced an increase in the apoptotic activity both at 2 and 6 mM $[Ca^{2+}]_o$ (apoptotic nuclei staining; Fig. 1C). The apparent different results obtained by the mean of the two different apoptotic activity measurements (caspase 3/7 activity vs. apoptotic nuclei staining) could be due to the chronologically different events in the apoptosis, since the activation of caspases precedes the nuclear fragmentation and condensation [30]. When the same experiments were performed in HT-29 RPMI cells, the results obtained appeared completely different in the case of CPP administration compared to EGTA administration. The proliferation rate, measured at 0.4 mM $[Ca^{2+}]_o$, which represents the physiological calcium concentration in the growth medium, and at 2 and 6 mM $[Ca^{2+}]_o$, calcium overload conditions, was increased by CPPs above all at 2 mM $[Ca^{2+}]_o$ ($P<.05$). EGTA determined a strong reduction of the proliferation rate at 0.4 mM $[Ca^{2+}]_o$, an event not observed at 2 mM $[Ca^{2+}]_o$, while at 6 mM $[Ca^{2+}]_o$, the proliferation rate was reduced by 43% ($P<.05$; Fig. 2A). The apoptotic activity of HT-29 RPMI cells was increased by EGTA at all the $[Ca^{2+}]_o$ considered, except at 6 mM $[Ca^{2+}]_o$, when the caspase 3/7 activity decreases, while CPP administration was without any effect (Fig. 2B, C).

Images of DAPI-stained nuclei (Figs. 3 and 4) before and after CPP and EGTA administrations are important to understand these results. Undifferentiated tumor DMEM cells are not sensitive to calcium overload (6 mM), in that no differences were observed either in the proliferation rate (see Fig. 1A) and in apoptosis activity compared to control cells (Fig. 3A, D), probably because they lack a part of the molecular machinery necessary to sense the variation in the extracellular calcium concentration able to modulate differentiation and apoptosis [33]. Differences were observed with the treatment with CPPs or EGTA both in standard condition 2 mM and in calcium overload (6 mM): while the proliferation rate does not change (see Fig. 1A), apoptosis activity increased (Fig. 3B, C, E, F), probably due to the CPP and EGTA action of binding calcium ions, thus subtracting the proliferative stimulus for tumor cells, without the possibility for them to differentiate [33]. In differentiated RPMI cells, after the addition of 500 μM EGTA in 0.4 mM $[Ca^{2+}]_o$, cell nuclei appeared smaller and rounded (Fig. 4B), a classical morphological sign of apoptosis, compared to control cells (Fig. 4A), according to the drastic fall observed in their proliferative rate (see Fig. 2A). Probably, under this experimental condition, EGTA has subtracted all the calcium necessary to cells for surviving. When 500 μM EGTA was administered in 2 mM $[Ca^{2+}]_o$, the appearance of DAPI-stained nuclei (Fig. 4C) was not different versus control cells, in accordance with a normal proliferative rate and a slight increase in the apoptotic activity (see Fig. 2A). CPPs, though displaying a strong ability to bind calcium ions, did not behave as a cytotoxic agent neither in 0.4 mM $[Ca^{2+}]_o$ (Fig. 4D) nor in 2 mM $[Ca^{2+}]_o$ (data not shown). At 6 mM $[Ca^{2+}]_o$, a condition in which a lot of cells were not viable (Fig. 4E), the CPP addition reversed the cells to the control condition (Fig. 4F), confirming previous observations about its role in maintaining calcium ions in a soluble form [3], in contrast to EGTA.

3.2. Effect of agonist/antagonist of voltage-operated L-type calcium channels on proliferation rate and apoptosis in differentiated HT-29 cells

The different effects observed in undifferentiated tumor HT-29 DMEM cells versus in differentiated HT-29 RPMI cells when CPPs or EGTA was administered could be possibly explained considering that CPPs are able to induce $[Ca^{2+}]_i$ rise only in differentiated intestinal cells [11]; thus the hypothesis that the CPP-induced increment in the proliferation rate of HT-29 RPMI cells could be associated to the influx of calcium ions activated by the same CPPs. To investigate this issue, experiments were performed in HT-29 RPMI cells with the use of agonist and/or antagonists of L-types calcium channels. First, the possibility that the activity of these channels could affect the cell proliferation rate and

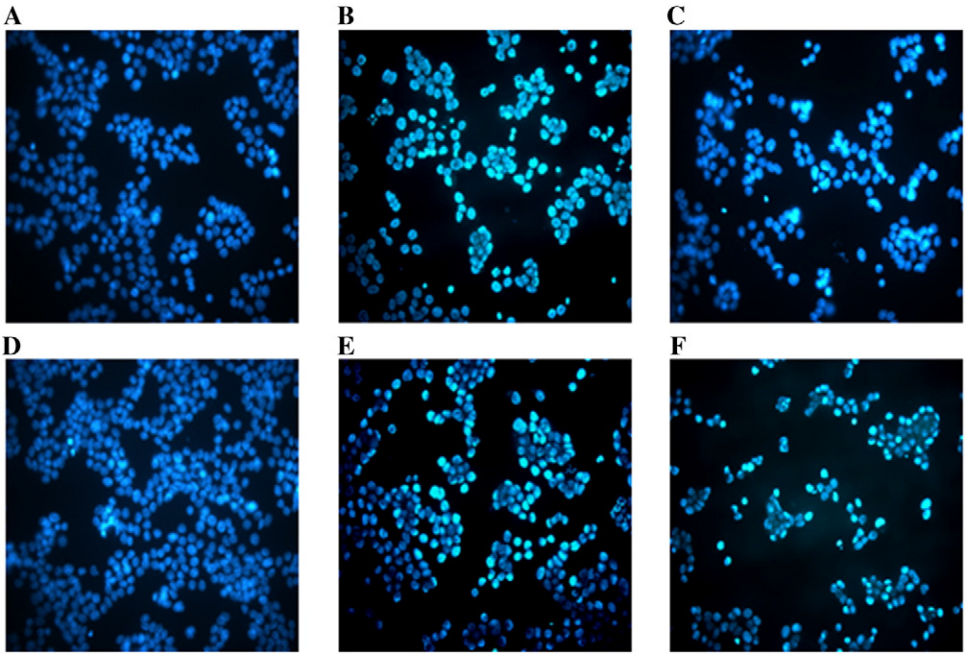

Fig. 3. Images of DAPI-stained nuclei in undifferentiated HT-29 DMEM cells. Photographs of control cells (Crt; A), 1280 μM CPP-treated cells in the presence of 2 mM $[Ca^{2+}]_o$ (B), 500 μM EGTA-treated cells in the presence of 2 mM $[Ca^{2+}]_o$ (C), control cells in the presence of 6 mM $[Ca^{2+}]_o$ (D), 1280 μM CPP-treated cells in the presence of 6 mM $[Ca^{2+}]_o$ (E) and 500 μM EGTA-treated cells in the presence of 6 mM $[Ca^{2+}]_o$ (F). Magnification was ×40.

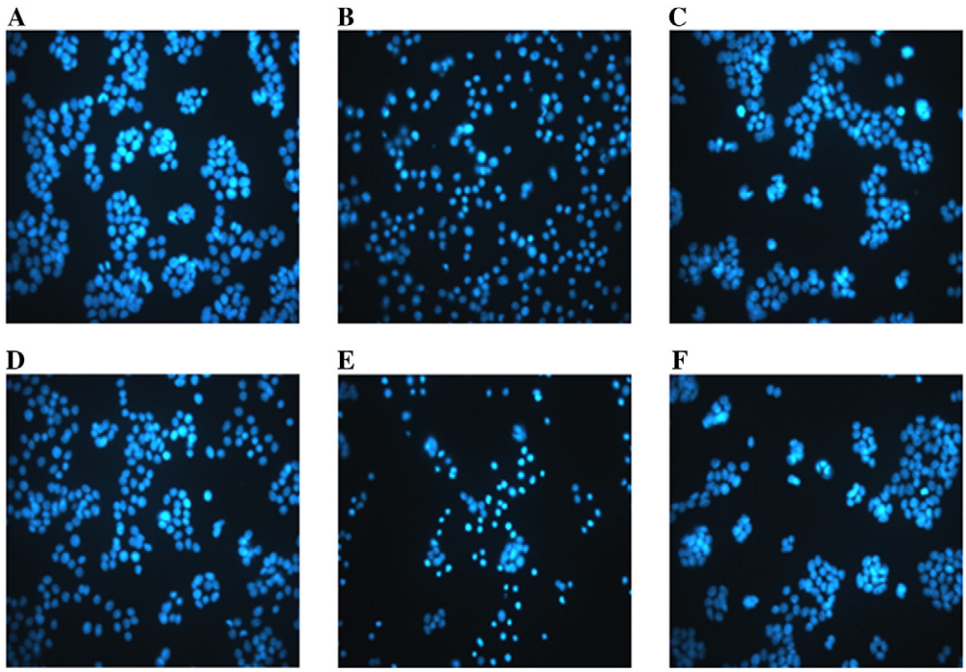

Fig. 4. Images of DAPI-stained nuclei in differentiated HT-29 RPMI cells. Photographs of control cells (Crt; A), 500 μM EGTA-treated cells in the presence of 0.4 mM $[Ca^{2+}]_o$ (B), 500 μM EGTA-treated cells in the presence of 2 mM $[Ca^{2+}]_o$ (C), 1280 μM CPP-treated cells in the presence of 0.4 mM $[Ca^{2+}]_o$ (D), control cells in the presence of 6 mM $[Ca^{2+}]_o$ (E) and 1280 μM CPP-treated cells in the presence of 6 mM $[Ca^{2+}]_o$ (F). Magnification was ×40.

apoptosis, as already described in different *in vitro* cell models [15,18,34,35], was studied at 0.4 and at 2 mM $[Ca^{2+}]_o$. Bay-K8644 is a known L-type Ca^{2+} channel activator widely used to evaluate the entity of the calcium influxes through the L-type channels in various cell lines and, among them, the HT-29 [18]. The cell treatment with the agonist Bay-K8644 increased the proliferation rate (Fig. 5A). The antagonists Nimodipine and Nifedipine, two dihydropyridine derivatives, which act as L-type Ca^{2+} channel antagonists [18,36], induced a significant reduction in the proliferation rate together with an increase in the apoptosis level (Fig. 5B).

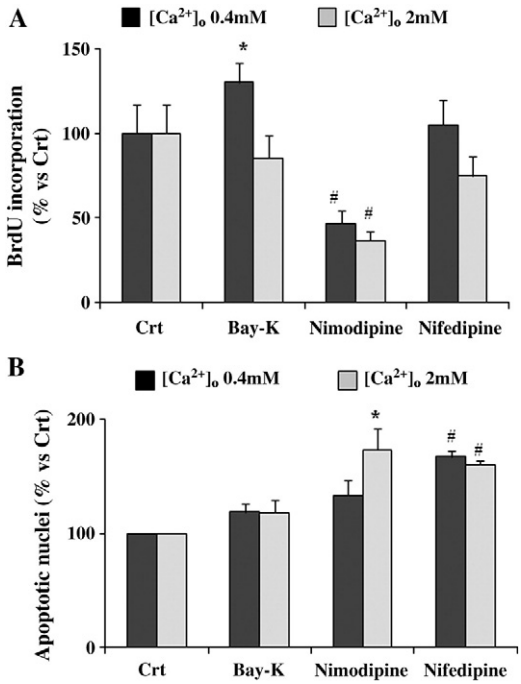

Fig. 5. Effect of agonist and antagonists of L-type Ca^{2+} channels in differentiated HT-29 RPMI cells. Proliferation rate (A) and apoptotic activity determined by DAPI staining (B) of control cells (Crt; 100%), cells treated with 400 nM Bay-K8644, or 30 μM Nifedipine or 30 μM Nimodipine in the presence of 0.4 and 2 mM $[Ca^{2+}]_o$. Each bar represents the mean±S.D. of three analogous experiments. Symbols indicate a statistically different value from Crt (*P<.05, #P<.01).

3.3. CPPs interact with the voltage-operated L-type calcium channels

The possibility that CPP activity in inducing the influx of calcium ions in differentiated HT-29 RPMI cells could involve the L-type voltage-operated calcium channels was evaluated. Cells were pretreated with the agonist or antagonists of these channels, and $[Ca^{2+}]_i$ changes were recorded after CPP administration. The behavior of HT-29 RPMI cells when stimulated with Bay-K8644 in the presence of 2 mM $[Ca^{2+}]_o$ is reported in Fig. 6A. The intracellular calcium rise recorded indicates the presence of active L-type Ca^{2+} channels in the cell population here used, according to the literature [18,37]. The administration of the same agonist, Bay-K8644, in CPP-responsive cells produced a higher response to the subsequent CPP administration (Fig. 6B), which accounts for a 100% increase in the $[Ca^{2+}]_i$ and a 50% increase in the percentage of responsive cells (Table 1). The effect of Nifedipine and Nimodipine on CPP bioactivity was thereafter evaluated. Both antagonists were able to totally or partially reduce the CPP-induced calcium rise in HT-29 RPMI cells, as shown in Fig. 7A and B. The percentage of cells that did not respond to CPP administration after Nimodipine or Nifedipine treatment was almost the same and accounts for about 36%–38%. The remaining 64%–62% of the cells responded to CPPs but with a marked reduction in the percentage of responsive cells (minus 45%) and in the intracellular calcium concentration (minus 52%) in the case of Nimodipine (Table 1), while in the case of Nifedipine, a marked reduction (minus 47%) was observed only in the intracellular calcium concentration (Table 1). A further demonstration of the involvement of the L-type Ca^{2+} channels

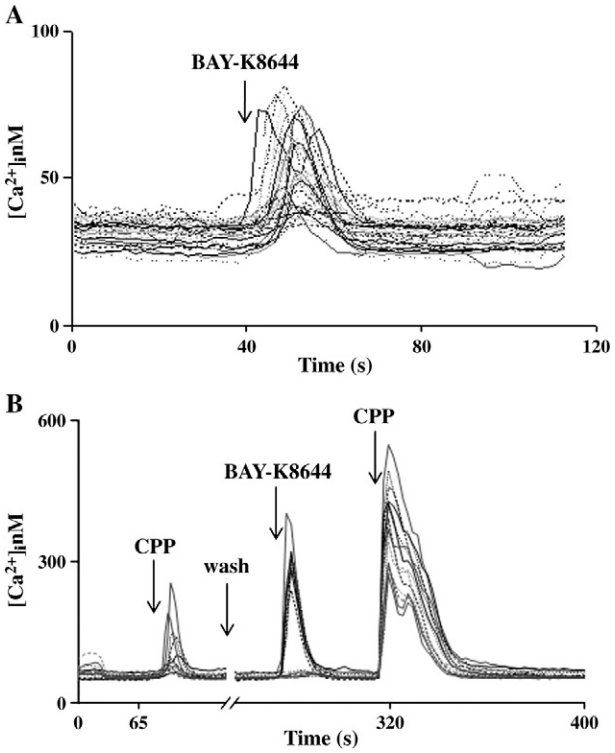

Fig. 6. Effect of Bay-K8644 on CPP-induced calcium rise in differentiated HT-29 RPMI cells. (A) 400 nM Bay-K8644 was administered in 2 mM $[Ca^{2+}]_o$. (B) 1280 μM CPP administration, cell washing and restoration of initial conditions (time axis break), followed by 400 nM Bay-K8644 and subsequently 1280 μM CPP. In the graphs, the intracellular calcium changes of some representative cells belonging to the chosen cellular field are reported. Each line in the graph refers to a single-cell behavior. Each graph is representative of at least five analogous experiments.

came from the use of La_2Cl_3, an inorganic inhibitor of calcium entry [29], which, when preadministered to cells, abolished the CPP bioactivity (Fig. 7C).

A direct evidence of the CPP action on L-type Ca^{2+} channels is also presented in Fig. 8, in which the membrane potential of HT-29 RPMI cells was measured by the use of $DiBAC_4(3)$, a probe whose fluorescence increases in depolarized conditions and decreases during hyperpolarization. CPPs increased the fluorescence activity (plus 16%) at the same entity of Gramicidin (plus 13%), a known depolarizing agent [38], while the hyperpolarizing KRH solution decreased fluorescence (minus 34%).

Table 1
Statistical analysis of the effects on $[Ca^{2+}]_i$ produced by CPP administration after cell treatment with Bay-K8644, Nimodipine and Nifedipine

Agonist/ antagonist	Preagonist/antagonist		Postagonist/antagonist	
	$\Delta[Ca^{2+}]_i$ (nM)	% Responsive cells	$\Delta[Ca^{2+}]_i$ (nM)	% Responsive cells
Bay-K8644	65.5±17	67±20.8	133±22#	100±0.5*
Nimodipine	92±48	91±9	44±19	50±12#
Nifedipine	91±40	61±23	48±21	54±22

The $[Ca^{2+}]_i$ rise was calculated for each single cell by subtracting the baseline from the peak value after CPP administration and averaging for all the analyzed cells. The percentage of responsive cells was the percentage of cells that responded to CPP administration with $[Ca^{2+}]_i$ increments equal to or above 20 nM. Each value represents the mean±S.D. of four analogous experiments, for a total of 500 analyzed cells. Symbols indicate a statistically different value between preagonist and postagonist/ antagonist administration: *P<.05, #P<.01.

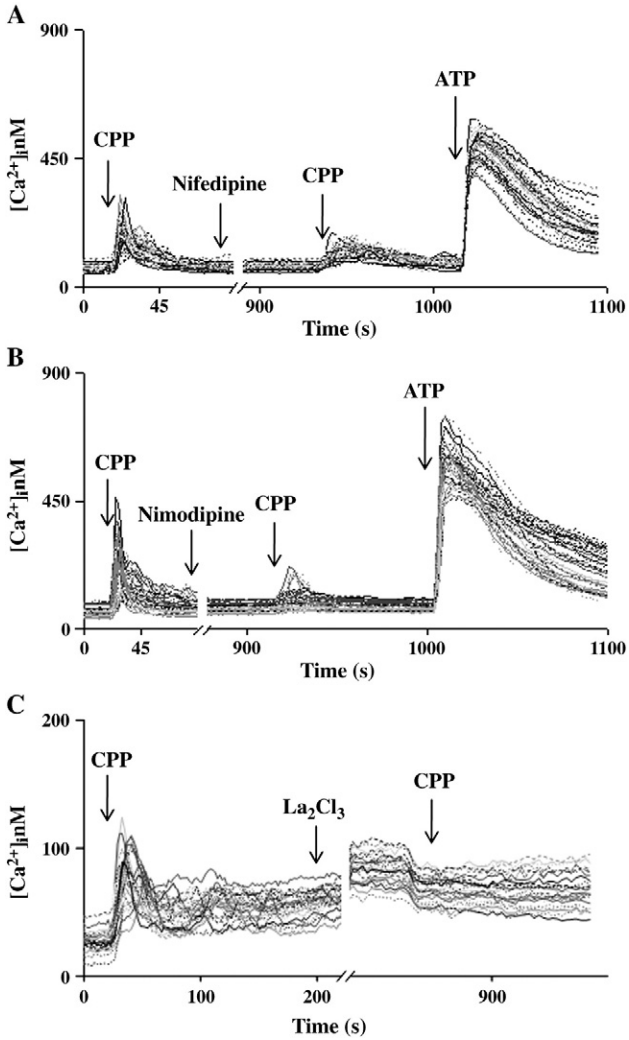

Fig. 7. Effect of Nifedipine, Nimodipine and La_2Cl_3 on CPP-induced calcium rise in differentiated HT-29 RPMI cell. (A) 1280 μM CPP administration, cell washing, 30 μM Nifedipine incubation (10 min, time axis break) and finally 1280 μM CPP and 100 μM ATP as a marker of full cellular viability. (B) 1280 μM CPP administration, cell washing, 30 μM Nimodipine incubation (10 min, time axis break) and finally 1280 μM CPP and 1 μM ATP. (C) Changes in the intracellular calcium concentration when cells were stimulated with 1280 μM CPP, washed and incubated with 250 μM La_2Cl_3 (5 min) and finally treated with 1280 μM CPP. In the graphs, the intracellular calcium changes of some representative cells belonging to the chosen cellular field are reported. Each line in the graph refers to a single-cell behavior. Each graph is representative of at least five analogous experiments.

4. Discussion

At the intestinal level, the extracellular calcium concentration can modulate processes such as proliferation and apoptosis, cellular events that are deeply and directly involved both in the maintaining of a cell-differentiated phenotype and in the development of a cancer phenotype [24]. Intestinal cells require small amounts of calcium ions to survive, about 0.05–0.1 mM [24,25], and when an overload of calcium takes place in the intestinal lumen, for instance, after a meal, they stop proliferating and begin the differentiation process that will finally lead them to apoptosis. The comprehension of these cellular events at molecular level has revealed the importance of dietary calcium, since the activation of CaSR, the calcium sensing receptor, by the mineral is the key that drives all the subsequent events toward the expression of the differentiated phenotype [33].

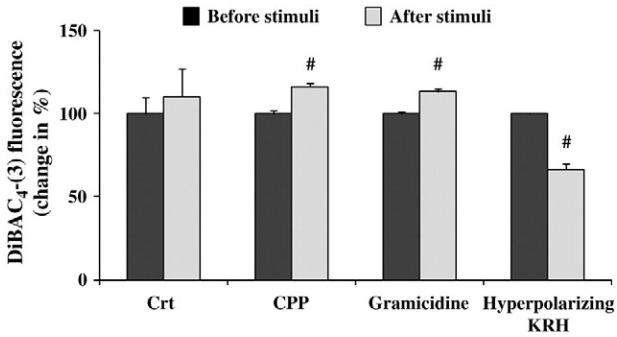

Fig. 8. Effect of CPP administration on membrane potential in HT-29 RPMI cells. Black bars represent fluorescence intensity of cells loaded with 500 nM $DiBAC_4(3)$ before the additions of stimuli (100%). White bars represent the change in fluorescence intensity (percentage vs, "before stimuli) of control cells (Crt) and cells treated with CPP (1280 μM), Gramicidin (1 μM) and hyperpolarizing KRH (5 mM KCl). Each bar represents the mean±S.D. of four analogous experiments. Statistically different value versus "before-stimuli" condition ($^{\#}P<.01$).

Otherwise, the calcium overload consequent to a meal exerts a proliferative stimulus on intestinal neoplastic transformed cells, due to the reduced expresson of CaSR in their membranes and the lack of the cellular mechanisms necessary to stop proliferation and start differentiation [39,40]. As a consequence, raising calcium with the diet may exert a chemoprotective role on normal differentiated intestinal cells, while it may behave as a carcinogenesis promoter in the presence of aberrant colonocytes and adenomatous crypts [24,41,42].

CPPs can be physiologically formed in the gastrointestinal tract and keep soluble a huge amount of calcium ions with which they are associated in milk and milk-derived food [3], hence the possibility that CPPs, previously demonstrated to increase intracellular calcium concentration in differentiated *in vitro* intestinal cell models HT-29 and Caco2 [11], could differently affect proliferation and/or apoptosis in differentiated/undifferentiated HT-29 cells.

In undifferentiated tumor HT-29 DMEM cells, CPPs do not affect proliferation but increase apoptosis, a result similar to that exerted by EGTA, the known calcium chelator, used to decrease the extracellular calcium concentration and thus diminish cell proliferation [20,43]; in fact, the entity of the increase due to CPPs was almost the same as that due to EGTA. Probably, CPPs and EGTA act under the same mechanism: binding calcium, they lower the mineral concentration in the extracellular space, causing a reduction pressure in the cell proliferation rate, but since these cells lack all the machinery necessary to start the differentiation process, they go on apoptosis. However, the usage of EGTA as antiproliferative agent in tumor regions is inadequate due to its toxicity for the neighboring differentiated cells, while CPPs never display a cytotoxic activity at the concentration here used.

In differentiated HT-29 RPMI cells, CPPs increase the proliferation rate with a maximal effect in the presence of 2 mM $[Ca^{2+}]_o$. It is worth remembering that CPPs induce an uptake of calcium ions in HT-29 RPMI cells in the presence of extracellular calcium ions and with a precise dose–response relationship [17], according to which their ability, due to the formation of aggregates with calcium ions, is higher at 2 mM compared to 6 mM $[Ca^{2+}]_o$. Thus, the effect of CPPs on the proliferation rate is in agreement with the demonstrated action on the induced calcium uptake by the same cells. EGTA drastically diminishes the proliferation rate at physiological $[Ca^{2+}]_o$, probably because it subtracts all the calcium available and necessary to cell survival, while at 2 mM, $[Ca^{2+}]_o$ has no appreciable effect, and at 6 mM $[Ca^{2+}]_o$, the effect is probably summed to the toxic effect exerted on cells by a such high $[Ca^{2+}]_o$. These results indicate a great difference between CPP versus EGTA outcomes on differentiated intestinal cells, since, although both able to bind calcium ions, CPPs do not behave toxic at physiological $[Ca^{2+}]_o$ and, in the presence of excess $[Ca^{2+}]_o$, do protect cells from death. No effects were determined on apoptosis by CPPs; on the contrary, EGTA increased caspase 3/7 activity at 0.4 and 6 mM $[Ca^{2+}]_o$ in analogy with the decreased proliferation. Also, the percentage of apoptotic nuclei is increased whichever the $[Ca^{2+}]_o$ tested. To summarize, CPPs protect differentiated intestinal cells from calcium overload toxicity and prevent their apoptosis favoring proliferation and at the same time induce apoptosis in undifferentiated tumor cells. These interesting and new results claim for a new potential role of CPPs as nutraceuticals and/or functional food, a role distinct from the mineral absorption but equally important. In fact, because molecules with an inhibitory activity toward apoptosis are considered tumor promoters [13], peptides displaying an apoptosis-inducing activity may be considered as probable anticarcinogens [44]. Previous studies have already demonstrated a protective, antiproliferative effect of the yogurt fraction on cultured mammalian cells Caco2 and IEC-6 [8], an inhibition of peripheral blood cell lymphocyte proliferation [9] and an influence on intestinal cell kinetics by casein peptides generated by dairy starter cultures [45]. Taken together, the experimental evidences here presented, whenever confirmed in further *in vitro* models and *in vivo*, could account for an anticancer role of CPPs.

The attempt to understand the mechanisms by which CPPs promote intracellular calcium increase and, at the same time, the proliferation in differentiated HT-29 RPMI cells has also revealed new insights on their role in the calcium absorption process in the intestine. Experiments performed in the presence of agonist/antagonists of voltage-operated L-types calcium channels, known to exert pro/antiproliferative effects in different *in vitro* cell cultures [15,18,34,35], demonstrate the existence of an interaction between CPPs and these calcium entry channels in differentiated HT-29 RPMI cells. CPPs were previously demonstrated to evoke calcium influx through L-type calcium channels in human osteoblast-like cells, being the predominant calcium channels type expressed in human osteoblast-like cells, also involved in cellular differentiation and function regulation [46]. Obviously, at present, we cannot exclude the contribution and therefore the interaction of CPPs with membrane channels other than L-types, since the correlation between ion channels and cancer prevention/development is a matter of intense studies as reported [47,48], and large bodies of evidence have revealed that a given ion channel can affect both cell proliferation and apoptosis depending on the properties of the cells. Moreover, the activity of a channel may depend on the interplay with other channels. In fact, experiments valuating the possible involvement of membrane channels coupled to cell calcium entry, as transient receptor potential cation channel subfamily V member 6 (TRPV6), are under investigation in our laboratory. Besides, it would be important to know the expression of the channels involved (L-types and others) between undifferentiated and differentiated cells. No evidences were found in the literature about the degree of expression for L-type calcium channels between HT-29 DMEM and RPMI cells. Zawadzki et al. [15] in their study described as the use of Bay-K8644 promoted calcium entry and apoptosis in HT-29 cells as well as in a human primary colon cancer cell line AZ-97; thus, we can presume that also in our HT-29 DMEM cells, voltage-gated channels can operate, but we do not know if the same subunit of the channel operates in undifferentiated and differentiated HT-29 cells, stated the different morphological features described for the two cell populations.

The notion here presented that CPPs can directly modify the membrane potential and interact with the depolarizing L-types calcium channels also in intestinal *in vitro* model cells assumes a great importance if we consider the recent findings on the $Ca_v1.3$ L-type channel. This channel is present in the apical membrane of rat intestine; its expression is more elevated in distal jejunum and

proximal ileum, where the calcium absorption is conventionally considered to be higher, about 85%, and to occur by the paracellular route, while at duodenum, where the active transport occurs, the TRPV6 channel is predominant. The two channels, apart from their localization, differ in their operating mode: (i) $Ca_v1.3$ is activated and mediates the calcium transport under depolarizing conditions, TRPV6 is activated by hyperpolarization; (ii) $Ca_v1.3$ is sensitive to the same agonist/antagonists for L-types calcium channels here used, while TRPV6 is not [49]. Moreover, all the calcium present in our body comes from the diet, and the principal digestion products, that is, glucose, amino acids and oligopeptides, are all depolarizing nutrients [50-52]. In this regard, the latest studies [53,54] have shed a new light about the comprehension of the intestinal calcium absorption, with the identification of $Ca_v1.3$ channel as the apical channel responsible for the glucose-stimulated calcium entry, being calcium and glucose signaling and absorption integrated [53]. Moreover, $Ca_v1.3$ channel is involved in prolactin-stimulated calcium absorption in pregnant and lactating animals [54]. After the ingestion of milk, CPPs are released by casein, the main protein fraction, and accumulate in ileum [44], where they can associate calcium ions but can also interact with the other nutrients here present and derived by the milk digestion as glucose from lactose and other peptides. The interaction of the different nutrients on calcium absorption constitutes an issue not completely understood, despite the recent findings here described [53,54], which deserves further studies, but the results here presented underline the importance of considering CPPs as biomolecules for the intestinal health.

Acknowledgments

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