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Caseinphosphopeptide-induced calcium uptake in human intestinal cell lines HT-29 and Caco2 is correlated to cellular differentiation ☆

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Abstract

Caseinphosphopeptides (CPPs) are considered as mineral carriers because of their ability to bind and solubilize calcium ions, with the possible role, yet to be definitely assessed, of improving calcium absorption at the intestinal level. Previous works demonstrated that CPPs improve calcium uptake, with increasing intracellular calcium concentration, by human differentiated tumor HT-29 cells, and that this effect correlates with the supramolecular structure of CPPs in the presence of calcium ions. The aim of the present study was to establish whether the CPP effect on calcium uptake is specific for HT-29 cells and depends on the differentiated state of the cells. To this purpose, HT-29 and Caco2 cells, two models of intestinal cells, were differentiated following appropriate protocols, including treatment with 1,25-$(OH)_2$ vitamin D_3. The CPP-dependent intracellular calcium rises were monitored at the single-cell level through fura2-fluorescence assays, and cell differentiation was assessed by biochemical and morphological methods. Results clearly showed that the ability to take up extracellular calcium ions under CPP stimulation is exhibited by both HT-29 and Caco2 cells, but only upon cell differentiation. This evidence adds novel support to the notion that CPPs favour calcium absorption, thus possibly acting as cellular bio-modulators and carrying a nutraceutical potential.

Keywords: Caseinphosphopeptides; Calcium uptake; HT-29 cells; Caco2 cells; Cell differentiation

1. Introduction

Casein, the major protein fraction of milk, is known to be an excellent source not only of amino acids but also of peptides with different modulatory activities [1]. Among these peptides, a growing interest has been devoted to phosphopeptides which are derived from casein cleavage by trypsin [caseinphosphopeptides (CPPs)]. Due to their properties for binding and solubilizing calcium ions, CPPs, especially CPP β-CN(1-25)4P, which corresponds to the first 25 amino acids of β-casein, and CPP α_{S1}-CN(59-79)5P, which corresponds to the sequences 59–79 of α_{S1}-casein [2], are considered as mineral carriers with the potential role to improve calcium absorption at the intestinal level. The CPP-mediated enhanced Ca^{2+} absorption observed in the rat ileum sacs or ligated segments [3–11] demonstrated a positive role by CPPs on calcium bioavailability in animals, suggesting that CPPs may act as functional foods. This hypothesis had some support in animal studies, whereas investigations on humans provided conflicting results [12–18]. In parallel with these studies in animals and humans, numerous works studied the aggregative properties of CPPs, starting from the notion that casein in milk is present as micelles with calcium and phosphate salts, and demonstrated that this supramolecular structure may be relevant for CPPs to exert a functional role. Casein micelles are stable structures composed of hundreds of smaller aggregates called calcium phosphate nanoclusters, or nanocomplexes, having a core of calcium phosphate surrounded by a shell of casein molecules [19–21]. The ability of bovine casein to form micelles was demonstrated to be retained also by the CPPs β-CN(1-25)4P and α_{S1}-CN(59-79)5P [22–25]. Efforts to understand the molecular mechanism by which CPPs function at the intestinal level were made in our laboratory using differentiated HT-29 cells as a model of cellular intestinal epithelium. We first observed

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that CPPs facilitate calcium uptake by these cells [26,27], and then that the effect strictly correlates with the supramolecular structure of CPPs [28]. Practically, the ability of CPPs to elicit their biological effect relies on two critical conditions: the presence of Ca^{2+}-CPP aggregates at the right conformation and concentration, and a proper ratio between Ca^{2+} and CPPs, this latter condition being in full agreement with data from *ex vivo* intestinal model studies [3].

The present work was undertaken with the aim to establish whether (i) the CPP effect on calcium uptake demonstrated in HT-29 cells is specific for this type of cells or is exhibited by other cellular models of intestinal epithelium; and (ii) the state of cell differentiation is relevant, or not, for the occurrence of the calcium uptake. To these purposes, we used, besides HT-29 cells, a second human intestinal cell line, Caco2 cells, and checked the CPP-mediated calcium uptake in both cell lines along with differentiation. Appropriate differentiation protocols were employed for both cell lines [29], including treatment with 1,25-$(OH)_2$ vitamin D_3, which is known to be an efficient differentiation agent and a modulator of the active calcium transport in intestinal cells [30,31].

2. Materials and methods

Cell culture media 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). 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, hereafter abbreviated as Vit.D_3), was from Calbiochem (La Jolla, CA, USA).

2.1. Casein phosphopeptides

The used CPP preparation (CPP DMV) is a casein-derived hydrolysate (CE 90 CPP III, DMV International, Veghel, The Netherlands), constituted by several components, each containing the characteristic CPP "cluster sequence" Ser(P)-Ser(P)-Ser(P)-Glu-Glu, which shows the following composition: 93.8% as dry matter; 96% purity; 10.8% total nitrogen content; 3.7% phosphorous content; nitrogen/phosphorous ratio of 3.1; P/Ser molar ratio of 0.85; average molecular weight of 2500. This CPP mixture was assessed to be calcium free as already reported [26]. For the intracellular calcium measurement experiments, CPP DMV was dissolved in doubly distilled water in stock solutions (1000× concentrated, with respect to the final concentration) and stored at −20°C.

2.2. Intestinal cell models

2.2.1. HT-29 Cells

The human colon carcinoma cell line HT-29 was obtained from Istituto Zooprofilattico Sperimentale di Brescia (Brescia, Italy). Cells were routinely grown in 75-cm^2 plastic flasks (Costar, Concorezzo, Italy) in high D-glucose (4.5 g/l) DMEM, supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 0.1 mg/L streptomycin, 100,000 U/L penicillin, 0.25 mg/L amphotericin-B (DMEM cells). 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. Under these experimental conditions, HT-29 DMEM cells are undifferentiated tumour cells [29].

2.2.2. Caco2 cells

The human colon carcinoma Caco2 cell line (BS TCL 87) was obtained from Istituto Zooprofilattico Sperimentale di Brescia. Cells were routinely grown in 75-cm^2 plastic flasks (Costar) in Eagle's minimum essential medium in Earle's BSS, supplemented with 15% fetal calf serum, 2 mM L-glutamine, 0.1 mg/L streptomycin, 100,000 U/L penicillin, 0.25 mg/L amphotericin-B and 1 mM sodium pyruvate. Cells were submitted to subcultivation and used as undifferentiated cells in the 7–12th passage. For brevity, the "passage number" is hereafter abbreviated as P.

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.

2.3. Cell differentiation protocols

2.3.1. HT-29 Cells

The differentiation of HT-29 cells was achieved by switching from the DMEM medium to low D-glucose (2 g/L) RPMI-1640 medium, supplemented as above [32]. These cells were termed RPMI cells. We experimented with two other alternative procedures: the replacement of glucose with galactose (HT-29 galactose cells) and the addition of 5 mM sodium butirate to the RPMI 1640 growth medium (NaBu HT-29 cells) [29].

2.3.2. Caco2 cells

The differentiation of Caco2 cells was achieved through successive subcultivations prior to reaching the post-confluent stage, as described [33]. In the 40–44th P, cells were well and fully differentiated.

2.4. Vit.D_3 Administration

After preliminary experiments to determine the Vit.D_3 concentration suitable for inducing changes in cellular morphology and shape, undifferentiated HT-29 and Caco-2 cells were fed with 100 nM Vit.D_3 (from a stock solution in DMSO, vehicle) added to their culture medium, for 48 h. The cell culture medium was freshly prepared without adding serum to avoid Vit.D_3–protein binding [34]. The cell density before Vit.D_3 feeding was carefully controlled so as not to overcome the 50% confluence, since a lack of differentiation effect by Vit.D_3 on confluent cells was reported [34].

In all cases, the differentiation process was monitored by assay of biochemical markers (alkaline phosphatase and sucrase-isomaltase), measurement of proliferation rate, and morphological examinations (by transmission electron microscopy), as reported elsewhere [28].

2.5. Ultrastructural analysis

For ultrastructural analysis, cells, plated in 35-mm Petri dishes and allowed to grow till the degree of confluence described above, were fixed for 60 min at room temperature with glutharaldehyde 2% in 0.1 M Sorensen phosphate buffer (pH 7.4), thoroughly rinsed with the same buffer, post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M Sorensen phosphate buffer, dehydrated through an ascending series of ethanols and embedded in Durcupan (Durcupan, Fluka, Milan, Italy). Ultrathin sections were obtained with an Ultracut ultramicrotome (Reichert Ultracut R-Ultramicrotome, Leika, Wien, Austria) and stained with uranyl acetate and lead citrate before examination by a Jeol CX100 electron microscope (Jeol, Tokyo, Japan).

2.6. Isolation of brush border fraction and enzyme assays

For the determination of alkaline phosphatase and sucrase-isomaltase activities, two well-known biochemical markers of intestinal cell differentiation, P2 subfractions, enriched in brush borders, were prepared as previously described [28,33]. Alkaline phosphatase activity (ALP) was assayed on samples of 20–50 μg of P2 subfractions resuspended to a final volume of 50 μl [35]. Sucrase-isomaltase was assayed following the one-step ultramicromethod on P2 subfractions (about 20 μg of protein) resuspended to a final volume of 20 μl [36]. Results were expressed as milliunit per milligram of protein, 1 U being defined as the enzyme activity that hydrolyses 1 μmol of substrate per minute. The protein content was measured following the Lowry method [37]. The results are reported as percentage with respect to undifferentiated cells.

2.7. Cell proliferation assay

Cells (1×10^4 cells/well), cultured in their medium in a Microtiter plate (96-well, Greiner bio-one, Cellstar, Frickenhausen, Germany), were incubated (24 h) with 1280 μM CPP DMV in their culture media devoid of fetal calf serum, submitted to a 2-h pulse with bromodeoxyuridine (BrdU) and BrdU incorporation into DNA quantified by the chemiluminiscent immunoassay (Roche Applied Science, Milan, Italy) following the manufacturer's instructions. The results are expressed as percentage with respect to undifferentiated cells.

2.8. Measurement of intracellular calcium concentration, $[Ca^{2+}]i$, at a single cell level

Cells grown at 70–80% of confluence were suspended with a trypsin/EDTA (final concentration 0.5/0.2 g/L) solution and seeded on a glass coverslip (24 mm diameter, thickness 0.13–0.17 mm, VWR International, West Chester, PA, USA) in Petri dishes (35 mm diameter, Costar) at 7.5×10^4 cells/cm^2. All the experiments were performed 48 h after seeding cells. Cytoplasmic calcium was measured according to the procedure described by Tsien and Poenie [38]. Briefly, cells on glass coverslips were incubated for 30 min at 37°C with 5 μM Fura-2/AM and 2.5 μM Pluronic F-127 in 1 ml Krebs-Ringer-HEPES solution (KRH) containing (in millimolars) 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 adjusted to pH 7.4. After incubation, cells were extensively rinsed with KRH and maintained for an additional 20 min at room temperature to allow de-esterification of the fluorescent probe. The glass coverslip was then mounted in a thermostatted (TC-202 A) perfusion chamber (PDMI-2) from Medical System Corporation (Harvard Apparatus, Holliston, MA, USA) and placed on the microscope stage (TE 200, Nikon, Tokyo, Japan) where the cells, incubated in 2 ml of KRH, were alternately excited at 340–380 nm through a 40× oil immersion objective (NA=1.3, Nikon, Tokyo, Japan). The emitted fluorescence at 510 nm was measured at 1- to 2-s intervals by a CCD intensified camera (Extended Isis, Photonic Science, Millham, UK), and ratio images of single cells, averaged over eight frames, within a chosen window of 50–200 cells, were collected and analyzed after background subtraction, using a fluorescence image

acquisition and data analysis system which was supplied by Applied Imaging (High Speed Dynamic Video Imaging Systems, Quanticell 700, Sunderland, UK). The amount of free calcium within the cells, $[Ca^{2+}]_i$, 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 [39]. The duration of each experiment never exceeded 2 min, a period of time during which the cells appeared to maintain full viability. The extracellular calcium concentration, $[Ca^{2+}]_o$, was kept at 2 mM and the intracellular calcium concentration was continuously monitored in single cells. CPP DMV was administered to cells in a single dose of 1280 μM from the stock solution through an appropriate syringe connected to the upper reservoir of the cell chamber. The single-cell analysis provided the following results: (i) percentage of responsive cells, i.e., the percentage of cells which responded to CPP DMV administration with $[Ca^{2+}]_i$ increments equal to or above 20 nM; (ii) the mean single maximum $[Ca^{2+}]_i$ rise, calculated for each single cell by subtracting the baseline from the peak value after CPP DMV administration and averaging all the analyzed cells; and (iii) the mean total $[Ca^{2+}]_i$ rise, calculated as the product of the percentage of responsive cells and mean single maximum $[Ca^{2+}]_i$ rise of a cellular field constituted by 100 cells.

2.9. Statistical analysis

The data reported in Fig. 2 and Table 3 are expressed as mean values of at least 5-7 parallel experiments±standard deviation. Statistically significant differences between two mean values were established by the Student's *t* test, independent two population *t* test, performed with Origin 6.0 (a P value<.01 was considered significant).

3. Results

3.1. Ultrastructural features of undifferentiated and differentiated HT-29 and Caco2 cells

These data are reported in Fig. 1 and Tables 1 and 2.

3.1.1. HT-29 cells

HT-29 cells, cultivated in DMEM medium, did not display signs of differentiation, apart from rare apical microvilli and desmosomes (Fig. 1A and Table 1), as already described [28]. The addition of Vit.D_3 to DMEM cells led to the formation (Fig. 1B, Table 1) of intercellular junctions, especially desmosomes, clearly detectable on the lateral side of the cell membrane (see asterisks in Fig. 1B) and of intercellular follicle-like structures (FLS) between adjacent cells, characterized by numerous and small microvilli as previously observed in galactose-differentiated HT-29 cells [28,40]. All these features are consistent with a differentiated phenotype. RPMI cultured cells displayed a much more differentiated morphological phenotype than Vit.D_3-treated DMEM cells, as documented by a complete junctional apparatus (tight junctions, adherens junctions, desmosomes) and very abundant microvilli (Fig. 1C, Table 1).

3.1.2. Caco2 cells

Caco2 cells in the 7–12th P did not feature signs of differentiation (Fig. 1D, Table 2), presenting only rare microvilli and very few glycogen deposits, a typical characteristic of Caco2 cell differentiation [41]. The addition of Vit.D_3 to the culture medium promoted differentiation of the same cells as shown by the appearance of abundant microvilli, tight junctions, adherens junctions and desmosomes in correspondence with the latero-apical side of the cell membrane and massive intracellular deposits of glycogen (Fig. 1E, Table 2). In Caco2 cells at higher passages (40–44th P), a well-developed brush border, with tight junctions, adherens junctions and desmosomes, as well as abundant glycogen deposits were present (Fig. 1F, Table 2), consistent with a well-differentiated phenotype and confirming previous evidence [33].

3.2. Brush border enzyme activities and proliferation rate in undifferentiated and differentiated HT-29 and Caco2 cells

These data are reported in Fig. 2.

3.2.1. HT-29 cells

No difference in the activities of alkaline phosphatase and sucrase-isomaltase between HT-29 DMEM and RPMI cells was observed (Fig. 2), as already reported [28]. On the contrary, the addition of Vit.D_3 to DMEM cells in culture induced a marked and statistically significant increase in both alkaline phosphatase (+58±15%) (Fig. 2A) and sucrase-isomaltase activities (+137.8±16%) (Fig. 2B). The proliferation rate was also negatively affected by the differentiation treatment, with the strongest reduction (−65 ± 3.9%) in Vit.D_3-treated DMEM cells, followed by RPMI cells (−24 ± 3.5%) (Fig. 2C).

3.2.2. Caco2 cells

Vit.D_3 treatment of undifferentiated Caco2 cells (7–12th P) produced a marked increase of alkaline phosphatase activity (+81.3±12%) (Fig. 2D) but not of the sucrase-isomaltase activity (Fig. 2E). In contrast, in well-differentiated Caco2 cells (40–44th P), alkaline phosphatase activity was reduced (−40±1.5%), (Fig. 2D) and sucrase-isomaltase activity strongly increased (+254±7%) (Fig. 2E) as compared to undifferentiated cells. The proliferation rate was reduced by both differentiation treatments, very strongly by Vit.D_3 treatment (about −83±4.9%), less by the 40–44th P (about −36 ±3.5%) (Fig. 2F), confirming previous observations [30].

3.3. CPP effect on $[Ca^{2+}]_i$ is correlated to cell differentiation

These data are reported in Fig 3 and Table 3.

3.3.1. HT-29 Cells

When CPP DMV was administered to undifferentiated DMEM cells, only a few cells (about 10%) were able to respond with a small (mean single rise, about 51 nM) and transient rise of $[Ca^{2+}]_i$, and a mean total rise of 529.4 nM (Fig. 3A and Table 3). In contrast, Vit.D_3-treated DMEM cells were reactive to CPP DMV with a sustained $[Ca^{2+}]_i$ increase (mean single rise, 88.2 nM), characterized by a great number of oscillations (Fig. 3B). The number of responding cells rose to 86.4% and the mean total increased to 7620.5 nM (Table 3). In RPMI cells, a prompt and transient rise of $[Ca^{2+}]_i$ was induced by CPP DMV administration (mean single rise, 77.4 nM; see Table 3), quite simultaneously in the majority of the cells (Fig. 3C), although with cellular responses not fully uniform in shape, amplitude or rapidity, as we previously reported [26,27]. The percentage of responsive cells was about 75%, and the mean total rise was about 5782 nM.

3.3.2. Caco2 cells

The 7–12th P Caco2 cells very rarely showed a responsiveness to CPP DMV administration (1.5% of responsive cells) (Fig. 3D and Table 3), with a mean single rise and mean total rise of $[Ca^{2+}]_i$ of 37.3 and 55.9 nM, respectively (Table 3). Vit.D_3 treatment of 7–12th P Caco2 cells determined a strong increase in the number of responsive cells (86.7%) accompanied by a huge uptake of calcium ions (mean single rise, 124.7 nM; mean total rise, 10811 nM; see Table 3), but with much less oscillations than in the case of HT-29 cells (Fig. 3E). The 40–44th P Caco2 cells showed a good response to CPP DMV treatment, although in a more modest way than Vit.D_3-treated cells, with a lower number of responsive cells (23.5%), mean single rise (52.5 nM) and mean total rise (1233.7 nM) (Table 3).

Control experiments were performed on HT-29 and Caco2 cells to which DMSO, the vehicle of Vit.D_3, was administered to cells, without monitoring any cell response. Parallel experiments were run

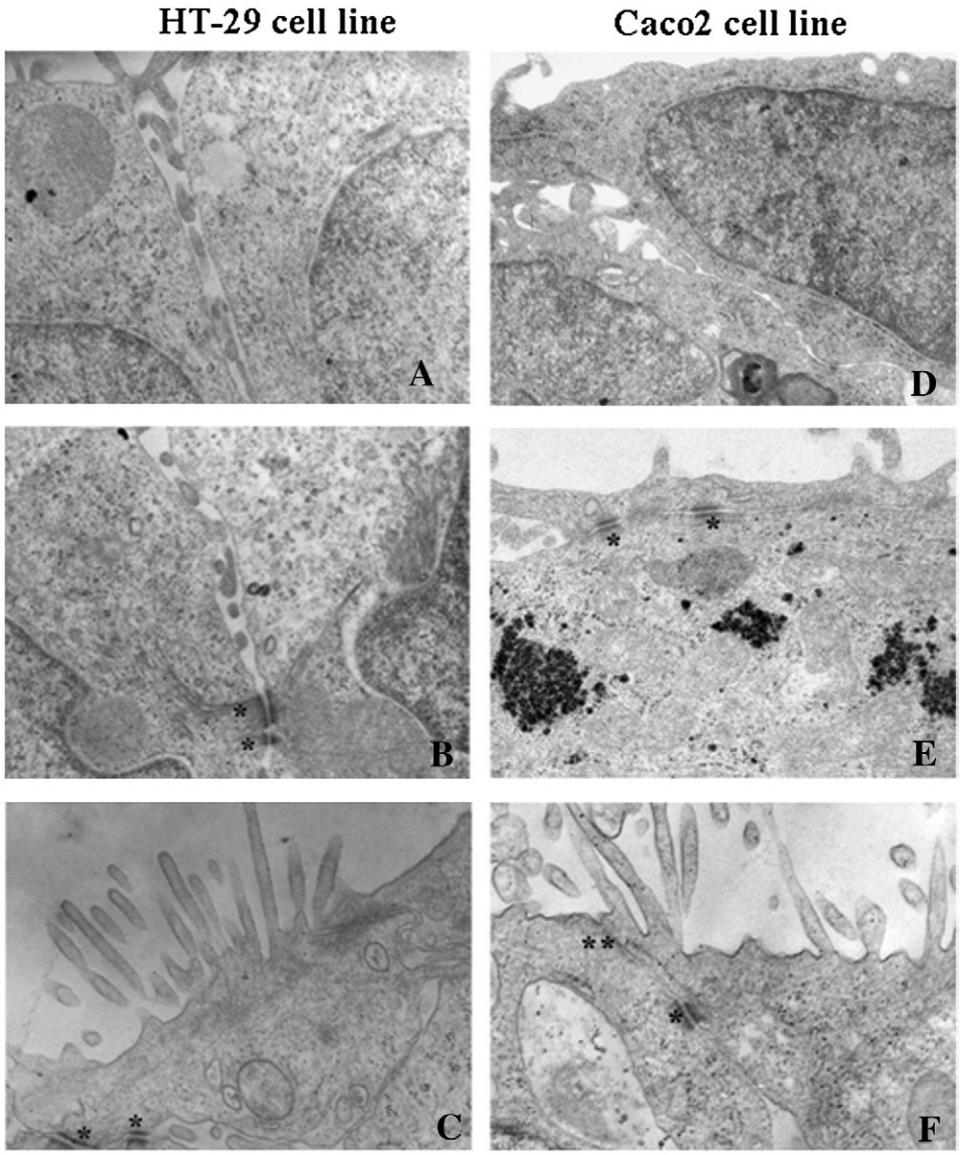

Fig. 1. Transmission electron microphotographs of undifferentiated/differentiated HT-29 and Caco2 cells. (A) DMEM HT-29 cells; (B) Vit.D_3-pretreated DMEM HT-29 cells; (C) RPMI HT-29 cells; (D) 7–12th P Caco2 cells; (E) Vit.D_3-pretreated 7–12th P Caco2 cells; (F) 40–44th P Caco2 cells. Single and double asterisks indicate respectively desmosomes and tight junctions on the lateral cell membrane. Original magnifications are as follows: Panel A, ×14,000; Panel B, ×19,000; Panel C, ×10,000; Panel D, ×15,000; Panel E, ×15,000; Panel F, ×20,000.

administering CPP DMV to HT-29 galactose and NaBu cells, since it was reported that the HT-29 cell line is a heterogeneous population, which possesses the ability to undergo different patterns of cell differentiation, depending on modifications of the culture medium or the addition of inducers of differentiation [29]. In both cases, the cellular responses to CPP DMV were very similar to those reported for RPMI cells (data not shown).

Table 1
Ultrastructural features of undifferentiated (DMEM) and differentiated (DMEM+Vit.D_3, RPMI) HT-29 cells (further details in Materials and Methods section)

	Undifferentiated DMEM cells	Differentiated DMEM cells+Vit.D_3	Differentiated RPMI cells
Apical microvilli	±	±	++
FLS	−	+	−
Junctional apparatus			
Tight junctions	−	−	++
Adherens junctions	−	−	++
Desmosomes	±	+	++

4. Discussion

In previous works [26,27], we reported and discussed the ability of CPPs to induce calcium uptake in HT-29 cells, with increasing $[Ca^{2+}]_i$. This biological effect was investigated in HT-

Table 2
Ultrastructural features of undifferentiated (7–12th P) and differentiated (7–12th P +Vit.D_3, 40–44th P) Caco2 cells (further details in Materials and Methods section)

	Undifferentiated Caco2 cells (7–12th P)	Differentiated Caco2 cells (7–12th P+Vit.D_3)	Differentiated Caco2 cells (40–44th P)
Apical microvilli	±	++	++
Junctional apparatus			
Tight junctions	–	±	++
Adherens junctions	–	+	++
Desmosomes	–	+	++
Glycogen	±	+++	+

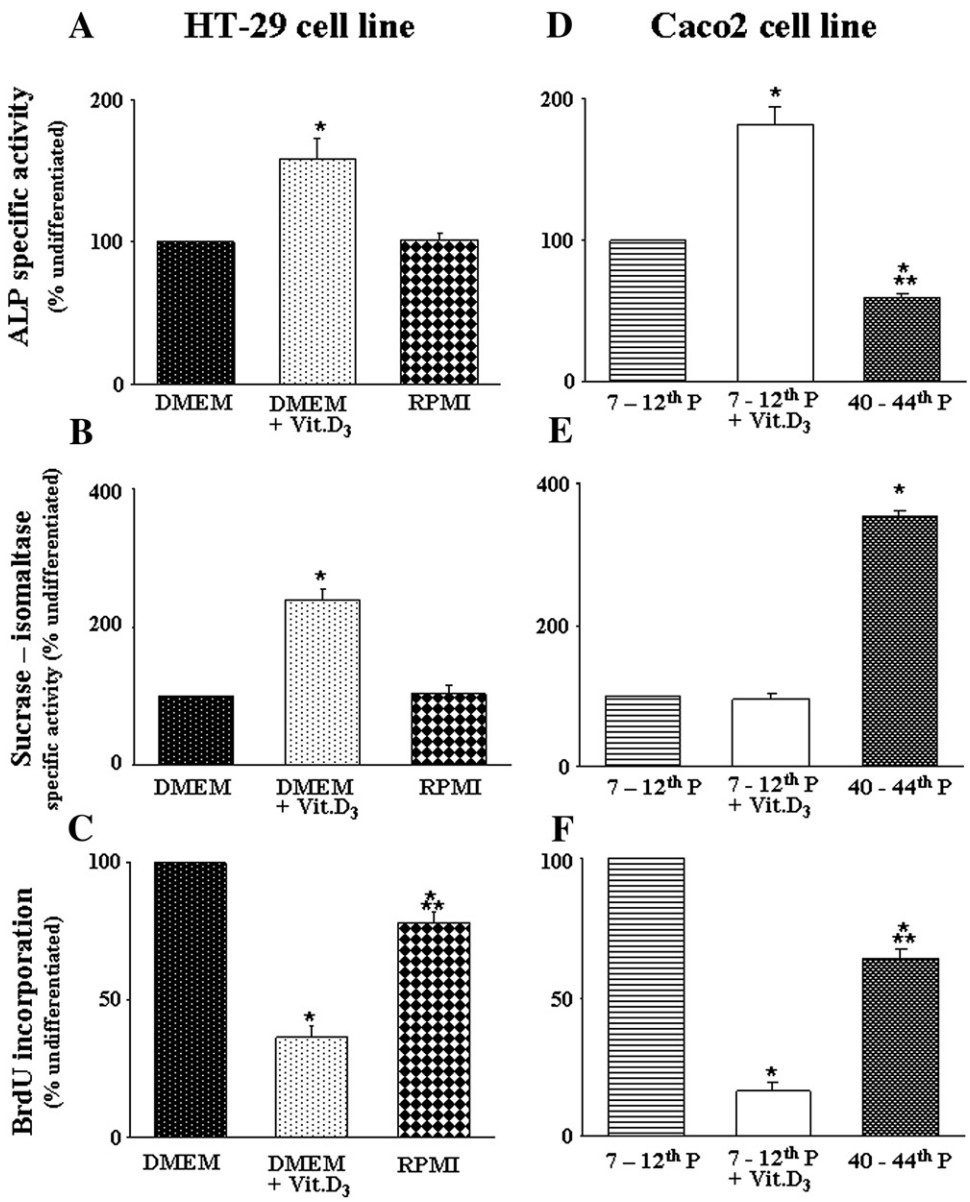

Fig. 2. Enzyme activities and proliferation rate in undifferentiated/differentiated HT-29 and Caco2 cell lines. HT-29 cell line specific activity of ALP (A); sucrase-isomaltase (B); proliferation rate (C). Caco2 cell line specific activity of ALP (D); sucrase-isomaltase (E); proliferation rate (F). Results are expressed as percentage with respect to undifferentiated cells (DMEM for HT-29 cell line; 7–12th P for Caco2 cell line). Each bar represents the mean±S.D. of five to seven analogous experiments. Single asterisk (*) means a statistically significant difference ($P<.01$), with reference to undifferentiated cells. Three asterisks (***) mean a statistically significant difference with $P<.01$ between Vit.D_3-treated and differentiated cells.

29 cells cultured in RPMI 1640 medium, a condition known to induce the features of cell differentiation towards intestinal epithelium, hence the use of these differentiated cells as a model of intestinal epithelium cells [29]. The results presented here clearly show that the same HT-29 cells, induced to differentiate by Vit.D_3 treatment, are able to respond to CPP DMV administration with an intracellular calcium rise, whereas they are very poorly responsive when cultured in DMEM, thus possessing the undifferentiated phenotype. Presumably, the very low percentage of DMEM cells responsive to CPPs represents the few spontaneously differentiated cells which are always present in the HT-29 cell culture [42]. Remarkably, another model of intestinal cells we employed, Caco2 cells, frequently used in studies of drug transport due to the tight monolayer composed of absorptive cells at full differentiation degree [33], when induced to differentiate by Vit.D_3 treatment or by prolonged subcultivations (40–44th P subcultures), has acquired the ability to respond to CPPs with a $[Ca^{2+}]_i$ rise, as it occurs in differentiated HT-29 cells, whereas they are almost totally unresponsive in the undifferentiated state, like undifferentiated HT-29 cells. Therefore, and regardless of the slight differences (mostly at the quantitative levels) between the two differentiated cell lines in their response to CPPs, a notion can be suggested that the ability of these cell lines of intestinal origin to take up extracellular calcium with increase of $[Ca^{2+}]_i$ under CPP stimulation is a molecular attitude expressed upon differentiation, that is, and intriguingly, a "marker" of differentiation for the same cells. In both cell lines, HT-29 and Caco2, the cell differentiation was achieved by pretreatment with Vit.D_3 or by following well-established procedures [29,30,34]. The morphological data obtained display undoubtedly the changes from an undifferentiated cell phenotype to a differentiated one. The biochemical data, i.e., the activity of the enzymes ALP and sucrase-isomaltase, known markers of intestinal differentiation, could seem contradictory. In the case of HT-29 cells, it is described and also confirmed by us elsewhere [28] that no differences were evident between

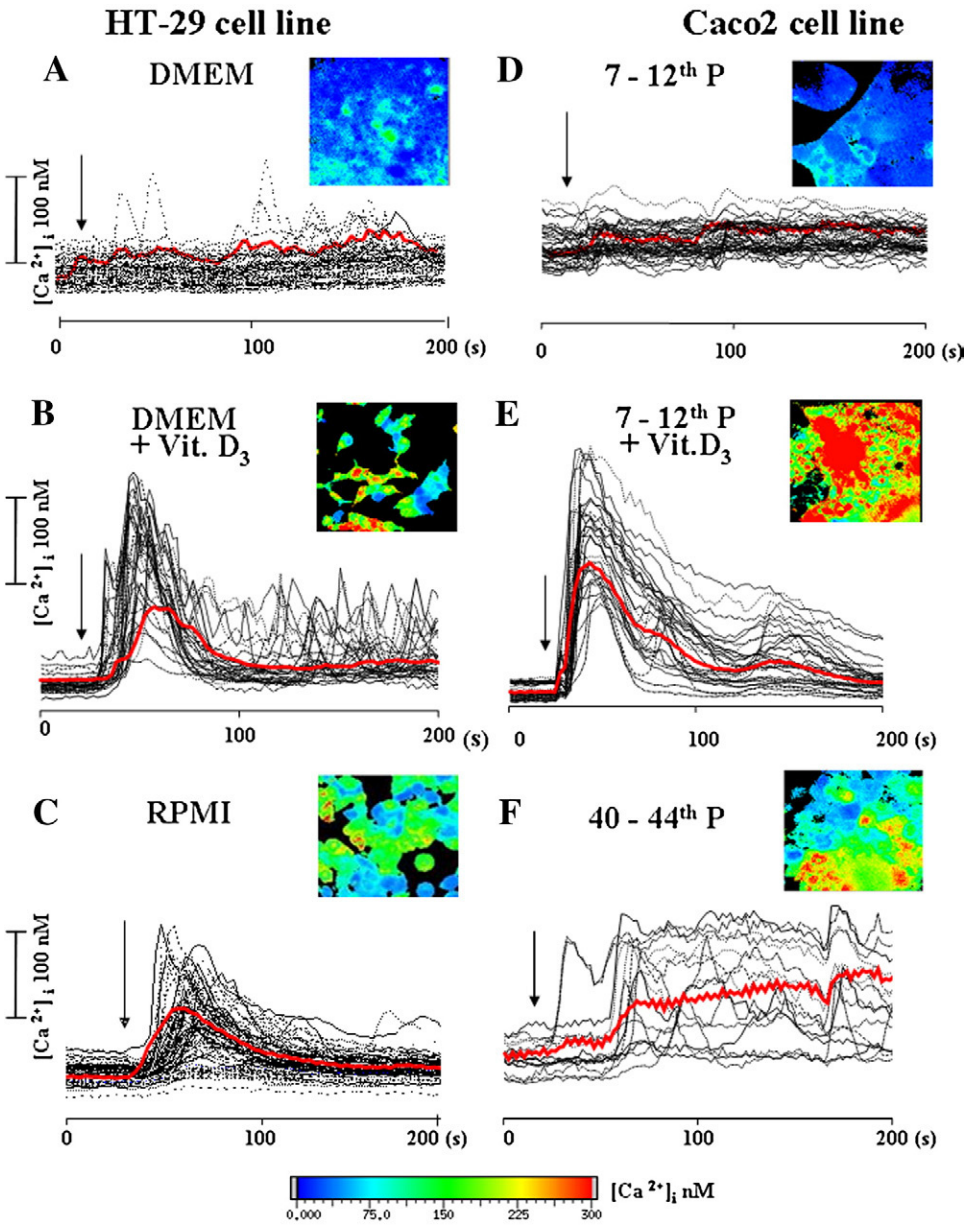

Fig. 3. Effect of CPP DMV on intracellular calcium in undifferentiated/differentiated HT-29 and Caco2 cell line. The time course of the intracellular calcium changes due to CPP DMV administration is shown for HT-29 cells (Panels A–C) and for Caco2 cells (Panels D–F) at different degrees of differentiation. Each line in the graphs represents the behavior of a single cell before and after 1280 μM CPP DMV administration (arrow) in a representative experiment chosen from five to seven analogous experiments. The red trace in each graph represents the mean time course of the intracellular calcium changes from all the experiments done for each cell population studied. The frame in each graph reproduces the 340/380 ratio images of a representative examined cellular fields at the peak after CPP DMV administration. Color changes are representative of different $[Ca^{2+}]_i$ values as indicated in the pseudo-color scale obtained by a calibration procedure described in Materials and Methods.

DMEM (undifferentiated) and RPMI (differentiated) cells, while the effect of the pretreatment with Vit.D_3 is much more evident (about twofold enzyme activities with respect to undifferentiated cells). This result is in agreement with data reporting a great number of the Vit. D_3 receptors in HT-29 undifferentiated cells (DMEM cells) and an increase in the sucrase-isomaltase activity [34,40]. In the case of Caco2 cell line, the effect of the differentiation procedure adopted here was an increase in the sucrase-isomaltase activity but not in the ALP activity [33]; on the contrary, the pretreatment with Vit.D_3 was characterized by an increment in the ALP activity instead of the sucrase-isomaltase activity, as already reported [30].

The observed differences between differentiated HT-29 and Caco2 cells in their responses to CPPs could be due to the different properties of the monolayer formed by the two cell lines, composed mainly of absorptive cells in the case of Caco2 cells, and of heterogeneous cell types in the case of HT-29 cells [43], and supported also by different enzyme activities [28,33].

It is worth remembering that in the present study we used mainly a commercial CPP preparation, CPP DMV, which is a mixture of peptides, but, as already described in our previous works [27,28], CPPs having different amino acid compositions, like β-CN(1-25)4P and α_{S1}-CN(59-79)5P, could exert a different ability to bind and promote calcium uptake by the cells, and these differences could become more evident when using differentiated instead of undifferentiated cells. Moreover, it could be considered that at the physiological level in the

Table 3
Statistical analysis of the effects produced by CPP DMV administration in undifferentiated/differentiated HT-29 and Caco2 cells

	HT-29 cell line			Caco2 cell line		
	Undifferentiated DMEM cells	Differentiated DMEM cells+Vit.D_3	Differentiated RPMI cells	Undifferentiated Caco2 cells (7–12th P)	Differentiated Caco2 cells (7–12th P+Vit.D_3)	Differentiated Caco2 cells (40–44th P)
% of cells responding	10.4±2	86.4±16.6*	74.7±19.3*	1.5±2	86.7±16.6**	23.5±4.5 **
Mean single maximum rise in $[Ca^{2+}]_i$ (nM)	50.9±10.3	88.2±28	77.4±20.3	37.3±4.9	124.7±89.7	52.5±28.3
Mean total rise in $[Ca^{2+}]_i$ (nM)	529.4	7620.5	5781.8	55.9	10811.5	1233.7

The mean single maximum rise and the mean total rise in $[Ca^{2+}]_i$ were determined as described in Materials and Methods. Asterisks indicate statistical significance: *P<.01 for DMEM +Vit.D_3, RPMI vs. DMEM; **P<.01 for the 7–12th P+Vit.D_3 Caco2 and the 40–44th P Caco2 vs. the 7–12th P Caco2.

human gut the casein hydrolysis produces a blend of CPPs similar to the used CPP DMV [26,27].

These novel results raise the questions as to the mechanism of action of CPPs and as to why a differentiated phenotype for the intestinal-like cells used is required for the expression of this action. Of course, only hypothetical lines of reasonings can be suggested. One possibility arises from the observation that both HT-29 and Caco2 cells only when fully differentiated feature a complete junctional apparatus, composed of tight junctions, adherens junctions and desmosomes, which is known to regulate the molecular traffic between the extracellular and intracellular compartments [44]. Recently, it was demonstrated that ingestion of various nondigestible saccharides increases intracellular calcium in Caco2 cells and opens their paracellular calcium transport under physiological conditions [45]. Due to a direct effect of CPPs on tight junction integrity reported to occur in goat mammary gland [46] and the claimed indication that the site of CPP action is the ileum [3,7], where the paracellular route of calcium absorption is predominant [47], a supposed mechanism of action of CPP could be the alteration of the tight junction permeability, thus linked to the presence of the junctional apparatus and therefore dependent on cell differentiation. However, more recent experiments carried on in our laboratory and measuring the transepithelial electric resistance before and after CPP administration in HT-29 and Caco2 differentiated cells showed that this is not the mechanism of action (our unpublished preliminary results).

A second hypothetical mechanism for the action of CPPs is that they can form a peculiar calcium-selective channel in the plasma membrane of responsive cells. It is worth remembering that Alzheimer's β-amyloid, human islet amylin and prion protein fragment PrP106-126, all featuring a β-pleated sheet structure, similar to that present in CPPs, were supposed to interact spontaneously with the plasma membrane of susceptible cells, forming unregulated Ca^{2+} channels [48]. Notably, CPPs can form aggregates in the presence of calcium ions, and these aggregates constitute the biologically active form of CPPs [28], possibly suitable to be inserted into the plasma membrane of the responsive cells. This possibility relies on the presence of an appropriate lipid environment favourable to the peptide insertion. The lipid and protein composition of discrete membrane areas could be different in HT-29 cells compared to Caco2 cells and more susceptible to the formation of the channels by the peptide itself, as it occurs for amyloid peptide in different cell types [48].

A third possible mechanism relies on the assumption that a molecular device, such as a receptor or a pore channel, expressed by responsive cells, interacts with CPPs allowing calcium entry into the cells. One of these possible structures could be the calcium sensing receptor (CaSR), a G protein-coupled receptor which is expressed in tissues involved in Ca^{2+} homeostasis, among them the human intestinal cells [49,50]. Both HT-29 and Caco2 cell lines were shown to express CaSR, but the expression of CaSR is higher in HT-29 than in Caco2 cells [51]; again this data could account for the different response to CPPs between the two cell lines. Vit.D_3 was reported to modulate CaSR expression in conjunction with cell differentiation [52]. Other possible candidates for this role are the calcium channels, like TRPV6 and the L-type, present on the membrane of intestinal cells and known to regulate apical calcium absorption and to be modulated by Vit.D_3 [53,54]. Caco2 cells are known to express the calcium channel TRPV6, while no information is available in the literature on this regard for HT-29 cells [53]; on the contrary, in HT-29 cells the calcium channel L-type is well characterized [55]. Experiments are ongoing in our laboratory to demonstrate the presence and the possible interaction by these channels with CPPs in both cell lines.

The results presented here shed new light on the involvement of CPPs in calcium absorption at the intestinal level, also in connections with Vit.D_3, and add novel support to the notion of CPPs as possible bio-modulators. It is worth remembering that recent clinical studies reported a lack of effects by CPPs on calcium absorption and balance [18,19], but these data are not conclusive, since a lot of factors, difficult to analyze and standardize, such as the presence of fibers in the meal, the different pH and the osmolality of the gastrointestinal tracts, can affect the calcium-CPP aggregates, leading to the loss of their biological action. Further studies are planned, first of all at the molecular level, using *in vitro* established cellular models under controlled experimental conditions [56] which can reproduce the physiology and functionality of the human gut and successively at the animal or human *in vivo* level, to completely clarify this important issue. Also, as it concerns the *in vivo* effects of Vit.D_3, several studies demonstrated that the vitamin regulates calcium transport and absorption [57], as well as protects against colorectal cancer risk in association with calcium [58]. However, in our study, due to the *in vitro* cell model we used under the experimental conditions adopted, the effect of Vit.D_3 was studied only as an inducer of cell differentiation [31,34,35]. Nevertheless, the presence of oscillations lasting all over the duration of the experiments only in Vit.D_3-pretreated DMEM HT-29 cells, and the pattern of the cell responses to CPP DMV in Vit.D_3-pretreated Caco2 cells, can suggest an activation of the cell signaling by the Vit.D_3 itself [59]. On the basis of these results, we can hypothesize the existence of an interplay between CPPs and Vit.D_3 in cell mechanism triggered and/or controlled by the intracellular calcium levels.

Investigations are already in progress in our laboratory aimed at elucidating how to couple CPP-induced calcium uptake, followed by increase of $[Ca^{2+}]_i$, to biological cell functions. Undoubtedly, these natural compounds are interesting not only as possible adjuvants to facilitate calcium uptake by intestinal cells, but also as promoters, through the activation of calcium signalling, of specific cellular functions.

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