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

Stefania Cosentino[a,1], Claudia Gravaghi[a,b,1], Elena Donetti[c], Bianca M. Donida[a], Giovanni Lombardi[d], Marzia Bedoni[c,e], Amelia Fiorilli[a], Guido Tettamanti[a], Anita Ferraretto[a,*]

[a]Department of Medical Chemistry, Biochemistry and Biotechnology, LITA Segrate, University of Milan, 20090, Milan, Italy
[b]Strang Cancer Research Laboratory, Division of Gastroenterology and Hepatology, Department of Medicine, Weill Medical College of Cornell University, New York, NY 10065, USA
[c]DMU, Department of Human Morphology, University of Milan, 20133 Milan, Italy
[d]IRCCS Istituto Ortopedico Galeazzi, 20133 Milan, Italy
[e]Fondazione Don Gnocchi ONLUS, Polo Tecnologico 20148 Milan, Italy

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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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* Corresponding author. Tel: +39 0250330341; fax: +39 0250230365.
E-mail address: anita.ferraretto@unimi.it (A. Ferraretto).
[1] These authors contributed equally to the study.

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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