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Casein derived bioactive phosphopeptides: role of phosphorylation and primary structure in promoting calcium uptake by HT-29 tumor cells

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Abstract Casein phosphopeptides β-CN(1–25)4P and α_{s1}-CN(59–79)5P, from β- and α_{s1}-casein, respectively, both carrying the characteristic 'acidic motif' Ser(P)-Ser(P)-Ser(P)-Glu-Glu, were chemically synthesized and administered to HT-29 cells differentiated in culture, which are a used model of intestinal epithelium for absorption studies. Both casein phosphopeptides caused an increase of $[Ca^{2+}]_i$ due to influx of extracellular Ca^{2+}. The response was quantitatively higher with β-CN(1–25)4P than α_{s1}-CN(59–79)5P. The synthetic peptide corresponding to the 'acidic motif' was ineffective and the dephosphorylated form of β-CN(1–25)4P almost inactive. The lack of the N-terminally located five amino acids, or sequence modifications within the N-terminal segment of β-CN(1–25)4P, caused a total loss of activity, whereas the lack of the C-terminal segment preserved activity. In conclusion, the influx of calcium into HT-29 cells caused by β-CN(1–25)4P appears to depend on the phosphorylated 'acidic motif' and the preceding N-terminal region.

Key words: Casein phosphopeptide; Fura-2; HT-29 cell; Calcium imaging

1. Introduction

Casein (CN) constitutes the major protein fraction of milk thus representing a good nutritional source of amino acids [1]. In addition to this function, peptides derived from casein by proteolytic digestion are known to exert various physiological effects [2,3]. Among these, a family of casein-derived peptides, enriched in phosphoseryl groups and named casein phosphopeptides (CPP), are mineral carriers and prevent the precipitation of calcium ions as calcium phosphate or phytate [4–7], thus increasing the fraction of calcium available for absorption in the small intestine. These peptides are produced in vivo from the digestion of α_{s1}-, α_{s2}- and β-caseins by gastrointestinal proteases [8,9], and in vitro by tryptic or chymotryptic fragmentation of bovine casein, followed by purification [6].

Despite their different length and amino acid composition, CPP share a common 'acidic motif', consisting of three phosphoserines and two glutamic acids, Ser(P)-Ser(P)-Ser(P)-Glu-Glu, a sequence fully conserved among the species, and representing the binding site for di- and trivalent minerals, including calcium [10–13].

Recently, we reported [14] that a CPP mixture of commercial origin (constituted by five different phosphopeptides) and the single CPP β-CN(1–25)4P, purified from a bovine casein proteolysate, elicited a marked and transient rise of intracellular free calcium concentration ($[Ca^{2+}]_i$) in human intestinal tumor HT-29 cells, differentiated in culture, which are used as a study model for intestinal absorption. The intracellular calcium rise provoked by CPP administration was due to uptake of extracellular calcium ions. These findings further stressed a possible active role of CPP in calcium absorption at the intestinal level, even if the reported experiments were not able to clarify the mechanism by which CPP induced calcium uptake, particularly to distinguish whether passive diffusion or active transport was affected. Moreover, the fact that the majority of calcium is present in milk as inorganic calcium phosphate complexed in the casein micelles [15] makes the understanding of CPP action on calcium uptake more difficult to elucidate, even though in the past several authors hypothesized that CPP could influence the passive calcium transport in the small intestine, as reviewed in [16].

Previous works already established the requirement of phosphate groups for the ion binding capacity of CPP. In fact, dephosphorylated derivatives of CPP do not bind minerals [5,17,18] and phosphorylation of the dephosphorylated forms of α_{s1}- and β-caseins enhances their capacity to bind calcium [19]. A more recent study, performed on sera from patients allergic to milk, showed that the IgE response to β-CN(1–25)4P was markedly reduced by dephosphorylation of the serine residues [20]. Therefore, the 'acidic motif' appears to hold a key role not only for calcium binding capacity but also for immunogenicity of CPP. Moreover, it has been established that the amino acid sequences upstream and downstream of this region also contribute to determine the CPP conformation suitable for calcium binding [20–23].

The present investigation was carried out with the aim to establish the overall structural requirements of CPP for their promoting action on calcium uptake by differentiated HT-29 cells. The used CPPs were β-CN(1–25)4P and α_{s1}-CN(59–79)5P [5], both prepared by chemical synthesis. These CPPs were recently investigated for their structural features in the

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; CPP, casein phosphopeptide; CN, casein; β-CN(1–25)4P, a CPP constituted by the 1–25 N-terminal fragment from β-casein carrying four residues of phosphorylated serine; α_{s1}-CN(59–79)5P, a CPP constituted by the 59–79 fragment from α_{s1}-casein, carrying five residues of phosphorylated serine; KRH, Krebs–Ringer–HEPES

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presence of calcium [24,25]. Notably, calcium binding to β-CN(1–25)4P was reported to cause conformational changes to the peptide backbone resulting in a loop-type structure of the residues 1–4 (Arg^1 to Glu^4) and β-turn structure of residues 8–11 (Val^8 to Glu^{11}), 17–20 ($Ser(P)^{17}$ to Glu^{20}), the 'acidic motif'), and 21–24 (Glu^{21} to Thr^{24}) [24]. In the case of α_{s1}-CN(59–79)5P calcium association leads to a β-turn structure of residues Pro^{73} to Val^{76}, and a loop-like structure of residues Glu^{61} to $Ser(P)^{67}$ [25]. Knowledge of these conformational properties guided us to chemically prepare derivatives of β-CN(1–25)4P, lacking or carrying modifications in the amino acid sequences featuring precise structural conformations.

2. Materials and methods

2.1. Natural casein phosphopeptides

As reference CPP, used to assess routinely the response ability of the cells, a preparation was employed known to elicit a $[Ca^{2+}]_i$ rise in HT-29 cells [14] and constituted by a mixture of five main components, each containing the 'acidic motif' (93.8% as dry matter; 96% pure; total nitrogen content, 10.8%; phosphorus content, 3.7%; nitrogen/phosphorus ratio, 3.1; P/Ser ratio, 0.85 mol/mol; average molecular weight, 2500). It was supplied by DMV International (Veghel, The Netherlands). This preparation, named CPP DMV, was assessed to be calcium-free. As a standard single CPP, β-CN(1–25)4P, prepared in pure form from a bovine casein proteolysate and kindly provided by Prof. H. Meisel (Kiel, Germany), was employed. This compound was also assessed to be calcium-free. The presence of calcium contaminants was ascertained by using a specific *o*-cresolphthalein complexone calcium detection reagent (Sigma, St. Louis, MO, USA) [26] or by the fura-2 spectrofluorometric method [27].

2.2. Chemical synthesis, purification and characterization of casein phosphopeptides and their derivatives

The individual CPPs used in this investigation were: (a) α_{s1}-CN(59–79)5P and β-CN(1–25)4P, and (b) some derivatives of β-CN(1–25)4P, chosen in order to provide information on the portion(s) of the CPP molecule that is (are) important for inducing the influx of Ca^{2+} into HT-29 cells. All these peptides were synthetically produced by Primm (Milan, Italy), using the PS3 synthesizer (Protein Technologies, Tucson, AZ, USA) and purified by semi-preparative reversed phase high pressure liquid chromatography (HPLC, Jasco series LC-1500, Tokyo, Japan) equipped with the column Phenomenex Juptir (Torrance, CA, USA) 10 μ C18 300A, 250×21.20 mm, the pump PU-1586 and the UV/Vis detector UV-975 and UV-1575. The elution gradient was composed of solution A (water/acetonitrile 95%/5% in 0.1% trifluoroacetic acid) and solution B (water/acetonitrile 20%/80% in 0.1% trifluoroacetic acid) and performed as follows: from 0 to 2 min, 100% solution A; from 2 to 5 min, 80% solution A, 20% solution B; from 5 to 35 min, 65% solution A, 35% solution B. Peptide purity was evaluated by analytical reversed-phase HPLC on the column Phenomenex Juptir 5 μ C18 300A, 250×4.60 nm, the pump was PU-1580 and the detector MD-1510. The elution gradient was performed from 5% solution B/95% solution A to 100% solution B in 30 min. The flow rate was 1 ml/min and the presence of peptides was monitored at 220 nm. As a reference standard pure natural β-CN(1–25)4P was employed. Chemical identification was performed for each peptide using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry on a Voyager-DE Biospectrometry Workstation (Perseptive Biosystems, Applied Biosystem, Foster City, CA, USA), see Fig. 1 for more details. All peptides were stored at −20°C until use, when they were dissolved in doubly distilled water in stock solutions (1000× concentrated, with respect to the final concentration) and brought to neutrality with 1 mM NaOH. The remaining solutions were then stored at −20°C for further experiments and used within a month. The primary structures and conformational features of α_{s1}-CN(59–79)5P and β-CN(1–25)4P, as well as the primary structure of the different β-CN(1–25)4P derivatives are reported in Table 1.

2.3. Cell cultures

The colon carcinoma cell line HT-29 was obtained from the 'Istituto Zooprofilattico Sperimentale di Brescia' (Brescia, Italy) and grown according to the procedure previously described [14]. In brief, cells were seeded in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, the medium was replaced with RPMI supplemented with 10% fetal calf serum 24 h later, and cells were subcultivated for at least 10 passages. Under these conditions HT-29 cells reach a high degree of differentiation and polarization [28] although exhibiting morphofunctional heterogeneity (features of absorptive and mucous-like cells) [29,30]. This condition was checked both morphologically (by microscopy) and biochemically (by measuring the activities of sucrase-isomaltase and alkaline phosphatase, two enzymes known to be markers of the intestinal differentiation process [31]). Noteworthy, HT-29 cells, cultivated as described, were widely used as a model of the epithelial intestinal mucosa [31–33]. Cell cultures were periodically checked for the presence of mycoplasma and were found to be free of contamination. Cell viability, assessed by the trypan blue exclusion test, and cell morphology, examined by optical microscope, remained unaffected by treatment with each one of the used synthetic peptides up to 40 mM for 24 h.

Table 1
Primary structure of the used synthetic peptides

α_{s1}-CN(59-79)5P	^{59}QMEAESΣSSSEEIVPNSVEQK^{79} (Loop-likestructure; β-turnstructure)
β-CN(1-25)4P	^{1}RELEELNVPGEIVESLSSSEESITR^{25} (a; b; Loop-likestructure; β-turnstructure; β-turnstructure)
β-CN(1-25)0P	RELEELNVPGEIVESLSSSEESITR
Head-Peptide β-CN(1-25)4P without the tail portion	RELEELNVPGEIVESLSSSEE
Tail-Peptide β-CN(1-25)4P without the head portion	SSSEESITR
β-CN(1-25)4P (-) β-CN(1-25)4P lacking the "acidic motif"	RELEELNVPGEIVESL SITR
β-CN(1-25)4P(inv) β-CN(1-25)4P with the "a" and "b" tetra peptides in inverted position	VPGEELNRELEIVESLSSSEESITR (b; a)
β-CN(1-25)4P (short) β-CN(1-25)4P with a shortened head portion	LNVPGEIVESLSSSEESITR
"acidic motif"	SSSEE
dephosphorylated "acidic motif"	SSSEE

The 'acidic motif' characteristic of CPP is indicated in bold characters. The underlined residues, also indicated with a and b, are the sequence portions inverted in β-CN(1–25)4P(inv).
Σ corresponds to phosphorylated serine.

2.4. Single-cell $[Ca^{2+}]_i$ measurements and calcium imaging

These experiments were performed with cells seeded on glass coverslips at 5.2×10^4 cells/cm^2. Cytoplasmic calcium was measured according to the procedure described by Tsien and Poenie [34] as specified in a previous work [14]. Briefly, 2–3 days after seeding, cells were loaded with 5 mM fura-2/AM (Calbiochem, La Jolla, CA, USA) and 2.5 mM Pluronic F-127 (Sigma Chemical, St. Louis, MO, USA) in Krebs–Ringer–HEPES solution (KRH) containing (in mM) 125.0 NaCl, 5.0 KCl, 1.2 KH_2PO_4, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 6.0 glucose and 25.0 HEPES, and adjusted to pH 7.4. After 30 min at 37°C, cells were rinsed extensively with KRH to allow deesterification of the fluorescent probe and the coverslip was mounted on the pre-heated stage of a microscope (TE 200, Nikon, Tokyo, Japan) equipped with the High Speed Dynamic Video Imaging Systems, Quanticell 700 (Applied Imaging, Sunderland, UK). 340/380 excitation ratio images with 510 emission fluorescence were collected through a 40× oil immersion objective. The amount of intracellular free calcium 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 [27]. All the experiments, employing 80–100 cells/optical field, were

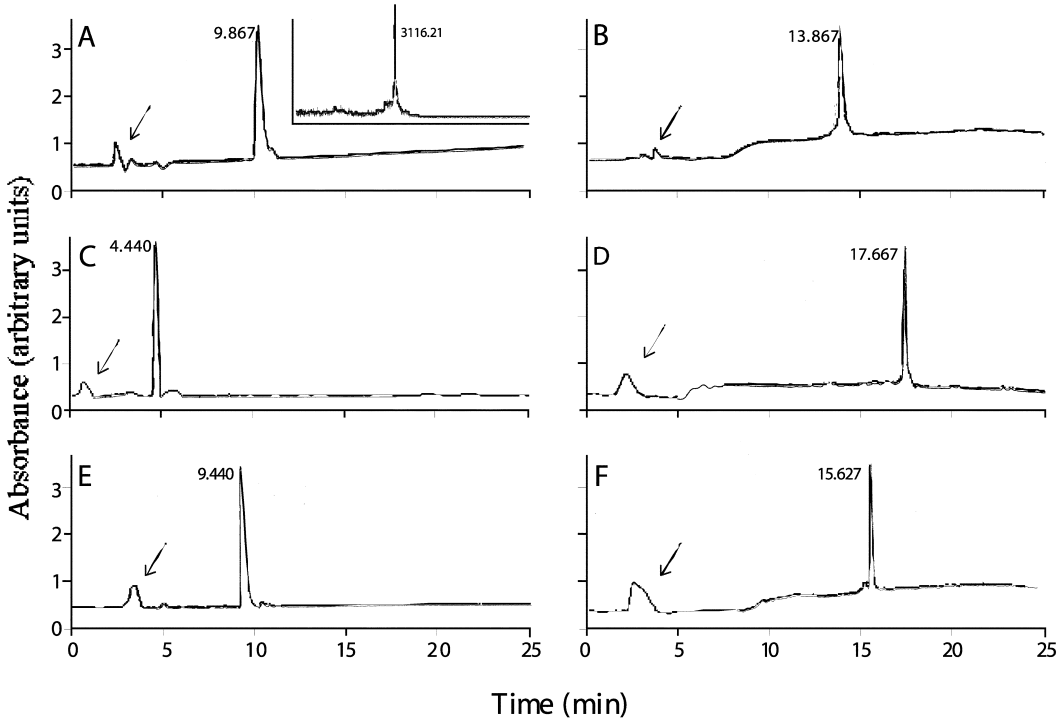

Fig. 1. Analytical HPLC profiles of the used synthetic peptides and mass spectroscopy identification of β-CN(1–25)4P peptide (inset in A). The panels show the elution profiles of β-CN(1–25)4P (A), α_{s1}-CN(59–79)5P (B), 'acidic motif' (C), β-CN(1–25)4P(−) (D), head peptide (E), tail peptide (F). Arrows correspond to injection peaks. For each peak corresponding to the synthetic peptide analyzed the elution time is reported expressed in minutes and hundredths of a minute. Mass spectroscopy determination (inset in A) was performed with samples crystallized on a matrix of α-cyano-4-hydroxycinnamic acid and at a laser power of 2700 kW/cm^2. As shown for the β-CN(1–25)4P peptide, the same mass spectroscopy identification was done for all the used synthetic peptides and the standard β-CN(1–25)4P CPP of natural origin. The results of mass spectroscopic analyses were as follows: β-CN(1–25)4P, 3116.21; α_{s1}-CN(59–79)5P, 3176.92; 'acidic motif', 777.2; β-CN(1–25)4P(−), 2362.2; head peptide, 2664.1; tail peptide, 1234.4.

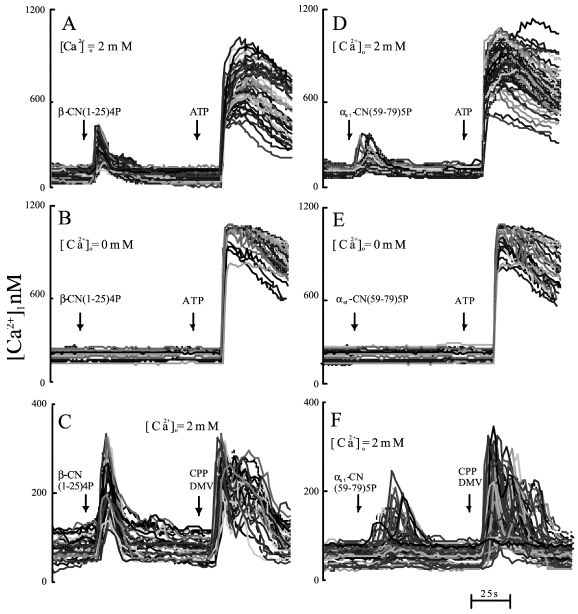

Fig. 2. Time course of intracellular calcium levels in single differentiated HT-29 cells in response to stimulation with synthetic CPPs. Cells loaded with fura-2 were alternately excited at 340–380 nm wavelengths and emission fluorescence recorded at 510 nm at 1–2 s intervals. A: β-CN(1–25)4P (50 mM) and ATP (100 mM) were successively administered in the presence of 2 mM extracellular calcium concentration. B: The same experiment as in A was repeated but in the absence of extracellular calcium. C: Single cell responses before and after β-CN(1–25)4P and CPP DMV (50 mM each) administration. D: Cellular responses to α_{s1}-CN(59–79)5P (50 mM) and ATP (100 mM) in the presence of 2 mM extracellular calcium concentration. E: The same experiment as in D was repeated but in the absence of extracellular calcium. F: Single cell responses before and after α_{s1}-CN(59–79)5P and CPP DMV (50 mM each) administration. Arrows indicate peptide additions. Each trace refers to the behavior of a single cell from a chosen field of 80–100 cells/optical field. The traces are representative of three or four experiments.

done at least in triplicate, at 37°C in KRH. For experiments requiring calcium-free solutions, $CaCl_2$ was omitted from KRH and 1 mM EGTA was added to complex any traces of contaminating Ca^{2+}.

2.5. Experimental design

The effect of all the individual synthetic peptides on $[Ca^{2+}]_i$ in differentiated HT-29 cells was assessed by monitoring $[Ca^{2+}]_i$ prior to, and after, application of each peptide. In order to check the maintenance of cell responsiveness, which was previously observed to be repetitive [14], after the application of each synthetic peptide, which induced a $[Ca^{2+}]_i$ rise, CPP DMV was added and the effect recorded. In the case of total absence of cell responses after peptide administration, ATP was added in order to assess the capacity of cells to release calcium from intracellular stores [34], thus verifying cell viability.

2.6. Statistical analysis

The data reported in the figures are records of individual responsive cells, using a range of 300–400 total analyzed cells collected from three or four experiments for each CPP tested. Student's *t*-test (independent two population *t*-test performed with Origin 6.0) was used to determine statistically significant differences between two mean values. A *P* value of < 0.05 was considered significant.

3. Results

As shown in Fig. 1 each of the synthetic peptides exhibited a single peak after analytical HPLC, with trace contaminants constituted by by-product peptide(s) of a length close to that of the principal peptide. No detectable metals, including calcium, were ascertained. The purity of each synthetic peptide was over 95%. MALDI-TOF mass spectrometry analysis provided a peak of molecular mass 3116.21 for synthetic β-CN(1–25)4P, as expected from the amino acid composition (see inset in Fig. 1A). Exactly the same peak was obtained with the pure sample of β-CN(1–25)4P of natural origin, which also displayed a HPLC behavior identical to that of the synthetic product. Each of the other synthetic peptides upon mass spectrometric analysis showed a peak of molecular mass corresponding to the designed peptide composition [3176.92 for α_{s1}-CN(59–79)5P; 777.2 for the 'acidic motif'; 2362.2 for the β-CN(1–25)4P(−); 2664.1 for the head peptide; 1234.4 for the tail peptide].

The time course of $[Ca^{2+}]_i$ recorded in individual HT-29 cells before and after administration of the synthetic β-CN(1–25)4P peptide is shown in Fig. 2A. This peptide induces a marked and transient cytosolic calcium rise that is very close to that obtained with the same peptide of natural origin [14]. The very slight differences in the cellular responses to the natural and synthetic β-CN(1–25)4P peptide, likely due to the cell heterogeneity in the two experiments, are statistically not relevant. The subsequent addition of ATP causes a marked increase of $[Ca^{2+}]_i$ indicating that the cytoplasmic calcium stores were not influenced by the CPP. As shown in Fig. 2B, in a calcium-free medium β-CN(1–25)4P peptide is not able to induce a cellular response, whereas a regular $[Ca^{2+}]_i$ rise is produced by addition of ATP. It is thus evident that β-CN(1–25)4P facilitates calcium influx from the external medium without affecting the intracellular stores, confirming previous findings [14]. Fig. 2C shows that the addition of CPP DMV after β-CN(1–25)4P causes a transient increase of $[Ca^{2+}]_i$ as expected [14]. However, the $[Ca^{2+}]_i$ rise following β-CN(1–25)4P administration features a narrower shape, a more uniform cell behavior and a shorter duration as compared to that displayed by CPP DMV. These differences are probably due to the fact that CPP DMV is a mixture of different peptides, each of them potentially able to induce an effect on $[Ca^{2+}]_i$ although somewhat different, resulting in an additive final effect. The administration of α_{s1}-CN(59–79)5P to HT-29 cells also induces a $[Ca^{2+}]_i$ rise (Fig. 2D), but this cellular response is reduced in height, more heterogeneous, and with a higher number of unresponsive cells compared to that obtained with β-CN(1–25)4P. However, the complete lack of effect in the absence of extracellular calcium and the regular cellular response upon addition of ATP (Fig. 2E) suggest that the influx of Ca^{2+} into HT-29 cells caused by the two peptides is based on the same mechanism. Also in the case of α_{s1}-CN(59–79)5P the subsequent administration of CPP DMV is followed by a regular $[Ca^{2+}]_i$ rise, strengthening the notion that the CPP effect on $[Ca^{2+}]_i$ enhancement follows a repetitive trend (Fig. 2F).

Fig. 3A shows that the large majority of cells are not responsive to the dephosphorylated form of β-CN(1–25)4P. Moreover, in the few responding cells, the amplitude of the $[Ca^{2+}]_i$ rise is drastically reduced. In all cases cell functionality is warranted by the finding that subsequent addition of CPP DMV is followed by the expected $[Ca^{2+}]_i$ rise. To assess the possible involvement of the 'acidic motif' per se in eliciting the influx of Ca^{2+}, the peptide Ser(P)-Ser(P)-Ser(P)-Glu-Glu and its dephosphorylated form, Ser-Ser-Ser-Glu-Glu, were administered to cells under the same experimental conditions. No $[Ca^{2+}]_i$ changes could be recorded in either case (Fig. 3B), also

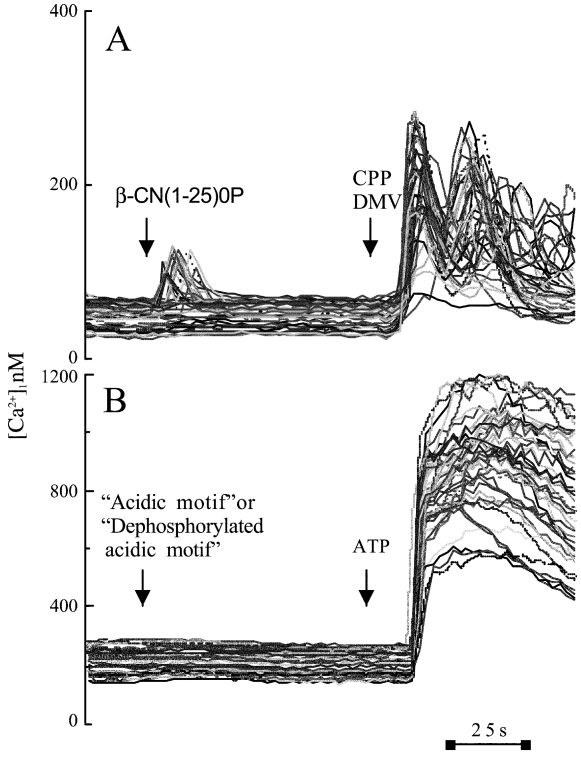

Fig. 3. Effect of dephosphorylated β-CN(1–25)4P peptide and the 'acidic motif' (or dephosphorylated acidic motif) on intracellular calcium levels in single differentiated HT-29 cells. Cells were prepared and images taken as described in Section 2 and in the legend to Fig. 1. A: Treatment with 50 mM of β-CN(1–25)0P and, successively, 50 mM of CPP DMV. B: Treatment with 50 mM of Ser(P)-Ser(P)-Ser(P)-Glu-Glu peptide and, successively, 100 mM ATP. Arrows indicate additions. The experiments were performed at 2 mM extracellular calcium concentration. Each trace refers to the behavior of a single cell from a chosen field of 80–100 cells/optical field. The traces are representative of three or four experiments.

by increasing up to 1 mM the peptide concentration, whereas a prompt response of cells to ATP was observed, indicating that cell functionality remained unchanged.

At this stage of the investigation the evidence was that: (a) both β-CN(1–25)4P and α_{s1}-CN(59–79)5P peptides are effective in inducing the $[Ca^{2+}]_i$ rise, the former compound providing a more intense response; (b) the dephosphorylated form of β-CN(1–25)4P is almost devoid of activity; and (c) the 'acidic motif' is totally ineffective. On this basis, we decided to focus on the more active β-CN(1–25)4P and try to clarify the structural requirements of this CPP for stimulating calcium uptake by HT-29 cells. As shown in Fig. 4A, the head peptide (50 mM), corresponding to β-CN(1–25)4P without the C-terminal segment, is able to elicit a $[Ca^{2+}]_i$ rise, although a little reduced in height as compared to that obtained with β-CN(1–25)4P at the same concentration. Conversely, no response at all (Fig. 4B,C) is observed with the tail peptide, that is the β-CN(1–25)4P lacking the N-terminal segment, and β-CN(1–25)4P(−) peptide, that simply lacks the 'acidic motif'. These results indicate that both the 'acidic motif' and the preceding long terminal sequence with its peculiar conformation are instrumental to the $[Ca^{2+}]_i$ enhancing action of CPP. In addition (Fig. 4D) β-CN(1–25)4P(inv), where the amino acids RELE, corresponding to positions 1–4 of the β-CN(1–25)4P primary structure, were exchanged with the VPGE residues, corresponding to positions 8–11, is not able to affect $[Ca^{2+}]_i$ level. The same negative result was obtained with β-CN(1–25)4P(short) (Fig. 4E), where the first five amino acids of β-CN(1–25)4P sequence were cut off. These results

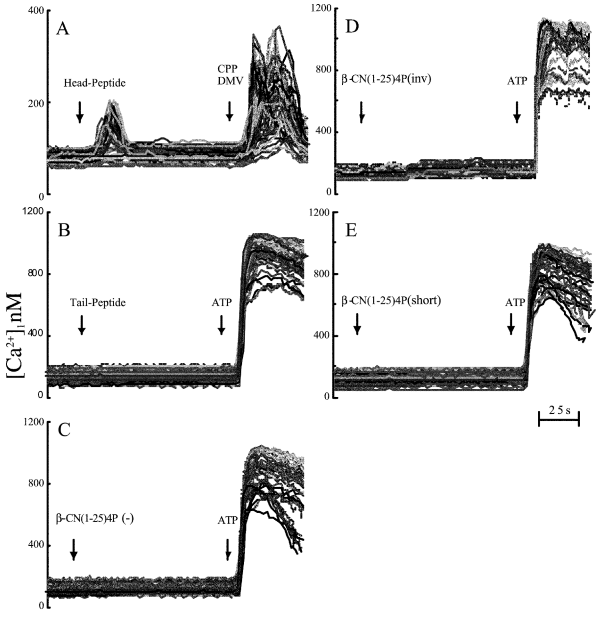

Fig. 4. Effect of modified β-CN(1–25)4P peptide and its fragments on intracellular calcium levels in differentiated HT-29 cells. Cells were prepared and images taken as described in Section 2 and in the legend to Fig. 1. $[Ca^{2+}]_i$ changes were monitored in single cells after administration of: (A) head peptide (50 mM) and CPP DMV (50 mM); (B) tail peptide (50 mM) and ATP (100 mM); (C) β-CN(1–25)4P(−) (50 mM) and ATP (100 mM); (D) β-CN(1–25)4P(inv) (50 mM) and ATP (100 mM); (E) β-CN(1–25)4P(short) (50 mM) and ATP (100 mM). Arrows indicate additions. The experiments were performed at 2 mM extracellular calcium level in KRH solution. Each trace refers to the behavior of a single cell from a chosen field of 80–100 cells/optical field. The traces are representative of three or four experiments.

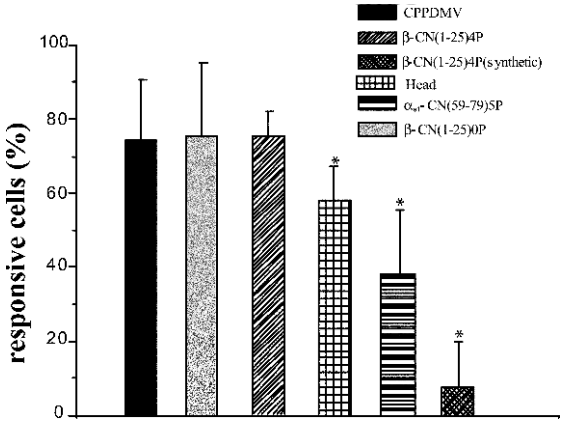

Fig. 5. Percentage of responsive HT-29 cells to administration of the following different CPPs (50 mM each): CPP DMV, natural and synthetic β-CN(1–25)4P, head peptide, α_{s1}-CN(59–79)5P, and β-CN(1–25)0P. The experimental conditions reported in the previous figures were used. Data were collected measuring the calcium response, at a single cell level, of 300–400 total cells for each stimulus. Each bar is the average percentage of responding cells (means ± S.D.). Asterisks mean that the values of β-CN(1–25)0P, α_{s1}-CN(59–79)5P and head peptide are significantly different from that of synthetic β-CN(1–25)4P value ($P < 0.05$).

point to the importance of the first four amino acid residues, with their loop-like structure, in the effect of CPP on calcium influx. In all the cases where peptides failed to evoke calcium uptake, the addition of ATP was followed by the expected $[Ca^{2+}]_i$ rise, indicating that cells were fairly viable and normally responsive to effective stimuli. Administration of tail peptide, β-CN(1–25)4P(−), β-CN(1–25)4P(short) and β-CN(1–25)4P(inv) up to 500 mM did not get any cellular response, indicating a lack of activity regardless of peptide concentration.

The effects of the different CPPs that were more or less active in enhancing intracellular free calcium level (CPP DMV, natural and synthetic β-CN(1–25)4P, α_{s1}-CN(59–79)5P, β-CN(1–25)0P and head peptide) were further evaluated by comparing the percentage of responding cells per optical field (Fig. 5). The histograms presented in Fig. 5 show that the percentage of responding cells is very similar and quite high with CPP DMV (74.5%), natural (75.2%) and synthetic β-CN(1–25)4P (74.9%), a little lower with head peptide (57.8%), lower in the case of α_{s1}-CN(59–79)5P (38.5%), and markedly reduced (7.3%) with β-CN(1–25)0P.

4. Discussion

The aim of this study was to recognize the structural features that underlie the reported ability of CPP to elicit a $[Ca^{2+}]_i$ rise in differentiated HT-29 cells, when buffered in 2 mM extracellular free calcium concentration [14]. In that study the promoting effect on $[Ca^{2+}]_i$ rise was observed with both a commercial mixture of CPP (CPP DMV) and a single CPP, the peptide β-CN(1–25)4P, purified from an hydrolysate of bovine β-casein and corresponding to the first 25 amino acids of this protein [9]. The hypothesis was launched that the characteristic 'acidic motif' Ser(P)-Ser(P)-Ser(P)-Glu-Glu present in both CPPs, and known to be the Ca^{2+} binding center of casein, might also be responsible for, or involved in, the observed effects on $[Ca^{2+}]_i$ rise in HT-29 cells. The approach adopted here to verify this hypothesis was to use

the synthetic CPPs β-CN(1–25)4P and α_{s1}-CN(59–79)5P, and derivatives of β-CN(1–25)4P properly engineered considering the segments of the starting molecule that possess [24,25] well-defined conformational features (segments 1–4, 8–11, 17–21 – the 'acidic motif' – and 21–24).

A first piece of evidence provided by the present investigation is that the synthetic CPP β-CN(1–25)4P has a behavior in promoting calcium uptake by HT-29 cells that is practically identical to that of β-CN(1–25)4P of natural origin, evidencing the good chance to use synthetic peptides. It is here also demonstrated that synthetic α_{s1}-CN(59–79)5P, corresponding to fragment 59–79 of bovine α_{s1}-casein, is effective in stimulating the $[Ca^{2+}]_i$ rise in HT-29 cells, although providing a smaller and a slightly more delayed peak rise than β-CN(1–25)4P (fragment 1–25 from bovine β-casein). Concerning these differences, it is worth remembering that different casein fragments carrying the 'acidic motif' show differences in their calcium binding properties, for instance β-CN(1–25)4P and α^{s1}-CN(59–79)5P display calcium binding constants at pH 7.0 of 0.63 mM^{-1} and 0.85 mM^{-1}, respectively [4]. Moreover, the calcium/peptide molar ratio of the two peptides is also different, being 4 for the β-CN(1–25)4P and 1 for the α_{s1}-CN(59–79)5P peptide [35]. Presumably, steric factors due to the amino acids flanking the 'acidic motif' contribute to the calcium binding ability, as well as other biological properties of CPP, like the $[Ca^{2+}]_i$ rise effect and immunogenicity [20,21].

A second piece of evidence is that dephosphorylation of β-CN(1–25)4P results in a remarkable loss of $[Ca^{2+}]_i$ rise with a largely reduced number of responding cells and a much lower rise of $[Ca^{2+}]_i$. All this is consistent with the notion that the phosphate groups of the 'acidic motif' are basic for calcium uptake by HT-29 cells, supporting the reported finding that the phosphorylated portion of casein is needed to enhance calcium absorption from the small intestine [17]. Moreover, a recent work by Farrell et al. [36] showed that the dephosphorylated form of β-CN(1–25)4P assumes a much more flexible and dynamic structure, which facilitates self-aggregation of the peptide. As a consequence, some motifs on the casein phosphopeptide might become cryptic, compromising the functionality of the peptide itself.

A third novel observation is that the pentapeptide constituting the 'acidic motif', Ser(P)-Ser(P)-Ser(P)-Glu-Glu, and the corresponding dephosphorylated peptide Ser-Ser-Ser-Glu-Glu are totally ineffective in promoting the $[Ca^{2+}]_i$ rise in HT-29 cells, regardless of their concentration. This tends to suggest that the interaction between CPP and the plasma membrane of HT-29 cells, presumably instrumental to favor a Ca^{2+} influx inside the cells, requires a precise peptide structure and conformation, where not only the 'acidic motif' but also some additional portions of the polypeptide play pivotal roles. The requirement of a crucial structural conformation of the peptide in order to be active is, in our opinion, supported by the following findings: (i) lack of any effects on Ca^{2+} influx by the peptide missing the acidic motif; (ii) equal lack of activity by the peptide carrying the 'acidic motif' but missing the preceding N-terminal portion; (iii) substantial maintenance of activity by the peptide carrying the 'acidic motif' and all the N-terminal portion, but missing the short C-terminal sequence following the 'acidic motif'; (iv) again lack of activity when the primary structure of the β-CN(1–25)4P peptide was modified by inverting the portions of the 1–4 tetrapeptide and 8–11 tetrapeptide or trimming the first five amino acids from the N-terminal portion. In this respect, the recent evidence by Cross and coworkers [24] might be illuminating. It was shown that the conformational features of β-CN(1–25)4P is calcium-dependent: the initial binding of calcium to the phosphoserine groups of the 'acidic motif' causes conformational changes to the peptide backbone, resulting in a loop-type structure of the segment Arg^1 to Glu^4, and in β-turn conformation of the segment Val^8 to Glu^{11}, $Ser(P)^{17}$ to Glu^{20} (the 'acidic motif') and Glu^{21} to Thr^{24}. As a consequence, some amino acids far from the 'acidic motif' also become able to interact with Ca^{2+}, thus achieving the maximal calcium binding capacity. Since the absence of the segment Arg^1 to Glu^4, or the exchange between segment Arg^1 to Glu^4 and segment 8–11 (Val^8 to Glu^{11}), or the lack of the 'acidic motif' which includes the segment $Ser(P)^{17}$ to Glu^{20}, causes a complete loss of the $[Ca^{2+}]_i$ rise, it should be inferred that the combination of these three conformational features is instrumental to the calcium rise effect. Furthermore, it should be remembered that casein in cow's milk is present as colloidal-sized casein micelles, where nanometer-sized amorphous particles of calcium phosphate are stabilized by surrounding casein phosphopeptide segments containing the 'acidic motif' [37]. These calcium phosphate casein phosphopeptide segments, named 'nanoclusters', were also shown to be formed with the β-CN(1–25)4P peptide [38,39], and constitute the molecular mechanism by which grossly supersaturated solutions of calcium and phosphate are prevented from undergoing uncontrolled precipitation. It is tempting to speculate that CPP calcium nanoclusters are involved in the CPP interaction with the plasma membrane of HT-29 cells, eliciting the flux of calcium inside the cell.

The notion that shortening of the N-terminal portion of β-CN(1–25)4P causes complete loss of Ca^{2+} influx into HT-29 cells may be relevant for human milk β-casein, known [40] to lack the first N-terminally located nine amino acid residues present in bovine β-casein. This would imply that no CPP from human β-casein should be expected to exhibit the promotion of Ca^{2+} uptake by HT-29 cells. This issue deserves to be inspected.

In conclusion, the present investigation provides evidence that the CPP promoting effect on $[Ca^{2+}]_i$ in differentiated HT-29 cells is dependent on a structural conformation conferred by both the phosphorylated 'acidic motif' and the preceding N-terminal portion, which is presumably instrumental to CPP interaction with the cell plasma membrane. The next research step would be to establish which is the mechanism by which CPP bind to the plasma membrane of HT-29 cells and how this binding leads to Ca^{2+} entrance into the cells.

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