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Milk peptides found in human jejunum induce enteroendocrine hormone secretion and inhibit DPP-IV†

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The strong effect of protein digestion products on gastrointestinal hormone release is well recognized. However, little is known about the specific characteristics of hormone inducing peptides. A detailed analysis of food-derived peptides remaining in the human intestinal lumen after protein ingestion would constitute a practical strategy for the targeted identification of hormone inducing and DPP-IV inhibitory peptides. In this study, *in vivo* gastrointestinal resistant peptides derived from casein and whey protein were evaluated in epithelial intestinal cells. The secretion of cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) was evaluated in the STC-1 enteroendocrine cell line and the DPP-IV inhibitory potential was assayed *in situ* using the Caco-2 cell line. Hydrophobic residues at N-terminal positions were crucial for the secretagogue and inhibitory activities, while the presence of multiple glutamic acid residues was shown to be a key trait for CCK secretion. The results appointed the sequence ^{126}TPEVDDEALEKFDK^{138} from β-lactoglobulin as a strong CCK inducer. Additionally, ^{94}KILDKVGINYWL^{105}, derived from α-lactalbumin, not only promoted GLP-1 secretion but also demonstrated significant DPP-IV inhibitory activity. These findings provide new insights into the functional potential of food-derived peptides, offering promising therapeutic avenues for regulating gastrointestinal hormones and improving metabolic health.

1. Introduction

Dietary proteins, beyond their nutritional role, elicit a range of metabolic effects both in the short and long term, including enhanced thermogenesis, reduced energy intake, and improved insulin sensitivity.[1] Among the factors mediating these metabolic effects, hormones secreted by the gut endocrine cells play a major role in the normal postprandial physiology, as part of the gut–brain–pancreas axis.[2] Protein digestion products are known to activate enteroendocrine cells through specific sensing receptors and sodium-coupled transporters. Various receptors involved in the detection of protein digestion products throughout the mouse and human gut epithelium have been described. Concretely, receptor GPR93, which is specialized in peptide sensing, shows high expression levels in the mid-intestine, peaking in the distal jejunum.[3]

Some studies using the enteroendocrine cell line STC-1 have shown that cholecystokinin (CCK) was maximally induced by peptides and to a lower extent by free amino acids or intact protein.[4,5] More recently, further evidence has been added on the prevailing role of food-derived peptides in the stimulating effects for both CCK and glucagon-like peptide-1 (GLP-1). Thus, whey-derived peptides longer than five amino acids were more effective than di- and tri-peptides on CCK secretion[6] and a casein digest elicited a strong GLP-1 response as compared with the same product subjected to complete hydrolysis.[7] A number of peptide sequences derived from food proteins *i.e.* milk proteins,[8] bovine haemoglobin,[9] egg white ovalbumin,[10] tilapia proteins,[11] soy β-conglicinin,[12] barley[13] or buckwheat[14] have demonstrated a secretagogue effect on the enteroendocrine cell lines STC-1 and GLUTag, being capable of inducing the release of CCK or GLP-1.

Concerning the characteristics of hormone inducing peptides, some authors have pointed out that peptide sequences with more than five free amino acids and aromatic residues were potent inducers of CCK secretion.[6,9] Likewise, we have previously shown that the peptide fraction in the 500–1500 Da range from egg white protein digests was mainly responsible

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for the GLP-1 secretory effect.[10] In order to elucidate the structure–function relationship, some studies have looked at the net charge and hydrophobicity of the peptides. Hence, peptides with hydrophobicity scores between 13 and 27 and isoelectric pH between 6 and 7.9 were reported to present a strong secretory effect on GLP-1.[6] Other authors have linked hydrophobicity with enhanced GLP-1 secretion. Komatsu *et al.*[8] suggested that high hydrophobicity scores would contribute to an efficient membrane permeability without the involvement of the peptide sensing mechanisms that have been described for L cells, namely, the peptide transporter 1 and calcium-sensing receptor.[15] Lastly, a number of studies have looked at the role of specific amino acids within the sequence. Some kokumi-active peptides containing γ-glutamyl residues have been shown to stimulate CCK and GLP-1 secretion in STC-1 cells by the activation of the calcium-sensing receptor.[16] Glutamic acid/glutamine-containing peptides have been recently postulated as important for the reduced fasting blood glucose in db/db diabetic-like mice produced by a casein hydrolysate. The intake of the hydrolysate increased the hepatic metabolites rich in glutamic acid, which acted as substrates for the Krebs cycle, enhancing hepatic glucose consumption.[17]

On the other hand, the incretin GLP-1 has a short half-life in the organism. Immunohistochemical studies revealed the conversion of GLP-1 (7–36) amide into the inactive (9–36) form by the enzyme dipeptidyl peptidase IV (DPP-IV) in the brush border epithelium as well as in the capillary endothelium supplying the L cells of the porcine intestine.[18] Thus, peptide sequences acting as DPP-IV inhibitors are expected to extend the half-life of this incretin. Examples of peptides with DPP-IV inhibitory action exerting glycaemic control through an increase in plasma GLP-1 are available.[19–21] Different food-derived peptides capable of inhibiting DPP-IV have been described from very diverse protein sources, with the sizes ranging from 2 to 15 amino acids.[22] The study of the position of selected amino acid residues in the peptide sequence and the side-chain properties of known inhibitors has allowed the proposal of some descriptors related to the inhibitory potency. Branched chain amino acids at the N-terminal position and proline at the second N-terminal position are the most prominent examples.[23] However, other amino acid residues such as tryptophan appear to play a relevant role. Tryptophan-rich oligopeptides from milk and fish sources have shown inhibitory activity against DPP-IV and high binding affinities to the DPP-IV enzyme by molecular docking.[21,24,25] Further experiments have linked these sequences with decreased postprandial blood glucose levels in humans.[21] The structure–function features can be used in *in silico* approaches to predict and facilitate the enzymatic release of these DPP-IV inhibitors.[26] However, evidence of these peptides to survive gastrointestinal digestion is still small.

Once the chyme enters the small intestine, the polypeptides are cleaved into oligopeptides and amino acids by proteases of different specificity. The products of endoproteases such as trypsin, chymotrypsin and elastase are further attacked by exopeptidases which remove amino acids from one or the other end of the chain. It has been reported that, in humans, the breakdown products of protein digestion comprise 30% free amino acids and 70% oligopeptides between 2 and 8 residues.[27] Although some of them are further hydrolysed by aminopeptidases from the brush-border membranes to free amino acids and di- or tripeptides, there is a great likelihood of resistant sequences to be sensed by specialized receptors in enteroendocrine cells. Thus, a detailed analysis of food-derived peptides identified in the gastrointestinal tract after ingestion would constitute a practical strategy for the targeted identification of hormone inducing and DPP-IV inhibitory peptides. In this study, peptides previously identified in human jejunum aspirates after the intake of milk proteins (casein or whey) with diverse amino acid arrangements were selected based on their occurrence in at least three volunteers.[28] The objective was to identify those sequences able to induce CCK and/or GLP-1 release in the enteroendocrine cell line STC-1 and to determine their capacity to inhibit the DPP-IV enzyme *in situ* using Caco-2 cells.

2. Materials and methods

2.1. Samples/synthetic peptides

Peptide sequences derived from β-casein (^{6}LNVPGEIVE^{14}, ^{7}NVPGEIVE^{14}, ^{8}VPGEIVE^{14}, ^{9}PGEIVE^{14}, ^{10}GEIVE^{14}, ^{81}PVVVPPFLQPE^{91}, ^{82}VVVPPFLQPE^{91}, ^{85}PPFLQPEV^{92}, ^{89}QPEV^{92}, ^{172}LPVPQ^{176}, ^{173}PVPQ^{176}), β-lactoglobulin (β-Lg) (^{41}VYVEELKPTPEGDLEIL^{57}, ^{94}VLDTDYK^{100}, ^{107}MENSAEPEQS^{116}, ^{125}TPEVDDEALEKFDK^{138}) and α-lactalbumin (α-La) (^{19}GGVSLPEWV^{27}, ^{80}FLDDDLTDD^{88}, ^{94}KILDKVGINYWL^{105}, ^{95}ILDKV^{99}, ^{96}LDKVG^{100}) previously identified in human jejunum after casein or whey ingestion[28] were chemically synthesized by CSBio Ltd (Shanghai, China). The purity of the peptides was verified by elemental analysis and reverse phase high-performance liquid chromatography coupled with mass spectrometry (LC-MS). The sequence length, theoretical isoelectric point and hydrophobicity of the peptides are given in Table 1.

2.2. Cell assays

STC-1 cells were supplied by the American Type Culture Collection (ATCC CRL3254, Manassas, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Merck, Darmstadt, Germany) containing 4.5 g L^{-1} glucose and 5 mM L-glutamine supplemented with 100 U mL^{-1} penicillin, 100 mg L^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin (Cytiva, HyClone, Utah) and 10% fetal bovine serum (ATCC, Manassas, VA, USA). Caco-2 cells were supplied by ATCC and cultured in DMEM supplemented with 100 U mL^{-1} penicillin, 100 mg L^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin, 10% fetal bovine serum (Merck, Darmstadt, Germany), and 1% of non-essential amino acids (Merck, Darmstadt, Germany). Both cell lines were grown at 37 °C under a 5% CO_2 humidified atmosphere,

Table 1 Characteristics of casein and whey peptides (parent protein, sequence, molecular weight average, sequence length, theoretical hydrophobicity and isoelectric point)

Protein	Sequence	M_w average	Sequence length	Hydrophobicity	Isoelectric point
β-Casein	^{6}LNVPGEIVE^{14}	969.11	9	24.97	3.1
β-Casein	^{7}NVPGEIVE^{14}	855.95	8	19.25	3.1
β-Casein	^{8}VPGEIVE^{14}	684.79	7	15.67	3.1
β-Casein	^{9}PGEIVE^{14}	642.71	6	11.65	3.1
β-Casein	^{10}GEIVE^{14}	545.59	5	9.32	3.1
β-Casein	^{81}PVVVPPFLQPE^{91}	1221.48	11	34.09	3.3
β-Casein	^{82}VVVPPFLQPE^{91}	1124.36	10	32.39	3.3
β-Casein	^{85}PPFLQPEV^{92}	926.09	8	27.64	3.3
β-Casein	^{89}QPEV^{92}	471.51	4	2.83	3.3
β-Casein	^{172}LPVPQ^{176}	552.68	5	11.06	6.0
β-Casein	^{173}PVPQ^{176}	439.52	4	3.36	6.0
β-Lactoglobulin	^{41}VYVEELKPTPEGDLEIL^{57}	1944.23	17	41.24	3.6
β-Lactoglobulin	^{94}VLDTDYK^{100}	852.95	7	12.62	3.9
β-Lactoglobulin	^{107}MENSAEPEQS^{116}	1121.15	10	6.59	3.0
β-Lactoglobulin	^{125}TPEVDDEALEKFDK^{138}	1635.76	14	25.40	3.8
α-Lactalbumin	^{94}KILDKVGINYWL^{105}	1461.78	12	38.52	9.7
α-Lactalbumin	^{95}ILDKV^{99}	586.74	5	14.77	6.8
α-Lactalbumin	^{96}LDKVG^{100}	530.63	5	7.87	6.8
α-Lactalbumin	^{19}GGVSLPEWV^{27}	943.08	9	31.47	3.3
α-Lactalbumin	^{80}FLDDDLTDD^{88}	1068.07	9	24.02	2.7

and once they reached 80% confluence, they were trypsinized and seeded according to each cell study requirement.

2.2.1. Cell viability. Cells were seeded into a black 96-well plate (5×10^4 cells per well) and cell viability was measured as described by Santos-Hernández *et al.*, 2018.[29] The cells were exposed to synthetic peptides at 2 mM and 1 mM in HEPES buffer (20 mM HEPES 1 M, 10 mM glucose, 140 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 7.4, Merck KGaA, Darmstadt, Germany) as previously described.[5,9] After 2 h of incubation, the medium was removed and replaced with Alamar Blue solution 1 : 10; v : v (AlamarBlue™ Cell Viability Reagent, Thermo Fisher Scientific, Waltham, MA, USA) followed by 1 h of incubation. Fluorescence was measured at 590/530 nm excitation/emission wavelengths. HEPES buffer and Triton™ X-100 diluted 1 : 30; v : v were used as the negative and positive control, respectively. Cell viability experiments were performed in triplicate as well as the fluorescence measurement.

2.2.2. Intracellular calcium assay. STC-1 cells were seeded in a black 96-well plate (2×10^4 cells per well) and incubated for 24 h at 37 °C. Intracellular calcium was measured using a Fluo-4 Direct™ Calcium Assay Kit (Thermo Fisher Scientific-Invitrogen, Waltham, VA, USA) as previously described by Santos-Hernández *et al.*, 2023.[30] Fluorescence was recorded every 5 seconds for 3 min measurement before and after adding each synthetic peptide in triplicate. Peptides were assayed at 1 mM in Hank's Balance Salt Solution (HBSS, Sigma Aldrich-Merck, Darmstadt, Germany) with 5.6 mM glucose and 20 mM HEPES (pH 7.4). HBSS buffer was used as a control. Results were expressed as relative fluorescence ($RFU_{max} - RFU_{min}$)/RFU_{min}. RFU_{min} is the basal RFU value and RFU_{max} is the maximum value recorded after the sample addition.

2.2.3. Secretion assays of CCK and GLP-1. Cells were cultured for 48 h under a 5% CO_2 humidified atmosphere at 37 °C in a 24-well plate (3×10^5 cells per well) and washed 3 times with HEPES buffer followed by 1 h of incubation. Cells were exposed to synthetic peptides at 1 mM and 0.25 mM in HEPES buffer, also used as a control. Each concentration was assayed in triplicate. After 2 h of incubation, supernatants were collected as described by Santos-Hernández *et al.*, 2018.[29] Measurement of GLP-1 and CCK concentrations was performed in duplicate with the commercial immunoassay CCK 26–33, non-sulphated EIA Kit (EK-069-04; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and Glucagon-Like Peptide-1 Active ELISA (EGLP-35K; Merck KGaA, Darmstadt, Germany), respectively, according to the manufacturer's instructions.

2.2.4. DPP-IV inhibition assay in Caco-2 cells. Caco-2 cells were seeded in a black 96-well plate (2×10^4 cells per well) and incubated for 24 h at 37 °C. The dipeptidyl peptidase-IV inhibition assay was performed as described by Santos-Hernández *et al.*, 2021.[31] Synthetic peptides were added in triplicate at 2 mM, 0.2 mM and 0.02 mM previously diluted in phosphate buffer (PBS, Sigma-Aldrich).[11] Ile-Pro-Ile (IPI) and PBS were used as positive and negative controls, respectively. Fluorescence was measured every 2 min for 1 h at 360/460 nm excitation/emission wavelengths. The percentage of DPP-IV inhibition was defined as the proportion of enzymatic activity suppressed by a specific concentration of peptide or IPI, compared to the enzymatic activity observed in the PBS control condition.

Statistical analysis

The intracellular calcium concentration was compared to the control and within samples by performing statistical analysis using one-way ANOVA with Tukey's *post-hoc* test for pairwise comparisons. CCK and GLP-1 release was compared to the control and within samples for both concentrations using one-

way ANOVA with Tukey's *post-hoc* test. DPP-IV inhibition was compared within samples and between using one-way ANOVA with Tukey's *post-hoc* test. The results were considered significant if $p < 0.05$. GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA) was used.

3. Results

The assayed peptides were selected among the large number of peptides previously identified in the human jejunum,[28] namely, 362 distinct sequences after casein and 313 distinct sequences after whey ingestion. From them, 185 sequences had been found in at least 3 subjects. Eleven peptides from β-casein, four from β-Lg and five from α-La with common amino acid motifs were selected. When this was not possible, the occurrence of aromatic and hydrophobic amino acids in combination with varying hydrophobicity or isoelectric points has been used as the selection criterion. Table 1 shows the protein fragment, molecular weight, sequence length, hydrophobicity and isoelectric point of the assayed peptides. Cell viability in the enteroendocrine cell line STC-1 was tested and none of the synthetic peptides affected cell viability at the assayed concentrations (ESI Fig. 1†).

3.1. Intracellular calcium mobilization of milk peptides identified in human jejunum samples

Prior to the hormone secretion assays, intracellular calcium mobilization was examined in STC-1 cells in the presence of selected peptide sequences, and intracellular calcium was expressed as $(RFU_{max} - RFU_{min})/RFU_{min}$. The sequence ^{41}VYVEELKPTPEGDLEIL^{57} could not be assayed due to its difficulty in dissolving at 10 mM of the necessary stock to reach 1 mM in the well. All β-casein-derived peptides (Fig. 1A–C) increased significantly the intracellular calcium levels after exposure, except fragment β-casein ^{7}NVPGEIVE^{14}. Greater calcium mobilization was observed for β-casein peptide sequences ^{9}PGEIVE^{14}, ^{85}PPFLQPEV^{92}, and ^{173}PVPQ^{175}. Regarding whey-derived peptides (Fig. 1D–F), all samples showed increased intracellular calcium levels except fragment α-La ^{96}LDKVG^{100}.

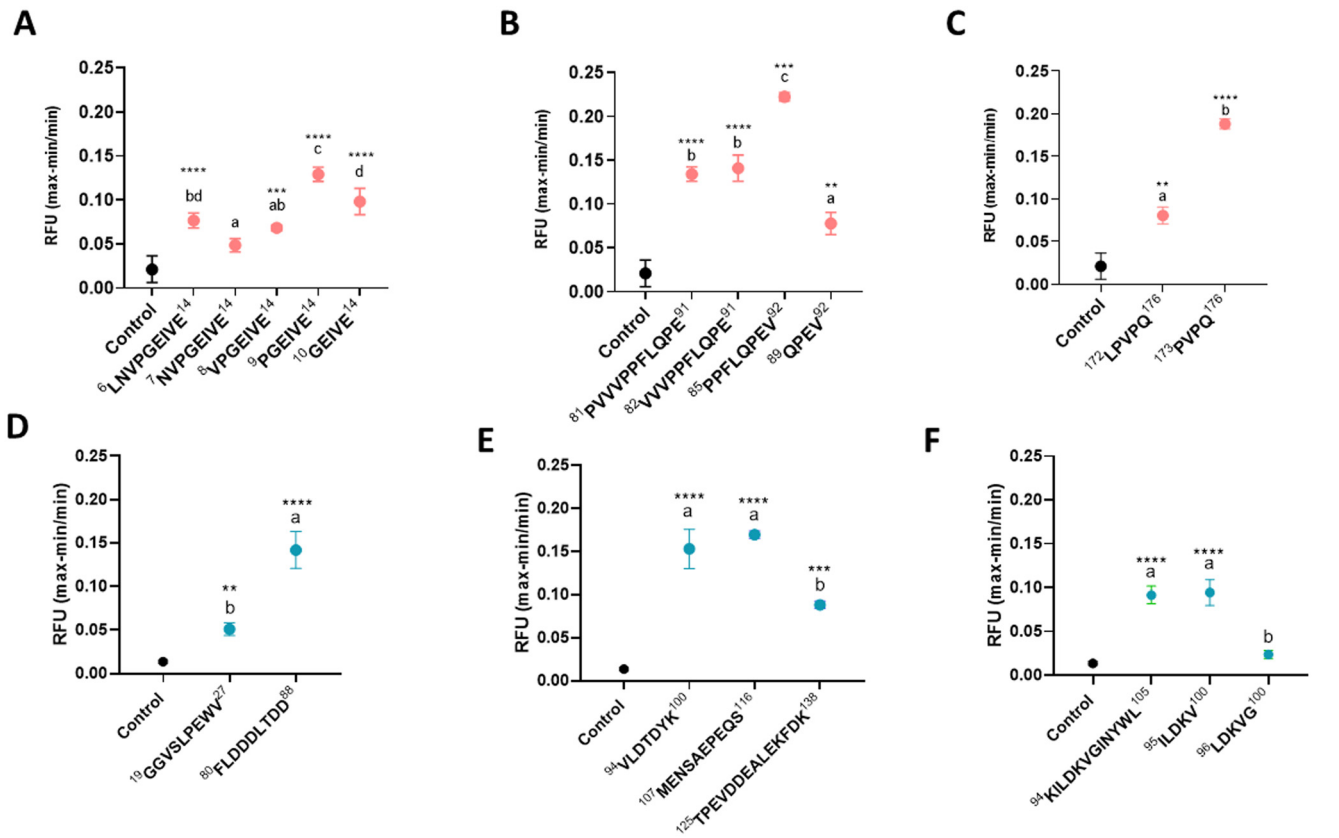

Fig. 1 Changes in the intracellular calcium concentration in STC-1 cells exposed to synthetic peptides. Intracellular calcium concentrations were measured by fluorescence. Cells were exposed to synthetic peptides at 1 mM in HBSS buffer containing HEPES and glucose (control), in triplicate. Intracellular calcium was expressed in relative fluorescence units (RFU) as $(RFU_{max} - RFU_{min})/RFU_{min}$ after exposure to casein (A–C) and whey protein derived peptides (D–F) at 0.5 mM. Error bars indicate SEM ($n = 3$). Statistical significance compared with the control is indicated (one-way ANOVA with Tukey's *post hoc* test) by $^{*}p < 0.05$ and $^{**}p < 0.01$. Different letters denote statistically significant differences ($p < 0.05$) between different peptides.

3.2. CCK and GLP-1 release in response to casein and whey peptides in STC-1 cell culture

Fig. 2 shows the CCK levels induced by STC-1 cells in response to peptides assayed at 1 and 0.25 mM. The β-casein peptide sequences ^{6}LNVPGEIVE^{14}, ^{7}NVPEGEIVE^{14}, and ^{8}VPGEIVE^{14} reached the highest levels of CCK, with values approximately 20-fold the control, showing that loss of the amino acids ^{6}L and ^{7}N at the N-terminal end did not produce alterations in CCK secretion. The subsequent loss of the amino acids ^{8}V and ^{9}P resulted in approximately twice less secretion than that of the longer forms, but still showed significant secretion (Fig. 2A). In other groups from casein-derived peptides, CCK release was moderate, about two-fold the control levels, with significant CCK hormone levels only at the 1 mM concentration as compared to the control. In the β-casein-derived peptide ^{81}PVVVPPFLQPE^{91} and related forms, the N-terminal loss of ^{81}P and various valine residues slightly reduced the observed levels of CCK. Regarding ^{85}PPFLQPEV^{92} and the comprised sequence ^{89}QPEV^{92}, the short form increased the response (Fig. 2B). In the β-casein peptide ^{172}LPVPQ^{176} the loss of ^{172}L increased the CCK release of the resulting fragment, as another example of induction augmented with a lower peptide size (Fig. 2C). Concerning the whey-derived peptides (Fig. 2D–F), all the assayed sequences were able to induce CCK secretion, except the β-Lg ^{107}MENSAEPEQS^{116} sequence (Fig. 2D). A fragment from β-Lg ^{125}TPEVDDEALEKFDK^{138} displayed the highest secretion of this hormone (40-fold the control), being the most potent inducer among all the forms tested, although β-Lg ^{41}VYVEELKPTPEGDLEIL^{57} also showed a remarkable inducing power with 15-fold the control CCK release. These two sequences have in common a relatively long size (14 and 17 amino acids, respectively), an acidic isoelectric point with multiple negative residues and elevated hydrophobicity. By contrast, the α-La-derived sequences showed moderate CCK secretion (Fig. 2E and F).

GLP-1 release was enhanced upon incubation of STC-1 cells with all the studied sequences at the highest concentration (Fig. 3). Remarkably, high values (over 5-fold the control) were observed for all β-casein related peptides derived from ^{6}LNVPGEIVE^{14}, except ^{7}NVPGEIVE^{14} (Fig. 3A). A less potent response was observed with the β-casein ^{81}PVVVPPFLQPE^{91} and related forms, all of which achieved levels between 2- and 3-fold the control release, except the 82–91 form (Fig. 3B). In the case of the related forms ^{172}LPVPQ^{176} and ^{173}PVPQ^{176}, the second one showed a more pronounced GLP-1 release, similar to the behaviour found for CCK secretion (Fig. 3C). Among the whey-derived peptides, the sequence with the highest

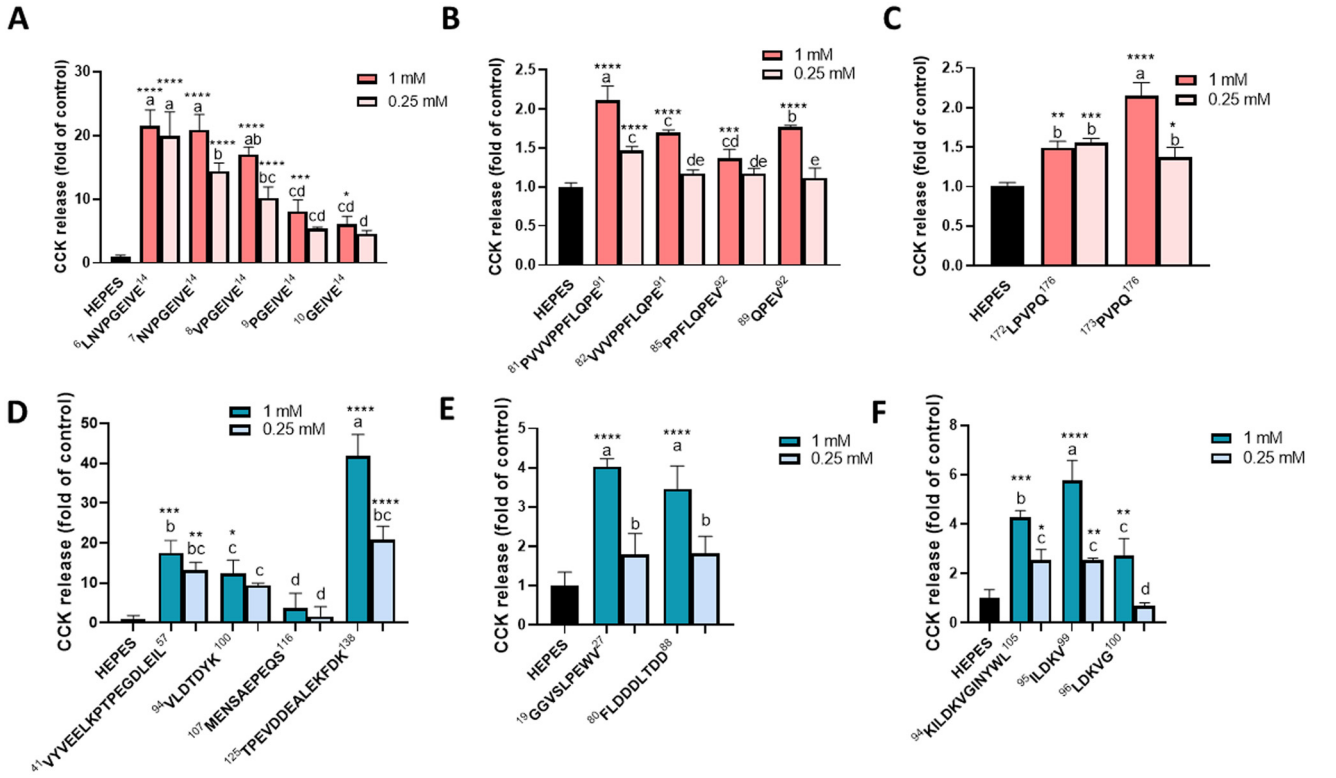

Fig. 2 CCK secretion after 2 h of incubation of STC-1 cells with synthetic peptides identified in human jejunum after taking a solution of casein (A–C) or whey (D–F). Synthetic peptides were tested at 1 mM and 0.25 mM. CCK secretion was determined by ELISA. Cell experiments were performed in triplicate, followed by technical duplicates. Error bars indicate SEM (n = 3). Statistical significance compared with the control (C) (one-way ANOVA with Tukey's *post hoc* test) is indicated by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Statistical significance (p < 0.05) in the comparison between different samples is indicated by different letters.

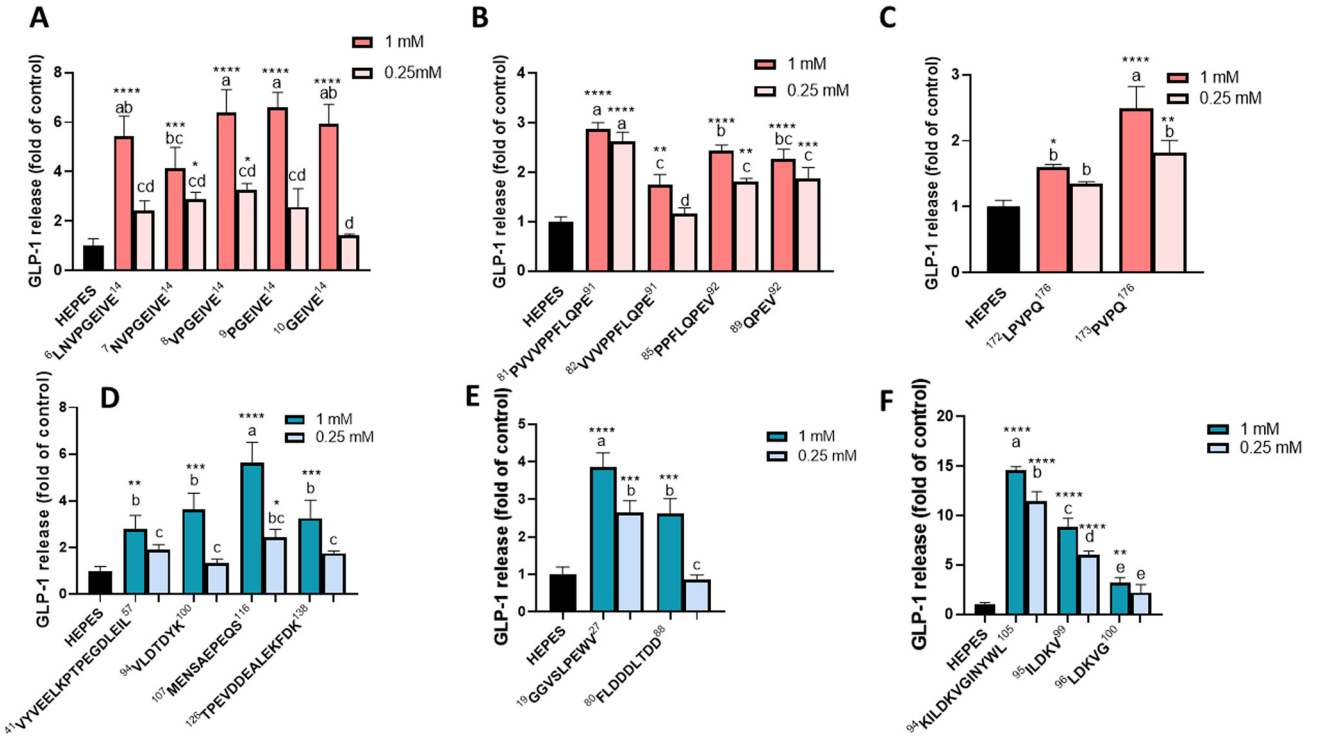

Fig. 3 GLP-1 secretion after 2 h of incubation of STC-1 cells with synthetic peptides identified in human jejunum after taking a solution of casein (A–C) or whey (D–F). Synthetic peptides were tested at 1 mM and 0.25 mM. GLP-1 secretion was determined by ELISA. Cell experiments were performed in triplicate, followed by technical duplicates. Error bars indicate SEM (n = 3). Statistical significance compared with the control (C) (one-way ANOVA with Tukey's *post hoc* test) is indicated by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Statistical significance (p < 0.05) in the comparison between different samples is indicated by different letters.

hormone secretion was the α-La fragment ^{94}KILDKVGINYWL^{105} that produced 15-fold the control release (*ca.* 800 pM of GLP-1) (Fig. 3F). Its derived form, ^{96}ILDKV^{100}, induced 10-fold GLP-1 release while ^{96}LDKVG^{100} further reduced the secretory activity. Other β-Lg or α-La derived peptides assayed reached levels of GLP-1 release between 2- and 5-fold the level of the control secretion (Fig. 3D and E).

3.3. DPP-IV inhibitory effect of casein and whey peptides in Caco-2 cell culture

The DPP-IV inhibitory effect of the assayed peptides was evaluated in cells using 2 mM, 0.2 mM, and 0.02 mM peptide concentrations in the Caco-2 cell culture (Fig. 4). Several peptides were shown to strongly inhibit the DPP-IV activity, namely ^{172}LPVPQ^{176} from β-casein, ^{107}MENSAEPQS^{116} from β-Lg, and ^{80}FLDDDLTDD^{88} and α-La ^{125}TPEVDDEALEKFDK^{138} from α-La, inhibiting more than 90% of the enzyme activity and the higher assayed concentration. Other sequences with notable DPP-IV inhibition were β-casein ^{6}VPGEIVE^{14} (88%), ^{85}PPFLQPEV^{92} (83%), and ^{89}QPEV^{92} (86%). Moreover, the inhibitory effect was assayed in four β-casomorphin-7 related forms which were also found to be resistant to *in vivo* digestion and described as CCK and GLP-1 inducers in our previous study.[7] Two of these sequences, β-casein ^{60}YPFPGPI^{66} and ^{60}YPFPGPG^{64}, showed 88% DPP-IV inhibition (ESI, Fig S2†).

4. Discussion

There is still limited knowledge regarding the specific characteristics of anorexigenic hormone-inducing peptides. An essential requirement to induce signalling in the intestinal epithelium is to be resistant to the digestive enzymes, a prerequisite that was fulfilled by the assayed sequences as they had been identified in human jejunum aspirates after casein or whey protein intake. In addition to that, peptide features developed in various studies for the induction of CCK and GLP-1 by enteroendocrine cells include size, net charge, hydrophobicity, or position in the chain of selected amino acid residues.

In our work, we have evaluated the response of sequences with lengths ranging from 5 to 17 amino acids and isoelectric points throughout a wide pH range, between 2.7 and 9.7. The highest hormone release has been observed for sequences that displayed high hydrophobicity, over 10 GRAVY units, regardless of the isoelectric point, a peptide feature that had been previously been linked to elevated hormone secretion *in vitro*.[8]

In some cases, a greater hydrophobic character was linked to a higher secretory effect, especially for GLP-1. Thus, the ^{95}ILDKV^{99} fragment of α-La exhibited a stronger GLP-1 secretory effect compared to the related ^{96}LDKVG^{100} fragment with the first displaying a hydrophobicity value twice as the second. This can also be supported by low intracellular

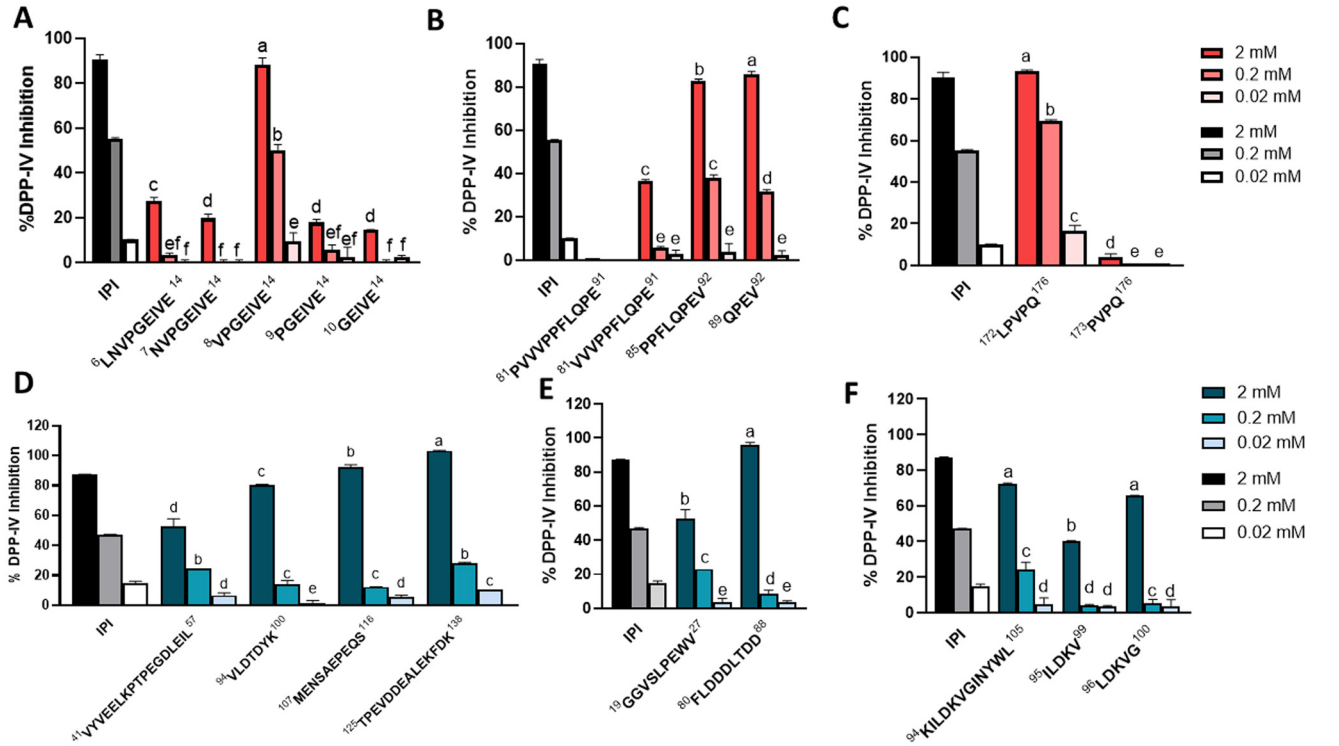

Fig. 4 DDP-IV enzyme inhibition in the presence of casein peptides (A–C) and whey peptides (D–F) at different peptide concentrations (2.00, 0.20, and 0.02 mM) using Caco-2 cells. Error bars indicate SEM (n = 3). Statistical significance compared between different samples is indicated by different letters (one-way ANOVA with Tukey's *post hoc* test). IPI is the positive control.

calcium mobilization for the last sequence. Interestingly, the sequence ^{94}KILDKVGINYWL^{105} comprising them displayed the highest GLP-1 inducing activity with four-fold theoretical hydrophobicity. All these fragments share the occurrence of the hydrophobic leucine and isoleucine as N-terminal amino acids, although the last form displays the basic residue lysine at the N-terminal. By contrast, the β-casein fragment ^{89}QPEV^{92} showed similar GLP-1 induction to its related sequences but displayed markedly lower hydrophobicity. This points to the contribution of other descriptors.

In terms of peptide length, a previous study by our group has shown an increase in GLP-1 secretion with decreasing size of peptides related to the opioid sequence β-casomorphin (β-casein 60–66) in which the ^{60}YPFPG^{64} motif, which includes the aromatic amino acids tyrosine and phenylalanine, was conserved.[7] The size did not correlate with the CCK inducing activity in these β-casomorphin-related fragments, although the smaller size fragment, with five amino acids, sharply decreased CCK induction. Interestingly, in the sequences of β-casein retaining the ^{10}GEIVE^{14} motif, the CCK induction trend points again to higher sensing with a size over seven amino acid residues. It should be noted that the maximal CCK response in this study has been observed for peptides with 14 and 17 residues. CCK is known to be induced by amino acids such as phenylalanine.[32] This hormone is also induced by a 35 amino acid luminal cholecystokinin releasing factor in the intestine and direct cellular effects have been shown in STC-1 cells in a calcium dependent manner.[33] Long food-derived peptides have similarly shown activity on CCK secretion. Thus, intraduodenal infusion of the 13 amino acid fragment 51–63 from β-conglycinin, VRIRLLQRFNKRS, inhibited food intake and raised the portal plasma CCK concentration in Sprague-Dawley rats. In the study of the mechanism, the authors remarked that this sequence strongly bound the brush border membrane and acted as a substrate of trypsin in the lumen due to multiple nonadjacent arginine residues in the peptide structure.[12]

Alternative sequence features such as the occurrence and location in the peptide chain of the amino acid residues, glutamic acid, and tryptophan have been assessed in the observed results. The above-mentioned group of β-casein fragments that shared the motif ^{10}GEIVE^{14} can be considered an outstanding set, in terms of both CCK and GLP-1 secretion. They present as common traits two glutamic acid amino acid residues in the peptide chain. Other potent secretors revealed in this study, especially for CCK secretion, are β-Lg ^{41}VYVEELKPTPEGDLEIL^{57} and ^{125}TPEVDDEALEKFDK^{138}, also with multiple glutamic acid residues. Other CCK inducers having survived gastrointestinal digestion with glutamic acid in the peptide chain derive from buckwheat (PAFKEEHL) or egg white (VLLPDEVSGL).[10,14] Moreover, some di- and tri-peptides containing either one or two consecutive γ-glutamyl residues have been shown to stimulate CCK and GLP-1 secretion in STC-1 cells.[16] These peptides share with the present studied

sequences a high resistance to gastrointestinal digestion through their unique γ-bond.[34]

As for tryptophan, the behaviour of α-La ^{94}KILDKVGINYWL^{105} in relation to GLP-1 secretion is striking. It shows a very potent inducing activity while none of the related forms, ^{95}ILDKV^{99} and ^{96}LDKVG^{100}, deprived of tryptophan, reaches the values observed for the parent sequence, which points to a prominent role of this aromatic residue. It is worth mentioning that tryptophan has been also found in other peptides with potent hormonal secretagogue effects. For instance, a seven amino acid long sequence showing N-terminal tryptophan, the lysozyme derived peptide^{123}WIRGCRL,^{129} has previously shown a potent GLP-1 secretory activity.[30] It is interesting to note that tryptophan in the penultimate C-terminal can also have a hormone secreting role, as the strongly inducing sequence ^{94}KILDKVGINYWL^{105} reveals. Another outstanding sequence, fragment ^{19}GGVSLPEWV^{27} from α-La, contains both tryptophan and glutamic acid at adjacent positions. It is remarkable that other surviving peptides in the human aspirates related to this sequence displayed at least one glutamic acid residue, such as fragments ^{17}GYGGVSLPE^{25} or ^{19}GGVSLPEW^{26}. The well-known metabolic effects of whey proteins[35] could be linked with the profile of surviving peptides at the intestinal level, with a prominent presence of glutamic acid in peptides, as previously observed.[28]

The assayed sequences have shown highly varying values of inhibition of the DPP-IV enzyme in the model system with Caco-2, ranging from no inhibition in the case of fragment ^{81}PVVVPPFLQPE^{91} from β-casein to complete inhibition for β-Lg ^{125}TPEVDDEALEKFDK^{138}. One of the structural features important for this inhibitory activity is the presence of proline at the second N-terminal position, and some of the peptides with the greatest effect, such as the β-casein derivatives ^{8}VPGEIVE^{14}, ^{85}PPFLQPEV^{92}, ^{89}QPEV^{92} or ^{172}LPVPQ^{176}, or the β-Lg derivative ^{126}TPEVDDEALEKFDK^{138}, showed this feature. There have been some studies aimed at identifying the structure–activity relationship from diverse peptides displaying DPP-IV inhibitory activity. In addition to proline at position 2, Tulipano and co-workers drew a consensus in the primary structure of inhibitory peptides that included a branched-chain amino acid or an aromatic amino acid at the first position.[23] Hence, QSAR models linking DPP-IV inhibitory activity and peptide descriptors suggest a greater inhibition with reduced hydrophilicity for the two N-terminal amino acids.[36]

In the present study, we have determined the DPP-IV inhibitory activity of four β-casomorphin-related sequences, of which our group had previously reported hormone-inducing activity.[7] Two of these sequences with 90% inhibition of DPP-IV showed the aromatic amino acid tyrosine at the first position in addition to proline at the second N-terminal position. Also, other sequences determined in this study as DPP-IV inhibitory showed the previously mentioned N-terminal features, such as β-Lg ^{94}VLDTDYK^{100} with the branched-chain amino acids valine and leucine, or the α-La sequence ^{80}FLDDDLTDD^{88} with the aromatic amino acid phenylalanine at the N-terminal position. In short, hydrophobic and aromatic residues seem to play a crucial role in the DPP-IV inhibitory activity. Hence, the presence of hydrophobic amino acids such as leucine, phenylalanine, and methionine has been reported to facilitate various structural and interactional factors that enhance binding stability with the enzyme.[36] The position of tryptophan in the peptide chain has been previously studied in the analysis of short peptide forms with anti-diabetic potential. In an overview of 55 food-derived dipeptides and tripeptides with DPP-IV or α-glucosidase inhibitory activity, analysis of the amino acid composition revealed that tryptophan was the most frequently occurring amino acid residue, followed by arginine. 72% of these oligopeptides had tryptophan at the N-terminus, while 25% displayed the same amino acid at the C-terminus.[37]

Another issue that would merit attention is the concurrence of CCK or GLP-1 hormone-inducing activity and DPP-IV inhibitory activity. In the family of β-casein peptides sharing the ^{10}GEIVE^{14} motif, with an important CCK and GLP-1 secretory capacity, only fragment ^{8}VPGEIVE^{14} exerted a relevant inhibitory activity, in accordance with the structural feature of proline at the second N-terminal position. Moreover, this sequence fulfilled another of the previously highlighted criteria, namely the presence of a branched amino acid at the first N-terminal position.[23] There were great differences in the inhibitory activity of the DPP-IV enzyme for overlapping sequences, such as the β-casein peptide ^{172}LPVPQ^{176} showing 93% inhibition compared to 4% in ^{173}PVPQ^{176}. The presence of the branched amino acid at the N-terminal position together with the presence of proline at the second N-terminal position is clearly related to the higher activity of ^{172}LPVPQ^{176}. A moderate inducing activity on both CCK or GLP-1 was shown for these sequences, although induction was higher for the short one, a peptide with null DPP-IV inhibitory activity. A similar case is the higher CCK and GLP-1 activity of α-La ^{95}ILDKV^{99} than that of the overlapping ^{96}LDKVG^{100} where greater DPP-IV inhibition is observed in the less active sequence.

There are several mechanisms by which peptides derived from dietary proteins can act as glucoregulatory compounds in the organism. Peptides can be involved in carbohydrate digestion, insulin secretion and function, glucose uptake, adipose tissue modification, and enteroendocrine hormone release.[38] Among the proposed mechanisms, the modulation of the endogenous intestinal hormone secretion is a highly promising strategy in obesity and type-2 diabetes[39] while the resistance to DPP-IV is known to prolong the metabolic stability of incretins *in vivo*.[18] In this study, milk peptides resistant to gastrointestinal digestion in humans have been assayed. The hydrophobic nature of the N-terminal end of the peptides has shown a crucial role in both the secretion of GLP-1 and the DPP-IV inhibitory activity, with the aromatic and branched chain residues at the N-terminal position being highly determinant, and proline at the second position key for the last activity. However, the hormone inducing and DPP-IV-inhibiting activities were not necessarily connected. While in the

assayed sequences, peptide length, hydrophobicity or charge could not be unequivocally linked to the activity, the occurrence of tryptophan or multiple glutamic acid residues in the peptide chain has been shown to be a key trait. In particular, the α-La derived sequence ^{94}KILDKVGINYWL^{105} displays an outstanding GLP-1 inducing activity together with a notable DPP-IV inhibitory activity, and the β-Lg peptide ^{125}TPEVDDEALEKFDK^{138} strongly induces CCK and fully inhibits DPP-IV. This points to both sequences as promising candidates for *in vivo* studies. The findings on hormone-inducing combined with the DPP-IV inhibitory activity are expected to shed light on the molecular characteristics associated with the metabolic signalling peptides of dietary origin.

Author contributions

Santiaga María Vivanco-Maroto: investigation, formal analysis, and writing – original draft. Cristina Gómez Marín: investigation, formal analysis, and writing – original draft. Beatriz Miralles: conceptualization, funding acquisition, supervision, and writing – review & editing. Isidra Recio: conceptualization, funding acquisition, supervision, and writing – review & editing.

Data availability

All data used for this study appear in the illustrated figures, and the raw data will promptly be made available upon request.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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