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Protein Hydrolysates Induce CCK Release from Enteroendocrine Cells and Act as Partial Agonists of the CCK_1 Receptor

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Protein has been reported to be the most satiating of all macronutrients. Upon gastrointestinal digestion, peptides are generated that stimulate the release of satiety hormones such as cholecystokinin (CCK) from enteroendocrine cells. As such, bioactive peptides could be the target of Functional Food ingredients with satiating effects. We set up an *in vitro* assay system to investigate if different protein hydrolysates exhibit varying CCK-releasing properties. Soy, pea, potato, casein, and whey protein hydrolysates were incubated with the enteric endocrine cell line STC-1 that endogenously expresses and secretes CCK. Release of CCK was measured by ELISA. All hydrolysates induced CCK release at low concentrations (>0.1 mg·L^{-1}); however, no significant differences in CCK-releasing properties between the different protein hydrolysates were found, suggesting a generic, nonspecific peptide-sensing mechanism in the STC-1 cells on hydrolyzed protein. As the ELISA exhibits sensitivity to all CCK isoforms possessing the C-terminal CCK octapeptide but varying in biological activity at the CCK_1 receptor (CCK_1R), a secondary module was added to the STC-1 cell assay. Intracellular calcium measurements were performed in CHO-CCK_1R cells. Following exposure of the STC-1 cells to the protein hydrolysates, the medium was tested on the CCK_1R assay. Released CCK was measured with higher sensitivity and lower variability than in the ELISA. Surprisingly, we found that some protein hydrolysates (soy > potato >> casein) also directly stimulated CCK_1R-expressing cells, while whey and pea protein hydrolysates were inactive. As CCK_1R is expressed in the GI tract, direct interaction of CCK_1R with dietary peptides may contribute to their satiety effects. Future experiments developing bioactive ingredients for Functional Foods for weight management could involve isolation of the active, CCK_1R-activating peptides in, for example, soy protein hydrolysates.

KEYWORDS: Cholecystokinin; CCK_1R; peptide hydrolysate; STC-1

INTRODUCTION

Gastrointestinal hormones such as cholecystokinin (CCK) are important in the regulation of food intake and maintaining energy homeostasis. The role of CCK as a major endocrine determinant in food intake regulation is well documented. CCK acts on a multitude of physiological processes such as gallbladder contraction, pancreatic secretion, intestinal motility, and gastric emptying. In addition, CCK modulates satiety via the activation of central as well as peripheral neurons. Secretion of CCK occurs in response to food intake by enteric endocrine I-cells that are scattered throughout the mucosa of the small intestine (*1, 2*).

Basal plasma CCK concentrations are generally in the low picomolar range in most species and increase approximately about 5- to 10-fold following meal ingestion with dietary fat and protein being shown to be the most potent stimulators of CCK release (*3*). Studies in animals and humans using different protein sources suggest that in order to stimulate CCK secretion efficiently, the protein needs to be hydrolyzed to short-chain peptides and amino acids (*4*). In addition, selected proteins and hydrolysates have been suggested to increase CCK secretion from enteroendocrine cells by protecting luminal CCK-releasing factors from proteolysis due to competitive peptidase inhibition (*5*). Very recently, a receptor, GPR93, was identified in the apical membrane of enterocytes and described to be activated by protein hydrolysates (*6*). When overexpressed in the enteroendocrine cell line STC-1, GPR93 activation by protein

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hydrolysates induced CCK secretion from STC-1 cells (*7*). Indeed, protein hydrolysate-induced satiety seems to be in part mediated by CCK, as specific inhibition of the CCK_1 receptor (CCK_1R) reversed the hydrolysate-induced effect in animal studies (*8, 9*). All in all, these data suggest that protein hydrolysates stimulate CCK release from enteroendocrine cells, which in turn induces satiety via the activation of CCK_1R.

Therefore, discovering either specific protein hydrolysates or other food-grade bioactive compounds with optimized CCK-releasing properties is an interesting target in the development of Functional Food products for weight management purposes. Identification of such food-grade ingredients that efficiently release CCK requires a suitable *in vitro* assay system to reduce the time-consuming and costly studies in animals and humans. A major limitation in studying the effect of potential satiety increasing ingredients on CCK release *in vitro* was for a long time the lack of a suitable, stable enteric endocrine cell line. A few studies investigating gastrointestinal hormone release used the cell lines GluTag and NCI-H716, which exhibit some characteristics of enteroendocrine cells (*10, 11*) but do not produce and release sufficient amounts of cholecystokinin. The mouse-intestinal, CCK-secreting cell line STC-1 has been established for studying mechanisms of cholecystokinin secretion from enteroendocrine cells (*12*). In both *in vitro* and *in vivo* studies, secretion of various isoforms of CCK from enteroendocrine cells has been reported (*13–15*), differing in activity at CCK_1R. All studies generally use RIA or ELISA for the quantification of CCK. In most assays, the used antibodies recognize a variety of CCK peptides with a sulfated tyrosine residue at the C-terminal octapeptide and do not discriminate between the different active isoforms of CCK. This has the drawback of the quantification of CCK not necessarily being related to the biological activity of the released peptides. An assay directly determining the effect of a nutritional intervention on activation of CCK_1R would be of more physiological relevance than measuring total CCK plasma concentrations. To our knowledge, such an assay is currently not available.

Therefore, the objectives of this study were (1) to develop a test system that predicts the effectiveness of potential satiety-inducing protein hydrolysates derived from dietary protein sources based on their ability to induce CCK release from STC-1 cells and in turn activate CCK_1R and (2) to identify protein hydrolysates that efficiently stimulate CCK release in this system.

MATERIAL AND METHODS

Cell Culture. A chinese hamster ovary cell line (CHO) functionally expressing rat CCK_1R (CHO-CCK_1-R) was kindly provided by Dr. R. Smeets (University of Nijmegen). Cells were grown in Dulbecco's modified Eagle's medium and Ham's F12 medium 1:1 (DMEM-F12) with 15 mM HEPES and L-glutamine supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 500 $\mu g \cdot mL^{-1}$ penicillin, 500 $U \cdot mL^{-1}$ streptomycin (Cambrex, Baltimore, USA), and 500 $\mu g \cdot mL^{-1}$ G-418 (Gibco, Paisley, UK). As negative control cells, native CHO-K1 cells (ATCC, CCL61) were used and grown in the same medium without G-418. Cells were used until passage 25.

The STC-1 cell line, a murine cell line of enteroendocrine origin, was purchased from Dr. Hanahan (University of California, San Francisco, USA). STC-1 cells were grown in RPMI-1640 medium with 25 mM HEPES and L-glutamine (Cambrex, Baltimore, USA) supplemented with 10% fetal bovine (Gibco, Paisley, UK) serum, 500 $\mu g \cdot mL^{-1}$ penicillin, and 500 $U \cdot mL^{-1}$ streptomycin (Cambrex, Baltimore, USA). Cells were used until passage 30. All cells were cultured at 37 °C in an incubator containing 5% CO_2/95% air.

Preparation of Peptide Hydrolysates. Whey protein hydrolysate (97% protein; <1% fat; <3% ash; <5% moisture; BioZate1, Davisco, USA), pea protein hydrolysate (90% protein; <0.5% fat; <4.5% carbohydrate; 5% ash; <5% moisture; Cosucra S.A., Belgium), casein protein hydrolysate (88% protein; 2% free amino acids, <1% fat; 5% ash; 4% moisture; NZMP, New Zeeland), soy protein hydrolysate (78% protein; 9% free amino acids; <1% fat; 6% ash; <6% moisture; Quest International, The Netherlands), and potato protein hydrolysate (66% protein, 12% free amino acids, <1% fat; <1% carbohydrate; <7% ash; <9% moisture; Organotechnie S.A., France) were tested. The concentration of the test hydrolysate solutions was prepared on the basis of the concentration of protein in each of the test materials determined by Lowry's protein assay. Samples were dissolved in the buffers mentioned in the sections CCK Secretion Studies in STC-1 Cells and Calcium Reporter Assay for the Determination of CCK_1R Activation, depending on the experiments performed. After dissolving in buffer, samples were centrifuged for 5 min at 1000*g*, and the supernatants were used in the experiments. Concentrations of the test hydrolysates are given in $mg \cdot L^{-1}$ and $g \cdot L^{-1}$.

CCK Secretion Studies in STC-1 Cells. For the measurement of CCK release, STC-1 cells were seeded in a 24 black clear bottom well plate ($1.6 \cdot 10^5$ cells$\cdot cm^{-2}$) (Wallac, Wellesley, USA). After 48–52 h of incubation the cells were washed twice with buffer containing D-PBS without Ca^{2+} and Mg^{2+} (Gibco, Paisley, UK). The cells were then incubated with the protein hydrolysate samples or the stimulants forskolin (50 μM) and the free fatty acid lauric acid (500 μM) (Sigma, Zwijndrecht, Netherlands) in HBSS with 10 mM HEPES at 37 °C for 120 min in a humified incubator at 5% CO_2. The supernatant was collected and stored at −20 °C. Cell viability was monitored by determinining ATP release according to the manufacturer's instructions (Vialight assay, Cambrex, Baltimore, USA). To each well, 200 μL of cell lysis solution was added and incubated on a plate shaker (5 min; 300 rpm). Subsequently, 100 μL of monitoring reagent was added, and the plate was measured on Wallac Trilux Microbeta 1450 (Wallac, Wellesley, USA).

Determination of CCK-8S in STC-1 Supernatants with ELISA. Supernatants from the STC-1 cells treated with protein hydrolysate were thawed from −20 °C and equilibrated to room temperature and tested for CCK-content by ELISA (Phoenix, Belmont, USA). A standard curve with concentrations between 0.01 and 100 $ng \cdot mL^{-1}$ CCK-8S was prepared in HBSS buffer. Samples (undiluted) or standards, primary antibody, and biotinylated peptide were added to the microtiterplate and incubated for 2 h at room temperature. After the initial incubation, the plate was washed twice, and streptavidin−horseradish peroxidase (SA-HRP) was added. The plate was incubated for 1 h at room temperature. Subsequently, SA-HRP was removed by washing the plate twice, and the TMB substrate (3,3′,5,5′-tetramethylbenzidine) was added. After 20 min, the reaction was stopped by the addition of 2 N HCl. The absorbance was measured at 450 nm on a microplate reader (Versamax, Molecular Devices, Sunnyvale, USA).

Calcium Reporter Assay for the Determination of CCK_1R Activation. CHO-K1 and CHO-CCK_1R were seeded in a 96 black, clear bottom well plate ($1.25 \cdot 10^5$ cells$\cdot cm^{-2}$) (Greiner, Frickenhausen, Germany). After a 24–36 h incubation period, the medium was removed, and cells were loaded in DMEM-F12 supplemented with 4, μM Fluo-4AM (Molecular Probes, Paisley, UK), 0.07% BSA (Sigma, Zwijndrecht, Netherlands), 1.6 mM probenecide (Sigma, Zwijndrecht, Netherlands), and 0.02% Pluronic F-127 (Molecular Probes, Paisley, UK) for 60 min at room temperature. Cells were washed twice with buffer containing 1× HBSS (Gibco, Paisley, UK), 20 mM HEPES (Sigma, Zwijndrecht, Netherlands), 2.5 mM probenecide (Sigma, Zwijndrecht, Netherlands), and 1 $mg \cdot mL^{-1}$ BSA (Sigma, Zwijndrecht, Netherlands) at pH 7.4.

Changes in intracellular Ca^{2+} concentrations were monitored by measuring changes in fluorescence signals with a microtiterplate fluorescence reader (BMG Novostar, BMG Laboratories, Offenburg, Germany). Fluorescence signals were measured before the addition ($f0$) of CCK_1R agonists for 7.2 s. After agonist addition, the fluorescence signal (f) was measured for an additional 30 s at 37 °C. In experiments with the CCK_1R antagonist, lorglumide cells were preincubated for 15 min with 50 μM lorglumide (Sigma, Zwijndrecht, Netherlands). Fluo-4 fluorescence signals (f) were expressed as $f/f0$ ratios to normalize to the fluorescence level at the start of the experiment ($f0$). Fluorescence

values were normalized against the *f*/*f*0 ratios measured after cell exposure to 1 nM sulfated CCK-8S (Bachem, Weil am Rhein, Germany), which caused a maximum increase in intracellular Ca^{2+}.

Determination of CCK-8S in STC-1 Supernatants Using CCK_1R Activation Assay. Quantification of CCK released into the supernatants of STC-1 cells upon stimulation with protein hydrolysates was determined by using the Ca^{2+} reporter assay. A CCK-8S standard curve was measured in the range of 1 pM–2.5 nM with 1 nM CCK-8S causing a maximum response in *f*/*f*0 ratios. STC-1 supernatants derived from CCK-release experiments were thawed from −20 °C, equilibrated to room temperature, and diluted twice in buffer containing 1× HBSS (Gibco, Paisley, UK), 20 mM HEPES (Sigma, Zwijndrecht, Netherlands), 2.5 mM Probenecid (Sigma, Zwijndrecht, Netherlands), and 1 $mg \cdot mL^{-1}$ BSA (Sigma, Zwijndrecht, Netherlands) at pH 7.4. CCK_1R activation was measured by monitoring fluorescence signals in CCK_1R-expressing CHO cells before (*f*0) and after the addition (*f*) of the STC-1 supernatants derived from CCK-release experiments as described above. As the negative control, identical concentrations of protein hydrolysates in HBSS were used that had not been in contact with STC-1 cells. As positive control 1 nM CCK-8S was used, which caused the maximum increase in Ca^{2+}.

Direct Activation CCK_1R by Protein Hydrolysates. As higher protein hydrolysates concentrations (>10 $mg \cdot L^{-1}$) in HBSS without being in contact with STC-1 cells caused changes in intracellular Ca^{2+}, higher concentrations of protein hydrolysates (10 $mg \cdot L^{-1}$−3 $g \cdot L^{-1}$) were tested for their potential to activate CCK_1R. As the negative control 1× HBSS and as the positive control 1 nM CCK-8S were used; this caused the maximum increase in Ca^{2+}.

Data Processing and Statistical Analysis. When measuring CCK in the CCK_1R Ca^{2+} reporter assay, baseline-corrected values were obtained by subtracting the *f*/*f*0 ratios measured for protein hydrolysates that had not been in contact with STC-1 (controls) from the *f*/*f*0 ratios observed for STC-1 supernatants. Data were expressed as the fold of control. Data derived from the standard curves with increasing concentrations of CCK-8S were used to compare the precision, sensitivity, and range of detection of the ELISA and CCK_1 [Ca^{2+}] assay. The values observed in the ELISA were fitted to the following model:

$$\text{value} = \text{value}_{max} \cdot e^{(\text{constant} \cdot \text{maximum} \langle \text{dose} - \text{threshold}, 0 \rangle)} \quad (1)$$

where the parameters constant and threshold were estimated by means of nonlinear regression. The $value_{max}$ was 4.0. The values observed in the CCK_1 [Ca^{2+}] were fitted to the following model:

$$\text{value} = \text{value}_{max} \cdot (1 - e^{(\text{constant} \cdot \text{maximum} \langle \text{dose} - \text{threshold}, 0 \rangle)}) \quad (2)$$

where the parameters, $value_{max}$, constant, and threshold were estimated by nonlinear regression. Comparisons were made on the basis of the residual standard deviation from the values predicted by the fitted model. To be able to compare the residual standard deviations between ELISA and the CCK_1 [Ca^{2+}] assay, those were expressed as the percentage of the maximum measurement value.

Differences in the potency of the different protein hydrolysates and protein sources to increase CCK-8 release from STC-1 cells and to increase $[Ca^{2+}]_i$ by activation of CCK_1 were analyzed using one-way ANOVA followed by a multiple comparison test. Differences were considered to be significant at $P < 0.05$. Data in the figures are presented as the mean ± SEM. All experiments were performed at least in quadruplicate.

RESULTS

Comparison of ELISA and CCK_1R Assay Using CCK-8S Standard Curves. Standard curves with increasing concentrations of CCK-8S (1 pM to 2.5 nM) were measured in the ELISA and the CCK_1R assay. CCK-8S-like immunoreactivity increased dose dependently with a linear range found between concentrations of 34 and 411 pM (**Figure 1**). In the ELISA, a large cross-reactivity with gastrin was observed (data not shown). Stimulation of CHO cells constitutively expressing CCK_1R with increasing concentrations of CCK-8S led to a dose dependent increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) with an EC_{50} of 62 ± 21 pM with 1 nM CCK-8S causing the maximum increase in $[Ca^{2+}]_i$ (**Figure 1**). The increase in $[Ca^{2+}]_i$ was completely abolished by the addition of 50 μM of the CCK_1R specific antagonist lorglumide, and concentrations up to 2.5 nM gastrin did not induce any change in $[Ca^{2+}]_i$ (data not shown).

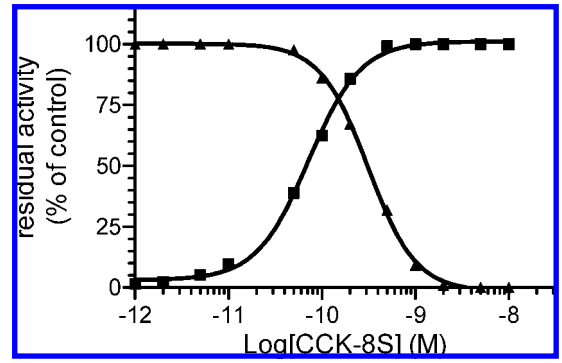

Figure 1. Comparison of standard curves obtained with increasing concentrations of CCK-8S (1 $pmol \cdot L^{-1}$−10 $nmol \cdot L^{-1}$) in the ELISA (▲) and CCK_1R $[Ca^{2+}]_i$ (■) assay. Curves were fitted by nonlinear regression (sigmoidal dose−response with variable slope). The results are expressed as normalized means from 11 experiments.

Effect of Protein Hydrolysates on CCK Release from STC-1 Cells: Results from ELISA Measurements. The release of CCK from STC-1 cells after 2 h of incubation with the control solutions forskolin (50 μM) and lauric acid (500 μM) was significantly stimulated by 2.8- and 1.9-fold respectively, as compared to the content of the HBSS control solution. Quantification of CCK-8S-like immunoreactivity in the cell culture supernatant of the STC-1 cells revealed a significant ($P < 0.05$) increase in CCK after 2 h of incubation with 10 $mg \cdot L^{-1}$ of pea protein hydrolysate (1.7 ± 0.9-fold), soy protein hydrolysate (2.1 ± 0.9-fold), potato protein hydrolysate (2.1 ± 0.7-fold), and casein hydrolysate (1.7 ± 1.0-fold) compared to that in in the saline control, whereas whey protein hydrolysate did not significantly change CCK concentrations in the cell supernatant (**Figure 2A**). No significant differences ($P > 0.05$) in CCK concentrations were observed between the different hydrolysates. Although a tendency toward dose dependent increase in CCK-8 immunoreactivity was observed for potato and soy protein hydrolysates (0.1, 1.0, and 10 $mg \cdot L^{-1}$), dose dependent effects were not significant ($P > 0.05$) for any of the test hydrolysates.

Effects of Different Protein Hydrolysates on CCK Release from STC-1 Cells Determined by CCK_1R Activation. To test the hypothesis that CCK released from STC-1 cells can be quantified using CCK_1R activation, intracellular Ca^{2+} levels were measured in CHO cells expressing CCK_1R in response to incubation with cell supernatants harvested from STC-1 cells exposed to different hydrolysates. As controls, protein hydrolysates of the same concentrations that had not been in contact with STC-1 cells were used. From these negative controls, only soy hydrolysate caused, without being in contact with STC-1 cells, at the highest hydrolysate concentration (10 $mg \cdot L^{-1}$) a weak increase in $[Ca^{2+}]_i$. Stimulation of CHO-CCK_1R cells with supernatant derived from STC-1 cells incubated for 2 h with 10 $mg \cdot L^{-1}$ of pea, potato, soy, casein, and whey protein hydrolysates significantly changed $[Ca^{2+}]_i$ to 2.1 ± 0.1-fold, 1.7 ± 0.2-fold, 2.6 ± 0.2-fold, 2.3 ± 0.1-fold, and 1.6 ± 0.3-fold, respectively, compared to that in the saline control (background corrected for $\Delta[Ca^{2+}]_i$ observed for hydrolysates

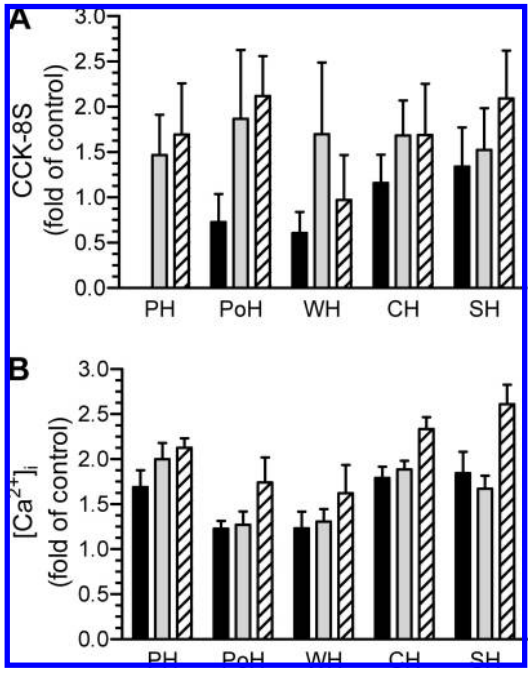

Figure 2. Effect of protein hydrolysate treatment on CCK-release from STC-1 cells. CCK release into STC-1 supernatants was measured after a 2-h incubation with increasing concentrations (0.1 mg·L^{-1}, black bar; 1.0 mg·L^{-1}, gray bar; 10 mg·L^{-1}, hatched bar) of pea (PH), potato (PoH), whey (WH), casein (CH), and soy (SH) hydrolysates. Released CCK was determined by ELISA (**A**) or by CCK_1R activation by monitoring intracellular Ca^{2+} levels in CHO-CCK_1R cells (**B**). The results are expressed in comparison to the saline control. As negative control, the direct effect of hydrolysates on CCK_1R was tested prior to incubation on STC-1 cells. Background correction was performed in B when the protein hydrolysate without being in contact with STC-1 cells caused a direct effect on CCK_1R. The results are expressed as the mean ± SEM ($n = 4$).

without being in contact with STC-1 cells) (**Figure 2B**). CCK release induced by low concentrations of hydrolysates (0.1 and 1 mg·L^{-1}) tended to increase the $[Ca^{2+}]_i$ response in all groups; however, significant differences ($P < 0.05$) to control were only observed after incubation with 0.1 and 1 mg·L^{-1} casein, pea, and soy hydrolysates (**Figure 2B**). At hydrolysate concentrations of 10 mg·L^{-1}, increase in $[Ca^{2+}]_i$ was significantly different ($P < 0.05$) from that of the control for all hydrolysates, with the largest effect observed in the presence of soy hydrolysate.

Direct Effects of Protein Hydrolysates on CCK_1R. As low concentrations of soy protein hydrolysates surprisingly activated CCK_1R directly, we tested whether this effect was specific and whether other hydrolysates also directly stimulate CCK_1R at higher hydrolysate concentrations. Changes in $[Ca^{2+}]_i$ were monitored in CHO-CCK_1R cells in response to incubation with increasing concentrations of the test hydrolysates. As shown in **Figure 3**, incubation of CHO-CCK_1R cells with either soy or potato protein hydrolysate at 10 mg·L^{-1} and higher concentrations resulted in a significant dose dependent increase in $[Ca^{2+}]_i$ that reached a plateau at 3 g·L^{-1}. Potato hydrolysate activated CCK_1R with an EC_{50} value of 225 mg·L^{-1}, whereas soy protein hydrolysate exhibited a much higher efficiency (EC_{50} 33 mg·L^{-1}) toward CCK_1R activation. Soy and potato protein hydrolysates exhibited a potency of 58 ± 2% and 37 ± 2%, respectively, for CCK_1R activation as compared to the maximum calcium increase induced by 1 nM CCK8-S. In contrast, casein hydrolysate caused a change in $[Ca^{2+}]_i$ only at high concentrations (3 g·L^{-1}), whereas pea and whey protein hydrolysates did not alter $[Ca^{2+}]_i$ levels, indicating no direct interaction of these hydrolysates with CCK_1R (**Figure 3**).

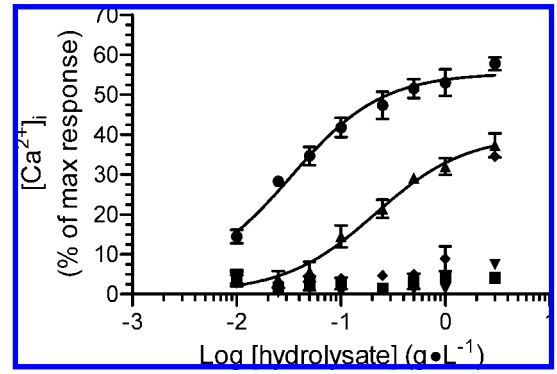

Figure 3. Protein hydrolysates are potent agonists of CCK_1R. The ability of increasing concentrations (0.1–3 g·L^{-1}) of pea (■), potato (▲), whey (▼), casein (♦), and soy hydrolysates (●) to activate CCK_1R was assessed in the $[Ca^{2+}]_i$ assay in CHO-CCK_1R cells. Cells were stimulated for 30 s with the protein and protein hydrolysates, and increase in $[Ca^{2+}]_i$ was quantified by monitoring fluorescence signals. The results are expressed as the percentage of the $[Ca^{2+}]_i$ increase measured after incubation with 1 nmol·L^{-1} CCK-8S, which caused a maximum increase in *f*/*f*0 ratios. The results are reported as the mean ± SEM ($n = 3$).

The CCK_1R antagonist lorglumide dose dependently inhibited the CCK-8S induced rise in $[Ca^{2+}]_i$ with complete inhibition observed at lorglumide concentrations of 50 μM (**Figure 4A**). Specificity of the lorglumide-mediated inhibition of CCK-8S induced CCK_1R activation in CHO-CCK_1R cells was demonstrated as lorglumide did not inhibit the calcium increase induced thrombin receptor-activating peptide (TRAP, 250 μM) (**Figure 4B**). Under the same experimental conditions, increasing concentrations of potato protein hydrolysate (0.05–3 g·L^{-1}) increased $[Ca^{2+}]_i$ up to 39 ± 6% compared to the CCK-8S induced maximum $[Ca^{2+}]_i$ increase (**Figure 4B**). However, in the presence of 50 μM lorglumide, the potato hydrolysate induced increase in $[Ca^{2+}]_i$ was inhibited only by 35 ± 5% as compared to the Ca^{2+} release observed in the absence of lorglumide. To exclude the nonspecific effect of the hydrolysate on $[Ca^{2+}]_i$, the effect of 3 g·L^{-1} potato hydrolysate was tested on CHO cells transfected with empty vector CHO cells and was found not to alter intracellular Ca^{2+} concentrations (data not shown).

DISCUSSION

Food intake and energy homeostasis is in part regulated by the secretion of gastrointestinal hormones such as CCK. Here, using a combined CCK release and CCK_1 receptor activation assay, we demonstrated that CCK is quantifiable by monitoring the increase in intracellular Ca^{2+} in response to CCK_1R activation. In addition, using this assay, we describe that selected protein hydrolysates efficiently stimulate CCK release from STC-1 cells, which in turn activates CCK_1R. Furthermore, we depict for the first time, to our knowledge, that selected hydrolysates such as soy and potato protein hydrolysates, stimulate CCK_1R-expressing cells and may mediate satiety in part by direct receptor stimulation.

Multiple molecular isoforms of CCK, varying in length up to 83 amino acids, have been described with the CCK_1R-activating domain residing in the amidated carboxyl octapeptide (*16*). All forms of CCK larger than CCK-8 have biological activity, but with different binding affinities at CCK_1R (*17*).

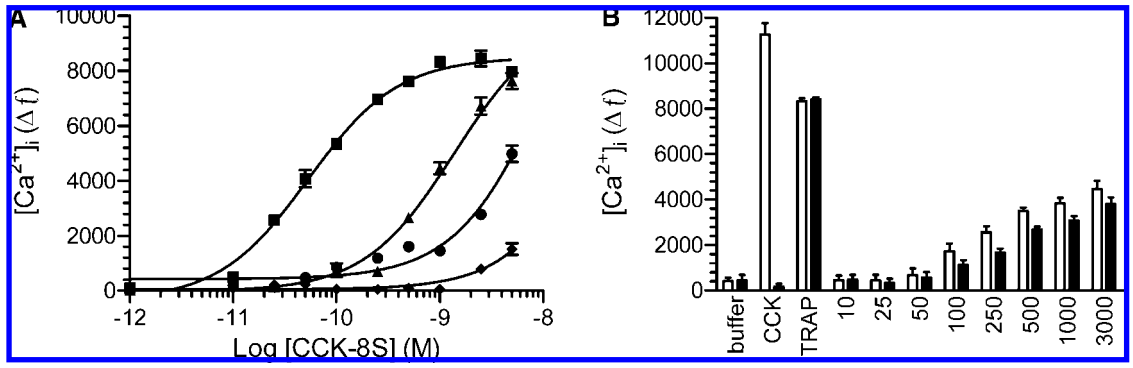

Figure 4. CCK_1R activation by potato protein hydrolysate is only partly mediated by direct interaction with the CCK-8S binding site. (**A**) CCK_1R activity induced by CCK-8S was dose dependently inhibited with increasing concentrations of lorglumide (0 μM, ■; 25 μM, ▲; 50 μM, ●). (**B**) Increase in $[Ca^{2+}]_i$ in the absence (empty bars) and presence (filled bars) of 50 μM lorglumide in response to incubation with HBSS, CCK-8S (1 nmol·L^{-1}), TRAP (250 μMol L^{-1}), and increasing concentrations (mg·L^{-1}) of potato hydrolysate. The results represent the mean ± SEM ($n = 3$).

Thus, ELISA or RIA, which detect the C-terminus of CCK, cover all molecular forms of CCK but do not discriminate between different bioactivities. The fact that the C-terminal pentapeptide of gastrin, which is not a ligand of CCK_1R, is identical with that of CCK further hampers CCK determination as gastrin plasma levels are 20 to 100 times higher than those of CCK (*18*). One aim of our study was, therefore, to develop an assay that would allow indirect quantification of CCK by means of CCK receptor activation. A direct comparison between CCK ELISA and the CCK_1R assay confirmed strong gastrin cross-reactivity in the former and absence in the latter assay, and thus higher specificity of the CCK_1R assay. Determinations of the sensitivity threshold values for CCK in the receptor assay revealed that this value did not significantly differ from zero, whereas in the ELISA, the average threshold was 40 pM, indicating a considerably higher sensitivity of the receptor assay. This may be important, in particular, at low basal plasma CCK concentrations. The calculated average rate constants of 0.0097 ± 0.0026 (CCK_1R assay) and 0.0025 ± 0.0012 (ELISA) at increasing CCK-8S concentrations (**Figure 1**) differed significantly from each other, indicating that the CCK_1R assay has a smaller operational range, but also the ability to detect smaller CCK differences. Relative standard deviations calculated from the rate constants of 11 individual CCK-8S standard curves were consistently and significantly ($P < 0.05$) lower in the CCK_1R assay (SD 27.2%) than in ELISA (SD 46.6%). Therefore, it is concluded that the CCK_1R activation assay is more suitable for rapid, relative quantification of CCK released from STC-1 cells but may also serve as a valuable tool for measuring total plasma CCK activity. Obviously, the advantage of this model is that the effects of CCK secretagogues can directly be translated into receptor activation, which is finally needed to trigger any physiological response.

Selected proteins and protein hydrolysates have been demonstrated to have eating inhibitory behavior and to be a major stimulus of CCK release *in vivo* (*19*). So far, systematic information on tailor-made peptide hydrolysates that effectively trigger CCK release from enteroendocrine cells is missing. To identify protein hydrolysates with CCK-releasing properties that may serve as satiety inducing food ingredients *in vivo*, we have chosen STC-1 cells as the test system. These cells possess phenotypical functions of enteroendocrine cells (*12*) and have high endogenous expression levels of CCK and glucagon-like peptide-1 (*14, 20*). Exposure of low concentrations (0.1–10 mg·L^{-1}) of protein hydrolysates for 2 h to STC-1 cells demonstrated the potency of soy, pea, and casein hydrolysates to induce CCK secretion already at concentrations of 0.1 mg·L^{-1}. Previous observations that CCK secretion from STC-1 cells increases with the dose of the hydrolysate (*21, 22*) was not confirmed in our study. In addition, no selectivity was found when the different hydrolysates were tested for their CCK-releasing properties. All tested hydrolysates induced CCK secretion up to 2.2-fold as compared to control. Although some studies using high hydrolysate concentrations (10 g·L^{-1}) show increase in CCK about more than 10-fold (*21, 22*), the modest increase in CCK in the present study is in line with *in vitro* studies in STC-1 cells, where CCK secretagogues as the CCK-releasing factor CRF increase CCK about 2-fold (*23*). Hydrolysate concentrations used in the CCK release assay were just as high so that they could not stimulate CCK_1R when directly exposed to the receptor. This may lead, however, to false negative results as some of the hydrolysates might show CCK-releasing potential in the STC-1 assay at higher concentrations. A CCK release assay using STC-1 cells grown on a Transwell filter system with apical application of the test compound and basolateral CCK sampling would be ideal. However, attempts to grow STC-1 cells in a Transwell system have so far not been successful.

In turn, the fact that low concentrations of pea, soy, potato, whey, and casein hydrolysates were effective in our *in vitro* assays suggests that it is likely that those hydrolysates are effective CCK secretagogues *in vivo*. In contrast, it is questionable whether the high concentrations tested in other *in vitro* studies can be achieved *in vivo*, when the dilutive capacity of the food matrix and more importantly that of the GI tract is taken into account. However, with respect to physiological in-body functionality of the *in vitro* tested hydrolysates, one has also to consider that other nutrients as fatty acids are potent stimulators of CCK secretion (*24*). Whether the observed effect of low amounts of protein hydrolysates on CCK secretion are additive to those of other nutrients will depend on the load of the different CCK-releasing stimulants. Additive effects of low concentrations of pea, soy, potato, whey, and casein on CCK secretion within a food matrix are most likely to appear *in vivo* in low energy−low fat foods. This, however, still needs to be determined.

A very sensitive peptide sensing mechanism seems to be present in STC-1 cells as indicated by CCK increase in response to 0.1 mg·L^{-1} of casein hydrolysate. Interestingly, very recently a protein hydrolysate sensitive, G-protein coupled receptor, GPR93, was found to be highly expressed in the mucosa of the small intestine (*6*) and with low expression levels in STC-1 cells (*7*). This receptor was shown, when overexpressed in STC-1 cells and activated by protein hydrolysates, to increase intra-

cellular Ca^{2+} levels from endoplasmatic Ca^{2+} stores and in turn to activate the CCK promoter, increase CCK message levels, and induce CCK release from STC-1 cells (*7*). GPR93 was suggested to be physiologically relevant, together with other cellular systems, to form the luminal protein sensory system. However, in these experiments, only high concentrations (1 g–50 $g \cdot L^{-1}$) of protein hydrolysates were able to induce a cellular response after overexpression of GPR93, whereas in our study, concentrations as low as 0.1 $mg \cdot L^{-1}$ provoked CCK release in native STC-1 cells. This suggests that beside GPR93 another protein hydrolysate sensing mechanism that is responsive to low concentrations of peptides seems to be present in STC-1 cells.

In contrast to the absence of any specificity of protein hydrolysates on their CCK-releasing properties, our data indicate that selected protein hydrolysates (soy and potato protein hydrolysate) do directly stimulate CCK_1R in a dose dependent manner with a minimal effective dose of 10 $mg \cdot L^{-1}$. High doses of soy protein hydrolysates (1 $g \cdot L^{-1}$) even evoked CCK_1R responses up to 50% of the maximum response evoked by 1 nM CCK-8S. This concentration is still far below the effective concentrations for the activation of GPR93 (*7*). The present data strongly indicate that the effect in CHO-CCK_1R cells is indeed mediated through direct interaction of soy and potato hydrolysates with CCK_1R as the controls, that is, CHO cells transfected with the empty vector, did not respond with any change in intracellular Ca^{2+} in reaction to the hydrolysate treatment (data not shown). The hydrolysates tested contained only very low amounts (<2%) of fat and carbohydrates, suggesting that the CCK_1R stimulatory effect is confined to the protein fraction. During protein hydrolysis, short-chain peptides are formed, which possibly act as bioactive peptides at CCK_1R. Further studies should focus on the fractionation of hydrolysates and identification of the CCK_1R-activating peptide structures in soy and protein hydrolysates.

The fact that pea and whey hydrolysates did not activate CCK_1R could be not only due to the absence of specific peptides that interact with the receptor but also due to the presence of CCK_1R inhibitory activities in the nonprotein fraction. Indeed, glycoproteins as lectins have been shown to inhibit CCK_1R activity *in vitro* (*25*). Although soy beans contain lectins, their presence in protein hydrolysates has so far not been demonstrated, but it is possible that residual lectin fractions were present in our hydrolysate. However, the tested soy hydrolysate was one of the most effective hydrolysates at CCK_1R. Therefore, it is suggested that the overall activity of the hydrolysate is defined by the fraction specific protein, that is, the presence of specific peptide sequences in the soy and potato hydrolysates that interact with CCK_1R.

Interestingly, the activity of potato protein hydrolysate on CCK_1R could only partially be blocked by the specific CCK_1R antagonist lorglumide, suggesting a different binding site or a nonspecific modulation of CCK_1R by short-chain peptides. CCK_1R is expressed on nerve terminals of the gastric and duodenal branch of the vagus nerve (*26*). Whether CCK_1R expression in the small intestinal tract is confined to the myenteric plexus (*27*) or also functionally expressed on vagal primary afferent fibers innervating the brush border membrane has not been fully established yet. However, afferent vagal neurons seem to innervate the gut, with some endings of their neurites spreading among the enteroendocrine cells (*28, 29*). Dietary peptides have been shown to be absorbed intact at low concentrations into circulation (*30*); thus, it is not unlikely that short-chain dietary peptides may also directly activate CCK_1R *in vivo*, contributing to their satiating effects.

In conclusion, in the present study, we have demonstrated that measuring CCK_1R activity by monitoring intracellular Ca^{2+} levels is suitable for the determination of CCK release. In addition, using this assay, we showed that low concentrations of protein hydrolysates nonspecifically induce the release of CCK from STC-1 cells. Although it remains to be determined whether soy and potato protein hydrolysate mediate both CCK release and direct CCK_1R activation *in vivo*, the present study demonstrated the potential importance of selected short-chain peptides to act in a dual mode on dietary satiety signaling.

ABBREVIATIONS USED

CCK, cholecystokinin; CCK-8S, sulfated CCK-8; CCK1R, cholecystokinin receptor 1.

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