Guanidinated Casein Hydrolysate Stimulates Pancreatic Secretagogue Release by Direct Action to the Intestine in Rats (44304)

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Abstract. **Previously, we observed that pancreatic exocrine secretion was strongly enhanced after duodenal administration of guanidinated casein peptic hydrolysate (HGC) in rats with chronic bile-pancreatic juice (BPJ) diversion. Using a perifusion system of isolated dispersed rat intestinal mucosal cells, we investigated whether this phenomenon depends on a direct effect of protein on the intestine to release pancreatic secretagogues, such as cholecystokinin (CCK). Amylase release from isolated pancreatic acini was used as an assay to measure CCK or other pancreatic secretagogues in the effluents. Mucosal effluent with HGC stimulated amylase release from acini with or without soybean trypsin inhibitor (SBTI). Perifused effluent with low-concentrated SBTI did not stimulate amylase release. These results indicate that HGC stimulates release of pancreatic secretagogue from the intestinal mucosal cells independent of remaining trypsin activity in the isolated mucosa. Effluents with intact casein, its peptic digest, and homoarginine, which is a unique amino acid contained in HGC, were unable to stimulate amylase release from acini. Effluent with a high concentration of SBTI, which is rich in arginine residues, stimulated amylase release, but not with the same tryptic inhibitory effect of lima bean trypsin inhibitor, which is poor in arginine residues. These findings suggest that guanidyl residues in protein structure are responsible for release of pancreatic secretagogues from isolated intestinal mucosal cells. Finally, the increment of amylase release from pancreatic acini in response to the perifused effluent with HGC was eliminated in acini treated with a potent CCK antagonist, FK480. We conclude that in rats with BPJ diversion, HGC stimulates CCK release from the intestine by direct action on intestinal mucosa.**
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In rats, dietary protein-induced pancreatic exocrine secretion is mediated by feedback control of pancreatic proteolytic enzyme activity, especially trypsin, in the proximal small intestine (1–3). The most important hormonal regulator in this mechanism is cholecystokinin (CCK) (4–6). CCK is produced by the I-cell of the proximal small intestine and secreted into the circulation. It is believed that trypsin-sensitive CCK-releasing peptides from the pancreas and intestine stimulate CCK release from the intestine (7). In a postprandial state, these endogeneous peptides may survive as intact active peptides while dietary protein occupies luminal trypsin activity. Therefore, the role of dietary proteins in this mechanism is indirect. Recently, Sharara *et al.* (8) demonstrated that casein and its digests did not stimulate CCK release directly from the rat small intestinal mucosa using an *in vitro* mucosal cell perifusion system.

We previously suggested that dietary proteins directly stimulate pancreatic secretagogue release from the intestine, according to observations that pancreatic exocrine secretion was enhanced by feeding a 25% protein, fat-free diet to rats with chronic bile-pancreatic juice (BPJ) diversion from the proximal small intestine (9). In chronic BPJ diverted rats, the indirect mechanism to regulate pancreatic secretion is

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eliminated. Additionally, we found that guanidinated casein stimulated pancreatic secretion much more strongly than intact casein in BPJ diverted rats (10). In chronic BPJ diverted rats, the stimulatory secretion of pancreatic enzymes after duodenal infusion of guanidinated casein peptic hydrolysate (HGC) solution was partially but significantly inhibited by a CCK receptor antagonist (11).

The purpose of this study was to examine the direct effect of protein on intestinal cells with regard to the release of pancreatic secretagogues. We used *in vitro* isolated dispersed rat intestinal mucosal cells as the perifusion system. In this system, pancreatic proteases were mostly removed as well as the intestinal innervations that participate in CCK release (12). Therefore, this system is suited to examine only the direct effect of protein on endocrine secretion from intestinal cells. We investigated pancreatic secretagogue release from intestinal cells using guanidinated casein, as a stronger stimulator of pancreatic secretion, with the perifusion system. Furthermore, to confirm the direct effect of HGC, contributions to feedback control caused by residual pancreatic proteases in the perifusion system were excluded by a perifused solution containing soybean trypsin inhibitor (SBTI) and by using mucosal cells prepared from the intestine of chronic BPJ diverted rats. Through these experiments, we evaluated the direct actions of dietary protein on the intestine for releasing pancreatic secretagogues.

Materials and Methods

Preparation of Guanidinated Casein Hydrolysate. Guanidinated casein, from which the lysyl residues were converted to homoarginine, was prepared by a previously described method (10). Guanidinated casein was hydrolyzed by 6 *N* HCl (110°C, 24 hr) and the amino acids analyzed by high-performance liquid chromatography (HPLC) as phenyl thiocarbamyl (PTC) derivatives with phenyl isothiocyanate (Wako Pure Chemical Industries, Osaka, Japan) (13). The HPLC was constructed with a mini-solvent delivery system, M-600 (Waters, Milford, MA), and a Wakopak WS-PTC column (4.0 × 200 mm; Wako Pure Chemical Industries, Osaka, Japan). The amino acid composition of the guanidinated casein is shown in Table I. The conversion rate of lysine to homoarginine was 99%, determined by the amounts of lysine and homoarginine in the guanidinated casein. The sum of both amino acids was compared with the amount of lysine in the intact casein. Then, guanidinated casein was hydrolyzed by pepsin (Sigma Chemical Co., St. Louis, MO) at pH 1.8 and 37°C for 1 hr. This hydrolysate was then neutralized, desalted, and lyophilized. The distribution of molecular weight in HGC is shown in Table II, which was analyzed twice by gel permeation chromatography using HPLC with a Protein Pak 60 column (7.8 × 300 mm; Waters, Milford, MA).

Animals. Male Sprague-Dawley rats (Japan SLC Inc., Hamamatsu, Japan), weighing 250–350 g, were kept in a temperature-controlled room at 23 ± 2°C and fed a semi-purified 25% casein-sucrose diet (stock diet). The rats were fasted the night before the experiment. The study was approved by the Hokkaido University Animal Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Table I. Amino Acid Composition of Guanidinated Casein

Amino acid	Content (mg/g protein)
Alanine	34.7
Arginine	31.2
Aspartic acid	41.8
Cysteine	10.8
Glutamic acid	167.9
Glycine	17.3
Histidine	31.9
Isoleucine	49.6
Leucine	89.4
Lysine	0.6
Methionine	24.4
Phenylalanine	48.2
Proline	98.5
Serine	35.6
Threonine	28.7
Tryptophan	N.D.*
Tyrosine	50.4
Valine	62.0
Homoarginine	85.5

Note. Guanidinated casein was hydrolyzed by 6N HCl at 110°C for 24 hr. Composition of guanidinated casein was analyzed by high-performance liquid chromotography with a Wakopak WS-PTC column (4.0 × 200 mm, Wako Pure Chemical Industries). Details are described in Materials and Methods. *: not detected.

Table II. Proportions of Peptide Molecular Weight Distribution of Intact and Guanidinated Casein Hydrolysates

Molecular weight	HIC	HGC
	%	%
<1,000	39.8	38.1
1,000–5,000	31.1	29.0
5,000–10,000	8.6	12.2
10,000–20,000	7.1	10.1
20,000>	13.4	10.6

Note. HIC: intact casein hydrolysate, HGC: guanidinated casein hydrolysate. Casein and guanidinated casein were hydrolyzed with pepsin at pH 1.8 for 1 hr. The distribution of molecular weight in these hydrolysates was analyzed twice by high-performance liquid chromatography with a Protein Pak 60 column (7.8 × 300 mm, Waters). Details are described in Materials and Methods.

Preparation and Perifusion of Mucosal Cell Column. The dispersed intestinal mucosal cell preparation and column perifusion were a modification of the technique of Sharara *et al.* (8, 14). Rats were sacrificed, and 20 cm of the small intestine were resected proximally beginning at the ampulla of Vater. The intestinal lumen was washed with saline and everted. Then, the intestine was incubated for 5 min under agitation in 15 ml modified Krebs-Henseleit bicarbonate buffer containing 2.5 m*M* EDTA at 37°C. This

buffer was enriched with mixed amino acids (MEM Essential Amino Acids (50X), Wako Pure Chemical Industries) and bubbled with 95% O_2: 5% CO_2 in advance (pH 7.4). After incubation, mucosa were immediately scraped from the intestine with a plastic bar and mixed with buffer. This suspension was centrifuged for 3 min at 500 rpm; the supernatant was discarded, and the pellet (containing mucosal cells) was resuspended in 2 ml of HEPES buffer containing 24.5 m*M* HEPES, 0.5 m*M* $CaCl_2$, 98 m*M* NaCl, 2.5 m*M* NaH_2PO_4, 6 m*M* KCl, 1 m*M* $MgCl_2$, 11.5 m*M* glucose, 0.2% bovine serum albumin, and mixed amino acids (pH 7.4). The cell suspension was mixed with 2 ml of preswollen Sephadex G-50 medium resin, and loaded into a perifusion chamber consisting of a 5-ml disposable plastic syringe covered with a glass filter. Its plunger was perforated with a 16-gauge needle connected to silicone tubing. The end of the tube was placed in a beaker containing perifusion solutions. Another silicone tube was connected to the tip of the column *via* a peristaltic pump. The perifusion solutions were introduced into the column in turn by motion of the pump. Each cell column was prepared from a different rat.

Cells were equilibrated for 1 hr by perifusion with HEPES buffer at a rate of 1 ml/min, and afterwards, the cells were perifused with test solutions. All test solutions were prepared in HEPES buffer and ranged between 60 and 500 mOsm/kg. Solutions were adjusted to pH 7.4, maintained at 37°C and bubbled with 95% O_2: 5% CO_2 in advance. The perifusion medium could be changed instantly. In this way, perifusion media containing different test substances were sequentially perifused through the column. The HEPES buffer was perifused between test periods to elute the solution that formerly flowed in the column. The flow rate of all solutions was 1 ml/min, and each fraction collected 3 ml. Finally, cells were perifused with 50 m*M* KCl in HEPES buffer to test the viability of the mucosal cells (CCK-secretory cells) (14). The high concentration of extracellular potassium depolarized the mucosal cells and released CCK in this system.

Pancreatic secretagogue levels in the perifused effluent were evaluated by the amylase release from isolated pancreatic acini. One ml of effluent per fraction was poured into a plastic vessel and lyophilized. Dispersed pancreatic acini were prepared from male Sprague-Dawley rats by enzymatic digestion of the pancreas with collagenase as previously reported (15). Acini were then incubated with a lyophilized sample for 30 min at 37°C. As an index of pancreatic secretion, the amylase activity released into the medium was assayed using procion yellow starch as the substrate (16). In this process, we did not extract and concentrate CCK because the perifused effluent might contain pancreatic secretagogues other than CCK.

Experimental Protocols

Effect of HGC on Pancreatic Secretagogue Release in Mucosal Cell Perifusion. To test whether HGC stimulated intestinal mucosal cells to release any secretagogues, cells were perifused with an HGC solution. After equilibration, cells were perifused with three varying doses (0.1, 1, 5% (wt/vol)) of HGC solution.

Effect of SBTI on Pancreatic Secretagogue Release in Perifusion. In perifusion, when using mucosal cells of normal rat intestine, pancreatic proteases may not be completely excluded from the mucus and may act as a negative feedback control when perifused with HGC in a cell column. To confirm that pancreatic secretagogue release by HGC perifusion was a direct action on intestinal cells, SBTI (Kunitz trypsin inhibitor, Sigma Chemical Co.) was added to a perifused solution. SBTI contains a strong trypsin-inhibitory activity and can completely neutralize pancreatic proteases in the mucus. In this experiment, cells were first perifused with 0.1% SBTI, 0.1% SBTI + 1% HGC, and 1% HGC solution. Second, the cells were perifused with two doses (0.1, 1%) of SBTI solution and 1% HGC solution.

Effect of Lima Bean Trypsin Inhibitor (LBTI) on Pancreatic Secretagogue Release in Perifusion. SBTI (Kunitz trypsin inhibitor) has reactive sites with trypsin consisting of an Arg-X bond (17). To confirm that pancreatic secretagogue release by SBTI perifusion was not an indirect action dependent on its trypsin inhibitory activity but a direct action on intestinal cells, lima bean trypsin inhibitor (LBTI, Sigma Chemical Co.) was added to a perifused solution. LBTI has reactive sites with trypsin consisting of a Lys-X bond (18). In this experiment, cells were perifused with a 1% LBTI and 1% SBTI. Amino acid analysis of LBTI and SBTI was performed with a previously described method.

HGC Perifusion Using Chronic BPJ Diverted Rat Intestines. To further confirm that pancreatic secretagogue release by HGC perifusion was a direct action on intestinal cells, HGC was perifused with mucosal cells from chronic BPJ diverted rat intestines. The operative procedure for the chronic BPJ diverted rats was described previously (19). Briefly, a polyethylene catheter was inserted into the common bile–pancreatic duct at the ampulla. A silicone catheter for BPJ returning to the lumen was placed in the small intestine 45 cm distal from the ligament of Treitz. These catheters were connected behind the neck. BPJ was diverted from the proximal small intestine for 7 days. In this experiment, cells were perifused with a 0.1% SBTI and 0.1% SBTI + 1% HGC solution.

Effects of Intact Casein and Intact Casein Hydrolysate on Pancreatic Secretagogue Release in Perifusion. Previous reports observed that casein solution and casein enzymatic digest solutions did not stimulate CCK release in the intestinal cells perifusion system (8). To confirm that these materials do not stimulate pancreatic secretagogues (containing CCK) release, cells were perifused with a 1% intact casein (casein sodium (Na.Cas), Wako Pure Chemical Industries), 1% casein hydrolysate (HIC) and 1% HGC solution. The preparation method for HIC and molecular weight distribution analysis in HIC was the same

as that for HGC preparation. The distribution of molecular weight in HIC is also shown in Table II.

Effect of HGC-Like Amino Acid Mixture on Pancreatic Secretagogue Release in Perifusion. To test whether the homoarginine in HGC exerted an effect on intestinal mucosal cells to release secretagogue, cells were perifused with a solution of amino acid mixture simulating HGC (HGC-A.A). The HGC-A.A solution was made based on Table I, with a final concentration of 0.91% (corresponding to 1% HGC).

Determination of Secretagogue Release from Intestinal Cells by HGC Perifusion. To determine if the released secretagogue from the mucosal cells by HGC perifusion was CCK or not, a CCK-A receptor antagonist, FK480, was added when amylase release from pancreatic acini was evaluated. FK480 (kindly gifted from Fujisawa Pharmaceutical Co., Osaka, Japan) is one of the most potent CCK-A receptor antagonists (20, 21). FK480 was dissolved in dimethyl sulfoxide (DMSO), and added to the acini suspension 30 min before reaction. The final concentration of FK480 in the suspension was 10 μM, and DMSO did not exceed 0.01%. Only DMSO was added to the FK480-nontreated acini suspension.

Statistical Analysis. Released amylase activity during each fraction period was expressed as a percentage of amylase activity, with the basal period (nonstimulating period) as 100. Results were expressed as mean ± SEM. Statistical analysis was performed by one- or two-way analysis of variance. Significant differences among means were determined by the least significant difference ($P < 0.05$). Experiments of HGC-dose response, using chronic BPJ-diverted rat intestines in the perifusion and HGC-A.A perifusion, were performed on two and four rat mucosa, respectively. These results showed representative data and were reproducible.

Results

Figure 1 shows the results when mucosal cells were perifused with 0.1%, 1%, and 5% HGC solutions. Doses of HGC perifused effluent with 1% and 5% HGC clearly increased amylase release, but 5% HGC effluent reached the highest level faster than the 1% HGC effluent. Perifused effluent with 0.1% HGC did not increase amylase release.

Figure 2 shows the results when mucosal cells were perifused with 0.1% SBTI and/or 1% HGC solution. The level of amylase released from acini by effluent with 0.1% SBTI did not significantly increase as compared with the basal level. Effluent with 0.1% SBTI + 1% HGC gradually increased amylase release from acini. For effluent with 1% HGC, a significant increase in amylase release from acini was also observed. Peak levels of amylase release between effluents with 0.1% SBTI + 1% HGC and 1% HGC were not significantly different. Trypsin activity was not detected in the perifused mucosa after experiments.

Figure 3 shows the results when mucosal cells were perifused with 0.1 and 1% SBTI. Similarly, Fig. 2 shows

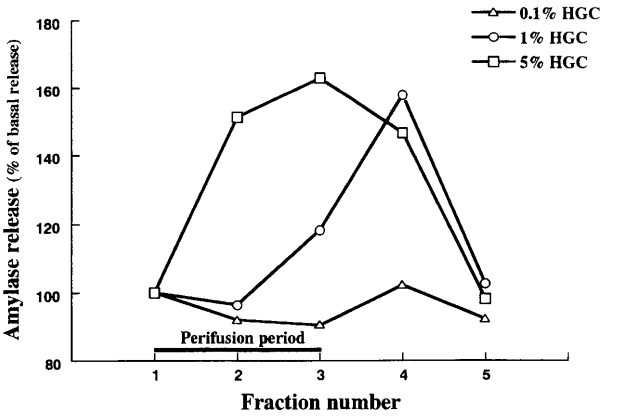

Figure 1. Amylase release from pancreatic acini was increased dose-dependently by effluents of 0.1% (△), 1% (○), and 5% (□) guanidinated casein hydrolysate (HGC) in perifusion with duodenojejunal mucosal cells. Studies began after HEPES buffer perifusion for equilibration. Each concentration of HGC solution was perifused at 2 periods (—). Results are representative of four different cell columns prepared from four rats.

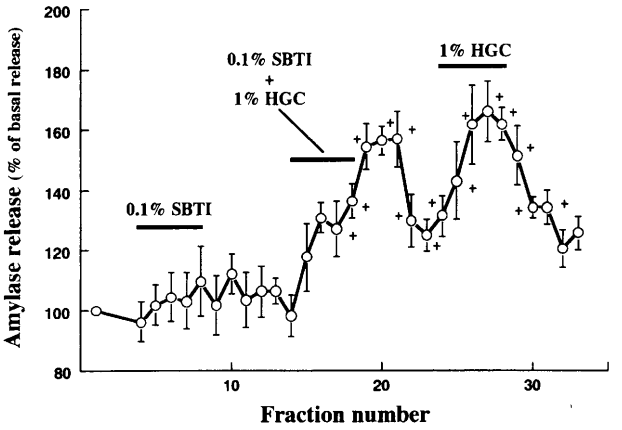

Figure 2. Amylase release from pancreatic acini was significantly increased by effluents with 0.1% SBTI + 1% HGC and 1% HGC but was not increased by effluent with 0.1% SBTI in perifusion. This study used six different cell columns prepared from six rats. Each value is mean ± SEM. Each test solution was perifused at 5 periods, in regular order and at time intervals. "+" shows a significant difference from the basal period (the first fraction) ($P < 0.05$).

thatthe released amylase level from acini by effluent with 0.1% SBTI was not significantly increased as compared with the basal level. In contrast, the level of amylase released from acini by effluent with 1% SBTI increased significantly. The increases in amylase release with SBTI effluent were comparable to that of the 1% HGC effluent. Trypsin activity was not detected in the perifused mucosa after experiments.

Figure 4 shows the results when mucosal cells were perifused with 1% LBTI and 1% SBTI. Effluent with 1% LBTI perifusion did not change amylase release from the pancreatic acini. In contrast, 1% SBTI significantly increased amylase release from the acini. Arginine content of LBTI and SBTI, which was calculated by amino acid analysis, is 25.7 and 59.8 mg/g protein, respectively.

Figure 5 shows the results when duodenojejunal mucosa originating from chronic BPJ-diverted rats were peri-

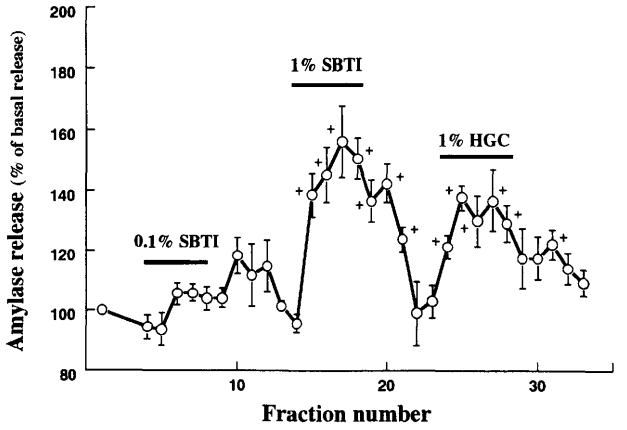

Figure 3. Amylase release from pancreatic acini was significantly increased by effluents with 1% SBTI and 1% HGC but was not increased by effluent with 0.1% SBTI in perifusion. This study used five different cell columns prepared from five rats. Each value is mean ± SEM. Each test solution was perifused at 5 periods, in regular order and at time intervals. "+" shows a significant difference from the basal period ($P < 0.05$).

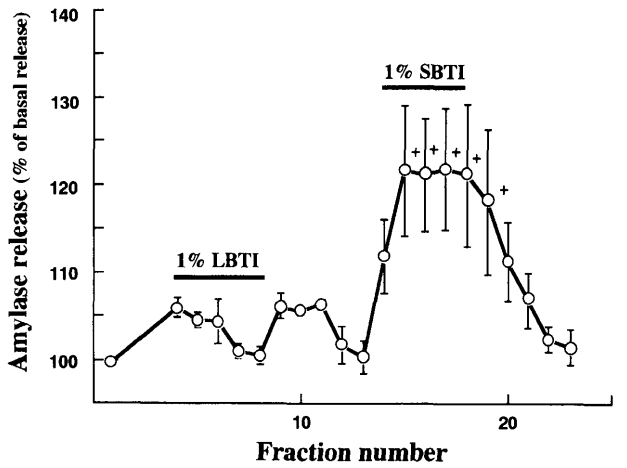

Figure 4. Amylase release from pancreatic acini was significantly increased by effluent with 1% SBTI but was not increased by effluent with 1% LBTI in perifusion. This study used four different cell columns prepared from four rats. Each value is mean ± SEM. Each test solution was perifused at 5 periods, in regular order and at time intervals. "+" shows a significant difference from the basal period ($P < 0.05$).

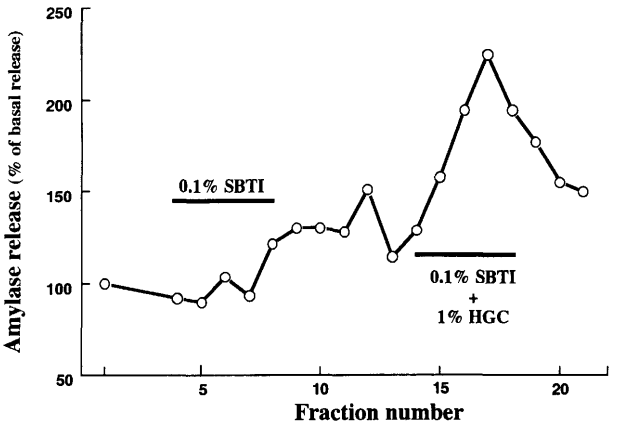

Figure 5. Amylase release from pancreatic acini was increased by effluent with 0.1% SBTI + 1% HGC but was not increased by effluent with 0.1% SBTI in perifusion with chronic BPJ-diverted rat intestinal cells. Each test solution was perifused at 5 periods, in regular order and at time intervals. Results are representative of two different cell columns prepared from two rats.

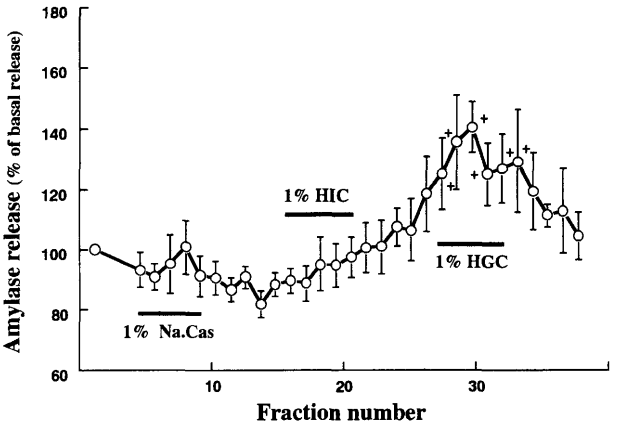

Figure 6. Amylase release from pancreatic acini was significantly increased by effluent with 1% guanidinated casein hydrolysate (HGC) but was not increased by effluents with 1% casein sodium (Na.Cas) and 1% intact casein hydrolysate (HIC) in perifusion. This study used five different cell columns prepared from five rats. Each value is mean ± SEM. Each test solution was perifused at 5 periods, in regular order and at time intervals. "+" shows a significant difference from the basal period ($P < 0.05$).

fused with HGC solution. Effluent with 0.1% SBTI perifusion did not change amylase release from the pancreatic acini. In contrast, 0.1% SBTI + 1% HGC greatly increased amylase release from the acini. Trypsin activity was not detected in the perifused mucosa after experiments.

Figure 6 shows the results when mucosal cells were perifused with Na.Cas solution and HIC solution. The effluents of both test solutions did not change amylase release from the pancreatic acini. In contrast, amylase release from the acini clearly increased with HGC perifused effluent.

Figure 7 shows the results when mucosal cells were perifused with HGC-A.A. The effluent with HGC-A.A did not increase amylase release from the acini. But, HGC effluent increased amylase release.

As shown in Figure 8, the level of amylase release from FK480-nontreated pancreatic acini by perifused effluent with HGC increased significantly as compared with the basal level, and the fourth fraction reached a maximum. However, the level of amylase release from FK480-treated pancreatic acini by perifused effluent with HGC did not change. KCl perifused effluent significantly stimulated amylase release from FK480-nontreated pancreatic acini but did not change amylase release from FK480-treated pancreatic acini.

Discussion

We previously reported that pancreatic exocrine secretion was enhanced after the administration of HGC in chronic BPJ-diverted rats (10, 11). The pancreatic stimulation of HGC does not depend on the feedback control of pancreatic protease activity in the proximal small intestine.

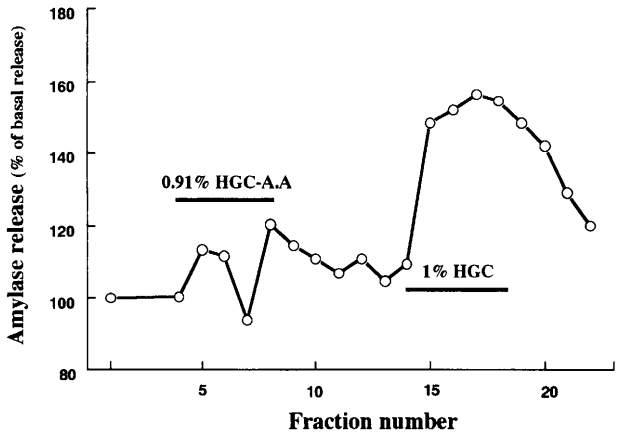

Figure 7. Amylase release from pancreatic acini was increased by effluent with 1% HGC but was not increased by effluent 0.91% HGC-like amino acids in perifusion. Each test solution was perifused at 5 periods prepared from five rats, in regular order and at time intervals. Results are representative of two different cell columns.

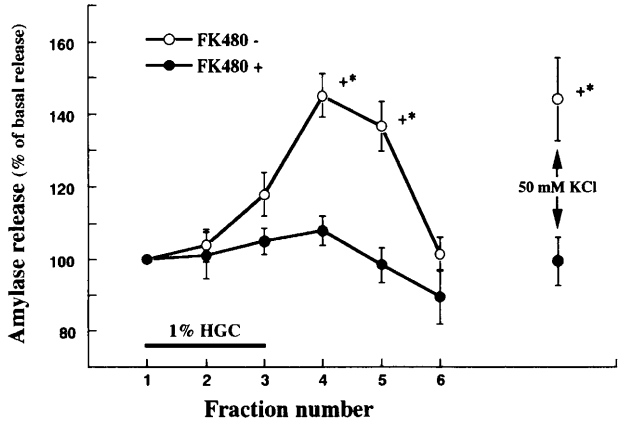

Figure 8. Amylase release from FK480-treated pancreatic acini (●) was not increased but FK480-nontreated pancreatic acini (○) was significantly increased by effluent with 1% HGC in perifusion. HGC was perifused at 2 periods. This study used four different cell columns prepared from four rats. Each value is mean ± SEM. From the results of two-way analysis of variance, P value of treatment was <0.0001 and of fraction was <0.0001. "+" and "*"show significant differences from the basal period in each group and between groups in each fraction, respectively ($P < 0.05$).

In this study, we examined whether HGC stimulates intestinal mucosal cells directly to release pancreatic secretagogue. As shown in Fig. 1, amylase release from pancreatic acini increased with the perifused effluent supplemented with 1% and 5% HGC. With 5% HGC perifusion, amylase secretion reached the peak level faster than with 1% HGC perifusion. HGC concentration may reach a sufficient level to stimulate intestinal cells faster with 5% HGC perifusion than with 1% HGC. These results indicate that pancreatic secretagogue release from the intestinal mucosa is maximally stimulated by 1% HGC perifusion, and secretagogue secretion from intestinal mucosal cells by HGC stimulation proceeds in a dose-dependent manner.

The masking capacity for trypsin activity with guanidinated casein was much less than that with intact casein (10). Therefore, guanidinated casein is a less potent stimulator of pancreatic secretion *via* feedback control with luminal trypsin activity. Guanidinated casein has a low affinity for trypsin; however, it inhibits trypsin to some extent as described above. Feedback control by proteases does not occur until luminal protease activity is reduced by >90% (22). When using normal rat intestine to prepare the mucosal cell column, residual pancreatic protease activity in the mucus may be higher than the threshold level required to exert feedback control. It is possible that the remaining trypsin was masked by HGC, and the reduction of trypsin activity in the column reached a threshold in the feedback control. Cells were therefore perifused with SBTI to exclude the remaining trypsin activity completely. SBTI is one of the most potent trypsin inhibitors, and it can strongly inhibit pancreatic trypsin activity even with a small dose (23). Additionally, if feedback control is active in this perifusion system, pancreatic secretagogue is released with SBTI perifusion, and amylase secretion from the acini is increased. As shown in Fig. 2, amylase release did not increase as a response of acini to perifused effluent with 0.1% SBTI. However, effluent of 1% HGC perifusion to the mucosa increased amylase release from the acini with or without SBTI. Trypsin activity was not detected in the SBTI perifused mucosa. These results demonstrate that the residual trypsin activity does not participate in the stimulatory release of pancreatic secretagogue from the intestinal cells by HGC, and that HGC itself stimulates the intestinal cells. Also, HGC stimulated pancreatic secretagogue release from chronic BPJ-diverted rat intestinal mucosa from which pancreatic trypsin was eliminated beforehand (Fig. 5). This result confirms the direct action of HGC in the release of pancreatic secretagogue from intestinal mucosa.

As shown in Fig. 3, amylase release from the pancreatic acini did not increase as a response of acini to perifused effluent with 0.1% SBTI, but it did increase with 1% SBTI. SBTI originates in soybean and has a protein structure. It is likely that the pancreatic secretagogue release with high-concentrated SBTI is caused by a direct action on intestinal cells as a protein and is not related to the feedback control by its trypsin inhibitory action. To confirm this idea, we performed 1% LBTI perifusion. LBTI is also a strong trypsin inhibitor and has a protein structure. As shown in Fig. 4, 1% LBTI effluent did not stimulate amylase release from the pancreatic acini. Therefore, the effect of high-concentrated SBTI to the pancreatic secretagogue release is not related to its trypsin inhibitory activity. The protein structure of LBTI is different from SBTI. The major difference is that LBTI is a Lys-trypsin inhibitor and SBTI is an Arg-trypsin inhibitor (17, 18). This point is thought to explain the difference between SBTI and LBTI in the pancreatic secretagogue release. The direct effect of HGC on intestinal cells to release pancreatic secretagogues is not a property of HGC but is a common character of protein, specifically the guanidyl residue in protein structure.

A previous report observed that casein and casein enzymatic digests did not stimulate CCK release from intes-

tinal mucosa in perifusion (8). In the present study, casein and casein digests did not stimulate the release of pancreatic secretagogues (Fig. 6), and HGC stimulated pancreatic secretagogue release. These results suggest that guanidination gives the stimulatory ability of casein to release pancreatic secretagogues from intestinal mucosa. This finding agrees with the previous result that guanidinated casein is a much stronger stimulant of pancreatic secretion in chronic BPJ-diverted rats than intact casein (10).

The guanidyl group of homoarginine in the modified casein may be responsible for binding to the mucosal receptor because homoarginine is a unique structure in guanidinated casein. The stimulatory effect of HGC on intestinal mucosa is possibly a pharmacological effect of homoarginine itself. However, as shown in Fig. 7, amylase release from the pancreatic acini did not increase in response to the perifused effluent with an HGC-like amino acid mixture containing homoarginine. This result indicates that the ability of pancreatic secretagogue release induced by HGC does not depend on homoarginine, and may depend on peptide structures containing guanidyl residues. This result also agrees with previous observations that amino acids do not stimulate CCK release and pancreatic secretion in rats (24–26).

We confirmed by using a potent CCK antagonist FK480 (Fig. 8) that the pancreatic secretagogue released from mucosal cells by HGC is CCK. Two possible mechanisms for CCK release from intestinal cells by the direct stimulation of HGC are proposed. In the first mechanism, HGC stimulates the I-cell directly. There is an endogeneous CCK-releasing factor, so-called monitor peptide, in the pancreas of rats (27, 28). The monitor peptide stimulates CCK release by acting on the I-cell directly (14). Yamanishi *et al.* (29) found that two sites of the monitor peptide, Arg^{23}-Ile^{24} and Arg^{57}-Arg^{58}, are specific binding sites on mucosal cells. It is characteristic that an arginine residue exists at both binding sites of the monitor peptide. Arginine has a guanidyl group, and guanidinated casein has many amino acids with guanidyl radicals (arginine, homoarginine), as shown in Table I. Peptides in HGC containing a guanidyl radical possibly bind to a putative monitor peptide receptor on the I-cell. We show that arginine-rich SBTI stimulates release of pancreatic secretagogue from the intestine but not arginine-poor LBTI as described above. In another proposed mechanism, HGC stimulates cells producing CCK-releasing factors in the intestine. Endogeneous intestinal CCK-releasing factors, so-called "CCK-releasing peptide," also exist in rats (30–32). Recently, Li and Owyang (12) reported that peptone in the duodenum stimulates CCK-releasing peptide secretion. Native and synthetic trypsin inhibitors also stimulate CCK-releasing peptide secretion (33). However, this mechanism is mediated by the submucosal neural plexus (12). The mucosal cell perifusion system used in this study does not involve the neural system.

In the present study, intact casein hydrolysate did not stimulate CCK release. This result contradicts our previous report in *in vivo* experiments (11). Possibly, intact casein hydrolysate may stimulate CCK-releasing peptide secretion *via* a neural pathway rather than act on the I-cell directly to stimulate CCK release. In an isolated vascularly perifused rat duodenojejunum model, which is a luminal trypsin-eliminated model, casein hydrolysate stimulated CCK release (34, 35). This result also suggests that intact casein hydrolysate stimulates CCK secretion *via* a neural pathway. Otherwise, we previously reported that a CCK-independent and vagally dependent mechanism partially contributes to enhancement of the pancreatic secretion by casein in BPJ-diverted rats (11). The finding also suggests that intact casein may stimulate pancreatic secretion *via* a neural pathway.

In conclusion, HGC acts on intestinal mucosal cells directly and stimulates CCK release from intestinal mucosa. However, there is insufficient information to demonstrate that the direct effect of HGC on intestinal cells to release CCK applies to dietary protein in general. Further studies are needed, especially to examine the participation of a peptide fragment containing amino acids with guanidyl radicals.

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