Effects of gastric digestive products from casein on CCK release by intestinal cells in rat

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We investigated the ability of gastric digestive products from casein to stimulate cholecystokinin release by intestinal cells using the isolated vascularly perfused rat duodenojejunum. Casein digests were prepared with an in vitro system simulating gastric digestion and emptying.

The luminal infusion of the digesta emptied from the artificial stomach for the first 10 minutes produced a sharp rise of portal cholecystokinin-like immunoreactivity to 300% of basal, followed by a well-sustained plateau secretion until the end of the infusion. The residual casein fraction of this digest brought about a modest cholecystokinin secretion, while the peptide component was as strong a stimulant as total digest. The peptide responsible for this effect was the glycomacropeptide that is a glycosylated fragment (106–169) of κ-casein. Only the slightly glycosylated forms of the peptide originating from variant A of κ-casein were active. The carbohydrate-free peptide did not alter basal cholecystokinin. The highly glycosylated forms of the peptide and the slightly glycosylated peptide from κ-casein variant B induced only a transient and low rise of portal cholecystokinin. The removal of N-*acetylneuraminic acid from the active peptide suppressed its effect, while the infusion of an* N-*acetylneuraminic acid solution induced only a very low response.*

It is concluded that the glycomacropeptide released from dietary casein during gastric digestion can stimulate cholecystokinin release by intestinal cells in the rat. A well-defined structure is required for the peptide activity. A part of the peptide chain and some glycosidic chains containing N-*acetylneuraminic acid, especially those bound to the amino acid residue threonyl 31 of caseinomacropeptide variant A, would be involved in this structure.*

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Keywords: cholecystokinin release; rat; dietary proteins; casein; glycomacropeptide; in vitro

Introduction

In rats, dietary casein has been reported as an inhibitor of gastric emptying[1,2] and as a stimulant of pancreatic secretion.[3,4] In these effects, casein is more potent than other proteins (whey proteins and soya).[1,2,4] Casein was also found to stimulate the release of cholecystokinin (CCK),[5,6] which is one of the major intestinal regulatory peptides. This peptide is the main regulator of pancreatic secretion in the rat[7,8] and is known to slow gastric emptying.[9] Cuber et al.[10] have developed a model based on an isolated vascularly perfused rat duodenojejunum for studying the effects of luminal nutrients on CCK release by I-cells. Using this model, Cuber et al.[11] have reported that hydrolysates of casein are stronger stimulants of CCK release than casein. However, the potency of casein hydrolysates varies with hydrolytic treatment. Therefore, the nature of hydrolysis products appears very important.

Because physiological effects are rapidly observed (15 to 30 min) after feeding casein,[1,2,4] products released from the stomach during the first 30 minutes of digestion are probably responsible for these effects. Contrary to hydrolysates used by Cuber et al.[11] these digestion products are weakly hydrolyzed.[12,13] Digesta similar to those emptied in vivo from the stomach can be prepared in vitro with a system that simulates gastric digestion.[14]

Therefore, we attempted to evaluate the effects of gastric digestive products prepared from casein on CCK release using an isolated duodenojejunum model of the rat. The

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hydrolysis products responsible for any effects were identified.

Methods and materials

Preparation and fractionation of digesta

We prepared casein digests using an in vitro system that simulates gastric digestion for 3 hr. This system has been previously described.[14,15] Briefly, a 1-L Erlenmeyer flask containing 500 mL of casein solution (30 g/L), simulating the stomach, was shaken in a 37° C water bath (Haake SWB 20 Munich, Germany). Acidification of the medium, addition of enzyme, and emptying of digesta were computer controlled. Acidification of the medium was ensured by a peristaltic pump and a three-way valve. During the digestion, the pH of the medium was continuously measured with a pH meter connected to the computer. Depending on the pH, the valve allowed automatic addition of either water or HCl (0.25 mol/L) so that the pH of the medium followed a predetermined curve. This curve was established by setting the initial pH at 6.7, the intermediate pH (90 min) at 3.2, and the final pH at 2.0 according to an exponential function. Porcine pepsin (2.8 g/L, 3,000 U/mg) was initially added to obtain an enzyme to substrate ratio of 1:500 (wt/wt). A diluted enzyme solution (56 mg/L) was then added at a variable flow rate following an exponential function, with initial and final flow rates of 8 and 1 mL/min, respectively, to compensate for the pepsin emptied with the digesta. A similar function was used for the emptying curve, with initial and final flow rates of 10 and 3 mL/min respectively. In this study, digestive products emptied during the first 30 minutes were collected by 10-minute periods: Cas10 (0 to 10 min), Cas20 (10 to 20 min), Cas30 (20 to 30 min). To stop enzymatic digestion, every sample was immediately raised to pH 8 with 2 mol/L NaOH.

Products of Cas 10 were separated on the basis of their molecular weight. The Cas10 digesta was precipitated at pH 4.6 (pHi of casein) and centrifuged at 10,000*g* for 20 min. The pellet contained casein and very large fragments of casein. Ultrafiltration of the supernatant on a YM5 Amicon membrane (cut off: 5,000 Da) provided two fractions: the residue contained mainly peptides and the ultrafiltrate contained small peptides, amino acids, and salts. From here on, residue will refer to the ultrafiltration residue.

Peptides of residue were further separated by reverse phase high performance liquid chromatography (RP-HPLC). Separation was carried out on a semi-preparative C18 nucleosil column (I.D. 9.2 mm; SFCC, Gagny, France). The elution system consisted of solvent A (0.115% trifluoroacetic acid [TFA] in water) and solvent B (0.10% TFA; 60% CH_3CN in water). The column was initially equilibrated in 90% solvent A and 10% solvent B. Peptides were eluted by increasing the gradient of solvent B (0 to 10 min, 2% solvent B/min; 10 to 50 min 1% solvent B/min) and detected at 220 nm. The column was kept at 40° C in a water bath and elution rate was 4 mL/min. Fractions were collected after several injections, pooled, and freeze dried.

The *N*-acetylneuraminic acid (NANA) of glycopeptides was liberated by neuraminidase from *Vibrio cholerae* (Boehringer, Mannheim, Germany). The enzyme was added to glycopeptides (25 mU/mg of peptide) in 50 mmol/L sodium acetate buffer (pH 5.5, 3.5 mmol/L $CaCl_2$), and incubation was performed at 37° C for 16 hr. Salts and NANA were then discarded by ultrafiltration on a YM2 Amicon membrane (cut off: 2,000 Da).

Characterization of tested fractions

The protein mass in every fraction was determined by amino acid analysis as the sum of all amino acids. Amino acid and amino sugar compositions were determined after hydrolysis (110° C; 5.7 mol/L HCl; under vacuum; 24 hr for amino acids and 3 hr for amino sugars) according to the method of Spackman et al.,[16] with a Biotronik LC 3000 amino acid analyzer (Munich, Germany).

Peptides isolated from the residue by HPLC were identified by amino acid composition and by determination of the *N*-terminal sequence using a 477 Sequenator connected to a 120A PTH HPLC analyzer (Applied Biosystems, Foster City, CA USA).

NANA was assayed by the thiobarbituric acid method.[17]

Duodenojejunum model

Surgical preparation. The isolated vascularly perfused rat duodenojejunum model used has been described previously.[10] Briefly, male Wistar rats (250 to 300 g) were anesthetized with pentobarbital sodium (40 mg/kg i.p.). The right and middle colic veins and arteries were tied and cut off near the serosa of the colon. A silastic cannula (I.D. 1.86 mm, O.D. 3.15 mm) was inserted 3 cm distal to the pylorus, and another one was positioned in the jejunum 20 cm beyond the ligament of Treitz. The lumen was then rinsed twice with 10 mL isotonic saline. A metal cannula (I.D. 0.6 mm, O.D. 0.8 mm) was quickly inserted in the superior mesenteric artery (within 15 sec of cutting the artery), and arterial perfusion was immediatly started at a rate of 0.5 mL/min with a Krebs-Henseleit solution (pH 7.4) containing 25% washed bovine erythrocytes, 3% bovine serum albumin, 8 mmol/L glucose, and 1% Azonutril (vol/vol). The mixture was continuously gassed by 95%/O_2-5%CO_2 and warmed to 37° C. Silastic tubing (I.D. 0.5 mm, O.D. 0.94 mm) was introduced in the portal vein, and the infusion rate was increased to 2.5 mL/min, thus allowing a perfusion pressure of 40 to 60 mm Hg. The duodenojejunum preparation was then transferred to a plastic box filled with isotonic saline at 37° C. Venous effluent was collected as 2-min fractions in glass tubes containing 250 µL of 200 mmol/L EDTA and was rapidly centrifuged. The supernatant was extracted with 2 vol of ethanol and evaporated to dryness for subsequent radioimmunoassay of CCK. The time required to make the preparation was about 20 min, and the viability of the preparation was estimated at 90 min.

Experimental protocol. The experiments consisted of a 20-min control basal period, during which isotonic saline was infused into the lumen of the intestine at a rate of 100 µL/min. This was followed by a 30-min period infusion. Solutions under study or isotonic saline were first administered as a 4-mL bolus over 1 min, followed by an infusion rate of 200 µL/min for 30 min. The lumen was then flushed with infusion of isotonic saline at a rate of 200 µL/min for 10 min. The amounts of all the digestion products studied corresponded to the amounts emptied from the artificial stomach after ingestion of 1.875 g of casein, which is usually contained in a rat's meal. After freeze drying, digestion products were diluted in 10 mL of isotonic saline. The pH was adjusted to 7, and the osmolality to 200 to 300 mosmol/kg H_2O. Six trials were performed for every digesta sample.

Radioimmunoassay of CCK (CCK-RIA). The CCK-like immunoreactivity (CCK-LI) in the ethanol extracted portal effluent was determined with an antiserum 39A, as previously described.[18] The antiserum 39A was obtained from a New Zeland white rabbit after injection of highly purified CCK33 coupled to bovine albumin and emulsified in complete Freund adjuvant. The antiserum 39A recognized CCK33 and CCK8 equally, while the reactivity is 12% for sulfated gastrin$_{17}$ and 5% for nonsulfated gastrin$_{17}$ and nonsulfated CCK8. The detection limit and the 50% inhibition dose (ID_{50}) were 0.5 and 3.6 pmol/L, respectively. CCK10 labeled with the monoiodated Bolton-Hunter reagent and purified by HPLC according to Fourmy et al.[19] was used as a tracer. CCK8, dissolved in isotonic saline solution containing 25 mmol/L sodium bicarbonate and 2% horse serum, was used as a standard. Ethanol extracts representing 1 mL of original venous effluent samples were reconstituted with

Table 1 Protein mass of the different fractions and peptides isolated from casein digesta

		Protein mass (mg)
Digesta	Cas10[a]	222
	Cas20[a]	122
	Cas30[a]	44
Cas10 fractions	Pellet[b]	210.9
	Residue[b]	9.8
	Filtrate[b]	1.3
Peptides isolated from Cas10 residue	GMP1[c]	1.4
	1-23[d]	1.5
	CMP[c]	2.4
	GMP2[c]	1.1
	β-lacto[e]	0.5

[a]Digesta Cas10, Cas20, and Cas30 = products of digestion emptied from artificial stomach during 0–10, 10–20, and 20–30 min periods, respectively.
[b]Pellet was obtained by precipitation of digesta Cas10 at pH 4.6. Residue and filtrate were obtained by ultrafiltration of the supernatant pH 4.6.
[c]CMP, GMP1, and GMP2 = carbohydrate-free, highly glycosylated, and slightly glycosylated-caseinomacropeptide.
[d]1–23 = fragment 1–23 of αs1-casein.
[e]β-lacto = β-lactoglobulin.

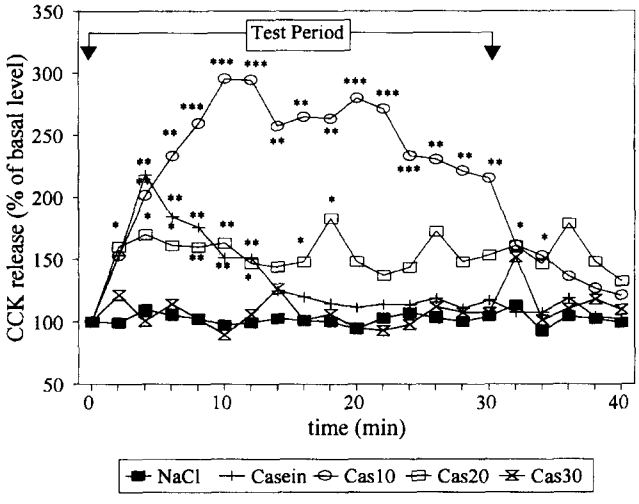

Figure 1 Effects of luminal infusion of casein solution (187.5 mg of protein) and digesta (Cas10, Cas20, and Cas30) on CCK-LI release. The amount of protein matter in each digesta corresponded to the amount emptied from the artificial stomach *(Table 1)*. Results are percent increase above basal and represent means for $n = 6$. Asterisks represent the significant differences from basal level (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

600 μL of assay buffer (phosphate buffer 0.05 mol/L, pH 7.5, containing 2% horse serum, 15.5% EDTA, and 3.3% sodium azide). Duplicate 100- or 200-μL samples were added to 600 or 700 μL of assay buffer. The antiserum was used at a final dilution of 1/200,000. After 24 hr at 4°C, the tracer (2,000 cpm) was added, and incubation was continued for a further 48 hr. Free and bound tracer were separated by centrifugation (1,500 rpm for 45 min) in presence of activated charcoal and the radioactivity of the pellet was measured.

Calculations and statistics. Data in figures are means of six trials and are expressed as percentage of increase above basal values. The basal secretion was the mean of five values obtained before the perfusion. The integrated responses of CCK-LI released by a given stimulus were calculated by subtracting the basal immunoreactivity release from the data upon stimulation during the same period. For statistical analysis of data, Student's *t* test was used.

Results

Effects of gastric digestion time of casein on the digesta potency to stimulate CCK-LI release

The protein masses of digesta obtained after in vitro gastric digestion of 1.875 g of casein are reported in *Table 1*.

During infusion of NaCl, basal plasma CCK-LI was un-

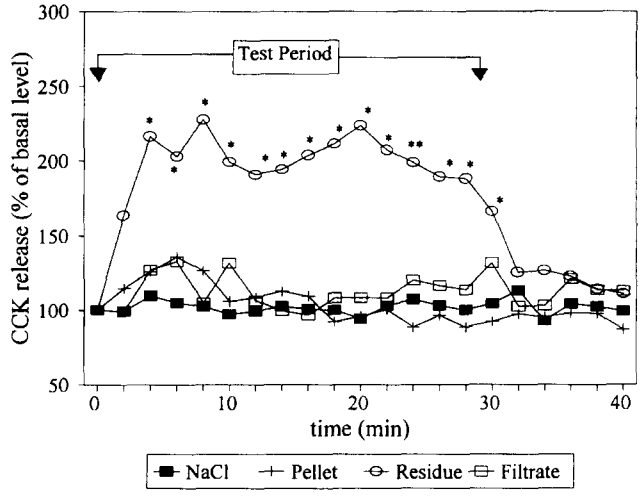

Figure 2 Effects of luminal infusion of Cas10 fractions (pellet, residue, and filtrate) on CCK-LI release. The amount of protein matter in each fraction corresponded to the amount emptied from the artificial stomach *(Table 1)*. Results are percent increase above basal and represent means for $n = 6$. Asterisks represent the significant differences from basal level (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

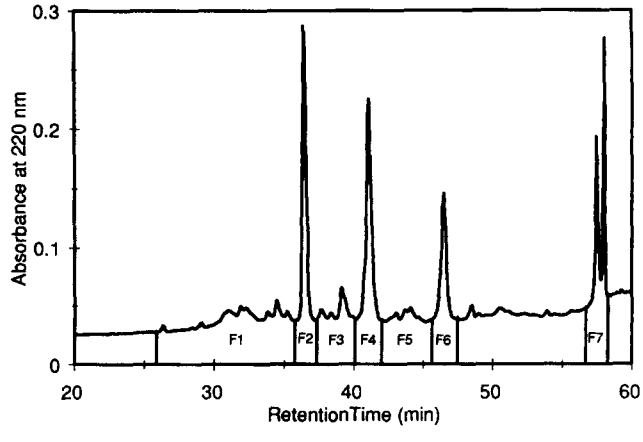

Figure 3 Separation of the residue peptides by reverse phase HPLC on a C18 nucleosil column. Detection at 220 nm.

Table 2 Identification of peptides isolated from the ultrafiltration residue of Cas10 digesta

Fraction[a]	F1		F2		F3		F4		F5		F6		F7	
Amino acid composition[b]														
Asx (B)	4.7	(5)	2	(2)	4.8	(5)	4.6	(5)	4.2	(4)	4	(4)	13	(15)
Thr (T)	9.7	(12)	1	(1)	9.6	(12)	11.7	(12)	8.8	(11)	10.7	(11)	8.5	(8)
Ser (S)	4.7	(6)	0.5	(0)	4.5	(6)	4.9	(6)	4.5	(6)	5.1	(6)	7	(7)
Glx (Z)	10.8	(10)	4	(4)	10.7	(10)	10.2	(10)	10.8	(10)	10.9	(10)	23	(25)
Pro (P)	7.8	(8)	3	(3)	7.7	(8)	6.8	(8)	7.4	(8)	6.5	(8)	5	(8)
Gly (G)	1.2	(1)	1	(1)	1.1	(1)	1.2	(1)	1.2	(1)	1.1	(1)	5	(3)
Ala (A)	5.4	(5)	0.5	(0)	4.7	(5)	5.4	(5)	5.2	(6)	5.6	(6)	10	(14)
Cys (C)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	2	(5)
Val (V)	5.7	(6)	1	(1)	5.5	(6)	5.9	(6)	5.6	(6)	6.1	(6)	8.5	(10)
Met (M)	1	(1)	0	(0)	0.7	(1)	1	(1)	0.7	(1)	1	(1)	2.5	(4)
Ile (I)	5.8	(6)	1.5	(1)	5.5	(6)	6.2	(6)	6.1	(7)	7	(7)	6.5	(10)
Leu (L)	1.7	(1)	3.5	(4)	1.6	(1)	1	(1)	1.7	(1)	1	(1)	15	(22)
Tyr (Y)	0.4	(0)	0	(0)	0	(0)	0	(0)	0.3	(0)	0	(0)	2	(4)
Phe (F)	0.2	(0)	1	(1)	0	(0)	0	(0)	0.3	(0)	0	(0)	1.5	(4)
His (H)	0.4	(0)	1.6	(2)	0	(0)	0	(0)	0	(0)	0	(0)	2	(2)
Lys (K)	3.5	(3)	2	(2)	3	(3)	2.9	(3)	3.6	(3)	3.1	(3)	10	(15)
Arg (R)	0.4	(0)	1.5	(2)	0	(0)	0	(0)	0.3	(0)	0	(0)	2.5	(3)
N-term sequence	MAIP		RPKH		MAIP		MAIP		MAIP		MAIP		LIV	
Sugar contents[c]														
Galactosamine	2.5				0.36				0.34					
NANA	2.6				1.1		0.10		0.8					
Identified Peptide[d]	Highly Glycosylated CMP or GMP1		1–23 αs1-casein		Slightly Glycosylated CMP or GMP2A		CMP A		Slightly Glycosylated CMP or GMP2B		CMP B		β-lacto	

[a]The fraction names refer to collected fractions in *Figure 3.*
[b]Results are mol residue/mol peptide and are obtained after 24 hr hydrolysis. Numbers in parentheses are theoretical number of residues in identified fragment.
[c]Results are mol residue/mol peptide.
[d]Identification of the peptide: CMP = caseinomacropeptide fragment 106–169 of κ-casein; 1–23 αs1-cas = fragment 1–23 of αs1-casein; β-lacto = β-lactoglobulin.

altered. Casein (187.5 mg/10 mL) produced a rise in portal CCK-LI, which peaked at the start of infusion (220%) and then decreased to near basal values at 15 min *(Figure 1).*

Luminal infusion of Cas10 immediately induced a rise in CCK-LI levels which reached a maximal value of 300% at 10 min, remained at this value during 15 min and then tended to decline at the end of infusion. A similar profile was observed with Cas20, but the maximal value was lower (160%). Cas30 did not stimulate the release of CCK-LI *(Figure 1).* Cas10 was the most potent CCK secretagogue, followed by Cas20 and Casein.

Fractionation, characterization and activity of products from Cas10

Firstly, digesta Cas10 was separated into three fractions (by precipitation and ultrafiltration) : pellet (intact casein or large fragments of casein), residue (peptides), and ultrafiltrate (amino acids and salts). The protein masses of the fractions are reported in *Table 1.*

Only infusion of the residue produced an increase in CCK-LI levels that was similar to the increase observed with infusion of Cas10. Pellet and ultrafiltrate did not alter the basal CCK-LI significantly *(Figure 2).*

Peptides of the residue were analyzed by RP-HPLC *(Figure 3).* Seven fractions were collected and analyzed for identification *(Table 2).* Comparing the amino acid composition, sugar contents, and chromatographic behavior of these peptides with those of known peptic cleavage products of casein was used to identify: highly glycosylated forms of caseinomacropeptide (fragment 106–169 of κ-casein) variants A and B in F1 (GMP1) (GMP means glycomacropeptide); fragment 1–23 of αs1 casein in F2 (1–23); slightly glycosylated forms of caseinomacropeptide variants A and B in F3 (GMP2A) and F5 (GMP2B), respectively; a carbohydrate-free form of caseinomacropeptide variants A and B in F4 (CMPA) and F6 (CMPB), respectively; and residual β-lactoglobulin in F7. Determination of the *N*-terminal sequence showed that F2, which contained fragment 1–23 of αs1 casein, was slightly contaminated; the contaminating peptide possibly being CMP. F3 and F5 were also slightly contaminated, but contamination (<5%) was not identified. In a first series of tests, fractions F2 and F4 (CMP) and fractions F3 and F5 (GMP2) were pooled to evaluate their activity. The protein masses of the fractions are reported in *Table 1.*

Infusion of the fraction containing GMP2 produced a similar CCK-LI release to infusion of the residue, whereas other peptides did not affect the release of CCK *(Figure 4).* Furthermore, we checked (data not shown) that the active

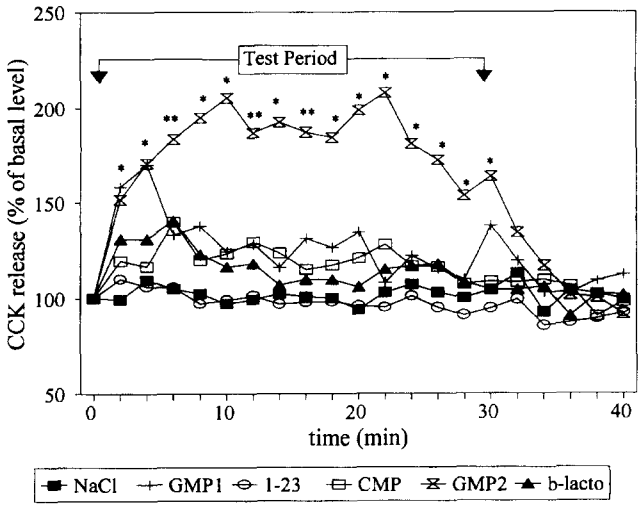

Figure 4 Effects due to luminal infusion of peptides isolated from the residue on CCK-LI release. CMP = carbohydrate-free CMP (A and B variants), GMP1 = highly glycosylated forms of CMP (A and B variants), GMP2 = slightly glycosylated forms of CMP (A and B variants), 1–23 = fragment 1–23 of αs1-casein, b-lacto = β-lactoglobulin. The amount of each peptide corresponded to the amount emptied from the artificial stomach *(Table 1)*. Results are percent increase above basal and represent means for $n = 6$. Asterisks represent the significant differences from basal level (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

casein fragment did not show any cross-reactivity in the CCK-RIA. The activities of GMP2A and GMP2B, which were constituents of the active fraction, were then each evaluated separately. The same peptide concentration (7.6 nmol/mL of peptide) was used for this experiment. Only the slightly glycosylated forms of CMP variant A (GMP2A) stimulated the CCK-LI release *(Figure 5)*. Finally, an *N*-acetylneuraminic acid solution and desialylated GMP2A were tested at the same concentration as GMP2A. Around 92% of original NANA was removed from GMP2A by the neuraminidase. The NANA solution induced a short and low response only, and desialylated GMP2A was inactive *(Figure 5)*.

The potency of fractions to stimulate CCK release in relation to purification step

Integrated response of potent fractions showed that the totality of Cas10 activity was recovered in the residue and then in GMP2 fraction, whereas the protein mass of these two fractions represented 4.4% and 0.6% of Cas10 protein mass, respectively.

Discussion

In vivo, caseinomacropeptide (CMP) is the first peptide released and emptied from the stomach after casein or milk feeding.[13,20] The in vitro system used to prepare digesta reproduced the kinetics of gastric emptying of digestive products observed in vivo both quantitatively and qualitatively.[14,15] Hence, CMP was also the main peptide in the digesta obtained during the first 10 minutes of in vitro digestion. The CMP fraction was heterogeneous because it contained all possible posttranslational modifications (phosphorylation and glycosylation) and mutations of major genetic variants A and B. Glycosylated CMP was reported to have physiological effects in digestion. It inhibits gastrin and gastric secretions in the stomach of rat or dog after intravenous, parenteral, or enteral injection.[21–23] In the preruminant calf, whole CMP added to meal partially inhibits gastric secretion and increases somatostatin secretion.[24] Thus, CMP seems to be a biologically active peptide.

In our study, only the fraction containing GMP2A strongly stimulated CCK release by gut cells in the rat. The other fractions containing either caseins (without CMP) or peptides were not active, though some of them contained a much higher protein mass than the active fraction. These results clearly demonstrate that the stimulation observed is due to a specific action of the peptide and not to a "protein effect." Besides, the activity of the three digesta collected during the first 30 min of digestion corresponded well to their CMP contents (788 nmol in Cas10, 480 nmol in Cas20, and 272 nmol in Cas30). One should notice that the stimulation of CCK release by whole casein was much lower and lasted a much shorter period than that of GMP2A, although casein contained the GMP2A sequence. However, the CMP content of tested casein solution (560 nmol) was lower than the CMP content of Cas10. Moreover, the active sequence could be buried or steric bulk could hamper the recognition by I-cells. Similar differences have previously been observed by Cuber et al.[11] between casein and various casein hydrolysates.

The carbohydrate moieties of GMP probably played an important role in the peptide activity because the carbohydrate-free form of CMP was not active. Five *O*-glycosidically linked carbohydrate chains have been identified in

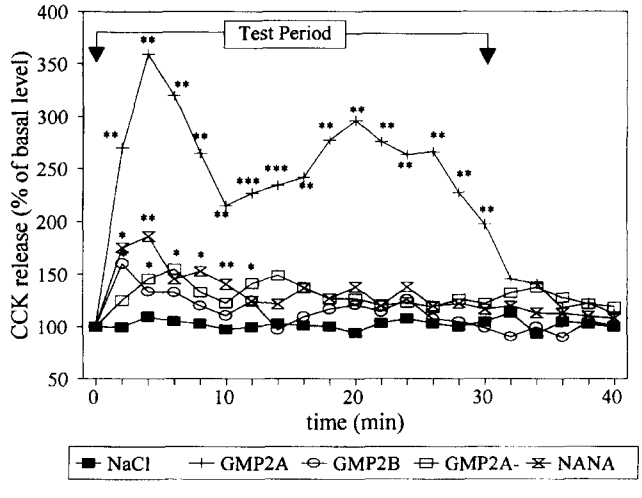

Figure 5 Effects due to luminal infusion of GMP2A (slightly glycosylated forms of CMP, A variant), GMP2B (slightly glycosylated forms of CMP, B variant), desialylated GMP2A (GMP2A-), and a NANA solution on CCK-LI release. GMP2A, GMP2B, and desialylated GMP2A were used in the same concentration (7.6 nmol/mL). The NANA concentration in the tested solution was the same as the GMP2A fraction. Results are percent increase above basal and represent means for $n = 6$. Asterisks represent the significant differences from basal level (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

bovine caseinomacropeptide[25] *(Figure 6)*, and their binding sites have been located on five threonyl residues[26] *(Figure 7)*. The carbohydrate chains are made of a linkage of different units (NANA, galactosyl, and *N*-acetylgalactosamine) and more often than not end in NANA. Because removal of NANA suppressed the stimulating effect of the active fraction, NANA is assumed to be essential for peptide activity. However, the highly glycosylated forms of CMP (GMP1) were inactive, and the NANA solution induced only a very low response. Consequently, the extent of glycosylation and the peptidic chain are also involved in activity. A recent study[27] has reported that glycosylated CMP inhibited the binding of cholera toxin to its receptor; the NANA at the end of the sugar moiety would play a major role by interacting with the toxin. The peptidic chain was also required because its hydrolysis by proteinases reduced the inhibitory activity of glycosylated CMP. Here NANA could also be involved in the recognition of GMP by I-cells that would lead to CCK release. Our results also show that the amino acid replacements of genetic variant A play a major role in recognition because slightly glycosylated forms of genetic variant B (GMP2B) were not CCK secretagogues. The genetic variants A and B differ only in two residues; Thr_{31} and Asp_{43} of variant A are replaced by Ile_{31} and Ala_{43}, respectively, in variant B *(Figure 7)*. The threonyl 31 residue of variant A may be more involved in the peptide activity because it is a glycosylation site.

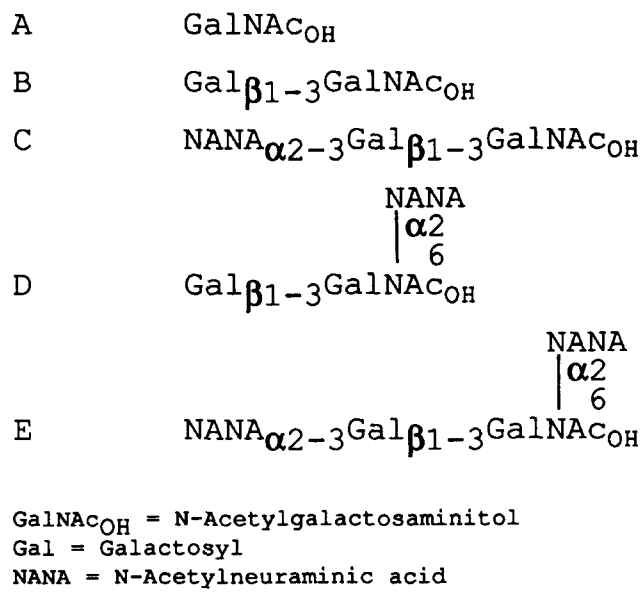

Figure 6 Chemical structure of the five carbohydrate chains isolated from bovine κ-casein taken 3 months after parturition. From Saito and Itoh.[26]

Therefore, a well-defined structure involving a peptidic chain and glycosidic chains, especially those bound to the replaced amino acid residue of variant A, is responsible for the GMP activity.

According to Cuber et al.[11] the luminal nutrients stimulate the release of CCK without activating the intramural neurons, but rather by direct action on the I-cells. Thus, GMP could stimulate CCK release through binding to intestinal receptors. In contrast, Sharara et al.[28] recently reported, using perifused CCK-cells, that proteins, protein digests, or amino acids did not act directly on I-cells. According to these authors, proteins stimulate CCK release postprandially via an indirect mechanism. Therefore GMP could act by stimulating secretion of the CCK releasing peptide.

In vivo, the high flow rate of gastric emptying just after feeding may allow the passage of GMP from stomach to the distal duodenum and jejunum, where CCK-containing cells are located, without further hydrolysis.[29] Moreover, the carbohydrates partially protect GMP from enzyme hydroly-

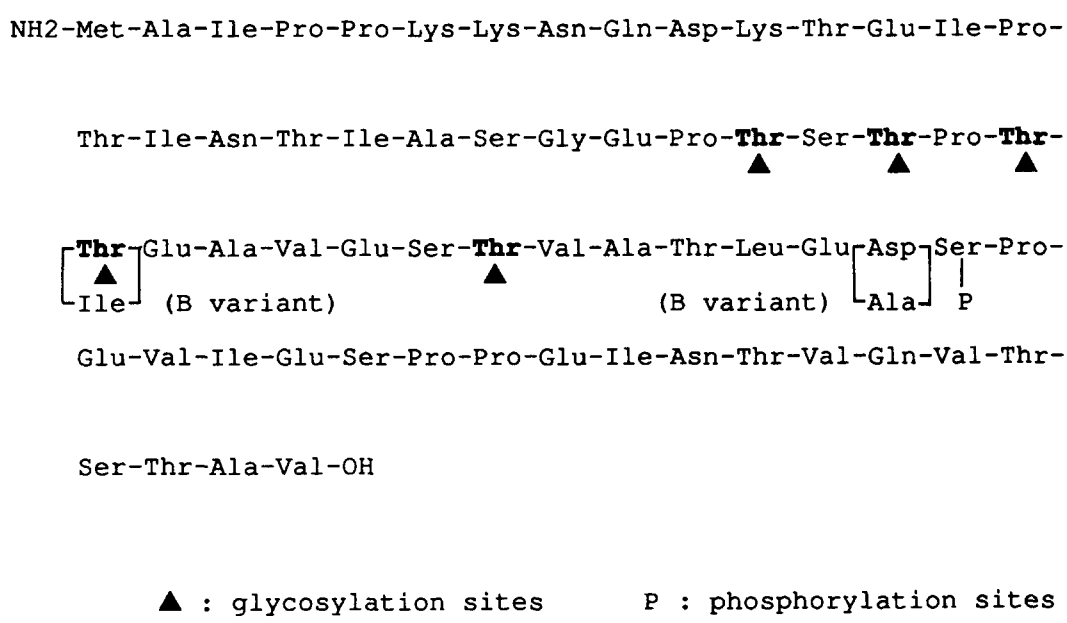

Figure 7 Primary structure of A and B genetic variants of bovine caseinomacropeptide.

sis by increasing its hydrophilic character and therefore hampering its binding by proteolytic enzymes.[30]

In conclusion, the inhibitory effect on gastric motility and the stimulating effect on pancreatic secretion of dietary casein observed in vivo in rats[1,2,4] may be partially related to the specific stimulating effect of GMP on the CCK release by I-cells. The GMP activity is due to a well-defined structure involving a peptidic chain, NANA, and probably glycosidic chains bound to residue Thr_{31}. Further studies should be carried out to determine the minimal sequence required for maintaining an effect, to find the exact structural characteristic required for the CCK releasing activity, and to elucidate the mechanism of this secretion.

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