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Nonsulfated cholecystokinins in the small intestine of pigs and rats

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ABSTRACT

Cholecystokinin (CCK) is a gut hormone that acts via two receptors. The CCK_A-receptor requires the tyrosyl residue in the C-terminal bioactive site of CCK to be O-sulfated, whereas, the CCK_B-receptor binds irrespective of sulfation. Consequently, unsulfated CCK peptides – if present – may constitute a hormone system that acts only through the CCK_B-receptor. Therefore, we have now examined whether, CCK peptides occur in nonsulfated form in the small intestine of pigs and rats. The concentrations of sulfated and nonsulfated CCK were measured by RIAs, one specific for sulfated CCKs and a new two-step assay specific for nonsulfated CCK. For further characterization, the intestinal extracts were subjected to size- and ion exchange-chromatography.

The intestinal concentrations of sulfated and nonsulfated CCK were highest in the duodenum and the proximal part of jejunum both in the pig and the rat. The porcine duodenal mucosa contained 193 ± 84 pmol/g sulfated CCK and 31 ± 10 pmol/g nonsulfated CCK, and the upper rat intestine 70 ± 19 pmol/g and 8 ± 2 pmol/g, respectively. The degree of sulfation correlated with the endoproteolytic proCCK processing. Thus, 38% of porcine CCK-58 was unsulfated, whereas, only 12% of CCK-8 was unsulfated.

The results show that a substantial part of intestinal CCK peptides in rats and pigs are not sulfated, and that the longer peptides (CCK-58 and CCK-33) are less sulfated than the shorter (CCK-22 and CCK-8). Hence, the results demonstrate that proCCK in the gut is processed both to sulfated and unsulfated α-amidated peptides of which the latter are assumed to act via the CCK_B-receptor.

1. Introduction

Amino acid derivatizations are common posttranslational modifications that diversify the cellular expression of proteins and peptides. The derivatizations are carried out by specific processing enzymes, and like other *in vivo* enzymatic processes, the derivatizations are rarely complete. For instance, tyrosyl-O-sulfation of gastrin peptides is only partial [1].

Cholecystokinin (CCK) and gastrin belong to a family of peptides with an identical α-amidated C-terminal sequence (Trp-Met-Asp-Phe-NH_2), which constitutes the core of the active site. The CCK gene is expressed in I-cells in the mucosa of the small intestine and in cerebral and peripheral neurons [1,2]. The CCK peptides in circulation originate from intestinal I-cells that secrete CCK to plasma in response to food. CCK peptides have several gastrointestinal actions such as gallbladder contraction, stimulation of pancreatic enzyme secretion and growth, initiation of satiety and inhibition of gastric acid secretion.

CCK was originally discovered as a gallbladder contracting substance [3], which has given the hormone its name [3,4]. A gallbladder contraction bioassay was used to monitor the original purification of CCK [4,5]. The structure-function identification showed that O-sulfation of the tyrosyl residue in position 7 (as counted from the C-terminus) was decisive for gallbladder contraction [4,5]. This observation suggested that CCK peptides per definition are completely sulfated. In the last decades, however, sporadic occurrence of nonsulfated CCK peptides has been noted. First, canine thyroid C-cells contain mainly nonsulfated CCK-8 and -5 [6], and CCK in human medullary thyroid carcinomas is all nonsulfated CCK-8 [7]. Later it was shown that CCK-58 in the porcine and canine small intestine occurs in both sulfated and nonsulfated form [8,9]. Thus, nonsulfated CCK peptides seem to occur *in vivo*. But the intestinal expression of CCK has not been examined systematically in terms of peptides with and without sulfation, neither have nonsulfated CCKs in gut tissue been quantitated. The structural similarities between the nonsulfated CCKs, sulfated CCKs and the gastrins have been a major barrier for development of an assay of sufficient specificity and sensitivity. Recently, however, we man-

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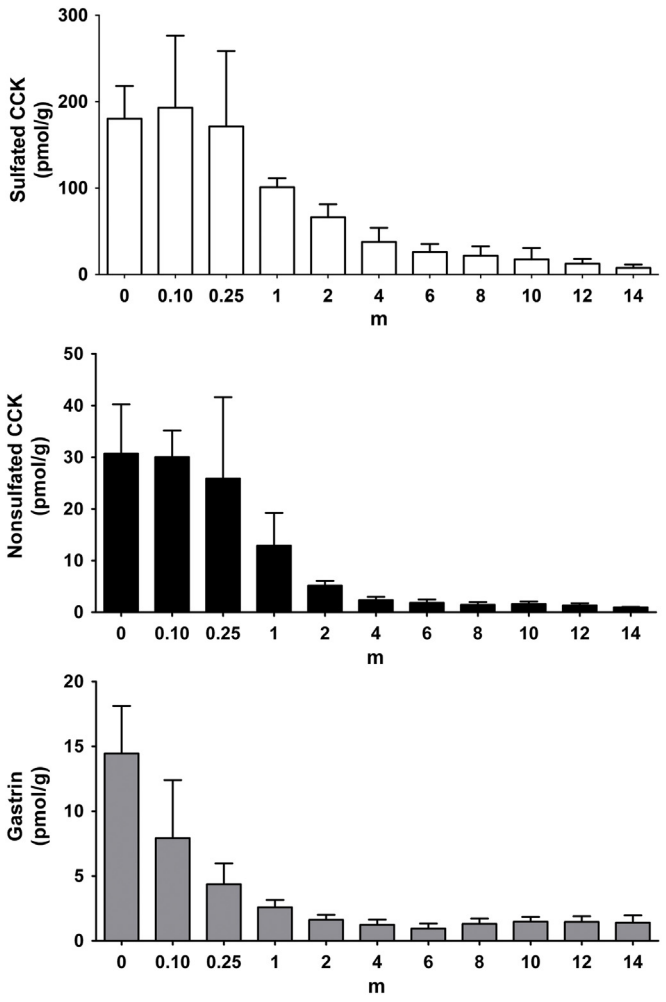

Fig. 1. Concentrations of sulfated and nonsulfated cholecystokinin (CCK) and gastrin in the small intestinal mucosa of pigs (mean ± SD, $n = 4$). Distance is measured from the pyloric sphincter.

aged to develop a highly specific two-step assay for nonsulfated CCK peptides [10]. Using this assay we first quantitated nonsulfated CCK in a single sample of the jejunum from 12 pigs in order to demonstrate that the new assay measured as planned [10].

In the present study, we have now characterized nonsulfated CCK in the small intestine of 12 normal pigs and 6 rats and extended the investigation to also measure nonsulfated CCK at multiple sites along the small intestine in 4 pigs and 6 rats. Moreover; these results have been compared with those of sulfated CCKs and the homologous gastrins.

2. Materials and methods

2.1. Tissues

Porcine tissues for characterizing the distribution of CCK and gastrin in the small intestine were obtained from four anaesthetized four-month-old pigs (The Panum Institute, University of Copenhagen, Denmark). The tissues were collected as 5 cm pieces at various distances from the pyloric sphincter; 0 cm, 10 cm, 25 cm, 1 m, 2 m, 4 m, 6 m, 8 m, 10 m, 12 m, 14 m. The mucosa was immediately stripped free from the remaining intestinal wall and all specimens were frozen on dry ice until storage at −80 °C. Jejunal mucosa of 12 pigs sampled earlier [10], were used for gel chromatography and FPLC. The samples were collected 50 cm from the pyloric sphincter.

The small intestine from 6 euthanized adult wistar rats (The Panum Institute, University of Copenhagen, Denmark) was divided into 10 cm pieces and immediately frozen on dry ice until storage at −80 °C. Notably, in the rat the mucosa was not stripped free of the intestinal wall, but the mucosa constitutes half of the weight of rat small intestine.

2.2. Extraction

Samples of 0.5–1 g were gathered in disposable plastic bags, immersed in liquid nitrogen and pulverized by a cryoPREP impactor. The pulverized samples were then boiled for 20 min in 10 mL/g (at least 1 mL) distilled water and homogenized. The extracts were centrifuged at 10,000 rpm and the supernatant was collected as the “neutral extract”. The pellet was then resuspended in 0.5 M acetic acid (10 mL/g), homogenized, and incubated for 20 min at room temperature. It was then centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant was collected as the “acid extract”.

2.3. Radioimmunoassays

The CCK peptides in the extracts were measured using two sequence-specific assays as previously detailed [10,11]. In addition, the homologous gastrin peptides were measured for control using gastrin antiserum no. 2604 [12]. The CCK-antiserum no. 92128 binds the α-amidated C-terminus of only sulfated CCK peptides, and was consequently used to measure sulfated CCK-58, -33, -22, and -8. This antiserum does not react with sulfated, nor with nonsulfated gastrins [11]. CCK-antiserum no. 94179 was used in our newly developed assay for nonsulfated CCK [10]. It binds the N-terminal pentapeptide sequence of nonsulfated CCK-8 [10]. Therefore, as a first step, tryptic cleavage of the binding between Arg and Asp in position 9 and 8 (as counted from the C-terminus) was necessary for quantitation of the longer nonsulfated CCK-peptides in the tissues (CCK-58, -33, and -22) [10]. Gastrin antiserum no. 2604 is entirely specific for the α-amidated C-terminus of gastrin [12]. It measures sulfated and nonsulfated gastrin-71, -34, -17, and -14 with equimolar potency [13], and its reactivity with CCK peptides is negligible [12,13].

2.4. Chromatography

The jejunal tissue extracts from 12 pigs were applied to Sephadex G50 superfine columns in order to separate the molecular forms of CCK according to size. The samples were applied to calibrated columns and eluted with 0.02 mol/L barbital buffer, pH 8.4, containing 1 g/L bovine albumin (Ortho) + 0.1 M NaCl at a flow rate of 4 mL/h. The fractions were collected in volumes of ∼1.3 mL and the concentration of CCK measured by the assays as described above [10–12].

The neutral extracts of the small intestine from wistar rats were also chromatographied with an ÄKTA purifier FPLC system on a MonoQ 5/50 ion exchanger column (Pharmacia, Sweden). Fractions were eluted with buffer A that contained 20 mM tris, at pH 8.2. Buffer B contained additional 1 M NaCl. The gradient of buffer B was linear, with 0% at fraction 6, 8% at fraction 27, and 28% at fraction 56. Fractions were collected in volumes of 1 mL and the concentration of CCK in each fraction measured by RIA as described above [10,11].

2.5. Estimation of the concentration of each form of CCK

The total concentration of sulfated and nonsulfated CCK in each tissue was measured by RIA in each of the neutral and acidic