PREPROENKEPHALIN A OCCURS IN THE ENTERO-CHROMAFFIN (EC) CELLS OF THE PORCINE INTESTINE: AN IMMUNOCYTOCHEMICAL STUDY USING ANTISERA TO MET-ENKEPHALIN-ARG⁶-GLY⁷-LEU⁸ AND TO SEROTONIN

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ABSTRACT

The porcine gut was immunocytochemically examined by using an antiserum against Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-enkephalin-8), which is included in the molecule of preproenkephalin A. Met-enkephalin-8-like immunoreactivity was recognized in dispersed endocrine cells as well as in intramural nerve fibers. The immunoreactive cells were most numerous in the duodenum, fewer in the pyloric antrum and still fewer in the lower small intestine. They all coincided with enterochromaffin (EC) cells, which were identified by an anti-serotonin serum applied to adjacent thin sections. In the duodenum, approximately 60% of the EC cells were also reactive to the anti-Met-enkephalin-8 serum. EC cells were also present in the gastric body and large intestine but they were not reactive to the anti-Met-enkephalin-8 serum. These findings indicate that not all EC cells, but only a subtype which is concentrated in the upper small intestine in the pig, contain preproenkephalin A.

The enterochromaffin (EC) cell represents the most numerous endocrine element in the mammalian gastrointestinal tract. Erspamer and Asero (3) showed that 5-hydroxytryptamine (serotonin) is contained in the EC cell. Various staining reactions are available for demonstrating this cell: chromaffin reaction, Masson-Hamperl's argentaffin reaction, diazonium reaction, and the formaldehyde-induced fluorescence histochemical method. These reactions are all due to the presence of serotonin. Recent reports have shown that immunocytochemistry using antisera against serotonin is the most useful and sensitive method for demonstrating this bioactive monoamine (8).

In 1973, Fujita and Kobayashi (6) predicted that EC cells would be found to possess a bioactive peptide or peptides in addition to the amine, serotonin, because it seemed the general rule for entero-endocrine and related cells, which later came to be called paraneurons (5), to

synthesize a peptidic messenger substance or substances, notwithstanding the fact that they may produce amines. Subsequently, motilin-like (19), substance P-like (15, 18), and Leu-enkephalin-like immunoreactivities (1) were reported to be present in the EC cells.

In searching for a bioactive peptide in the EC cell, we found positive immunoreactivity to antisera against enkephalins, especially against Met-enkephalin-Arg⁶-Gly⁷-Leu⁸. Recently, nucleotide sequence analysis of the cloned cDNA has revealed the whole amino acid sequence of an enkephalin-precursor contained in the bovine adrenal medulla. This opioid precursor, called preproenkephalin A, includes four molecules of Met-enkephalin, and one molecule each of Leu-enkephalin, Met-enkephalin-Arg⁶-Phe⁷ (Met-enkephalin-7), and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-enkephalin-8) (16). Although Met-enkephalin and Leu-enkephalin molecules are also contained in the

other two known opioid precursors, ACTH/ β -LPH precursor (14) and preproenkephalin B (9), respectively, Met-enkephalin-8 is a molecule specific for preproenkephalin A. In mammalian adrenomedullary cells and carotid body chief cells, Kobayashi *et al.* (12) suggested the presence of preproenkephalin A by demonstrating the immunoreactivity of the cells to an anti-Met-enkephalin-8 serum. This paper will demonstrate the Met-enkephalin-8-like immunoreactivity, and therefore the probable occurrence of preproenkephalin A in the EC cells of the porcine gut.

MATERIALS AND METHODS

An antiserum (R 0171) against Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-enkephalin-8) was raised in a rabbit using Met-enkephalin-8 conjugated with ascaris protein by glutaraldehyde. Radioimmunoassay studies revealed that the antiserum recognizes mainly the 4-6 sequence of Met-enkephalin-8 and shows no cross-reactivity with α -endorphin, β -endorphin, Met-enkephalin and Leu-enkephalin.

An antiserum against serotonin was raised by Dr Y. Endo and Dr J. Nishiitsutsuji-Uwo (Shionogi Research Laboratories, Shionogi & Co., Osaka, Japan) in a rabbit using serotonin conjugated with bovine serum albumin.

Two adult (body weight, about 90 kg) and three infant pigs (body weight, 5-10 kg), females and castrated males, were used in this study. The adult specimens were obtained from a slaughterhouse, and the infant animals were purchased elsewhere. Various regions of the gastrointestinal tract were immersed in Bouin's fluid for 6-8 h. The tissues were dehydrated and embedded in paraffin. Paraffin sections were cut at 4-5 μ m. A part of the sections was made serially at about 2 μ m.

Dewaxed paraffin sections were subjected to immunostaining by the peroxidase-antiperoxidase (PAP) method of Sternberger (23). The antisera against Met-enkephalin-8 and serotonin were used at a dilution of 1: 5,000 and 1: 8,000, respectively.

The specificity of immunoreactivity was checked by the conventional staining controls according to Sternberger (23). In addition, an absorption test was carried out using antisera pretreated with various synthetic peptides related to Met-enkephalin-8. Antiserum R 0171 was preincubated for 24 h at 4°C with Met-enkephalin, Leu-enkephalin, and Met-enkephalin-8 (10 μ g/ml diluted antiserum).

RESULTS

Clear and selective immunoreactivity to antiserum R 0171 was recognized in dispersed endocrine cells and intramural nerve fibers of the porcine gut. Immunoreactive endocrine cells were most numerous in the duodenum (Fig. 1); there were fewer in the pyloric antrum and still fewer in the lower small intestine. The duodenal immunoreactive cells were numerous in crypts but the cells in the lower small intestine were localized mainly in the epithelium of the villi. They were typical open-type basal-granulated cells with a thickened perikaryal body and a slender process reaching the gut lumen. No immunoreactive cells were found in the oxyntic area of the stomach or the large intestine. Immunoreactive nerve fibers were distributed exclusively in the myenteric and submucous nerve plexuses. The detailed distribution is described elsewhere (Nihei, in preparation).

The immunoreactivity was completely inhibited by preincubation of the antiserum with synthetic Met-enkephalin-8 but was not influenced by pretreatment with synthetic Leu-enkephalin and Met-enkephalin.

On the other hand, the antiserum against serotonin stained numerous endocrine cells throughout the gastrointestinal tract, including the stomach and large intestine.

Observation of the serial thin sections stained alternately by the antisera against Met-enkephalin-8 and serotonin revealed that about 60% of the serotonin cells in the porcine duodenum were also reactive to the anti-Met-enkephalin-8 serum (Fig. 2). Every Met-enkephalin-8-immunoreactive cell showed immunoreactivity to the anti-serotonin serum. In the pyloric antrum and lower small intestine the number of Met-enkephalin-8-immunoreactive cells was approximately 20-40% and 10-15% the number of serotonin cells, respectively.

DISCUSSION

Preproenkephalin A is believed to liberate the amino acid sequences of Met-enkephalin, Leu-enkephalin, Met-enkephalin-7, and Met-enkephalin-8 by proteolytic processing, because these sequences are bound by a pair of basic residues, either Lys-Arg, Lys-Lys, or Arg-Arg, which is known to be the site of cleavage of a large molecule into small ones (16). The sequence of Met-enkephalin is also contained in the ACTH/ β -LPH precursor, and Leu-

Fig. 1 Numerous Met-enkephalin-8-immunoreactive cells are seen in the crypts of porcine duodenum. $\times 250$

Fig. 2 Two serial thin (about $2\text{ }\mu\text{m}$ in thickness) sections from the porcine duodenum. Four Met-enkephalin-8-immunoreactive cells in a coincide with serotonin-immunoreactive cells in b. One serotonin cell (arrow) shows only weak reactivity to anti-Met-enkephalin-8 serum. $\times 1,000$

enkephalin also occurs in the β -neo-endorphin/dynorphin precursor called preproenkephalin B (9, 14). It is reasonable to presume that these two precursors and their end-products are present not only in the brain tissues, but also in the gastrointestinal tract, because ACTH-like immunoreactivity has been recognized in the gut (13, 20) and the dynorphin molecule has been isolated from the porcine gut (24). Accordingly, the antisera against Met-enkephalin and Leu-enkephalin used in the previous studies might have recognized the common sequences contained in or derived from the different precursors. On the other hand, the antiserum R 0171 used in the present study is specific to the Met-enkephalin-8 molecule, because its immunoreactivity was not influenced by preincubation of the antiserum with Met-enkephalin or with Leu-enkephalin. The Met-enkephalin-8-immunoreactivity in the EC cells strongly suggests the presence of preproenkephalin A in the cells.

Besides antiserum R 0171, we also used anti-Leu-enkephalin and anti-Met-enkephalin sera (UCB-Bioproducts, Brussels, Belgium). Although we could confirm that the cells reactive to the antiserum R 0171 were also reactive to these antisera, the anti-Leu-enkephalin serum showed considerable cross-reactivity to Met-enkephalin and Met-enkephalin-8; similarly, the anti-Met-enkephalin serum cross-reacted with Leu-enkephalin. Cytochemical discrimination of Leu- and Met-enkephalin needs more specific antisera.

What bioactive peptides might co-exist with serotonin in EC cells is one of the most important questions for researchers on the GEP (gastro-entero-pancreatic) endocrine system. Pearse and his co-workers (19) reported occurrence of motilin-immunoreactivity in the EC cells. However, this result was contradicted by later immunocytochemical studies, especially using antisera against synthetic motilin (4, 11). Motilin is now believed to be stored in a different type of endocrine cells of a smaller population, called 'Mo cells' (22). Pearse *et al.* (18) and Nilsson *et al.* (15) reported that substance P might be contained in the EC cells. However, pronounced species differences seem to exist in the substance P-immunoreactivity in the EC cells (22). Our preliminary immunocytochemical study using substance P antiserum showed numerous positively stained nerve fibers in the porcine gut, but no immunoreactive endocrine cells.

Alumets *et al.* (1) reported the distribution of

Leu-enkephalin-immunoreactive cells in the gut of various mammals including the pig. They were most numerous in the pyloric antrum and proximal duodenum of the pig. In this species Alumets and associates demonstrated that the Leu-enkephalin-immunoreactive cells coincided with a major population (approximately 70%) of EC cells, which were identified by formaldehyde-induced fluorescence reaction in one section and by electron microscopy in adjacent ultra-thin sections. It is most likely that these Leu-enkephalin cells correspond to our Met-enkephalin-8-immunoreactive cells, because preproenkephalin A includes one molecule of Leu-enkephalin in addition to Met-enkephalin-8.

Now we can consider preproenkephalin A as one of the EC cell's peptides. It must be stressed, however, that major parts of extra-duodenal EC cells do not react to the anti-Met-enkephalin-8 antiserum. These are presumed to contain other unknown peptides. The heterogeneity of EC cells has been suggested on the basis of conspicuous differences in their granule size due to the sites of their distribution both in man (10, 17) and animals (2, 7, 26). Solcia *et al.* (21) have divided the population of EC cells into three types, intestinal, duodenal, and gastric, on the basis of ultrastructural observations of human gastrointestinal argentaffin EC cells. It is reasonable to assume that EC cells include different subtypes corresponding to different peptides. The heterogeneity of EC cells becomes more evident if one considers the cells outside of the GEP endocrine system. In the pulmonary epithelium of human fetuses our research group discovered that part of the serotonin-immunoreactive cells contained gastrin-releasing peptide (GRP) (25). This is a clear indication that EC cells may contain various peptides in addition to with serotonin.

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