Separation of the gut hormone endocrine-cell storage granules of human jejunum using analytical subcellular fractionation

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SUMMARY Analytical subcellular fractionation techniques in combination with specific radio-immunoassays have been used to investigate the properties of the hormone-containing granules in human jejunal biopsy specimens. After gentle homogenisation, for all hormones except VIP, over 90% of the immunoreactivity was released in intact granules into the post-nuclear supernatant. Approximately 50% of the VIP immunoreactivity sedimented with the low speed pellet, probably reflecting the presence of this peptide in nerve fibres. The following hormone granules (equilibrium densities between parentheses) were separated by isopycnic centrifugation on the sucrose density gradients: VIP (1.17), motilin (1.20), and secretin (1.24). Gastrin and GIP granules were not resolved, both having the same modal density of 1.22 g/cm.³ This combination of procedures thus provides a new quantitative technique for studying the properties of the gastrointestinal hormone-containing granules, and should therefore be of value in investigating pathophysiological problems in a variety of gastrointestinal diseases.

With the development of accurate methods for measurement of gut hormones it has become apparent that there are characteristic abnormalities of the plasma gut hormone profile associated with various diseases of the human gastrointestinal tract.¹ Very little information, however, is as yet available about such fundamental aspects as the synthesis, storage, and mode of secretion of these hormones at the cellular level. Using the combined techniques of specific immunocytochemistry and electron microscopy it is now possible to identify the cells of origin of the gut hormones and recognise the hormone storage granules characteristic of each endocrine cell-type.² However, while such studies give information as to the relative size and appearance of the granules, quantitative studies are not possible.

It has recently been shown that analytical subcellular fractionation procedures permit the quantitative assessment of the properties of individual subcellular organelles both in healthy and diseased tissues.³ The development of a suitable single-step fractionation technique, coupled with highly sensi-

tive organelle-marker enzyme assays, has further allowed analytical fractionation techniques to be applied successfully to human biopsies, even in the small quantities of tissue obtained by jejunal biopsy.⁴ The purpose of this present study is to evaluate the use of these fractionation procedures to investigate the physical properties of gut hormone storage granules.

Methods

ANALYTICAL SUBCELLULAR FRACTIONATION Jejunal biopsies were obtained with a Crosby capsule from patients undergoing routine investigation for possible gastrointestinal disease. The tissues used in this study were found to be entirely normal on histochemical examination. Approximately 5 mg (wet weight) of biopsy material was collected in 3 ml of ice cold sucrose solution (0.3 mol/l) containing 1 mmol/l Na₂ EDTA pH 7.2 and 20 mol/l ethanol (SVE medium). The tissue was disrupted with 10 strokes of a loose-fitting (type A) pestle in a small Dounce homogeniser (Kontes Glass Co Vineland, NJ) and centrifuged at 800 g for 10 minutes. The pellet was resuspended in a further 2 ml of SVE medium with three strokes of the pestle and recentrifuged.

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These post-nuclear supernatants (PNS fraction) were combined and the low speed pellet comprising nuclei, large brush-border fragments, and undisturbed cells (nuclear fraction) was resuspended in 2 ml of SVE medium with a tight fitting (type B) pestle. Approximately 3.5 ml of PNS fraction was layered onto 28 ml of a sucrose density gradient extending linearly with respect to volume from a density of 1.05 to 1.28, resting on a cushion of density 1.32 in an automatic zonal rotor.⁴ All solutions contained 1 mmol/l Na₂ EDTA pH 7.2 and 20 mmol/l ethanol. The rotor was accelerated to 35000 rev/min and run for 35 minutes with an integrated force of 3.3×10^{10} rad² sec⁻¹. The rotor was then slowed to 8000 rev/min for automatic unloading and collection of the gradient fractions. Some 15 fractions were collected into tared tubes, thoroughly mixed, reweighed and their density determined indirectly with an Abbe refractometer.

Aliquots of the gradient fraction were mixed with an equal volume of 0.2 mol/l HCl to minimise proteolytic degradation of the hormones and kept deep frozen at -20°C until radioimmunoassay of hormone content. Freezing and thawing of the gradient samples in 0.1 mol/l HCl after fractionation released the granule-bound hormones for radioimmunoassay.

RADIOIMMUNOASSAYS

Full details of radioimmunoassays for the gut hormones motilin,⁵ gastrin,⁶ secretin,⁷ gastric inhibitory peptide (GIP),⁸ and vasoactive intestinal peptide (VIP)⁹ have been reported elsewhere. All assays, except that for gastrin, were developed to the pure natural porcine hormones. In the case of gastrin, synthetic human gastrin I (ICI) was used. Antibodies to each hormone were raised in rabbits with the peptide coupled to bovine serum albumin by carbodiimide condensation. All antisera were tested for specificity by addition of up to 2 nmol per assay tube of all available gut hormones. In no case was any significant degree of cross-reactivity observed. The antibody dilutions routinely used and the assay sensitivities achieved are summarised in Table 1. VIP, secretin, motilin, and GIP were all radioactively labelled with the lactoperoxidase

technique,^{10,8} whereas a modification of the chloramine T method was used for gastrin.¹¹

Because the quantities of tissue used in this study were extremely small all assays were specially modified and optimised for small sample addition using a total reaction volume of 200 µl. Each centrifugation gradient fraction was assayed at three separate dilutions (1/200, 1/20, and 1/4).

Protein content of the homogenates was assayed by the technique of Lowry *et al.*¹² Protein in the subcellular fraction was assayed by the fluorimetric technique of Hiraoka and Glick.¹³ Bovine serum albumin (Sigma, London) was used as a standard.

Results

In all cases the immunoreactive hormone content of the sucrose density gradient fractions diluted in parallel fashion to the respective standard curves. Sucrose at concentrations found in the density gradient fractions was found to have no effect on the assays.

Table 2 *Specific activity of hormones in total jejunal biopsy homogenates and percentage of hormonal immunoreactivity released by homogenisation procedure*

	Specific activity	Percentage hormonal immunoreactivity in PNS
Motilin	4.0 ± 0.9	94.6 ± 1.5
Secretin	3.2 ± 0.8	97.0 ± 0.8
VIP	1.9 ± 0.7	56.6 ± 1.0
GIP	2.4 ± 0.6	98.4 ± 0.7
Gastrin	2.7 ± 0.5	98.9 ± 0.4

Protein content of the biopsies was 2.7 ± 0.6 mg (mean ± SEM) with 70.0 ± 1.6% being recovered in the PNS fraction.

Table 2 shows the hormone concentrations in the biopsy extracts and the amount of immunoreactivity in the PNS fraction as a percentage of the total activity in the tissue homogenate. Whereas most of the hormonal immunoreactivity in the cases of gastrin, GIP, motilin, and secretin was recovered in the PNS fraction, considerable quantities of VIP immunoreactivity remained in the low speed pellet (nuclear fraction).

The Figure shows frequency-density histograms for the individual gut hormones and also for the protein content of the biopsies. In the case of VIP and secretin, each had distinct particulate components of immunoreactivity of modal density 1.17 and 1.24 respectively with little or no immunoreactivity detectable in the soluble region of the gradient. The GIP and gastrin distribution patterns were similar, each having a small quantity of a soluble component and a well-defined granule component of modal density 1.22. Motilin had a distinct

Table 1 *Antibody dilutions used and sensitivities achieved for the individual radioimmunoassays*

Antiserum	Final dilution (× 10 ⁴)	Assay sensitivity (fmol)
Gastrin	18	0.6
Secretin	28	0.4
Motilin	24	0.4
VIP	32	0.4
GIP	9.6	1.0

Figure Isopycnic centrifugation of 8000g-min supernatants from five jejunal biopsy homogenates. Graph shows frequency-density histograms for gastrointestinal hormones and for protein. Frequency (mean $\pm$ SEM) is defined as the fraction of total recovered activity present in the subcellular fraction divided by the density span covered. The cross-hatched area represents, over an arbitrary abscissa interval, the activity remaining in the sample layer and is presumed to reflect soluble activity.

particulate component at a density of 1.20 with some activity remaining in the sample layer.

Discussion

This study is the first to report the separation of the various hormone-containing storage granules of the endocrine cells of the human gastrointestinal tract by zonal sucrose density gradient centrifugation. Both secretin¹⁴ and gastrin-like immunoreactivity¹⁵ have, however, previously been shown to be present in particulate structures by subcellular fractionation procedures. It would seem clear from the data presented here that a considerable difference exists in the density of the different hormone granule of the human jejunum and that for the most part they can be separated with ease. Only GIP and gastrin have

granules of such similar density as to prevent clear separation under these conditions.

The conditions used here for the homogenisation of the biopsies and the subsequent intracellular fractionation appear to have been highly successful. In all cases, except for VIP, over 90% of the hormonal immunoreactivity was recovered from the post-nuclear supernatant fraction with little or no immunoreactivity being detectable in the low speed pellet, thus indicating that the cells were adequately disrupted to allow release of the hormone granules. Similarly, very little immunoreactivity was recovered in the low density 'soluble' region of the gradients, indicating that conditions were not so harsh as to cause significant disruption of the released granules. The presence of considerable quantities of VIP-like immunoreactivity in the low speed pellet could be explained by the fact that VIP occurs for the most part in fine nerve fibres and plexi both in the gut wall and mucosa.¹⁶⁻¹⁸ Thus the homogenisation conditions may not have been completely effective in the disruption of all the VIP-containing tissue. The VIP immunoreactivity seen here in the low speed pellets may thus reflect that present in nerve terminals, which would be expected to escape disruption under the conditions chosen and so would sediment with the larger cell debris.

There appears to be little correlation between the hormone granule densities reported here and the sizes of the granules determined by electron microscopy. Of those examined here, GIP granules in the human are the largest (350 nm)³ and intestinal gastrin granules the smallest (180–190 nm)¹⁹ whereas these two granule types have similar centrifugation densities (1.22). Similarly secretin granules have a diameter of 250 nm² and high modal density of 1.24 and motilin a diameter of 180–190 nm²⁰ and density 1.20. However, in view of the fact that granule size depends on the fixation procedure employed, this lack of correlation is not surprising.

The data presented here are derived from jejunal biopsies taken from fasting subjects but clearly these techniques are readily applicable to other situations and other tissues. Thus it should be possible to study the dynamic changes in granule density in response to physiological stimuli. Similarly, possible granule alteration occurring in diseased states of the gut can also be readily studied. In view of the existence of different molecular forms of some of the gut hormones, in particular gastrin²¹ and enteroglucagon,²² these techniques have provided a unique opportunity to assess whether they are stored as single or multiple granules.

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