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Full Papers

Intracellular topography of immunoreactive gastrin demonstrated using electron immunocytochemistry

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Summary. Gastrin (G)-producing cells from the mammalian gastric antrum have been investigated using computer-assisted morphometry and a novel double colloidal gold-labeled-immunoglobulin electron immunocytochemical procedure. Correlation analysis of human antral G-cells indicates ($p < 0.001$) that a single population of granules exists with small (160 nm) electron-dense and large (240 nm) electron-lucent forms representing the extremes. Non-crossreacting region-specific antisera have been used to visualize G-17 and G-34 (progastrin) to the small electron-dense granules and G-17 to the other intermediate forms. From the results we propose a topographic segregation of immunoreactive gastrins within 2 apparently distinct granule subclasses and suggest that this may represent the pathway of granule maturation.

Introduction

Post-translational cleavage of precursor to smaller molecular forms of bioactive peptides has been established in recent years[18,19,31]. The majority of endocrine cell types exhibit a range of secretory granule morphology[12] which could reflect storage of different molecular structures. One such example is the mammalian antral G-cell which has been claimed to contain 2 main granule populations, 1 large electron-lucent and 1 small dense-cored[9,13,32]. The observed variations in granule ratio has been suggested by different groups to represent the functional state of the secretory cycle[8,13], the effect of fixation[20,21], the co-existence of 2 unrelated products[16] or of 2 molecular forms of gastrin[13,36] within the same cell. Recent advances in electron immunocytochemistry particularly the introduction of region-specific antisera[17] and immunogold procedures[5,8,28–30,37] have allowed the ultrastructural topographic distribution of separate regions of peptide molecules to be visualized[26,33]. In this study we have combined newly developed electron immunocytochemical procedures with computer-assisted morphometry in order to characterize the granule population in mammalian antral G-cells.

Materials and methods

Fresh antral mucosa was obtained from adult human subjects ($n = 6$) at surgery, and from adult cats ($n = 10$) following sodium pentobarbitone anesthesia ('Euthatal'; 200 mg/ml, 1 ml/kg). The tissue was processed for conventional electron microscopy or for electron immunocytochemistry[37]. Electron micrographs of cells fulfilling the criteria acknowledged for the classification of G-cell granules[7] were analyzed

Characteristics of antisera used for electron immunocytochemistry

Antiserum	Raised in	Hapten	Immunocytochem. specificity	Optimal dilution	Absorption controls (nmoles/ml diluted antiserum)* G_{1-34}	G_{1-15}	G_{17-34}	CCK-8	$ACTH_{1-39}$	$FMRF \cdot NH_2$
655	Rabbit	G_{27-34}	Carboxy term	1:4000	1	-	0.1	0.1	10 (−)	20 (−)
687	Guinea-pig	G_{17-34}	Amino term. G-17	1:2000	20 (−)	-	1	10 (−)	-	-
819**	Guinea-pig	G_{1-15}	Amino term. G-34	1:5000	1	1	-	-	ND	ND
800	Rabbit	G_{1-15}	Amino term. G-34	1:4000	1	1	-	-	-	-

*Amount of peptide required to partially abolish immunostaining by diluted antiserum.-, Not absorbed at 30 nmoles/ml; 10 (−), partially absorbed at 10 nmoles/ml but not fully absorbed at 30 nmoles/ml; ND, test not performed.**Gly-Lys-gastrin (synthetic).

using an IBAS II computerized image analyser as described elsewhere[14]. Granule size (diameter: nm, intramembranal area: nm^2), density (monochromatic grey value: 0-255 grey levels, black to white) and numerical frequency (granule number per cell field) were measured for each type. Three-dimensional representations of the data obtained were displayed and interpreted using statistical packages incorporating correlation analysis and paired Student's t-test. A new double immunogold staining technique[34], applied at the ultrastructural level and based on a modification of the colloidal gold-labeled immunoglobulin procedure[5,10,37] was used in concert with non-crossreacting region-specific antisera recognizing the amino- or carboxy-terminal of gastrin-17 or the amino-terminal of gastrin-34, the characteristics of which are presented in the table. It is important to note that the antiserum raised to N-terminal gastrin (G)-17 (687) does not significantly cross-react with G-34 in this system, presumably because the antigenic sequence is masked by tertiary folding[4].

Briefly, ultrathin sections of non-osmicated tissue mounted on nickel grids, were 'etched' with 10% hydrogen peroxide, incubated with normal goat serum (1:30 dilution) followed, for example, by a mixture of rabbit anti-gastrin-34 and guinea-pig anti-gastrin-17 diluted to optimal titres for 1 h at room temperature. The sections were rinsed with 0.05 M Tris-buffered saline (pH 7.3) containing 0.2% bovine serum albumin (Sigma Type V), before incubation with a mixture of 40-nm gold-labeled goat anti-rabbit IgG and 20-nm gold-labeled goat anti-guinea-pig IgG. Homogeneous populations of 20- or 40-nm gold particles (non-overlapping size ranges) were prepared by the controlled chemical reduction of chloroauric acid with sodium citrate and these particles were adsorbed with goat gamma-globulins following well documented procedures[4,5,7,14,25,26,33,34,37]. Absorption controls with the respective antigens, antigenic fragments and homologous molecular species were carried out as indicated in the table. In addition, immunogold staining procedures employing 1 primary antiserum

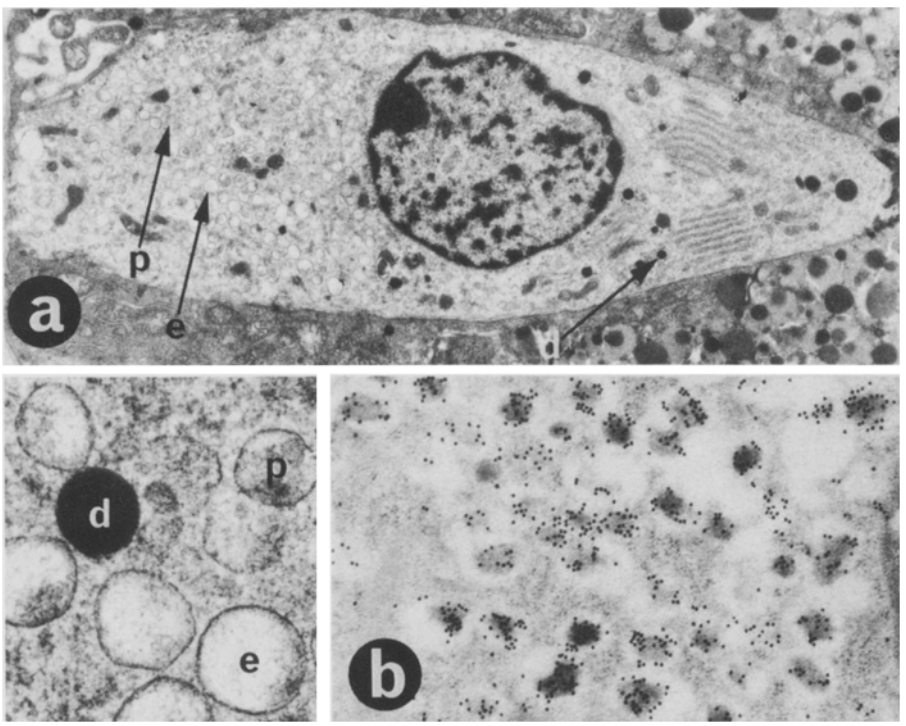


Figure 1. *a* Electron micrograph of human antral G-cell, secretory granules arrowed. d, Electron-dense (160 nm), e, electron-lucent (240 nm), p, pale-cored intermediate forms (200–220 nm). Magnification ×5980. Inset exhibits a range of secretory granule types from human antral G-cell, magnification ×44,000. The human dense-cored G-cell granules closely resemble those in the D_1 cell type[22,24]. *b* Ultrathin section of cat antral G-cell immunostained with anti-C-terminal gastrin serum. Antigenic sites are visualized by labeling with 20 nm gold-adsorbed immunoglobulins (arrowed). Magnification ×30,000.

alone, or a mixture of both primary antisera followed by 1 gold-labeled antiserum were run simultaneously to check for reaction specificity. The sections were counterstained with uranyl and lead salts and viewed with a Zeiss 10CR transmission electron microscope.

Results

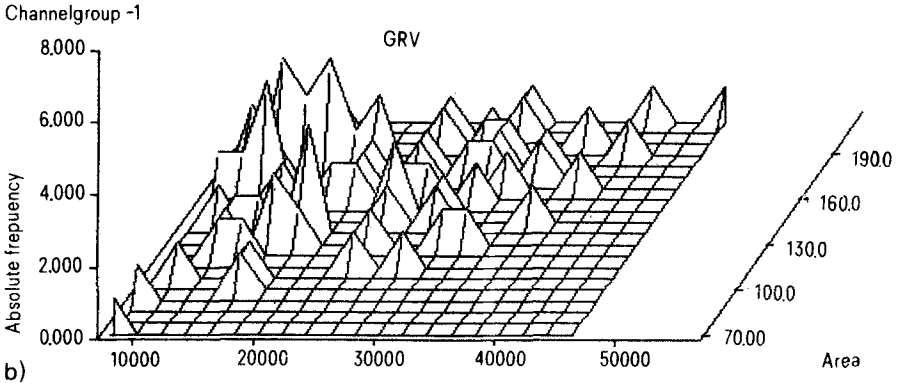
Our electron microscope observations corroborate previous findings[7,32] that the antral G-cells of cat and man contain at least 2 morphologically distinct types of secretory granules (large electron-lucent: 220–240 nm in man, 310–330 nm in cat; and small electron-dense: 150–170 nm in man, 220–240 nm in cat) with a range of intermediate forms (pale-cored: 200–220 nm in man, 280–300 nm in cat) (fig. 1, a), the latter 2 forms of which were found to be immunoreactive to C-terminal gastrin antisera (fig. 1, b). Large electron-lucent granules were not immunostained using C-terminally-directed anti-gastrin. This finding is in agreement with a recent report by Håkanson and co-workers[13]. The occurrence of a single granule population in the antral G-cells of both species, with electron-lucent and dense-cored representing the extreme forms was demonstrated by morphometric analysis of granule area correlated with granule density (grey value) from calibrated electron micrographs (fig. 2, a). The results indicated a continuous granule population for each cell with profiles ranging from small (160 nm D_{max}) electron-dense to large (240 nm D_{max}) electron-lucent with many intermediate (pale-cored) forms. This was confirmed by correlation analysis of the bivariate-normal data. The sample correlation coefficient (r) obtained was 0.39 (df = 78) indicating a statistical degree of significance (p) at the population level between paired area and density of less than 0.001 (fig. 2, b).

Application of the immunogold staining procedure using antisera directed to the amino-terminal of gastrin-34 revealed antigenic sites localized exclusively to the small electron-dense granules (fig. 3, a) which closely resemble those of the D_1-cell type[22,24] found throughout the gut and pancreas, and gastrin-17-like immunoreactivity visualized by the use of N- and C-terminally-directed antisera also to the electron-dense granules but predominantly to the pale-cored secretory granules. Profiles of the large electron-lucent secretory granules were not significantly immunostained. Simultaneous ultrastructural localization of both immunocytochemically identified gastrin terminals with region-specific antisera using the double immunogold staining procedure confirmed this pattern of distribution and demonstrated the co-existence of 2 separate regions of the gastrin molecule in the dense-cored secretory granules (fig. 3, b and c).

Discussion

For more than a decade the mechanisms of gastrin storage and release from the mammalian gastric antrum have been theorized upon[7,8,13,20,23,35]. Forssmann and Orci[8] proposed that bioactive gastrin release was mediated by intracytoplasmic molecular dispersion, the electron-lucent vesicles merely representing 'empty' secretory organelles. Mortenson and co-workers[21] demonstrated that the duration and pH of fixation largely determined the morphological appearance of the G-cell granule population. Biochemi-

a)



b)

Correlation analysis for channels – 1

Paired data of single group: counts 79, in range 79.

Parameter	Mean	SD
Area	21112.3	8909.49
GRV	160.871	29.8682

Coefficient of correlation: 0.39

There is a significant correlation at 99.95% confidence level between the 2 parameters mentioned. (Test value 3.69, degrees of freedom 77)

Figure 2. *a* Three-dimensional representation of grey value (GRV), area (nm^2) and absolute frequency of secretory granules for the human G-cell depicted in figure 1, a. *b* Correlation analysis of above data indicating a significant correlation between grey value and area at 99.95% confidence level.

cal analysis has established that gastrin-17, the C-terminal heptadecapeptide of gastrin-34 is the predominant molecular form in extracts of mammalian antra, whereas much lower amounts of gastrin-34 are extracted[6,11,27]. Although several groups hypothesized on the proteolytic cleavage of gastrin-34 to gastrin-17 in individual gastrin cells and proposed that the dense-cored secretory granules were probably immature, containing a precursor molecule which is subsequently split to yield the active hormone[13,15,20,36], the sub-cellular distribution of both molecular forms has not been demonstrated. Similar reports on the biosynthesis of other peptides[24,33] have suggested to some authors that D_1-type granules may contain precursor molecules which are subsequently processed to active forms, therefore supporting the view that a common undifferentiated endocrine granule type exists at least in mammals. By morphometric analysis of antral G-cells we have characterized the secretory granules and conclude that the dense-cored and electron-lucent granules represent extreme forms of a single population. Electron immunocytochemistry using a double immunogold labeling procedure in combination with region-specific antisera demonstrates topographic segregation of gastrin-like immunoreactivities with immunoreactive gastrin-34 apparently present only in the dense-cored secretory granules. If proteolytic cleavage of gastrin-34 occurs in the dense-cored granule it is conceivable that the N-terminal product may be bound in the tissue such that the tertiary structure is not available to the applied antibodies. Perhaps more likely is the elution of immunoreactive N-terminal G_{1-15} during tissue processing. In either case it is possible that post-translational cleavage of a pro-form of gastrin (G-34) to the bioactive hormone[36] is reflected by the morphological change in secretory granule structure from dense-cored to electron-lucent. Similarly, as gastrin-17 immunoreactive material was not observed in the large electron-lucent granules this may merely represent elution of the granule contents during processing. Alternatively, the absence of gastrin-like immunoreactive material within the electron-lucent granules may indicate that these molecules have been released into the cytoplasm. The absence of immunostaining in the cytoplasm of the gastrin cells would seem unsupportive of this, the molecular dispersion theory of gastrin release[7,8] unless a smaller molecular form of gastrin, unreactive to the region-

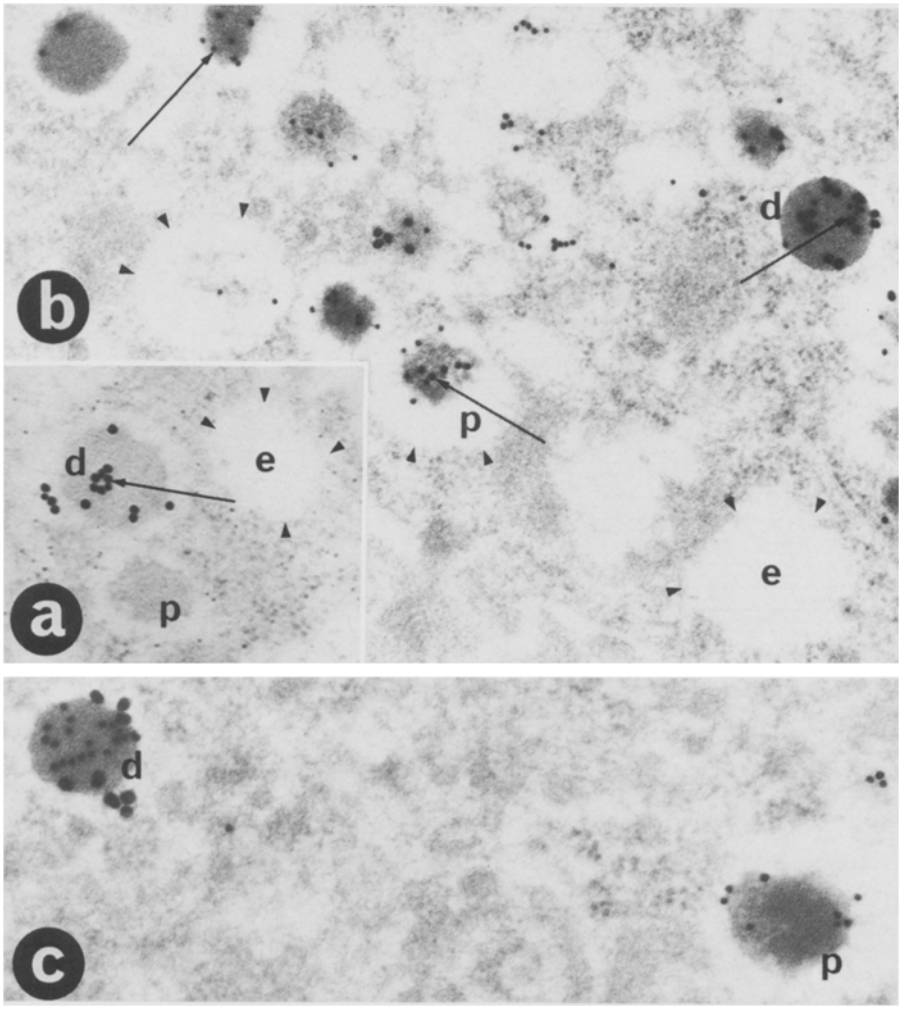


Figure 3. Electron micrographs of ultrathin sections of human antral G-cells following application of single (a) and double (b, c) immunogold staining procedures. Antisera reactive to N-terminal of G-34 are visualized using 20-nm (a) or 40-nm (b, c) gold particles and those reactive to N-terminal of G-17 are visualized using 20-nm gold (b, c; small arrows). Only the dense-cored secretory granules are immunostained with 40-nm gold, whereas 20-nm gold particles label each granule type. As non-osmicated tissue has to be used for the electron immunocytochemical localisation of gastrin immunoreactive material to date the membranes of the electron-lucent granules are indistinct. For this reason the position of the granule membrane has been indicated by arrowheads. Magnifications: *a* ×65,000; *b* ×53,600; *c* ×74,000.

specific antisera applied, co-exists in the secretory granules as the ultimate bioactive molecule.

We have thus been able to demonstrate a topographic segregation of immunoreactive gastrins within 2 apparently distinct granule sub-classes using a combination of region-specific antisera with electron immunocytochemistry. Furthermore, computerized morphometry indicated that these 2 separate sub-classes of secretory granules represent 2 extreme forms of a unimodal granule population. Our investigations are suggestive of a pathway of granule maturation and thus possibly of intragranular gastrin biosynthesis.

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