Introduction

Chromogranin A is the main component of the family of highly acidic proteins that are co-stored and co-released with the catecholamines from adrenomedullary chromaffin cells (Winkler et al., 1986; Blaschko et al., 1967). Recent investigations have shown that chromogranin A is co-stored in the different cells of the endocrine pancreas. Immunohistochemical studies of bovine endocrine pancreas have demonstrated that chromogranin A is present in cells producing insulin (B-cells), glucagon (A-cells), and somatostatin (D-cells) and in a few cells containing pancreatic polypeptide (PP-cells) (Yoshie et al., 1987; Ehrhart et al., 1986). Chromogranin A is co-stored in the same secretory granules as the established hormones (Ehrhart et al., 1986).

A hormonal role for chromogranin A has yet to be established. Within the adrenomedullary chromaffin secretory granules, chromogranin A is presumably involved in osmotic pressure regulation (Helle et al., 1985) and in the binding of Ca²⁺ (Reiffen and Gratzl, 1986a, 1986b; Bulenda and Gratzl, 1985). More recently, chromogranin A has been shown to contain a sequence of amino acid residues (Eiden, 1987; Huttner and Benedum, 1987; Benedum et al., 1986; Iacangelo et al., 1986) that appears homologous to pancreastatin, which inhibits glucose-stimulated insulin secretion in rat pancreas (Tatemoto et al., 1986). Therefore, a role for chromogranin A in the regulation of hormone secretion is likely.

For further elucidation of the functional aspects of the pancreatic chromogranin A, it is essential to quantify this protein relative to the well-established hormones. In the present study we report on the amounts of chromogranin A relative to the insulin content of the pancreatic islet and on the localization of chromogranin A primarily to the insulin-storing B-cells.

Materials and Methods

Immunohistochemistry. The immunohistochemical stainings were performed in serial semi-thin sections cut at 0.25 μm and 0.5 μm from tissue blocks that had been freeze-dried, fixed by vapor-phase β-formaldehyde, and embedded in epoxy resin (Araldite). Details of the methods applied have been published previously (Ehrhart et al., 1986; Grube and Kusumoto, 1986). The antisera used included anti-insulin (A 564; Dakopatts, Hamburg, FRG) diluted 1:2000, anti-glucagon (A 565; Dakopatts) diluted 1:3000, anti-somatostatin (kindly provided by Dr. Etzrodt, Ulm, FRG) diluted 1:4000, anti-bovine pancreatic polypeptide (bPP; from Dr. Chance, Indianapolis, IN) diluted 1:8000, and anti-chromogranin A (CGA; provided by Dr. D. Aunis, Strasbourg, France) diluted 1:16,000. The serial semi-thin sections were sequentially immunostained for chromogranin A and the established hormones by the peroxidase-antiperoxidase (PAP) technique of Sternberger (1986).

Quantitative Immunocytochemistry. Taking into account the basic prerequisites for quantification in immunocytochemistry (see Sternberger, 1986), we developed a technique for quantitative determinations of optical densities in immunocytochemistry-stained semi-thin sections by computer-assisted analyses. The principles of the present method have been pub-

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lished previously (Grube and Kusumoto, 1986), and details will be published elsewhere (Grube et al., in preparation). Briefly for quantification we found the most useful sections to be semi-thin sections, cut at 0.25 μm, which were immunostained by highly diluted antisera using a standardized immunohistochemical protocol. Optical density measurements were performed with the "Interactive Image Analysis System IBAS" (Zeiss-Kontron, FRG) equipped with a Photomicroscope II (Zeiss, FRG) and a video camera. The regions or cells to be measured were entered into the system at a final magnification of 1:1600. The corresponding images were processed by a 15-step measuring program; optical densities were given as differential histograms in arbitrary units (0 = black, 255 = white; see Figure 2). The densities of chromogranin A immunoreactivities were determined in 174 endocrine cells which, in appropriately immunostained adjacent semi-thin sections, had been identified as 103 B-cells, 47 A-cells, and 24 D-cells.

Immunohistochemical Analyses. Fresh pancreatic tissues from calves were obtained at the local slaughterhouse in Ulm. The tissues were placed in an ice-cold medium (20 mM Mops, 0.5 mM EGTA, 340 mM sucrose, 1 mM PMSF, pH 7.3) and transported to the laboratory. Chromogranin extraction was carried out in acidic medium to prevent proteolysis. The tissue was homogenized at approximately pH 1 (10 g tissue in 5 ml medium + 20 ml 1 N HCl) using an Ultra-Turrax (Janke and Kunkel KG; Stetten, FRG) and was centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was used for SDS-PAGE.

Soluble proteins of chromaffin granules were isolated as described previously (Reiffen and Gratzl, 1986a). For quantification of pancreatic chromogranin A, chromaffin vesicle content was used as reference; 40% of its protein is chromogranin A (see Winkler and Westhead, 1980). A calibration curve was constructed using dilutions containing 1.2 μg/ml-15 μg/ml of protein. The reference probes and the pancreatic extracts were dissolved in extraction medium containing 5% mercaptoethanol, 2% SDS, 0.001% bromphenol blue and 10% sucrose in 62.5 mM Tris-HCl, pH 6.8. Twenty μl of each sample were subjected to SDS-PAGE (Laemmli, 1970) in 11% acrylamide gels. Immunoblotting was carried out following the method of Burtonette (1981). The proteins were transferred to nitrocellulose (Schleicher and Schüll; Dassel, FRG) in the electrode buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, at 15 V for 16-18 hr. The nitrocellulose sheets were incubated in TBS-Tween (150 mM NaCl, 50 mM Tris, 0.05% Tween-20, pH 7.6) containing 2.5% lipid-free instant milk instead of 5% BSA as blocking reagent, four times for 5 min at room temperature, and were washed for 5 min in TBS-Tween containing 0.5% instant milk. The sheets were then incubated overnight at 4°C with the rabbit anti-bovine chromogranin A antiserum diluted 1:5000 in TBS-Tween. The antiserum, characterized in a previous investigation (Ehrhart et al., 1986), was kindly provided by M.F. Bader and D. Aunis (Centre de Neurochimie, Strasbourg). The nitrocellulose was washed four times for 15 min in TBS-Tween and was then immersed in TBS-Tween containing 1 μCi/ml of iodinated protein A for 1 hr (prepared according to Greenwood et al., 1963, using 125I). After washing in TBS-Tween three times for 1 hr, the nitrocellulose was dried and exposed to Kodak X-Omat AR film overnight. For quantification, the labeled bands were cut out of the nitrocellulose and counted for radioactivity in a gamma counter. The values obtained for the pancreatic extracts were compared with the calibration curve constructed using bovine chromaffin vesicle contents.

Insulin extraction was carried out in acidified ethanol (Romans et al., 1940): 5 g of pancreatic tissue were homogenized in 50 ml of acid/alcohol solution (80% ethanol, 1.5% conc. HCl (v/v), kept for 1 hr on ice, sonicated for 5 min, and centrifuged for 30 min at 100,000 × g for 30 min at 4°C. The supernatant was used for SDS-PAGE.

Results
Confirming our previous findings (Yoshie et al., 1987; Ehrhart et al., 1986), chromogranin A immunoreactivities were regularly found in B-, A-, and D-cells, whereas only a minority of PP-cells showed less dense immunostaining. In sections immunostained by highly diluted chromogranin A antiserum (1:16,000), it was obvious that most of the B-cells were heavily immunoreactive and that A-cells

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Figure 1. Three serial semi-thin (0.25 μm) sections through a bovine pancreatic islet. Sections were immunostained for chromogranin A (A), glucagon (B), and insulin (C). All glucagon and insulin cells contain chromogranin A immunoreactivities. Glucagon cells (arrows in A and B) are less densely stained than insulin cells. Phase-contrast microscopy. Original magnification × 495. Bar = 30 μm.
Figure 2. Computer-assisted analyses of optical density of chromogranin A immunoreactivities in bovine pancreatic insulin and glucagon cells. Two serial semi-thin (0.25 µm) sections were immunostained for chromogranin A (A) and for insulin (B). The histogram in (C) corresponds to the whole visual field as shown in (A). The histogram in (D) depicts the optical densities in non-immunoreactive areas (= background staining). Optical densities were measured in four endocrine cells, indicated by 1, 2, 3, and 4 (A). Cells 1, 2, and 3 correspond to insulin cells, as shown in (B); cell 4 is a glucagon cell, as identified in an adjacent semi-thin section immunostained for glucagon (not shown). Among the insulin cells, the densities of chromogranin A immunoreactivities are heterogeneous. (E) Histogram of cell 1, mean value of the optical density 118 (arbitrary units). (F) Histogram of cell 2, mean value 126. (G) Histogram of cell 3, mean value 145. (H) Histogram of cell 4, mean value 149 (A, B): brightfield illumination. Original magnification x 1125. Bar = 10 µm.

were less densely stained (Figure 1). Likewise, D-cells were faintly stained (not shown).

At higher magnifications (Figures 2A and 2B), B-cells turned out to be heterogeneous in their densities of chromogranin A immunoreactivities (Figures 2E–2G, Figure 3). Chromogranin A immunoreactivities in A- and D-cells were weaker and more uniform (Figures 2A and 2H, Figure 3). Therefore, as observed immunohistochemically and quantitatively, chromogranin A in the pancreas is mainly localized to B-cells.

With the antibody raised against bovine chromogranin A, a strong reaction was obtained in the immunoblots. The main immunoreactive bands of the adrenomedullary vesicle content and
of pancreatic extracts moved identically. Both have an apparent molecular weight of 74 KDa. In the adrenal medulla, a band of about 60 KDa is also present. This band is not prominent in the pancreas (Figure 4).

For quantification, nitrocellulose strips containing the two major chromogranin A bands (74 KDa and 60 KDa) were cut out (Figure 5) and counted. The radioactive labeling was logarithmically proportional to the bovine chromaffin granule protein, in the range of 2.5 ng protein/ml to 15 ng protein/ml (Figure 2).

Using this standard curve, 96.7 ng chromogranin A/mg protein (± 24.1; n = 12) was found in the pancreatic extracts. Per gram wet weight, the pancreas contained 3.2 μg chromogranin A (± 1.3 μg; n = 12) and 918 μg insulin (± 65.9 μg; n = 12). Therefore, the bovine pancreas contained 3.5 μg chromogranin A per mg of insulin (Table 1).

Chromogranin A has an apparent molecular weight of 74 KDa in SDS-PAGE; however, an actual molecular weight of 48 KDa has been obtained from sequence analyses of the cDNA encoding bovine chromogranin A (Benedum et al., 1986; Iacangelo et al., 1986). Therefore, on the molar level the endocrine pancreas contains 460 μmol chromogranin A per mol insulin.

Discussion

Our analysis of the chromogranin A immunoreactivities of pancreatic endocrine cells indicates that chromogranin A is mainly localized in the insulin-containing B-cells. Taking into account that about 70–80% of the islet cells in bovine pancreas are accounted for by B-cells (Falkmer et al., 1984), it seems reasonable to assume that most of the pancreatic chromogranin A is co-stored with insulin in the secretory granules of the B-cells. Our quantifications indicate a ratio of chromogranin A:insulin of 0.35% by weight for the whole tissue, i.e., the B-cells account for about 3.0 μg chromogranin A per mg of stored insulin. From the work of Hutton (1984), it is known that the secretory granules in rat B-cells contain about 500 μg insulin per mg of protein, suggesting a chromogranin A concentration of 0.15% by weight of granule protein. Since rat insulin-containing secretory granules contain about 46 nmol Ca²⁺ per mg of protein (Anderson et al., 1982), this would give a ratio of Ca²⁺:chromogranin A of 33 μmol/mg. Such a ratio is more than
100-fold greater than the maximal binding of $\text{Ca}^{2+}$ to adrenomedullary chromogranin A (Reiffen and Gratzl, 1986a, 1986b), which indicates that calcium binding proteins other than chromogranin A may also be present in the periphery of the protein core of the insulin-storing granules to account for the peripheral concentration of $\text{Ca}^{2+}$ (Ehrhart et al., 1986; Lenzen and Klöppel, 1984; Ravazzola et al., 1976).

The glandular content of insulin presently obtained (918 µg per g wet weight of pancreatic tissue) is in accordance with the value reported by Burgermeister et al. (1975). The islets represent about 5% of the total tissue weight (Bonner-Weir and Like, 1980). Consequently, the average concentration of insulin in the islet would be approximately 3 mmol/liter. Analogously, the content of chromogranin A (3.2 µg per g wet weight; Table 1) would correspond to 1.4 µmol/liter, assuming a $\text{MW}$ of 48 KD (Benedum et al., 1986; Iacangelo et al., 1986). Tatetomo et al. (1986) reported on the inhibitory effects of pancreastatin on glucose-induced insulin secretion at peptide concentrations of 10 nmol/liter. Hence, if no more than 1% of pancreatic chromogranin A was released, processed to yield its pancreastatin-like sequence, and distributed throughout the whole islet tissue, its concentration would be in the range reported to inhibit insulin secretion.

With the present anti-bovine chromogranin A serum, the immunoreactivity was associated with the 74 KD molecular form of pancreatic chromogranin A (see Results). Other groups have found chromogranin A-immunoreactive proteins of about 20 KD in extracts of bovine and rat pancreatic tissue (Hutton et al., 1985, 1987a, 1987b; Nolan et al., 1985), with little or no unprocessed forms present. However, when proteolysis is avoided by addition of an inhibitor of proteolysis and homogenization in acid medium (see Methods), the unprocessed 74 KD chromogranin A form dominates. Synthesis and storage of chromogranin A in its high molecular weight form (74 KD) by the pancreatic endocrine cells have also been found with another chromogranin A antiserum (Yoshie et al., 1987). In the latter study, possible proteolytic breakdown by endogenous proteases was avoided by immediate heating of the extracts. Despite these precautions, a processed form of secretogranin II (chromogranin C) has been detected in the same samples. This indicates that another type of chromogranin, which is present mainly in the pancreatic polypeptide-containing cells of the pancreatic islet, is effectively processed (Yoshie et al., 1987).

Further analysis of the cellular distribution and processing of the chromogranin/secrectogranin family in the pancreatic islet cells may provide a molecular basis for understanding of their role in endocrine secretory processes.

### Table 1. Concentration of chromogranin A (74 KD) and insulin in bovine pancreas

<table>
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<tr>
<th></th>
<th>Chromogranin A</th>
<th>Insulin</th>
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<tr>
<td>per gram wet weight</td>
<td>(n = 12)</td>
<td>per mg insulin</td>
</tr>
<tr>
<td>$\mu$g/g (± 1.3)</td>
<td>3.2 (± 1.64)</td>
<td>22.05 (± 1.64)</td>
</tr>
<tr>
<td>$\mu$g/mg</td>
<td>3.5</td>
<td>460</td>
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<tr>
<td>$\mu$g/g</td>
<td>918</td>
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Data are expressed as the mean ± SEM of the number (n) of experiments.

Acknowledgments

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Figure 5. Quantification of chromogranin A. (A) Immunoblotting of the two main bands of chromaffin vesicle content. Samples contained 290 ng, 145 ng, 73 ng, 48 ng, 36 ng, 22 ng protein/slot. They were subjected to PAGE (11% acrylamide) followed by blotting and immunostaining with the chromogranin A antiserum. (B) Calibration curve for chromogranin A. The labeled bands were cut out of the nitrocellulose and counted in a gamma counter.


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