Glycine, GABA and their transporters in pancreatic islets of Langerhans: evidence for a paracrine transmitter interplay

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Summary

To elucidate the possible roles of the CNS neurotransmitters glycine and GABA in neuroendocrine paracrine signalling, we investigated their localizations, and those of their transport proteins, by confocal immunofluorescence and quantitative post-embedding immuno-electron microscopy in the pancreatic islets of Langerhans. We show that A-cells contain glycine in synaptic-like microvesicles as well as in secretory granules. A-cells express the macromolecules necessary to: (1) concentrate glycine within both organelle types before release (the vesicular GABA/glycine transporter VGAT=VIAAT); and to (2) take up the transmitter from the extracellular space (the plasma membrane glycine transporter GLYT2). Also B-cells have glycine in their microvesicles and granules, but the microvesicle/cytosol ratio is lower than in A-cells, consistent with the presence of GABA (which competes with glycine for vesicular uptake) in the cytosol at a much higher concentration in B-cells than in A-cells. Both A- and B-cells contain GABA in their microvesicles and secretory granules, and the membranes of the two organelle types contain VGAT in both cell types. A-cells as well as B-cells express a plasma membrane transporter GAT3 that mediates uptake of GABA. The localization of VGAT in the cores of A-cell secretory granules, and in the secretory granule membranes in both cell types, indicates novel aspects of the mechanisms for release of glycine and GABA. The discovery that both A- and B-cells possess the molecular machinery for the evoked release of both glycine and GABA from synaptic-like microvesicles suggests that both of the principal inhibitory transmitters in the brain participate in paracrine signalling in the pancreas.

Key words: γ-aminobutyric acid, Glycine, Pancreas, Paracrine, Uptake

Introduction

GABA and glycine are considered to be the major inhibitory neurotransmitters in the central nervous system (Moss and Smart, 2001). They are stored in presynaptic nerve endings and released by exocytosis from synaptic vesicles to activate postsynaptic receptors. Specific transporter proteins situated in neuronal and glial plasma membranes subsequently remove the transmitters from the extracellular fluid surrounding the receptors thereby terminating receptor activation.

GABA is also suggested to have a paracrine signal function in neuroendocrine tissues, including the pancreatic islets of Langerhans. The main cell types in the islets are the peripherally localized glucagons-secreting A-cells and the centrally localized insulin-secreting B-cells. Several components of a GABA signalling system have been found in islet tissue, particularly in B-cells.

Most immunocytochemical studies have shown that GABA,, and the GABA-synthesising enzyme glutamate decarboxylase (GAD), are found selectively in B-cells (Vincent et al., 1983; Garry et al., 1986; Garry et al., 1988; Reetz et al., 1991). However, some investigators have localized GABA (Gilon et al., 1988; Chessler et al., 2002) and GAD (Li et al., 1995) also to A-cells. Moreover, the intracellular localization of GABA is not unequivocally resolved. In B-cells, one immunofluorescent study indicates that GABA is stored in synaptic-like microvesicles (SLMVs) rather than in secretory granules (SG) (Reetz et al., 1991), but there are no previous studies on GABA at the electron microscopical level. Isolated SLMVs from B-cells show ATP-dependent uptake of GABA (Thomas-Reetz et al., 1993), but similar uptake data from A-cells are lacking. Recently, evidence for ATP-dependent uptake of GABA in intracellular organelles in permeabilized clonal A-cells as well as B-cells has been presented (Hayashi et al., 2003). Upon glucose stimulation, GABA and insulin are released from B-cells (Anhert-Hilger et al., 1996). GABA is reported to inhibit glucagon secretion from A-cells (Rorsman et al., 1989), as well as to inhibit insulin secretion from B-cells (Gu et al., 1993) via

GABA-A and GABA-B receptor mediated mechanisms, respectively.

The role of glycine in the endocrine pancreas is less well known. Glycine-mediated receptor responses (strychnine-sensitive) are present on a cell line derived from pancreatic B-cells (GK-P3 cells) (Weaver et al., 1998). The cellular and subcellular distribution of glycine in islet tissue is still unknown.

Inactivation of ligand-receptor interaction by cellular uptake of fast acting transmitters is important for the maintenance of a high signal-to-noise ratio in the target cells. In fact, such uptake is critical to synaptic transmission in the brain. Uptake through the plasma membrane might also serve to supply nerve endings and cells with transmitter for release. Four high-affinity plasma membrane GABA transporters (GAT1, GAT2, GAT3 and GAT4) (Borden et al., 1992; Liu et al., 1993a), and two main subtypes of glycine transporters (GLYT1 and GLYT2) (Smith et al., 1992; Guastella et al., 1992; Liu et al., 1993b) (for a review, see Zafra et al., 1990) have been identified in the central nervous system. Whether cells in the islets of Langerhans express the plasma membrane transport proteins for GABA or glycine has not previously been established.

Exocytotic release would require prior packaging of the amino acids into intracellular vesicles. A vesicular GABA transporter (VGAT; also termed inhibitory amino acid transporter, VIAAT) was cloned (McIntire et al., 1997) and shown to transport both GABA and glycine into synaptic vesicles in the brain (Sagné et al., 1997; Chaudhry et al., 1998). Recently it was shown that VGAT is present in abundance in islet mantle cells, with a lower expression level in the islet core cells (Chessler et al., 2002), whereas other reports show that VGAT expression is exclusive to mantle A-cell Sgs (Hayashi et al., 2003). As the latter authors found ATP-dependent GABA uptake into intracellular vesicles in both types of cells, the question of the identity of the vesicular GABA transporter in B-cells remains unresolved.

To explore the roles of GABA and glycine as signalling molecules in the endocrine pancreas we used immunocytochemical methods, and specific antibodies recognising GABA, glycine and their transport proteins in aldehyde-fixed tissue. The cellular distributions of GABA, glycine, GAT1, GAT3, GLYT1, GLYT2 and VGAT immunoreactivities were investigated by confocal immunofluorescence microscopy, identifying A- and B-cells with antibodies to glucagon and insulin, respectively. Taking advantage of postembedding immunogold labelling, the organellar localizations of VGAT, GABA and glycine were studied quantitatively by electron microscopy (EM).

Materials and Methods

Materials and equipment

Glutaraldehyde (25%, EM), glycine, paraformaldehyde and picric acid were from Merck (Whitehouse Station, NJ). β-Alanine, γ-amino-*n*-butyric acid (GABA), and L-glutamic acid were from Sigma (St Louis, MO). Pentobarbital (50 mg/ml) was from Haukeland Hospital Pharmacy (Bergen, Norway). Equithesin [chloral hydrate (42.5 mg/ml) and pentobarbital (9.7 mg/ml)] was from Rikshospitalets Apotek (Oslo, Norway). Dextran D-70 was from Amersham Corporation (Buckinghamshire, UK). All other chemicals were of analytical grade.

Axiophot light microscope was from Zeiss (Germany). Cryocut 1800 was from Reichert-Jung (Wien, Austria). Kryostat 1720 was from Leitz GmbH (Stuttgart, Germany). Vibratome series 3000 sectioning systems was from Technical Products International (O'Fallon, MO). DM RXA confocal laser scanning microscope and MPS 60 camera and fluorescence filters A, N2.1 and L5 from were from Leica (Wetzlar, Germany). Vanox AHBT3 light microscope was from Olympus (Hamburg, Germany).

Antibodies

The polyclonal antibodies against amino acids (GABA and glycine) were raised in rabbits and used essentially as originally described (Storm-Mathisen et al., 1983; Dale et al., 1986; Ottersen and Storm-Mathisen, 1987) by immunizing with the amino acids coupled to bovine serum albumin by a glutaraldehyde-formaldehyde mixture. The anti-GABA (GABA990) and the anti-glycine (Gly290) sera have been extensively characterized previously (Gundersen et al., 2001; Kolston et al., 1992). The antibodies do not bind to amino acid residues in proteins. To block slight cross-reactivities, the GABA990 antiserum was preabsorbed (overnight at 4°C) with glutaraldehyde/formaldehyde complexes (weight proportion 2.5:1.0) of β-alanine (300 μM) and L-glutamic-acid (200 μM). The Gly290 glycine antiserum was pre-absorbed with complexes of β-alanine (300 μM) and GABA (200 μM). Addition of GABA and glycine complexes (500 μM) abolished labelling of tissue sections by the GABA990 and Gly290 antisera, respectively.

Anti-GAT1 serum (Chaudhry et al., 1998) was obtained from rabbit 68514 immunized with the C-terminal peptide EQPQAGSSASKEAYI (amino acid residues 584-599 of rat GAT1). The anti-GAT3 antibodies were produced as described (Danbolt et al., 1998) by immunizing rabbit 7D4318 with a synthetic peptide CEAKVKGDGTISAITEKETHF corresponding to the C-terminal amino acid residues (607-627) of rat GAT3. The ensuing antibodies were affinity purified as described and labelled one band with the appropriate molecular mass on immunoblots of SDS-PAGE separated whole rat brain tissue proteins. Three different antibodies against different parts of the VGAT protein were used. Antibodies against the N-terminal 99 amino acids of VGAT were raised in a rabbit immunized with a glutathione-S-transferase-VGAT fusion protein (N-terminal VGAT antibody), and antibodies against the C-terminal of VGAT were obtained from a rabbit immunized with the keyhole-limpet hemocyanin (KLH)-conjugated synthetic peptide of the C-terminal 17 amino acids of VGAT (C-terminal VGAT antibody) (Chaudhry et al., 1998). A VGAT antibody raised in guinea pig against synthetic peptide from rat, human and monkey VGAT protein was purchased from Chemicon. (Temecula, CA) (Chemicon antibody). The N-terminal VGAT antibody was used for immunofluorescence, immunoperoxidase and immunogold experiments, whereas the C-terminal VGAT antibody and the Chemicon antibody were used only in immunogold experiments. The N-terminal VGAT anti-serum was preabsorbed with rat liver acetone powder (40 mg/ml) overnight at 4°C, before the supernatant was transferred to a microcentrifuge tube with filter insert and spun in a microcentrifuge for 5 minutes at 7280 ***g***. The affinity purified anti-GLYT1 antibody (Zafra et al., 1995) was from rabbit 80748 immunized with the synthetic C-terminal peptide IVGSNGSSRLQDSRI (amino acids 623-638 of GLYT1). The GLYT2 antibody (Zafra et al., 1995) was obtained from a rabbit immunized with a glutathione-S-transferase-GLYT2 fusion protein containing 193 amino acids of the (intracellular) amino-terminus of GLYT2. This antiserum was absorbed with immobilized GST, the antibodies concentrated on a protein A-Sepharose column, and finally affinity purified on a column with immobilized fusion protein (Zafra et al., 1995). The specificities of the antibodies to the transporter proteins have all been documented in our laboratory by immunoblots, as described in the publications quoted in this paragraph.

Mouse monoclonal antibodies to glucagon, insulin, synaptophysin

and GABA were from Sigma (St Louis, MO), Zymed Laboratories (San Francisco, CA), Boehringer Mannheim Biochemica (Mannheim, Germany) and ICN Biomedicals (Costa Mesa, CA), respectively. Polyclonal guinea pig anti-insulin antibodies were from Sigma (St Louis, MO). Polyclonal anti-synaptophysin antiserum from rabbit was from Euro Diagnostica (Malmö, Sweden). Anti-rabbit Ig (biotinylated from donkey), streptavidin-biotinylated horseradish peroxidase complex, Cy3 coupled anti-rabbit IgG from goat and Cy3 coupled anti-mouse IgG from goat were from Amersham Pharmacia Biotech (Uppsala, Sweden). Cy3-coupled anti-rabbit IgG from donkey and FITC-coupled anti-mouse IgG from donkey, FITC-coupled anti-mouse IgG [$F(ab')_2$ fragment from donkey] and FITC-coupled anti-guinea pig IgG [$F(ab')_2$ fragment from donkey] were from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-rabbit Ig F(ab) coupled to 15 nm gold particles were from Europrobe (Lyon, France), and Goat anti-mouse Ig coupled to 10 nm gold paticles was from Aurion (Netherlands).

Perfusion fixation

Wistar rats (150-350 g) were obtained from M&B (Ry, Denmark) and Møllergaard-Hansen (Vejby, Denmark), while BDIX rats (150-350 g) were obtained from Harlan (Oxon, UK). The animals had free access to food (standard low-protein pellet diet) and tap water. They were deeply anesthetized with intraperitoneal injections of Equithesin (5 ml/kg) or pentobarbital (50-75 mg/kg). Before cessation of spontaneous respiration the rats were perfused for 15 min through the left ventricle with different fixatives in 0.1 M sodium phosphate buffer pH 7.4 (NaPi) (4°C or room temperature, 50 ml/min) (see below), preceded by a brief flush (10-15 sec) of 4% (w/v) dextran (M_r 70,000) in the same buffer without fixative (for details, see Danbolt et al., 1998). The pancreas and the brain were dissected free and postfixed (2-3 hours at room temperature) in the same fixative as used for perfusion. For amino acid immunoperoxidase cytochemistry a mixture of 2.5% glutaraldehyde and 1% formaldehyde in NaPi was used. For immunofluorescence either a mixture of 3-4% formaldehyde and 0.5% glutaraldehyde in NaPi or a pH-shift protocol [4% formaldehyde in 0.2 M sodium acetate buffer (pH 6.0) followed by 4% formaldehyde in 0.2 M sodium carbonate buffer (pH 10.5) with 0.2% picric acid added to both solutions] was used. For electron microscopic studies, a mix of either 4% formaldehyde and 0.1% glutaraldehyde, or 1% formaldehyde and 2.5% glutaraldehyde was used. Formaldehyde was freshly made from paraformaldehyde, and the aldehydes were added to the buffers immediately before fixation. The tissues were stored at 4°C in the fixative diluted 1:10 in NaPi until they were processed for light or electron microscopic immuncytochemistry.

Immunofluorescence and immunoperoxidase microscopy

After fixation, the tissues were sectioned on a vibratome (40 μm) or on a cryostat (7-20 μm) after cryoprotection in 30% sucrose or 25% sucrose and 10% glycerine at 4°C overnight. The cryostat sections were collected on SuperFrost®Plus or gelatin-coated glass slides and stored at –22°C until they were processed with the antibodies according to an indirect immunofluorescence method (Veruki and Wässle, 1996). The vibratome sections were incubated with the antibodies in immunoperoxidase experiments using a streptavidin-biotin-peroxidase method (Hsu and Raine, 1981).

The sections were incubated with the primary antibodies over night at 4°C or at room temperature. The antibodies were used at the following dilutions: anti-GABA (no. 990) 1:500-1:1000, anti-GABA (monoclonal) 1:200, anti-GAT1 0.3-0.6 μg/ml, anti-GAT3 0.3-0.6 μg/ml, anti-glucagon 1:2000-1:2400, anti-glycine 1:300-1:3000, anti-insulin 1:40-1:100, anti-synaptophysin (polyclonal) 1:600, anti-synaptophysin (monoclonal) 1:5-1:200, anti-GLYT1 2 μg/ml, anti-GLYT2 6 μg/ml and anti-VGAT (N-terminal VGAT antibody) 1:100-1:300. The Cy3-conjugated secondary antibodies were used at dilutions of 1:50-1:1000. The FITC-conjugated secondary antibodies were used at dilutions of 1:50-1:200. The biotinylated secondary antibodies (anti-rabbit Ig) and the streptavidin biotinylated horseradish peroxidase complex were used at dilutions of 1:100.

For immunofluorescence microscopy, double-labelling experiments were performed. The sections were first incubated with a mixture of primary antibodies from two different species, followed by a mixture of species specific secondary IgG antibodies that were coupled to different fluorochromes. In control experiments, in which the primary antibodies were omitted or substituted with pre-immunesera, there was no staining of the tissue sections.

As a specificity control in amino acid immunoperoxidase experiments, cellulose nitrate/acetate filters with blots of different amino acids conjugated to brain macromolecules by glutaraldehyde-formaldehyde were processed along with the tissue sections (Dale et al., 1986; Kolston et al., 1992). This test system ensured that the GABA and glycine labelling of tissue sections was highly specific. When aldehyde complexes of GABA or glycine (0.3 mM) were added to the respective antisera, the staining of tissue and test sections was abolished.

The distributions of immunoreactivities for GABA, glycine, GAT, GLYT, VGAT, synaptophysin, insulin and glucagon were analysed with one of three microscopes: (1) an Olympus Vanox light microscope using differential interference contrast (DIC) illumination; (2) a Zeiss Axiophot light microscope using different combinations of fluorescent filters (BP 450-490 and FT 510 for FITC, and BP 546, FT 580 and LP 590 for Cy3), and an additional green filter (BP515-565) blocked strong crosstalk of Cy3 fluorescence; (3) a Leica DM RXA confocal laser scanning microscope using a FITC/TRITC filter set. Leica TCS NT software was used to obtain digital images of fluorescent sections using fluorescence filters A, N2.1 and L5 and an MPS 60 camera from Leica, and to take photomicrographs using DIC illumination.

Quantitative immunogold electron microscopy

Tissue for electron microscopy was low temperature embedded in Lowicryl HM20 and immunogold labelling was performed as described (Chaudhry et al., 1995; Chaudhry et al., 1998). Slices of perfusion-fixed pancreatic tissue (see above) were microdissected into rectangles containing islets of Langerhans, cryoprotected with increasing concentrations of glycerol (10%, 20% and 30% in NaPi, 30 minutes each), before freezing in liquid propane (190°C) in a KF80 Universal Cryofixation System (Leica, Vienna, Austria). Then the samples were substituted with anhydrous methanol containing 0.5% uranyl acetate in a Cryo Substitution Apparatus (Leica) precooled to –90°C. The samples were infiltrated at –45°C with a gradient of increasing concentrations of Lowicryl HM20 in methanol and subsequently with pure Lowicryl HM20 overnight before polymerization by ultraviolet light (360 nm) for 2 days at -45°C. Ultrathin sections (gold) were cut on a Leica ultramicrotome and placed on nickel mesh grids.

To investigate the ultrastructural localization of VGAT and amino acids in islet cells, the ultrathin sections were processed with the VGAT or amino acid antibodies with or without glucagon antibody in double-labelling immunogold incubations. The three different VGAT antibodies (see above) were used at the following dilutions: the N-terminal VGAT antibody 1:100, the C-terminal VGAT antibody 1:300 and the Chemicon antibody 1:500. The anti-GABA antibody (GABA990) and the anti-glycine antibody (Gly290) were from rabbit and were diluted 1:300. The ultrathin sections were first treated with a mixture of either of the antibodies to VGAT or amino acids (from rabbit or guinea pig) and the glucagon antibody (mouse, dilution 1:2000), before the bound immunoglobulins were visualized with species-specific secondary antibodies coupled to gold particles with different diameters (15 nm for VGAT, GABA and glycine, and 10 nm

for glucagon). Other ultrathin sections were single labelled for VGAT or amino acid. Ultrathin test sections (Ottersen, 1987) with different amino acids fixed to brain macromoloecules were processed along with the islet sections, ascertaining that the antibodies to GABA and glycine labelled only the amino acids they were raised to, in the conditions of the individual experiment.

The sections were viewed in a Philips CM10 electron microscope. The cells were identified by immunolabelling for glucagon and on morphological grounds according to Weiss (Weiss, 1988). Briefly, B-cells were identified by the presence of numerous secretory granules (SGs) with an electron-dense core surrounded by an electron-lucent halo within the limiting membrane. The A-cells have SGs with a uniform diameter (that is slightly smaller than that of B-cell SGs) and an electron-dense core that is closely surrounded by the vesicular membrane. Pictures were taken of A- and B-cells adjacent to each other.

The quantifications were performed at ×48,000 final magnification. Immunogold particles signalling VGAT (only the N-terminal antiserum was used for quantitative analysis), glycine and GABA, and grid points for area determination (see below) were recorded over SGs (also known as large dense core granules, LDCG), cytoplasm free of SGs and mitochondria (here called residual cytoplasm) and over mitochondria in A- and B-cells. In addition, the residual cytoplasm was divided into areas of SLMVs and of cytosol. Endoplasmic reticulum components, filaments and microtubules were included in cytosol. Other organelles were rarely encountered and were excluded from the analysis. Particles and grid points were included in the SLMV when the particle centres or grid points were within a 30 nm distance from the outer border of the SLMV. This is a distance within which most of the immunogold particles are expected to occur (Chaudhry et al., 1995; Nagelhus et al., 1998). These criteria are illustrated in Fig. 1. To correct for the contamination of cytosol labelling to the SLMV labelling, the gold particle densities recorded over cytosol were subtracted from the gold particle densities recorded for SLMVs. Similarly, to avoid contamination, particles or grid points closer to a mitochondrion or an SG than 30 nm were excluded from recording as localized over residual cytoplasm, cytosol or SLMV. The values for glycine and GABA were corrected for background labelling over empty resin (average 2 gold particles/μm^2). VGAT associated with the membranes limiting SGs was also analyzed. Gold particles with centres within 30 nm on either side of the limiting membrane were recorded (see above), but ones closer than 15 nm to the SG core were excluded to minimize contamination from the core (Fig. 1), which is densely labelled in A-cells. The areas of the profiles were determined by point counting using an overlay screen (Gundersen et al., 1988; Gundersen et al., 1998) using the same inclusion criteria as for gold particles. The results were statistically evaluated by a non-parametric test (Mann-Whitney-U) (Statistica) and a Student's *t*-test (Excel).

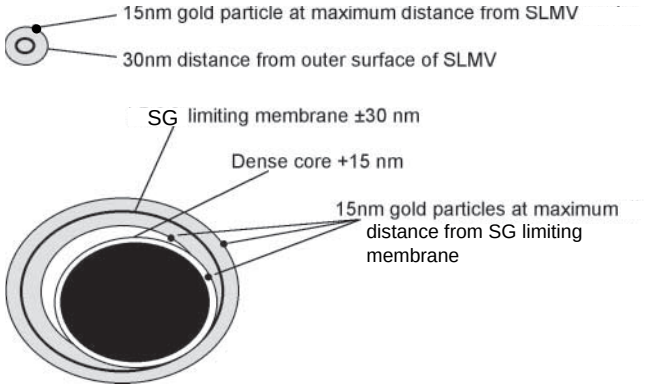

Fig. 1. Criteria for recording of immunogold particles as associated with synaptic-like micro vesicles (SLMVs), or secretory granule (SG) limiting membranes (thick contours). Gold particles (and grid points for area determination) with centres within 30 nm of the limiting membranes of SLMVs or SGs were included (grey areas), omitting ones closer to SG dense cores than 15 nm. Particles within 30 nm of the surface of mitochondria (or other, rarer organelles) were excluded from recording (see text).

Results

GABA and glycine in neuroendocrine cells in the islets of Langerhans

To establish the cell-specific localization of GABA, cryosections of rat pancreas with islets of Langerhans were processed for double-labelling immunofluorescence and examined by confocal laser scanning microscopy. We show that GABA is strongly concentrated in a majority of the insulin-positive cells in the islet core (B-cells) (Fig. 2A,B). There is very weak GABA-immunoreactivity in a few core cells as well as in the majority of the glucagons-positive cells in the islet mantle (A-cells) (Fig. 2C,D).

The subcellular localization of GABA was explored by electronmicroscopy by postembedding immunogold labelling. We demonstrate that the GABA immunogold particles are localized with a high density in areas of cytoplasm free of mitochondria and SGs (Fig. 2E,F). Confirming and extending the immunofluorescence results, the mean density of GABA immunogold particles over B-cell SGs was similar to the density over residual cytoplasm (Fig. 2F), suggesting that GABA is present both in SGs and other cytoplasmic components. In A-cells too, gold particles signalling GABA are situated both over cytoplasm and over SGs, but at a much lower labelling density than in the B-cells (Fig. 2F).

Closer electron microscopic analysis (Fig. 2G,H) revealed that immunogold particles situated over residual cytoplasm were preferentially associated with small vesicular structures, i.e. SLMVs. Thus, after dividing the residual cytoplasm into areas of SLMVs and cytosol (organelle-free cytoplasmic matrix), we found that the observed gold particle ratio (SLMV/cytosol) for GABA (see Materials and Methods) was about 1 both in A- and in B-cells (Fig. 2G). As the inside of the SLMV (the diameter of the SLMVs is 34.1±9.4 nm, mean±s.d., n=92) is much smaller (ca. 1/8, Fig. 1) than the area over which SLMV-associated immunogold particles are distributed, we estimate that the concentration of GABA inside the SLMVs is about 8 times higher than indicated by the observed gold particles (Fig. 2H). This is similar to the concentration of glutamate in synaptic vesicles compared with cytosol in glutamatergic terminals in the brain (Nicholls and Attwell, 1990). As the area of SGs is only slightly smaller than the area containing SG-associated gold particles, the observed density of GABA immunogold particles closely represents the concentration of GABA inside SGs. Hence, the GABA concentration inside SGs is similar to that in cytosol in B-cells, but slightly higher than that in cytosol in A-cells (Fig. 2F).

Although the presence and releasability of GABA is well established in a number of different neuroendocrine cells, other neuroactive amino acids might serve similar paracrine functions as well. Our results with glycine specific antibodies show the presence of this amino acid within most or all neuroendocrine cells of the islets of Langerhans, at much higher levels than in the exocrine cells (Fig. 3A). Both insulin containing core cells and glucagon containing mantle cells

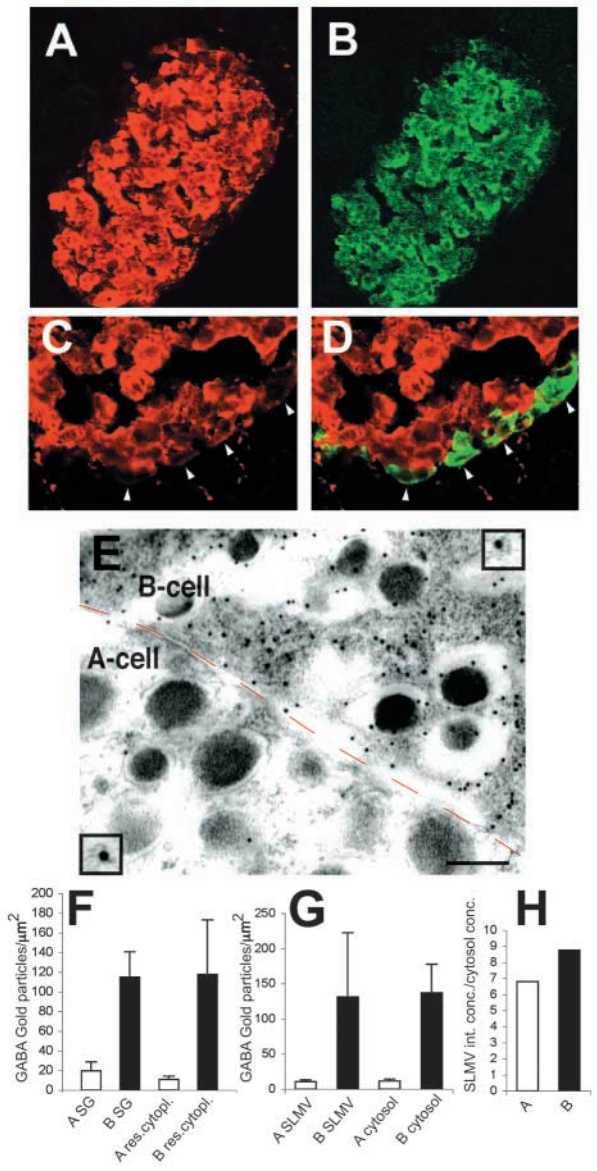

Fig. 2. GABA (red A,C) is in core cells containing insulin (green B), i.e. B-cells. GABA occurs, at lower concentrations, also in mantle cells (C, arrowheads) co-localized with glucagon (green D, arrowheads), i.e. in A-cells, as shown by double labelling immunofluorescence confocal microscopy. Note lack of labelling (black) of exocrine pancreas. Electron microscopic post-embedding immunolabelling (E) shows a higher concentration of GABA in a B-cell (E top) than in an A-cell (E below). Insets: high magnification examples of SLMVs labelled for GABA, from the two cell types. Quantification (F) of immunogold particles shows the distribution of GABA intracellularly in A- and B-cells (presented as gold particles representing GABA per μm^2; background over tissue-free plastic, ca 2 per μm^2, subtracted). The SGs and residual cytoplasm (mitochondria etc. excluded) contain GABA at about 6 and 11 times higher concentration in B-cells than in A-cells, respectively (P=0.01, n=3) (F). Further analysis shows (G,H) that SLMVs in the residual cytoplasm also have about 10 times higher particle densities in B-cells than in A-cells (P<0.001, n=4). SLMVs have similar net densities of GABA particles as cytosol both in A-cells and in B-cells (the GABA particle densitiy over cytosol was subtracted from that over SLMVs; cf. Fig. 1 and Materials and Methods). However, as the area of the SLMVs (average diameter 34.1 nm) is much smaller (ca 1/8) than the area over which SLMV associated immunogold particles are distributed, we estimate that the internal SLMV concentration of GABA is about 8 times higher than indicated by the observed gold particles (A-cells P=0.001, B-cells P=0.01, for gross particle densities of SLMVs versus cytosol, n=4). Hence, the GABA concentration inside the SLMVs is nearly an order of magnitude higher in the cytosol in both A- and B-cells (H). Scale bar E: 200 nm (scale for insets: 15 nm gold particles).

are clearly glycine positive (Fig. 3B,C). Corroborating the immunofluorescence data, immunoelectron microscopy shows that gold particles signalling glycine are present in both A- and B-cells (Fig. 3D,E). However, in contrast to GABA, glycine is more concentrated in SGs than in the residual cytoplasm, in A-cells as well as B-cells (Fig. 3E). Further electron microscopic analysis of the residual cytoplasm revealed that, like GABA, glycine is preferentially associated with SLMVs, with a SLMV/cytosol ratio of about 2 in A-cells, significantly higher than in B-cells (about 0.5) (Fig. 3F). The estimated glycine concentration inside A-cell SLMVs (cf. above on GABA) is some 15 times higher than in cytosol, whereas the concentration inside B-cell SLMVs is 5 times that in cytosol (Fig. 3G). The glycine concentration inside A- and B-cell SGs is about 2.5 times that in residual cytosol (Fig. 3E,F).

Vesicular transport of GABA and glycine in islet neuroendocrine cells

If an amino acid is concentrated within vesicles (SLMV or SG) for subsequent exocytotic release, an amino acid transport protein must be present in the vesicle membrane. The accumulation of GABA and glycine in SLMVs as well as in SGs prompted us to investigate the islet distribution of VGAT, which is the obvious candidate for a vesicular GABA and glycine transporter protein (McIntire et al., 1997; Sagné et al., 1997; Chaudhry et al., 1998).

To this end, we first applied immunofluorescence microscopy with specific antibodies against VGAT, in combination with antibodies against insulin, glucagon, GABA or synaptophysin to reveal the cellular distribution of VGAT in the islet cells. A high fluorescent signal for VGAT is present in glucagons-positive mantle cells (Fig. 4A-C), questioning the role of VGAT for vesicular GABA transport in B-cells. However, in double-labelling experiments with antibodies against VGAT and GABA, using the confocal microscope, we could show that the strongly GABA-positive core B-cells also express VGAT, although at a much lower level than mantle A-cells (Fig. 4D). The SLMV marker synaptophysin appears to label all islet cells (Fig. 4E).

With the expression of high levels of VGAT in A-cells and the presence of glycine in SLMVs as well as SGs in these cells, the question arises whether A-cells express the transport protein in both types of organelle, rather than in SLMVs like in the brain (Chaudhry et al., 1995). Post-embedding immunogold electron microscopy was applied for addressing this question. Immunogold labellings with three different antisera against VGAT showed that A-cells express VGAT in SGs (Fig. 4G-J) at a level that is an order of magnitude higher than the expression level in B-cell SGs (Fig. 4G,J), the latter being close to background levels (see below). However, the densities of VGAT

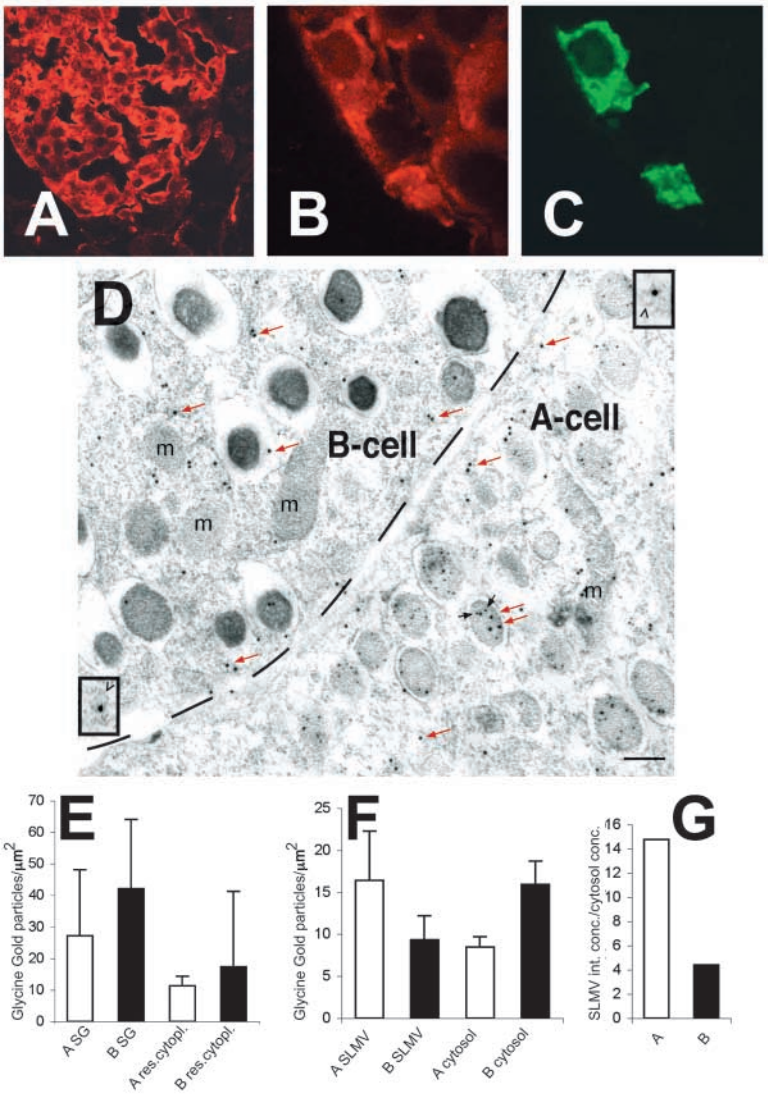

Fig. 3. Glycine (red) is found both in core and in mantle cells of the islet (A,B) and is co-localized with glucagon (green) in mantle cells (C). Electron microscopic immungold analysis (D) shows glycine (15 nm gold particles, red arrows) distribution in a B-cell (left) and an A-cell (right, double labelled with 10 nm gold particles representing glucagon, short arrows). Insets: high magnification examples of SLMVs (arrowheads) labelled for glycine in the two cell types. Quantification of immunogold particles (E-G) shows the intracellular distribution of glycine. Note that the numbers cannot be directly compared with those for GABA (Fig. 2), as the labelling efficiencies are not the same. The densities (number of 15 nm gold particles per μm^2, background over tissue-free plastic, ca 2 per μm^2, subtracted) of glycine immunoreactivity over SGs and residual cytoplasm (free of mitochondria and SGs), show that glycine occurs at higher concentrations in SGs than in the residual cytoplasm (P=0.05, n=5) both in A- and in B-cells (E). (F) In A-cells, SLMVs have higher net densities of glycine particles than cytosol, whereas in B-cells, cytosol has a higher net labelling density than SLMVs (subtraction as in Fig. 2G; P=0.005 for gross particle densities, n=4, for both A- and B-cells). However, owing to the small diameter of the SLMVs compared with the diameter in which the glycine gold particles are observed, the concentration of glycine inside A-cell SLMVs is about 15 times higher than in cytosol, whereas the concentration inside B-cell SLMVs is about 5 times higher than in cytosol (A greater than B at P=0.002) (G). Scale bar D: 200 nm (scale for insets: 15 nm gold particles).

immunogold particles associated with the limiting membranes of SGs are similar in A- and B-cells (Fig. 4K), most of the SG labelling in A-cells being situated over the SG dense cores. The SG membrane-associated particle densities are much higher than the densities over cytoplasm distant from the SG membrane. However, after dividing the cytoplasm free of SGs and mitochondria into areas of SLMVs and cytosol, we could show that both in A- and in B-cells, high VGAT immunoreactivity is associated with SLMVs (Fig. 4L). Both in A- and in B-cells, the particle densities over cytosol are low and similar to those over mitochondria (Fig. 4J), or empty plastic (about 3 particles/μm^2), i.e. background noise. Geometrical considerations (cf. Fig. 1) suggest that the length of sectioned limiting membrane per area of observation is similar for SLMVs and SGs. Although no exact comparison can be made, the data therefore suggest that the VGAT density is somewhat higher in SLMV membranes (Fig. 4L) than in SG membranes (Fig. 4K) (observed particle density ratio SLMV/SG ≈1.5 both in A- and in B-cells).

Plasma membrane transporters of GABA and glycine are present in neuroendocrine islet cells

The plasma membrane GABA transporters GAT1 and GAT3 are present in the brain, in presynaptic terminals and in astrocytes, respectively (Itouji et al., 1996). They serve distinct roles in ensuring temporal limitation of synaptic transmission and recycling of the transmitter. Although neuroendocrine cells do not have the same demands for temporal resolution of their extracellular signalling processes, it is probable that plasma membrane transport of released transmitter amino acids are an integral part of their paracrine regulation. In accordance with this, we found that GAT3 is present throughout the islet, both in insulin- and in glucagons-positive cells (Fig. 5A). The GAT3 labelling shows a granular pattern in the cytoplasm, i.e. it is not confined to the plasma membrane, suggesting the presence of intracellular reservoirs of the transporter. The GAT1-specific antibody did not produce staining of pancreatic sections (though it does specifically stain neuroendocrine cells in the stomach antrum (S. Davanger, unpublished).

In view of our observation that the amino acid transmitter glycine is present in neuroendocrine cells in the islets of Langerhans, we performed immunolabelling for the brain glycine plasma membrane transporters GLYT1 and GLYT2, which (in the brain) are localized in astrocytes and glycinergic neurons, respectively (Zafra et al., 1995). GLYT2 is strongly present in glucagon-positive mantle cells (A-cells) (Fig. 6), while most of the core cells (including B-cells) showed slight immunoreactivity. Antibodies against GLYT1 produced no immunostaining of pancreatic sections (not shown).

Exocrine pancreas

The antibodies used in this study did not stain the exocrine

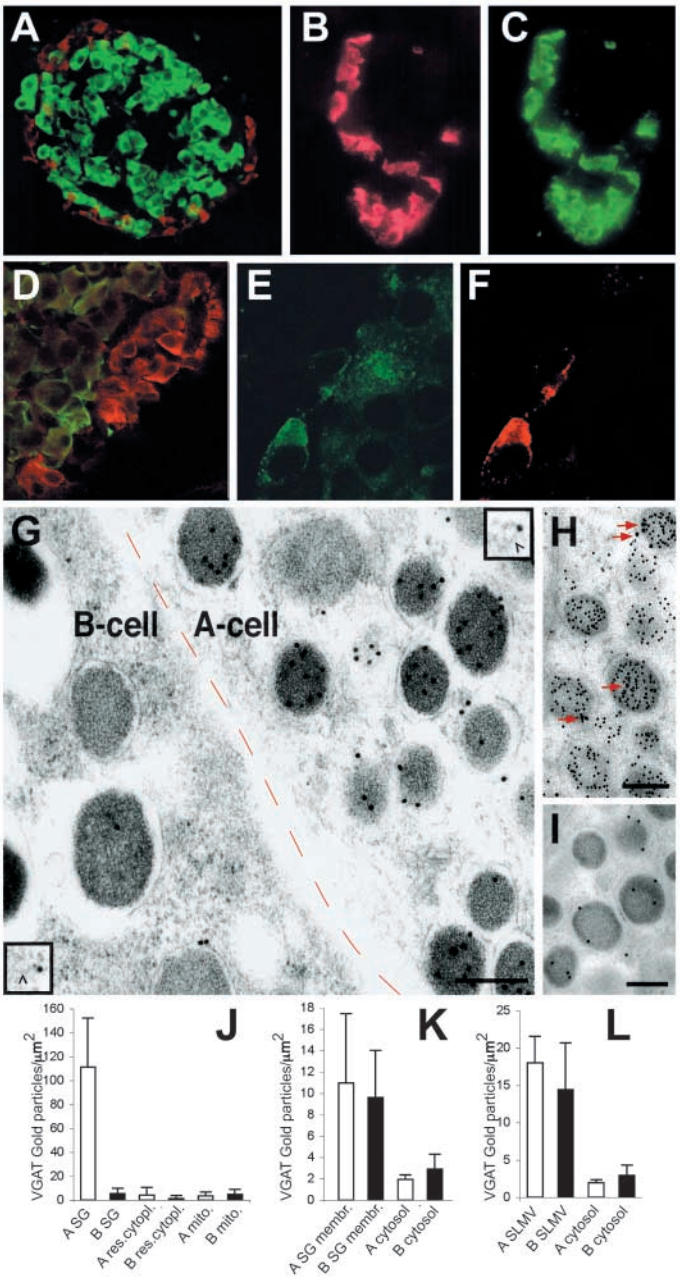

Fig. 4. VGAT (red A,B,D,F) co-localizes with glucagon (green C) in A-cells. Insulin-positive (green A) and GABA-positive (green D) core cells, are very slightly labelled for VGAT (red D). Synaptophysin (green E) is in both A- and B-cells. Electron microscopic immunogold labelling with three different VGAT antibodies (G,H,I) shows strong signal in the SGs of A-cells (G,H,I) but not B-cells (G). The identity of the A-cell SGs is confirmed by double labelling (H) with 10 nm gold particles for glucagon and 15 nm particles (red arrows) for VGAT. Insets: higher power photographs of SLMVs (arrowheads) labelled for VGAT, from the two cell types. Electronmicroscopic quantification (J) shows that VGAT immunogold density over A-cell SGs is >10-fold higher than that over B-cell SGs, or those over mitochondria or cytoplasm of either A- or B-cells ($P<0.01$). Further analysis (K,L) of the distribution of VGAT was conducted as shown in Fig. 1 (see also Materials and Methods). The density of VGAT immunogold associated with the membrane of SGs (K) is only slightly higher in A-cells than in the B-cells, but is significantly higher than cytosol background ($P=0.01$ in both A- and B-cells, $n=5$). Analysis of the distribution of VGAT in the residual cytoplasm (free of SGs and mitochondria) shows a many times higher density of VGAT immunogold associated with SLMVs than over cytosol (L) ($P<0.001$ in A-cells, $P=0.007$ in B-cells, $n=5$), which is close to the background noise over tissue-free plastic (2-3 part/µm^2, not subtracted). SLMV-associated VGAT relative to cytosol background is slightly higher in A-cells than in B-cells ($P=0.008$, $n=5$). Scale bars G-I: 200 nm (scale for insets: 15 nm gold particles).

pancreas, except for the glycine antibodies, which produced a weak labelling (Fig. 3A), consistent with the roles of glycine in metabolism and protein synthesis.

Control immunoperoxidase labelling

To confirm that the labelling patterns obtained with the immunoflourescence method could be reproduced with another immunocytochemical method, we used immunoperoxidase with diaminobenzidine to visualize the location of the different primary antibodies in islet vibratome sections. The two gave concordant labelling patterns.

Control brain sections

The staining patterns of GABA, glycine, VGAT, GAT1, GAT3, GLYT1 and GLYT2 in the brain were similar to those described previously (Ottersen et al., 1988; Chaudhry et al., 1998; Ikegaki et al., 1994; Itouji et al., 1996; Zafra et al., 1995). In particular, in spite of the lack of GLYT1 and GAT1 staining in the islets, the antibodies to these proteins selectively stained astrocytes and GABAergic nerve endings, respectively, in the brain.

Discussion

The main findings in the present study are that glycine and GABA are concentrated in SLMVs relative to cytosol both in A- and in B-cells. SGs concentrate the amino acids to a lesser extent. Whereas the two cell types have similar concentrations of glycin, GABA is some 10 times higher in B- than in A-cells. The cores of the glucagons-positive SGs in A-cells contain very high levels of the vesicular GABA/glycine transporter VGAT. However, the VGAT concentrations in the limiting membranes of the organelles are somewhat higher in SLMVs than in SGs (SLMV/SG ratio ≈1.5), and are similar in A- and B-cells. The plasma membrane GABA transporter GAT3 (astroglial in brain) is expressed equally by A-cells and B-cells, while the plasma membrane glycine transporter GLYT2 (in glycinergic neurons in brain) is expressed more highly in A-cells.

The facts that the concentrations of VGAT in the membranes of SGs and SLMV are similar in A- and B-cells, and that VGAT transports both glycine and GABA (Sagné et al., 1997; Chaudhry et al., 1998), are in agreement with the observation that glycine and GABA are accumulated in SGs as well as SLMVs in both types of cell. This suggests that glycine and GABA are both transported by VGAT to be stored in A- and B-cell SLMVs, as well as SGs.

Thus, VGAT is the propbable transporter that provides B-

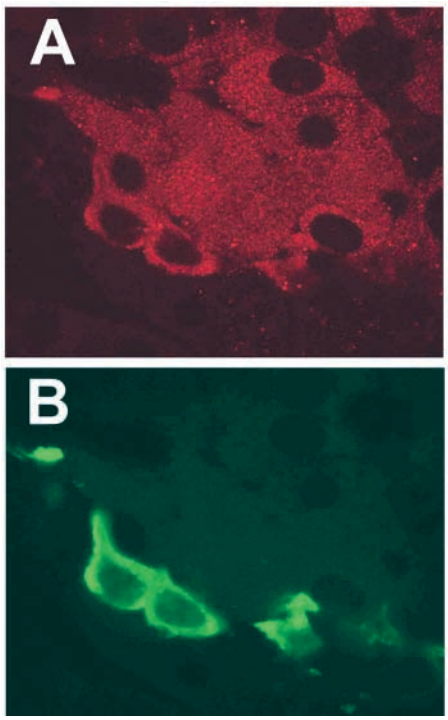

Fig. 5. The GABA transporter GAT3 (red A) is expressed at similar levels in core B-cells and mantle A-cells, identified by double labelling for glucagon (green B). GAT3 is concentrated in intracellular compartments.

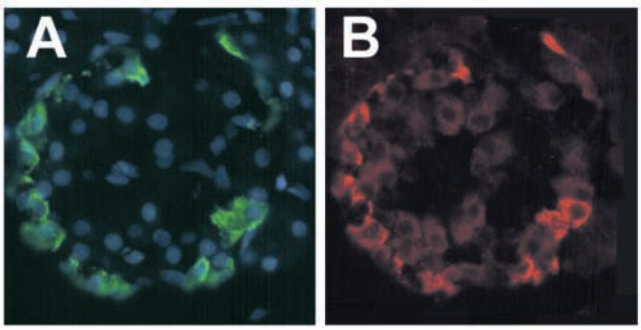

Fig. 6. The glycine transporter GLYT2 (red B) co-localizes with glucagon (green A, with blue nuclei) in A-cells of islets of Langerhans. There is also a low signal in core B-cells, whereas exocrine cells (blue nuclei, e.g. at upper corners of frame) have hardly any GLYT2 signal.

cell SLMVs with GABA in pancreatic islets, like in the brain. The finding of GABA and VGAT in SLMVs in B-cells is in agreement with previous reports indicating that GABA could be taken up in and released by exocytosis from SLMVs in B-cells (Thomas-Reetz et al., 1993; Ahnert-Hilger et al., 1996). The expression of GAT3 in A- as well as B-cells, together with the fact that these cells express GABA receptors (Borboni et al., 1994; Brice et al., 2002), means that the machinery for vesicular storage, release, signal detection and inactivation of GABA is present in the islets of Langerhans and further supports an auto- and paracrine role of GABA in islet cells. It is noteworthy that pancreatic islet cells express GAT3 rather than GAT1, in which respect they resemble brain astroglia rather than GABAergic neurons.

Although B-cells have been considered to be the major GABAergic cells in the islets, our results suggest that A-cells may have a similar function, inasmuch as GABA and VGAT are co-localized in A-cell SLMVs and SGs. However, owing to the lower GABA concentration in A-cells, glycine might compete favourably with GABA for transport by VGAT into A-cell SLMVs and SGs. Furthermore, A-cells also contain the plasma membrane glycine transporter GLYT2, which in the central nervous system (CNS) localizes to glycinergic nerve endings (Zafra et al., 1995). Thus, we show that A-cells express the molecular machinery necessary for a glycinergic signal system to occur. They express the transporter that concentrates glycine in vesicles (VGAT), as well as the transporter responsible for removal of glycine from the extracellular space after release (GLYT2). GLYT2 enables the A-cells to limit the stimulation of glycine receptors temporally, which are localized on B-cells (Weaver et al., 1998). At the same time, GLYT2 might contribute to recapturing glycine and to providing A-cells with glycine for transport into the vesicles. This makes glycine a strong candidate for an A-cell-mediated paracrine transmitter in the endocrine pancreas. However, B-cell SLMVs and SGs have significant concentrations of glycine, suggesting that even B-cells might release glycine.

We know that the vesicular inhibitory amino acid transporter (VIAAT=VGAT) is exclusively present in A-cell Sgs (Hayashi et al., 2003). This is in line with our finding that A-cell SGs express VGAT at very high levels. In addition, we find that VGAT is localized also in the membranes of SGs, as well as in those of SLMVs in both A- and B-cells, explaining the finding that there are intracellular ATP-dependent uptake sites for GABA both in A- and in B-cells (Hayashi et al., 2003). Our high-resolution immunogold quantification was necessary to make this discovery. Besides, the immunogold labelling efficiency achieved in the present work is higher than that obtained in a previous study (Hayashi et al., 2003), where 5 particles/μm^2 for A-cell SGs versus our 110 particles/μm^2 were reported (Fig. 4J).

Co-secretion of different amino acid transmitters from islet cells has been incompletely studied. Our results suggest co-release of GABA and glycine from both A- and B-cells, though in different proportions. Glutamate is co-secreted with glucagon from pancreatic A-cells to evoke GABA release from B-cells (Hayashi et al., 2002). Thus, the A-cells might co-release glutamate with glycine and GABA, adding to the complexity of the regulation of para/autocrine signal transduction in the islets of Langerhans. In fact, glycine, like glutamate, gives a depolarizing receptor response in B-cells, mediating an increase in the intracellular Ca^{2+} concentrations (Weaver et al., 1998). This means that the glutamate and glycine released from A-cells might regulate Ca^{2+}-dependent secretions from B-cells.

The co-localization of glycine, GABA and glutamate in the endocrine pancreas is in line with the growing evidence for co-release of two or more transmitters at the same synapse in the brain. Although co-release of glycine and glutamate has not been directly demonstrated in the brain, co-localization of glycine and glutamate has been shown in nerve terminals in the retina, brain stem and spinal cord (Davanger et al., 1994;

Davanger, 1996; Somogyi and Llewellyn-Smith, 2001). There is firm evidence for co-release of glycine and GABA from the same nerve terminal in the spinal cord (Jonas et al., 1998) as well as of glutamate and GABA from the same nerve terminal in the hippocampus (Walker et al., 2001; Bergersen et al., 2003).

The obvious role of VGAT localized in SLMV and SG membranes in A- and B-cells is the sequestration of glycine and/or GABA in the corresponding organelles for storage before exocytosis. The release of glycine and GABA from two types of organelles in the same cell could contribute to the fine-tuning of hormone secretion to changes in, for instance, the glucose concentration. However, SG glycine might serve other functions than that of a releasable signal molecule. Uptake of amino acids, such as GABA and glycine, could reduce the SG membrane potential and potentiate the secretion from SGs, as has been suggested for glutamate (Maechler and Wollheim, 1999; Hoy et al., 2002), or could contribute, as osmolytes, to the swellling of SGs, a phenomenon that enhances the fusion of zymogen granule membrane with the plasma membrane in the exocrine pancreas (Jena et al., 1997).

The important question of what might be the role of VGAT in the dense core of SGs in A-cells is a matter of conjecture. The SG core might possibly serve as a reservoir and/or 'sink' for the transporter, or the amino acid binding site of VGAT might contribute to the storage of glycine and GABA. Answering these questions is beyond the scope of the present study. In conclusion, glycine may be exocytotically released from SLMVs and SGs in both A- and B-cells, whereas GABA may be released from SLMVs and SGs in B-cells and to a lesser extent in A cells.

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