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GABA and pancreatic β-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion

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GABA, a major inhibitory neurotransmitter of the brain, is also present at high concentration in pancreatic islets. Current evidence suggests that within islets GABA is secreted from β-cells and regulates the function of mantle cells (α- and δ-cells). In the nervous system GABA is stored in, and secreted from, synaptic vesicles. The mechanism of GABA secretion from β-cells remains to be elucidated. Recently the existence of synaptic-like microvesicles has been demonstrated in some peptide-secreting endocrine cells. The function of these vesicles is so far unknown. The proposed paracrine action of GABA in pancreatic islets makes β-cells a useful model system to explore the possibility that synaptic-like microvesicles, like synaptic vesicles, are involved in the storage and release of non-peptide neurotransmitters. We report here the presence of synaptic-like microvesicles in β-cells and in β-cell lines. Some β-cells in culture were found to extend neurite-like processes. When these were present, synaptic-like microvesicles were particularly concentrated in their distal portions. The GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), was found to be localized around synaptic-like microvesicles. This was similar to the localization of GAD around synaptic vesicles in GABA-secreting neurons. GABA immunoreactivity was found to be concentrated in regions of β-cells which were enriched in synaptic-like microvesicles. These findings suggest that in β-cells synaptic-like microvesicles are storage organelles for GABA and support the hypothesis that storage of non-peptide signal molecules destined for secretion might be a general feature of synaptic-like microvesicles of endocrine cells.

Key words: β-cell/diabetes/GABA/glutamic acid decarboxylase/synaptic vesicle

Introduction

GABA, the major inhibitory neurotransmitter of the brain, is thought to play an important role as a signal molecule also outside the nervous system (Erdö and Wolff, 1990). In particular, evidence for a role of GABA as a paracrine signal molecule in pancreatic islets has been reported (Rorsman *et al.*, 1989; for a review see Okada, 1986). Insulin-secreting β-cells of the islets, like GABA-secreting neurons (Oertel *et al.*, 1981a,b; Nagai *et al.*, 1984), contain high levels of GABA (Briel *et al.*, 1972; Okada *et al.*, 1976; Vincent *et al.*, 1983), of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) and of GABA-transaminase (Vincent *et al.*, 1983). The function and characteristics of the GABA system in the pancreas have become the focus of considerable attention after the demonstration that GAD is a dominant autoantigen in insulin-dependent diabetes mellitus (Baekkeskov *et al.*, 1990).

GABA-A receptors have been found on α-cells and it has been suggested that these receptors are involved in the inhibition of glucagon release mediated by β-cells (Rorsman *et al.*, 1989). A modulatory action of GABA on δ-cells has also been reported (Taniguchi *et al.*, 1980). The direction of blood flow in the islet is consistent with this hypothesis because blood capillaries perfuse first the core of the islet, composed of β-cells, and then the periphery (mantle) of the islet, composed of α- and δ-cells (Bonner-Weir and Orci, 1982). However, the mechanism of GABA secretion from β-cells is unknown. A previous immunocytochemical study carried out with antibodies directed against GABA had failed to reveal the presence of GABA in insulin-containing granules. This finding suggested that GABA was secreted via a non-vesicular mechanism (Garry *et al.*, 1987).

It is currently accepted that in the nervous system GABA is concentrated in, and secreted from, synaptic vesicles (Hell *et al.*, 1988), although a non-vesicular release of GABA at certain specialized regions has been proposed (Schwartz *et al.*, 1987). Several intrinsic membrane proteins of synaptic vesicles are also present in peptide-secreting endocrine cells (Matthew *et al.*, 1981; Buckley and Kelly, 1985; Wiedenmann and Franke, 1985; Navone *et al.*, 1986; Baumert *et al.*, 1989, 1990). Studies carried out on the pituitary and the adrenal medulla, and on cell lines derived from these tissues, have shown that in endocrine cells these proteins are localized in the membranes of a population of microvesicles distinct from peptide-containing secretory granules (Navone *et al.*, 1986; De Camilli and Navone, 1987; Wiedenmann *et al.*, 1988; Johnston *et al.*, 1989; Baumert *et al.*, 1989, 1990; Clift-O'Grady *et al.*, 1990; Cutler and Cramer, 1990; Schweitzer and Paddock, 1990; for a review see De Camilli and Jahn, 1990). These vesicles, referred to as 'synaptic-like microvesicles' (SLMVs) appear to have at least some functional similarities in common with synaptic vesicles of neurons, including the ability to undergo exocytosis, endocytosis and recycling (Johnston *et al.*, 1989; Clift-O'Grady *et al.*, 1990). The function of SLMVs remains unknown. In view of their similarities with synaptic vesicles, the possibility that SLMVs store and secrete neurotransmitter-like molecules should be considered. Given the proposed paracrine role of GABA in pancreatic islets,

β-cells appeared to be a useful model system to explore this possibility.

The working hypothesis of this study was that secretion of GABA from β-cells is mediated by SLMVs. If this were the case, SLMVs of β-cells should share with synaptic vesicles of GABA-secreting neurons specific properties related to GABA uptake and storage. In this paper evidence supporting this hypothesis is presented. We have demonstrated the existence of SLMVs in β-cells and have shown that, in analogy to the colocalization of GABA and GAD with synaptic vesicles in GABA-secreting neurons, GABA and GAD are colocalized with SLMVs in β-cells. These results represent the first indication that, at least in some endocrine cells, SLMVs might store neurotransmitter-like molecules. This work has been presented previously in abstract format (Reetz *et al.*, 1990a,b).

Results

SLMVs are present in β-cells

Expression of synaptic vesicle membrane proteins in pancreatic islets had been previously reported (Buckley and Kelly, 1985; Navone *et al.*, 1986; Baumert *et al.*, 1989, 1990), but their precise subcellular localization had not been investigated. An important preliminary step of our study was to demonstrate that in β-cells these proteins are localized on SLMVs, a population of microvesicles distinct from secretory granules. To demonstrate this we used isolated islets as well as cell lines derived from islet cells.

As markers for SLMVs we have used antibodies directed against synaptophysin and p29, two major integral membrane proteins of synaptic vesicles of neurons (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Navone *et al.*, 1986; Baumert *et al.*, 1990). As markers for secretory granules we have used antibodies directed against insulin and secretogranin I. Secretogranin I (also known as chromogranin B) is a peptide stored in secretory granules together with peptide hormones in a variety of peptide-secreting endocrine cells (Rosa *et al.*, 1985b).

Figure 1a and b shows RINm5F cells (Gazdar *et al.*, 1980; Bhathena *et al.*, 1982), a commonly used rat insulinoma cell line, double-labeled for p29 and secretogranin I. Secretogranin I immunoreactivity was represented by puncta sparse throughout the cytoplasm and particularly concentrated at the enlarged tips of short thick cellular processes. Electron microscopy immunocytochemistry (not shown), demonstrated that each of these puncta corresponded to individual secretory granules (Rosa *et al.*, 1985a; and not shown). p29 immunoreactivity had a distribution very different from that of secretogranin I (Figure 1a) but virtually identical to that of synaptophysin (Figure 1c and d). Both p29 and synaptophysin were present at low levels throughout the cell but were particularly concentrated at a paranuclear area, which included the Golgi complex as demonstrated by double-labeling for synaptophysin and GIMPt, an intrinsic membrane protein of the 'trans' region of the Golgi complex (Yuan *et al.*, 1987) (Figure 1e and f). The same results were obtained by similar double-labeling experiments carried out on another β-cell line, βTC3 (Efrat *et al.*, 1988) (Figure 1g and h). In both cell lines, synaptophysin was not accumulated in the short thick processes rich in secretory granules (Figure 1b and not shown). However, some of the βTC3 cells were found to extend relatively long, often beaded, neurite-like processes. When this was the case,

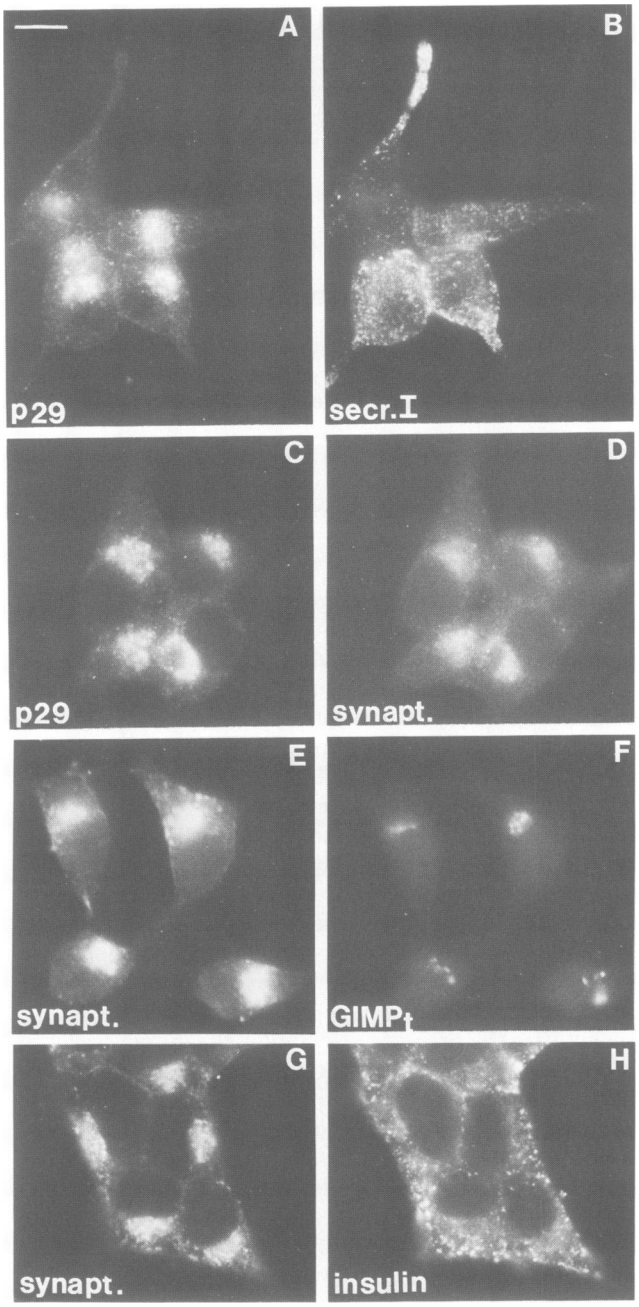

Fig. 1. Distribution of secretory granule proteins, of synaptic vesicle proteins and of a marker antigen of the trans-Golgi (GIMPt) in insulinoma cell lines. **a–f**: RINm5F cells double-labeled by immunofluorescence for p29 and secretogranin I (secr I), for p29 and synaptophysin (synapt.), and for synaptophysin and GIMPt; **g** and **h**: βTC3 cells double-labeled by synaptophysin and insulin. Puncta of secretogranin I and insulin immunoreactivities, which correspond to secretory granules, have a distribution very different from that of synaptophysin and p29 immunoreactivities (compare a with b and g with h). Both synaptophysin and p29 are primarily concentrated at a paranuclear region which includes the Golgi complex (compare e with f), where their distribution is very similar (compare c and d). The calibration bar in the upper left corner is the equivalent of 10 μm (a and b), 7.6 μm (c and d), 8.2 μm (e and f) and 8.4 μm (g and h).

synaptophysin as well as secretory granule markers, were concentrated in the distal portion of the processes (see Figure 5c, e and g, Figure 8a and not shown).

The distribution of synaptophysin, p29 and secretogranin I was studied by subcellular fractionation. A postnuclear

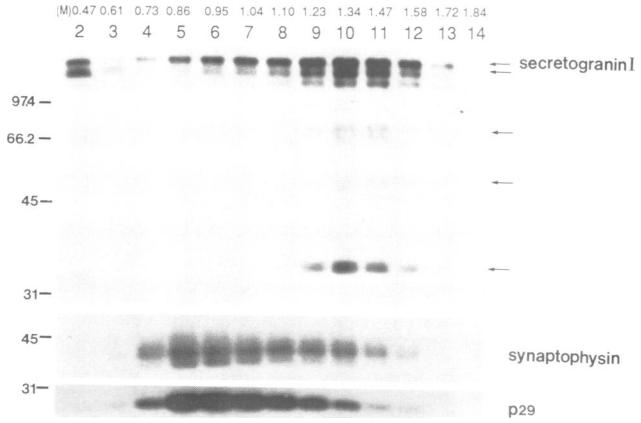

Fig. 2. Separation of synaptic vesicle proteins from secretory granules in a continuous sucrose density gradient. A 5000 *g* supernatant of a total homogenate of RINm5F cells was loaded onto a sucrose gradient ranging from 0.45 to 2 M sucrose. Fifteen fractions were collected starting from the top and equal volumes of such fractions were analyzed by SDS–PAGE and Western blotting with primary antibodies directed against secretogranin I, synaptophysin and p29. Fractions 1–3 correspond to the 'load' of the gradient and contain soluble proteins. Synaptophysin and p29 peak in a lighter portion (0.86–0.95 M sucrose) of the gradient than do secretory granules (1.34–1.47 M sucrose) represented by secretogranin I immunoreactive bands. Some endoproteolytic cleavage of secretogranin molecules is known to take place in parallel with granule maturation (Rosa *et al.*, 1985; Benedum *et al.*, 1987). Thus, lower mol. wt secretogranin immunoreactive bands reflect the position in the gradient of mature granules.

supernatant of RINm5F cells was subjected to centrifugation either on a Percoll gradient or on a continuous sucrose density gradient. Fractions were collected and analyzed for their content of synaptophysin, p29 and secretogranin I by a Western blot procedure. Results obtained with the sucrose gradient are shown in Figure 2. Synaptophysin and p29 immunoreactivities peaked near the top of the gradient [density 0.86–0.95 M sucrose, similar to the density previously described for neuronal synaptic vesicles (Navone *et al.*, 1989)]. Secretogranin I immunoreactivity was accounted for by several bands as typically seen for proteins of the chromogranin/secretogranin family which undergo partial proteolytic processing during granule condensation (Rosa *et al.*, 1985b; Benedum *et al.*, 1987). Mature secretory granules, represented by the proteolytically processed form of secretogranin I, peaked in the dense portion (1.34–1.47 M sucrose) of the gradient and were well separated from the peak of synaptophysin. Qualitatively similar results were obtained when subcellular fractionation was performed on a Percoll gradient (not shown). This distribution confirms that synaptophysin and p29 are localized on an organelle distinct from secretory granules.

The morphology of synaptophysin-positive organelles in the endocrine pancreas was investigated by immunogold electron microscopy. Figure 3 shows agarose-embedded fragments of rat islets labeled for synaptophysin. Fragments of β- and α-cells were identified by the typical morphological features of their secretory granules (Williams and Porte, 1974). These have a characteristic dense core surrounded by a clear halo in β-cells and a homogeneous granular content in α-cells (Williams and Porte, 1974). As demonstrated in Figure 3a, b and c, mature insulin-containing granules were

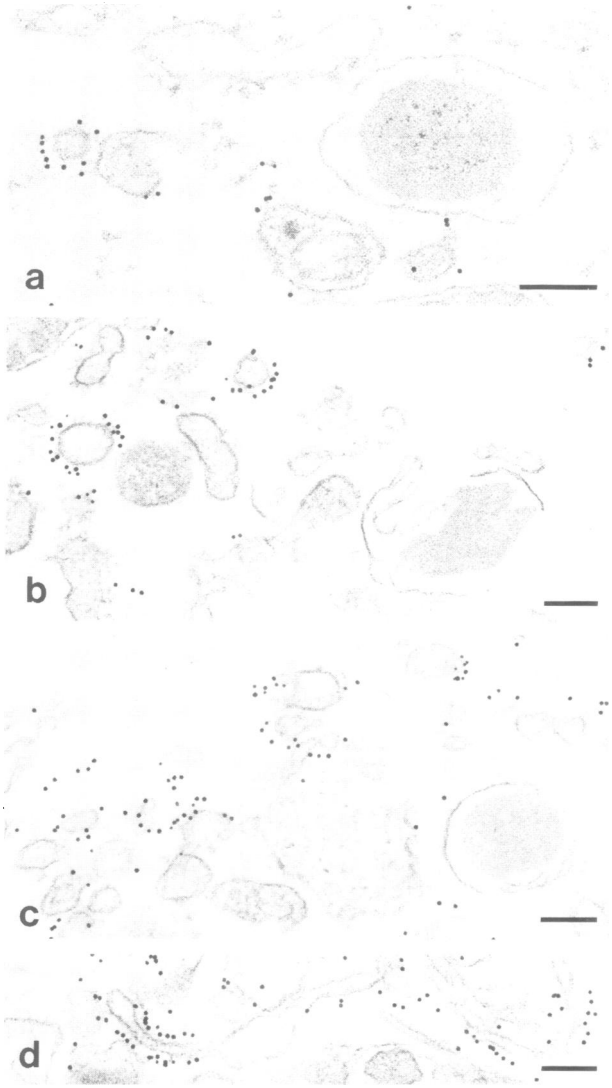

Fig. 3. Gallery of electron micrographs showing the localization of synaptophysin in fragments of rat pancreatic β-cells as demonstrated by an immunogold technique. *a–c*: gold labeling of pleiomorphic microvesicles with a clear core. **d**: gold labeling of a cisternal element in the region of the Golgi complex. Note lack of labeling of insulin-containing secretory granules characterized by the typical unstained halo around the granule core (a and c) which may have a crystal-like shape (b). Calibration bars = 100 nm.

devoid of synaptophysin immunoreactivity. The predominant pool of synaptophysin immunoreactivity was concentrated on the membranes of pleiomorphic clear microvesicles and tubules, with the same characteristics of SLMVs previously identified in other endocrine cells. When fragments of the Golgi complex were visible, synaptophysin immunoreactivity was always present, but confined to its 'trans' side, where it was localized both on vesicles and on cisternal and tubular elements (Figure 3d). Synaptophysin was not detected at a significant concentration on the plasma membrane. In conclusion, the localization of synaptophysin in β-cells was similar to that previously observed in other endocrine cells (Navone *et al.*, 1986; Baumert *et al.*, 1990). Synaptophysin was found to have a similar distribution also in α-cells (not shown).

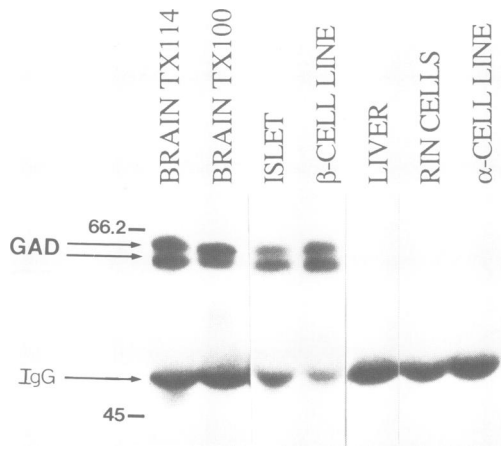

Fig. 4. Selective expression of GAD in brain and in pancreatic β-cells demonstrated by immunoprecipitation followed by Western blotting. GAD was immunoprecipitated from detergent extracts (500 μl each) of cell lines and tissue using a sheep serum directed against GAD (Oertel *et al.*, 1981a). The immunoprecipitate was subjected to SDS–PAGE and its GAD content analyzed by Western blotting using the rabbit antibody directed against GAD described in Katarova *et al.* (1990). A GAD doublet is visible in the extracts of brain, islets and βTC3 cells, but not in extracts of liver, RINm5F cells or αTC1 cells. (No GAD bands were detectable in the last three lanes irrespective of the autoradiographic exposure time.)

GAD is concentrated around SLMVs in β-cells and around synaptic vesicles in neurons

The major pathway of GABA synthesis in neurons and in β-cells involves GAD (Wu *et al.*, 1976; Oertel *et al.*, 1981a), an enzyme represented by two similar isoforms of mol. wt ~63 and 59 kd (Legay *et al.*, 1987; Chang and Gottlieb, 1988; Erlander *et al.*, 1990) which are expressed both in the brain and in the pancreas (Baekkeskov *et al.*, 1990). GAD is not an integral membrane protein (Kobayashi *et al.*, 1987; Julien *et al.*, 1990; Katarova *et al.*, 1990; Wyborski *et al.*, 1990) but exhibits some hydrophobic properties which might be consistent with membrane association (Baekkeskov *et al.*, 1990; Christie *et al.*, 1990). Within GABA-secreting neurons, GAD was found by immunofluorescence and by immunoperoxidase to be concentrated in regions containing synaptic vesicle membranes, i.e. nerve terminals and the region of the Golgi complex (Oertel *et al.*, 1981b; Mugnaini and Oertel, 1985). If SLMVs of β-cells and β-cell lines had a function similar to that of GABA-containing synaptic vesicles, a colocalization of GAD with SLMV membranes would be expected. This was investigated by light and electron microscopy immunocytochemistry.

In preliminary experiments we determined that the β-cell line βTC3 expresses GAD. This was demonstrated both by immunoprecipitation and by immunofluorescence experiments (see below and not shown). For the former experiments cell extracts were immunoprecipitated with antibodies directed against GAD (or control antibodies) and the GAD content in the immunoprecipitates was determined by Western blotting with a different anti-GAD antibody. A doublet of immunoreactive bands with mol. wts ~63 and 59 kd, corresponding to the two isoforms of GAD, was detected in brain, pancreatic islets and in βTC3 cells (Figure 4). As expected, GAD was not detected in a liver extract or in αTC1 cells, a glucagonoma cell line (Powers *et al.*, 1990) (Figure 4). GAD was also not detected in RINm5F cells (Figure 4). While they are often used as a model for β-cells, recent evidence suggests that RINm5F cells exhibit some characteristics typical of α-cells (Halban *et al.*, 1988). It is noteworthy that RINm5F cells were previously found to be negative for the 64 kd antigen of insulin-dependent diabetes (Colman *et al.*, 1987), now known to be identical to GAD (Baekkeskov *et al.*, 1990).

The distribution of GAD in the βTC3 cell line was investigated by immunofluorescence and compared with the distribution of a marker protein of SLMVs, synaptophysin, GAD immunoreactivity was detected only in a subpopulation of cells which included all the cells with neurite-like processes (Figure 5a–h, see also Figure 8). Within GAD-positive cells, the distribution of GAD and of synaptophysin was very similar (Figure 5a–h). GAD, like synaptophysin, was concentrated in the Golgi complex area and in the distal portions of neurite-like extensions. The virtually identical distribution of the two antigens was particularly impressive in the tips of the processes as shown at higher magnification in Figure 5e–h. The colocalization of GAD with synaptophysin visible in the processes was similar to the colocalization of the same two antigens in axons and growth cones of primary neuronal cultures double-labeled by the same procedure (Figure 5i–l).

The subcellular localization of GAD in pancreatic β-cells was further investigated using electron microscopy colloidal gold immunocytochemistry on agarose-embedded tissue fragments of pancreatic islets. Gold particles were found to be concentrated in proximity to the membranes of pleiomorphic microvesicles and of tubular and cisternal elements (Figure 6). The latter were, in general, in close proximity to the Golgi complex. This localization of GAD was similar to the localization of synaptophysin in the same cells. However, in the case of GAD, a low level of GAD immunolabeling was observed throughout the cytoplasm.

When the same immunocytochemical technique was applied to cerebellar tissue fragments, a low level of immunolabeling was observed throughout the cytoplasm of GABA-secreting neurons. Very intense immunolabeling was observed in nerve terminals. Within nerve terminals gold labeling was primarily associated with synaptic vesicles, while other nerve terminal membranes were virtually unlabeled (Figure 7).

The interaction of GAD with brain synaptic vesicles and with SLMVs of β-cells was investigated by subcellular fractionation. However, GAD could not be recovered on synaptic vesicles or on SLMVs purified by immuno-isolation (Burger *et al.*, 1989), and GAD did not copurify with synaptic vesicles or with any other membranous organelle in sucrose density gradients or in glycerol velocity gradients (unpublished observations). These findings suggested that the interaction between GAD and vesicles is either indirect or highly sensitive to homogenization conditions.

GABA is colocalized with SLMVs in β-cells

Antibodies specific for GABA have proved to be a useful tool to identify sites where GABA is present at high concentration by immunocytochemistry. Immunostaining of brain sections with antibodies directed against GABA results in the selective labeling of GABA-ergic nerve terminals, i.e. of nerve terminals containing synaptic vesicles specialized for the storage of GABA (Storm-Mathisen *et al.*, 1983). If

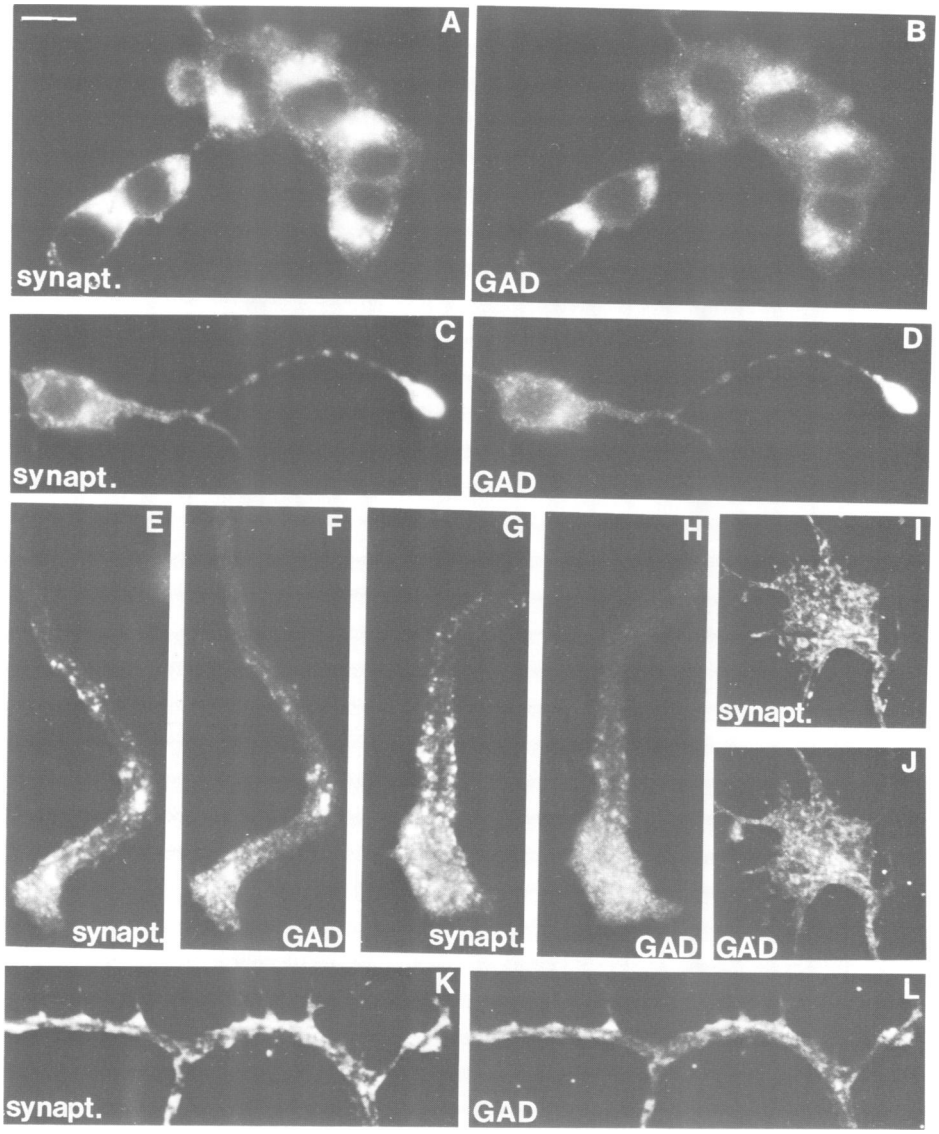

Fig. 5. Colocalization of synaptophysin (synapt.) and GAD in βTC3 cells and in primary cultures of hippocampal neurons as demonstrated by double-immunofluorescence. **a–h**: βTC3 cells. The two antigens colocalize both in the Golgi complex area (a–d) as well as in neurite-like processes (c and d). The tips of two such processes are shown at higher magnification (e and f, g and h). *i–l*: neurons. Colocalization of the two antigens in axonal growth cones (i and j) and in segments of isolated axons (k and l) is shown. The calibration bar in the upper left corner is the equivalent of 8.7 μm (a–d), 7.0 μm (e–h) and 8.3 μm (i–l).

SLMVs of β-cells contain GABA in a concentrated form, then GABA immunoreactivity should colocalize with SLMVs. When βTC3 cells were labeled with antibodies directed against GABA, immunoreactivity was found to be particularly high in cells with neurite-like processes, and in these cells it was preferentially localized in these processes (Figure 8b and d). Double-staining of these cells for GABA and for synaptophysin resulted in a very similar immunostain within the processes (Figure 8a and b), while a detectable accumulation of GABA immunoreactivity was not seen in the region of the Golgi complex. Double-staining of the same cells for GABA and for GAD demonstrated that the cells containing high levels of GABA were the same as those expressing high levels of GAD (Figure 8c and d). GABA immunoreactivity was not detected in the αTC1 cells, the GAD-negative α-cell line (see above).

Discussion

Over the last several years growing evidence for a paracrine role of GABA in pancreatic islets has accumulated. GABA is thought to be released from β-cells and to act on α- and possibly δ-cells, but until now its mechanism of secretion was unknown (Taniguchi *et al.*, 1980; Robbins *et al.*, 1981; Cavaginini *et al.*, 1982; Passariello *et al.*, 1982; Rorsman *et al.*, 1989). We report here results supporting the hypothesis that GABA might be secreted from β-cells via SLMVs. These findings provide an initial insight concerning the possible function of SLMVs in endocrine cells.

SLMVs are organelles of a vesicular pathway distinct from the typical 'regulated secretory pathway' of endocrine cells and defined by the presence in their membranes of several of the major membrane proteins of synaptic vesicles (Navone

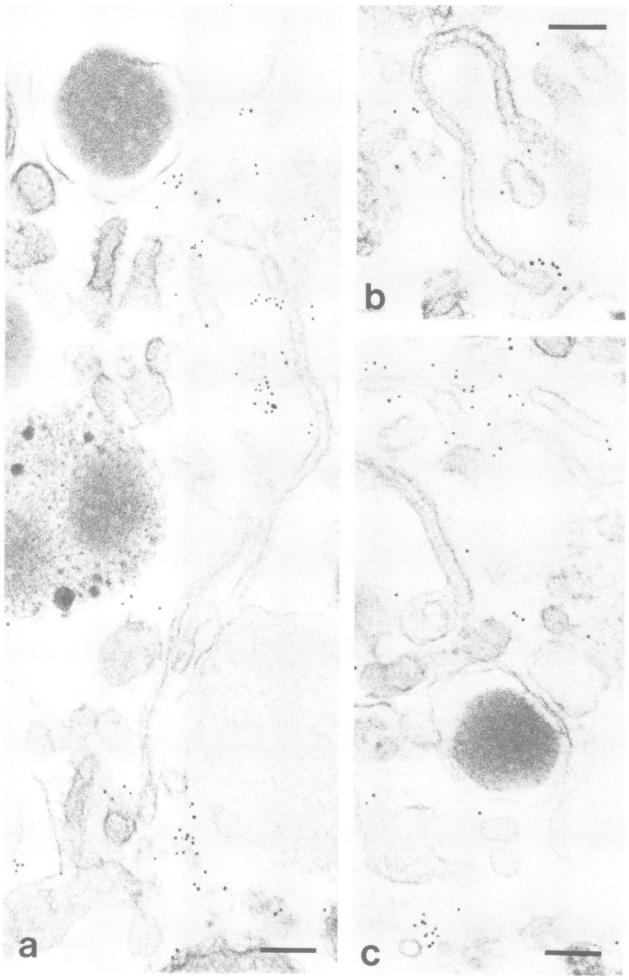

Fig. 6. Gallery of electron micrographs showing the localization of GAD in β-cells as demonstrated by an immunogold technique. Fragments of rat islets were agarose embedded and immunolabeled as described (De Camilli *et al.*, 1983; Mignery *et al.*, 1989). Portions of β-cells are shown in panels a–c. Calibration bars = 100 nm.

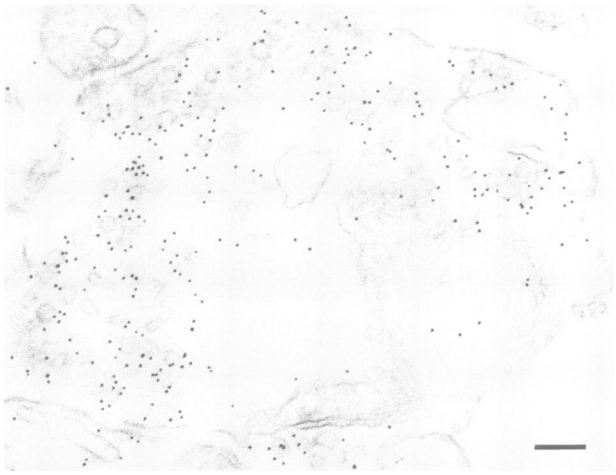

Fig. 7. Electron micrograph showing the localization of GAD in a brain nerve terminal demonstrated by immunogold. Gold particles are concentrated around synaptic vesicles. The plasma membrane and other membranes of the ending are unlabeled. Calibration bar = 100 nm.

et al., 1986; De Camilli and Navone, 1987; Wiedenmann *et al.*, 1988; Baumert *et al.*, 1989, 1990; Clift-O'Grady *et al.*, 1990; Cutler and Cramer, 1990; Fischer von Mollard *et al.*, 1990; Schweitzer and Paddock, 1990). Like synaptic vesicles they undergo exocytosis, endocytosis and recycling (Navone *et al.*, 1986; Johnston *et al.*, 1989; Clift-O'Grady *et al.*, 1990). SLMVs were previously described in chromaffin and pituitary cells and are shown by this study to be present in cells of the endocrine pancreas.

The similarities between SLMVs of endocrine cells and neuronal synaptic vesicles strongly suggest that the function of the two organelles is somehow related. The function of synaptic vesicles is to store and secrete non-peptide neurotransmitter molecules (Hökfelt *et al.*, 1984). In this study we have addressed the possibility that this function might be shared by SLMVs. To do so, we have taken advantage of the known property of pancreatic β-cells to secrete the neurotransmitter GABA and we have used β-cells as a model endocrine cell.

Our present results show that synaptic vesicles of GABA-secreting neurons and SLMVs of β-cells share, in addition to properties common to all synaptic vesicles and SLMVs, an important property relevant to GABA uptake and storage, namely the concentration of the GABA-synthesizing enzyme GAD at their cytoplasmic surface. An association of GAD with synaptic vesicles had been inferred previously by electron microscopy immunoperoxidase studies (McLaughlin *et al.*, 1975), but in those studies the peroxidase reaction product was found on all organelles of nerve terminals. Non-specific labeling of all membranous compartments adjacent to antigenic sites is a known limitation of immunoperoxidase techniques (De Camilli *et al.*, 1983). We have now provided strong evidence for a specific localization of GAD around synaptic vesicles using an electron microscopy immunogold procedure on brain tissue and immunofluorescence on primary neuronal cultures. Likewise, we have shown a localization of GAD around SLMVs of β-cells by electron microscopy immunogold labeling of pancreatic islets and by immunofluorescence on β-cell lines.

The distribution of GAD both in neurons and in endocrine cells suggests a direct or indirect interaction of at least a significant pool of the protein with vesicle membranes. However, a direct association of GAD with synaptic vesicles or with SLMVs could not be demonstrated by subcellular fractionation studies. Although a pool of GAD, variable depending upon the homogenization conditions, was found to be sedimentable by high speed centrifugation (100 000 *g* for 1 h). However, this pool could not be recovered on synaptic vesicles or on SLMVs by either immuno-isolation experiments or by sucrose density gradient centrifugation. It appeared to represent insoluble GAD not bound to membranes (unpublished observations). These findings indicate either that the interaction of GAD with membranes is indirect or that our conditions of homogenization abolish the interaction. The primary sequence of GAD does not include typical transmembrane regions (Kobayashi *et al.*, 1987; Katarova *et al.*, 1989; Julien *et al.*, 1990; Wyborski *et al.*, 1990). However, a significant pool of GAD partitions in the detergent phase after Triton X-114 extraction (Baekkeskov *et al.*, 1990), suggesting the existence of post-translational hydrophobic modifications of the molecule. These modifications may mediate a membrane attachment labile to homogenization conditions. The nature of the association between GAD and vesicle membranes needs to be further investigated. This issue deserves special interest

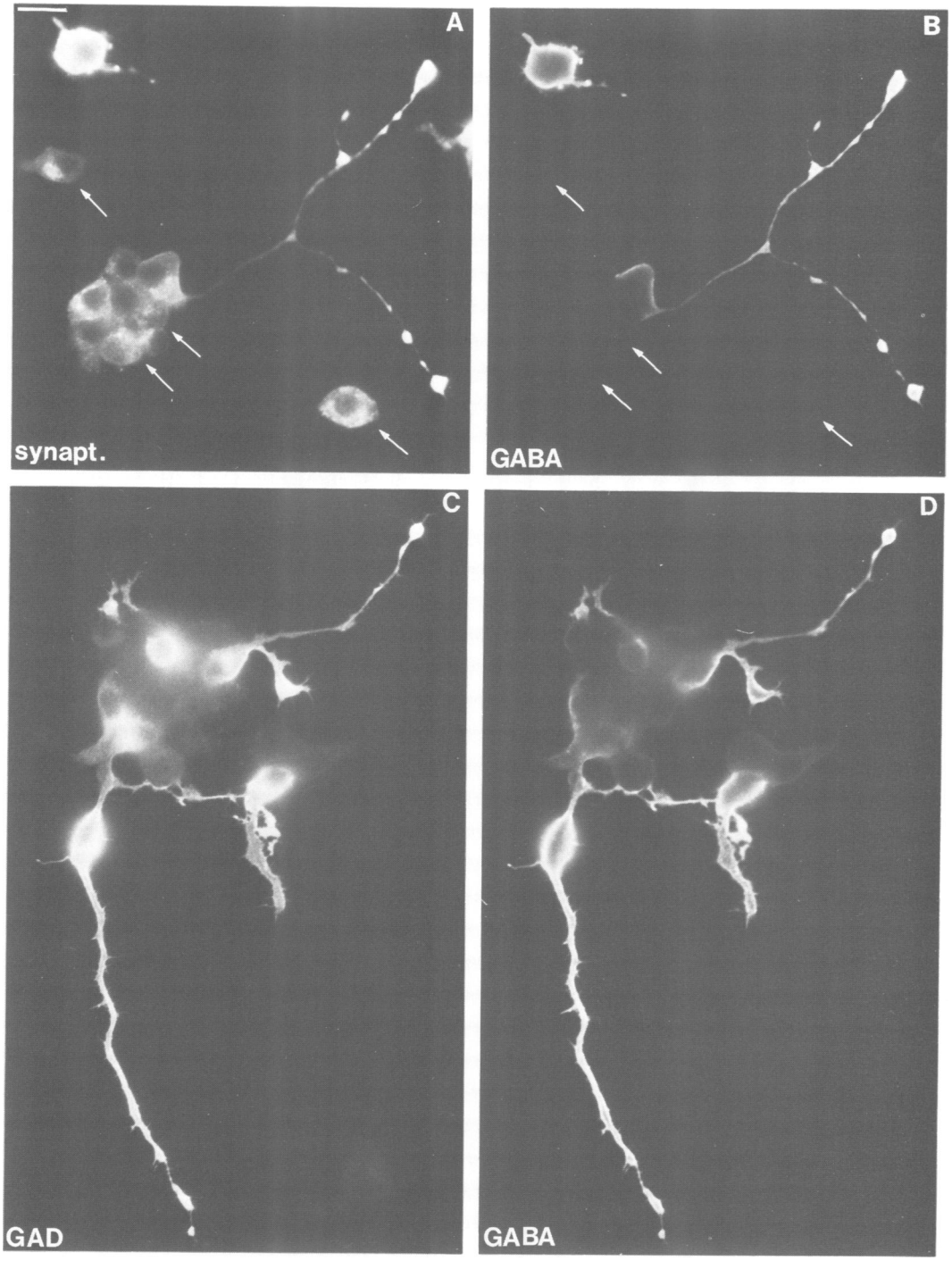

Fig. 8. Colocalization of GABA with synaptophysin (synapt.) and with GAD in βTC3 cells demonstrated by double-immunofluorescence. Note that GABA is present at high concentrations only in cells that express high levels of GAD (compare **c** with **d**) and that these cells include all the ones with neurite-like processes. GABA is particularly concentrated in the processes where it is colocalized both with synaptophysin and with GAD. Calibration bar = 10 μm.

because it may provide some clues concerning the properties of GAD as a major autoantigen in Stiff–Mann syndrome and insulin-dependent diabetes mellitus (Solimena *et al.*, 1990; Baekkeskov *et al.*, 1990).

We have also demonstrated here that GABA is concentrated in regions of β-cells which are enriched in SLMVs, as would be expected if SLMVs contain GABA. A similar colocalization of GABA with synaptic vesicles can be observed in neurons (our study and Storm-Mathisen *et al.*, 1983).

Morphologically, as assessed by electron microscopy immunocytochemistry, SLMVs are small, translucent, pleiomorphic vesicles and tubules (Navone *et al.*, 1986; Baumert *et al.*, 1990; this study). In endocrine cells *in situ*, and in cultured endocrine cells devoid of neurite-like processes, SLMVs are scattered throughout the cytoplasm

but are primarily concentrated in the region of the Golgi complex. In these regions they are localized at the *trans*-side and occupy a space which includes the *trans*-Golgi network (Navone *et al.*, 1986; Baumert *et al.*, 1990; Schweitzer and Paddock, 1990; this study). The localization of SLMVs in the Golgi complex area is thought to reflect the transit of recycling SLMV membranes, because after incubation of PC12 cells in the presence of extracellular tracers, tracer-positive SLMVs rapidly appear in this region (Johnston *et al.*, 1989; De Camilli and Jahn, 1990). If SLMVs are indeed involved in a regulated release of signal molecules, lack of a significant accumulation of SLMVs under the plasmalemma suggests that regulation of SLMV exocytosis differs from regulation of synaptic vesicle exocytosis. This is not unexpected given the different characteristics of endocrine and synaptic signalling. A large reserve pool of vesicles in the proximity of release sites might be needed in nerve terminals but not in endocrine cells.

In cultured endocrine cells which extend neurite-like processes, SLMVs are concentrated in the distal portions of the processes. This property, which was shown previously for other endocrine cells (Tooze *et al.*, 1989; Schweitzer and Paddock, 1990), and is shown here for β-cells, further emphasizes the relationship between SLMVs and synaptic vesicles, because synaptic vesicles accumulate in the distal axonal arbour. It has been shown that axonal differentiation of a neuronal process occurs only after it has reached a critical length (Goslin and Banker, 1989). Thus, it was of interest that in β-cells, SLMVs were concentrated only in the distal portions of processes with neurite-like characteristics (long, often beaded processes) but not in short, thick cellular extensions. In contrast, both types of processes were enriched in secretory granules, as typically seen in processes of endocrine cells (Tooze and Burke, 1987). Accumulation of SLMVs in the distal portions of neurite-like processes might reflect a local exo–endocytotic recycling similar to the well known local exo–endocytotic recycling of synaptic vesicles in neuronal axons.

The colocalization of GABA with SLMVs was particularly impressive in the neurite-like processes where both GABA and SLMVs were present at the highest concentrations. In contrast, GABA was not colocalized with synaptic vesicle markers in the region of the Golgi complex. This observation may suggest that the property to take up GABA operates primarily in SLMVs located at the cell periphery, possibly SLMVs ready for exocytosis.

Concluding remarks

The existence of a variety of important biochemical and functional similarities between peptide-secreting endocrine cells and neurons is well known (Grazl and Langley, 1990). These similarities had led in the past to the speculation that most peptide-secreting endocrine cells and neurons have a common embryological origin from the neural crest (Pearse, 1977). It is now clear that at least some endocrine cells, including cells of pancreatic islets, are of endodermal origin (Le Douarin, 1988). Yet, a close relationship between pancreatic endocrine cells and neurons has been shown (Polak *et al.*, 1984; Navone *et al.*, 1986; Eisenbarth *et al.*, 1987; Teitelman and Lee, 1987; Teitelman *et al.*, 1987a,b; Alpert *et al.*, 1988; Baumert *et al.*, 1989, 1990). The identification of SLMVs in endocrine cells is a novel important element of similarity between endocrine cells and neurons. We have previously hypothesized that synaptic vesicles may represent a neuron-specific adaptation of SLMVs (De Camilli and Navone, 1987; De Camilli and Jahn, 1990). Our present results, suggesting a role of SLMVs of β-cells in the storage and secretion of the neurotransmitter GABA, strengthen this hypothesis. Studies aimed at providing a direct biochemical demonstration of the presence of GABA and GABA-uptake mechanisms in SLMVs of β-cells are currently under way in our laboratory.

It will be of interest to determine whether SLMVs of all endocrine cells contain non-peptide substances with paracrine/endocrine functions. Different neuronal subpopulations differ in their neurotransmitter phenotype. Expression of the GABA system only in a subpopulation of endocrine cells, and, more specifically, of a subpopulation of cells of the endocrine pancreas, is consistent with the possibility that a similar heterogeneity applies to endocrine cells. The property to co-secrete peptide and non-peptide signal molecules might be a general characteristic of neurons and endocrine cells.

Materials and methods

Animals

Sprague–Dawley rats weighing ~150 g were purchased from Charles River.

Antibodies

The following antibodies were obtained as generous gifts: affinity-purified polyclonal antibodies directed against p29 (Baumert *et al.*, 1990) and monoclonal and affinity-purified polyclonal antibodies directed against synaptophysin (Navone *et al.*, 1986) from R.Jahn (Münich); polyclonal and monoclonal antibodies directed against secretogranin I (Rosa *et al.*, 1985b) from W.Huttner (Heidelberg); a sheep serum directed against GAD (Oertel *et al.*, 1981a) from E.Mugnaini (Storrs, CT); a monoclonal antibody against GAD (Chang and Gottlieb, 1988) from D.Gottlieb (St Louis, MO); a rabbit serum directed against GAD (Katarova *et al.*, 1989) from Z.Katarova and R.J.Greenspan (Nutley, NJ); a rabbit antibody directed against GABA (Wenthold *et al.*, 1986) from R.J.Wenthold (Washington, DC); a monoclonal antibody directed against GIMPt (Yuan *et al.*, 1987) from Dr C.A. Suarez-Quian (Washington, DC). Human antibodies directed against GAD were obtained from the cerebrospinal fluid of a patient affected by Stiff–Mann syndrome (Solimena *et al.*, 1990). The following immunological reagents were purchased: anti-insulin (chicken) and anti-chicken IgG (goat) sera (Sera Lab, Westbury, NY); anti-rabbit IgG (goat), anti-sheep IgG (goat) and normal (goat) sera (Cappel, Westchester, PA); anti-mouse IgG (goat) (Sigma, St Louis, MO). Protein A–colloidal gold conjugates (4–8 nm) were prepared as described in Slot and Geuze (1983). 5 nM gold-conjugated anti-human IgGs (goat) were from E-Y Laboratories (San Mateo, CA).

Pancreatic cell lines

RINm5F cells (Gazdar *et al.*, 1980; Bhathena *et al.*, 1982) were a kind gift from L.Vallar (Milano) and K.Wollheim (Genève). They were grown in RPMI 1640 medium supplemented with 10% fetal calf serum as described (Bhathena *et al.*, 1982). βTC3 (Efrat *et al.*, 1988) and αTC1 cells (Powers *et al.*, 1990) were a kind gift from D.Hanahan (San Francisco, CA). These cell lines were grown in Click medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% fetal calf serum.

Islet isolation

Islets were isolated from rats as described by Bowen *et al.* (1980).

Neuronal cultures

Primary neuronal cultures were prepared from the hippocampi of 18-day-old fetal rats as described by Banker and Cowan (1977) and Bartlett and Banker (1984). Briefly, hippocampi were dissociated by treatment with trypsin (0.1% for 15 min at 37°C), followed by trituration with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-$_{\gamma}$-lysine-treated glass coverslips in MEM supplemented with 2 mM glutamine, 1% HL1 (Ventrex, Portland, ME), 10% horse serum, 5% fetal calf serum at densities ranging from 16 000 to 50 000 cells/cm^2. The following day, coverslips were transferred (upside down) to dishes containing a monolayer of cortical glial cells, so that they

were suspended over the glia but not in contact with them (Bartlett and Banker, 1984). Cells were maintained in MEM without sera, supplemented with 1% HL1, 2 mM glutamine and 1 mg/ml BSA.

Immunofluorescence

Pancreatic cell lines grown on poly-ornithine (100 μg/ml)-coated glass coverslips (50–70% confluency) and primary neurons after 5 days *in vitro* were used. Cells were fixed for 1 h at 4°C with 4% paraformaldehyde (freshly prepared from paraformaldehyde) in 0.12 M Na-phosphate buffer, pH 7.4. When cells were to be stained with antibodies directed against GABA, glutaraldehyde (0.1% final concentration) was added to the fixative and immunostaining was preceded by a 5 min incubation in 1% sodium borohydride to quench glutaraldehyde-induced autofluorescence.

The cells were rinsed with phosphate buffer and then permeabilized for 30 min in goat serum dilution buffer (GSDB: 17% goat serum, 0.3% Triton X-100, 0.45 M NaCl, 20 mM Na-phosphate buffer, pH 7.4). They were then double-stained by immunofluorescence using standard procedures involving secondary antibodies coupled to fluorescein and rhodamine (De Camilli *et al.*, 1983; Johnston *et al.*, 1989). All antibody incubations were performed in GDSB for 2 h at room temperature. Coverslips were mounted in 70% glycerol, 10 mg/ml phenylenediamine in PBS, observed in a Zeiss Axiophot microscope and photographed with T-Max 100 (Kodak).

Equilibrium sucrose density gradients

RINm5F cells were grown in ten 100 mm tissue culture dishes to ~70% confluency (~5 × 10^6 cells/dish). Cells were detached from the dishes by a 20 min incubation in PBS containing 10 mM EDTA at 37°C, pelleted by centrifugation, resuspended in 500 μl of homogenization buffer [250 mM sucrose, 1 mM $MgCl_2$, 0.005% DNase (Sigma, St Louis, MO), 4 mM HEPES, pH 7.4, and the following protease inhibitors: 0.4 mM PMSF (Boehringer-Mannheim, Indianapolis, IN), 10 mM benzamidine (Sigma, St Louis, MO) and 4 μg/ml each of pepstatin A, leupeptin, antipain and Aprotinin (Sigma)] and passed four to six times through a ball-bearing homogenizer (clearance: 0.0028 inches; Berni-Tech, Saratoga, CA). The homogenate was centrifuged (5000 *g* for 10 min at 4°C) and the supernatant [post nuclear supernatant (PNS)] recovered. EDTA was added to the PNS to a final concentration of 1.5 mM. The PNS was then loaded onto the top of a continuous sucrose density gradient (0.45–2 M sucrose, 1.5 mM EDTA, 4 mM HEPES, pH 7.4) and centrifuged at 30 000 r.p.m. for 18 h at 4°C in a SW41 rotor (Beckman, Fullerton, CA). Fractions (750 μl) were collected from the top of the gradient. The sucrose molarity of each fraction was determined by measuring its refractive index. Fractions were then analyzed by SDS–PAGE and Western blotting.

Immunoprecipitation assays

Preparation of cell and tissue extracts. Cells grown in 100 mm dishes were harvested and pelleted as described for sucrose density gradients. The cell pellet was resuspended in (200 μl/dish) ice-cold buffer A (150 mM NaCl, 10 mM HEPES, pH 7.4, protease inhibitors as described above) containing 2% Triton X-100 and incubated on ice for 2 h. Insoluble material was removed by centrifugation (16 000 *g* for 30 min, 4°C). The remaining supernatant was stored at −20°C and used for immunoprecipitations. The protein concentration of the extracts varied between 1.0 and 1.5 mg/ml for the different cell lines. Rat brain and liver were homogenized in 10 vol (w/v) of buffer A using a polytron homogenizer (30 s on ice). The homogenate was then spun at 100 000 *g* for 60 min at 4°C and the pellet was resuspended in 40 ml of ice cold homogenization buffer containing either Triton X-100 (2% w/v) or Triton X-114 (2% w/v) and incubated on ice for 2 h. Insoluble material was removed as described for cells. The Triton X-114 supernatant was further processed as described (Bordier, 1981). Both Triton X-100 and Triton X-114 were diluted 1:1 in homogenization buffer before use; the final protein concentration was 0.5 mg/ml (Triton X-114 brain extract), 1.2 mg/ml (Triton X-100 brain extract) and 0.625 mg/ml (Triton X-114 liver extract). Approximately 500 freshly dissociated rat islets were pelleted and resuspended in 500 μl buffer A with 2% Triton X-100. They were further processed as described for cells, above.

Immunoprecipitation. Preclearing of detergent extracts was performed as follows. Control human serum (25 μl) was added to 0.5 ml of each extract. After 1 h incubation, 100 μl of 50% protein A–Sepharose (Pharmacia, Uppsala, Sweden) was added to each sample, the samples were incubated for an additional hour and centrifuged at 750 *g* for 1 min. The resulting supernatants were incubated for an additional hour with 100 μl of 50% protein A–Sepharose and centrifuged as described above.

For immunoprecipitation, the following additions were made in sequential order to precleared extracts incubated with rotation: 1.5 μl of sheep serum directed against GAD or preimmune sheep serum (Oertel *et al.*, 1981a) (16 h), 20 μl of rabbit IgGs directed against sheep IgGs (1.5 h), 125 μl of 50% protein A–Sepharose (1.5 h). The samples were then centrifuged (750 *g* for 1 min) and the resulting protein A–Sepharose pellets were washed three times in 150 mM NaCl, 10 mM HEPES, pH 7.4. All preclearing and immunoprecipitation steps were carried out at 4°C. The final washed pellet was solubilized in 75 μl of gel sample buffer (Laemmli *et al.*, 1970) (0.0625 M Tris–Cl, pH 6.8, 2% SDS, 10% glycerol, 30 mM DTT, bromophenol blue), heated at 100°C for 3 min, and centrifuged at 16 000 *g* for 10 min. The supernatants were removed and stored at −20°C. Immunoprecipitates were analyzed by SDS–PAGE and Western blotting.

Miscellaneous analytical procedures

Proteins were measured by the BCA assay (Pierce). SDS–PAGE was performed according to Laemmli *et al.* (1970). Western blotting was performed as described by Towbin *et al.* (1979) using a [^{125}I]protein A (Amersham, Arlington Heights, IL) radioimmunolabeling procedure.

Electron microscopy

Electron microscopy immunocytochemistry was performed essentially as described by De Camilli *et al.* (1983) and modified by Mignery *et al.* (1989). Briefly, fresh dissociated rat islets or rat brains were mildly homogenized in 0.25 M sucrose, 25 mM KCl, 10 mM Na-phosphate buffer, pH 7.4, by passing them a few times through a loosely fitting glass–Teflon homogenizer. The resulting tissue fragments were fixed, embedded in agarose, labeled by an immunogold procedure and finally embedded in plastic and processed for electron microscopy observations as described. Affinity-purified antibodies directed against synaptophysin followed by protein A–gold were used for the anti-synaptophysin immunostain. A human CSF containing autoantibodies specifically directed against GAD (Solimena *et al.*, 1980) followed by gold-conjugated goat anti-human IgGs was used for the anti-GAD immunostain. The specificity of these antibodies was previously reported [case no. 161 of Solimena *et al.* (1990) identical to case no. 2 of Schwartzmann *et al.* (1991)].

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