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A γ-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic β cells

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ABSTRACT **A variety of peptide-secreting endocrine cells contain a population of recycling microvesicles that share several major membrane polypeptides with neuronal synaptic vesicles (SVs). The function of these synaptic-like microvesicles (SLMVs) remains to be elucidated. It was previously suggested that SLMVs of pancreatic β cells may store and secrete γ-aminobutyric acid (GABA). GABA, the major nonpeptide inhibitory neurotransmitter of the central nervous system, is stored in and secreted from SVs. GABA uptake into SVs is mediated by a transporter that is driven by a vacuolar proton ATPase. GABA is also present at high concentration in the endocrine pancreas where it is selectively localized in insulin-secreting β cells, the core cells of pancreatic islets. GABA is not present in peripheral islet cells (mantle cells), represented primarily by glucagon-secreting α cells. In this study, an immunoisolation procedure was used to purify SLMVs from cell lines derived from mouse β cells and α cells. SLMVs obtained from the β-cell line, but not those obtained from the α-cell line, displayed a GABA-transport activity dependent upon a proton electrochemical gradient generated by a vacuolar proton ATPase. These data support the hypotheses that (*i*) SLMVs have a secretory function similar to that of SVs and (*ii*) β-cell SLMVs are involved in the secretion of GABA, which in turn may have a paracrine function on mantle cells of the islet.**

In the nerve terminal, nonpeptide neurotransmitters are stored in and secreted from highly specialized secretory organelles known as synaptic vesicles (SVs). Neurotransmitters are loaded into these vesicles by specific transporters that are driven by an electrochemical gradient that is generated by a proton vacuolar ATPase (V-ATPase) present in the vesicle membrane (1). Recent studies have shown that organelles closely related to SVs and referred to as synaptic-like microvesicles (SLMVs) are present in a variety of peptide-secreting endocrine cells (2–4). Both SLMVs and SVs undergo cycles of exocytosis/endocytosis at the cell surface (5–9). It is still unclear whether SLMVs, like SVs, store and secrete neurotransmitters or neurotransmitter-like molecules. The pancreatic β cell is a particularly well-suited model to address this question. β cells contain high concentrations of the neurotransmitter γ-aminobutyric acid (GABA) and of the enzymes that catalyze the biosynthesis [glutamic acid decarboxylase (GAD)] and metabolism (GABA transaminase) of GABA (10–14). Furthermore, a paracrine role of GABA in the islets has been suggested (15–19). It has been demonstrated that GAD, which in GABA-secreting neurons is concentrated in close proximity to SVs, is also concentrated in close proximity to SLMVs of pancreatic β cells (20). In this study, an immunoisolation procedure was used to obtain highly purified SLMVs from pancreatic β- and α-cell lines. SLMVs obtained from β cells, but not those obtained from α cells, contain a GABA transport activity driven by a V-ATPase that is similar to that of SVs.

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MATERIALS AND METHODS

Antibodies. The following antibodies were previously described: affinity-purified polyclonal antibodies directed against p29 (21), monoclonal antibody (mAb) C.7.2 and polyclonal antibodies directed against synaptophysin (2), polyclonal antibodies directed against synaptobrevin (22), human serum containing autoantibodies directed against GAD (14), two antibodies directed against the $\beta_2\beta_3$ subunit of the $GABA_A$ receptor [mAb bd17 (23, 24) and mAb 62-3G1 (26, 27)], an antibody directed against ribophorin II (28), and an antibody directed against SV2 (29). The following immunological reagents were obtained from commercial sources: anti-insulin (chicken) and anti-chicken IgG (goat) sera (Sera Lab, Westbury, NY), anti-rabbit IgG (goat) and normal goat serum (Cappel Laboratories), and anti-mouse IgG (goat) (Sigma).

Pancreatic Cell Lines. βTC3 (30) and αTC9 cells (31) were grown in Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% (vol/vol) fetal calf serum.

Organelle Immunoisolation. Nonimmune bovine immunoglobulins (Sigma) or purified mAb C.7.2 directed against synaptophysin was conjugated to Eupergit CIZ methacrylate microbeads (Rohm Pharmaceuticals, Darmstadt, Germany) as described (32, 33). βTC3 cells were grown to about 70% confluency in either 100-mm tissue culture dishes (≈5×10^6 cells per dish) or 175-mm flasks (≈15×10^6 cells per flask). Cells were detached from the dishes by a 10-min incubation in phosphate-buffered saline (PBS)/10 mM EDTA at 37°C, pelleted by centrifugation, resuspended in 1 ml of homogenization buffer [250 mM sucrose, 1 mM $MgCl_2$, 0.005% DNase (Sigma), 4 mM Hepes at pH 7.4 containing the following protease inhibitors: 0.4 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim), 10 mM benzamidine (Sigma), and pepstatin A, leupeptin, antipain, and aprotinin (each at 4 μg/ml; Sigma)], and passed four to six times through a ball-bearing homogenizer (clearance of 0.0028 inch; Berni-Tech, Saratoga, CA). The homogenate was centrifuged (10,000 × *g* for 10 min at 4°C), and the supernatant [low speed supernatant (LSS)] was recovered. EDTA was added to the LSS at a final concentration of 1.5 mM. The LSS was incubated with immunobeads for 60 min at 4°C with constant rotation. For electron microscopy and [^{3}H]GABA uptake experiments, an excess of LSS relative to immunobeads was used. In this case, a LSS obtained from 30 to 40 175-mm flasks was incubated with 200 μl of an immunobead pellet. The beads were sedimented by centrifu-

Abbreviations: SV, synaptic vesicle; SLMV, synaptic-like microvesicle; V-ATPase, vacuolar proton pump ATPase; LSS, low speed supernatant; GABA, γ-aminobutyric acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; GAD, glutamic acid decarboxylase; $\Delta\mu H^+$, proton electrochemical gradient; mAb, monoclonal antibody.

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gation at 5000 × g for 30–60 sec at 4°C, washed three times in homogenization buffer, and further processed for gel electrophoresis and Western blotting, electron microscopy, or ΔpH measurement and [^{3}H]GABA uptake experiments as described below. At the end of each uptake experiment, the recovery of SLMVs on the beads was evaluated by gel electrophoresis followed by immunoblotting for synaptophysin. This control was also used to confirm that comparable recoveries of synaptophysin were obtained in different experiments and for both βTC3 and αTC9 cells. Typically, about 40–60% of the synaptophysin in the LSS of both βTC3 and αTC9 cells was recovered on the immunobeads.

Measurement of ΔpH. ΔpH was measured as described (33) with minor modifications. Briefly, the washed immunobeads were resuspended in 320 mM sucrose/10 mM Mops-KOH, pH 7.3, so that a 50-μl bead suspension was equivalent to a 1-μl bead pellet. (This bead suspension was used for ΔpH measurements and [^{3}H]GABA uptake experiments.) ΔpH was measured using the pH-sensitive dye acridine orange. Changes in absorbance at 492 nm were measured with an Aminco DW 2000 dual wavelength spectrophotometer, using 530 nm as a reference wavelength. A 100-μl bead suspension was added to 900 μl of standard assay buffer (160 mM KCl/4 mM $MgCl_2$/10 mM Mops-KOH, pH 7.3/10 μM acridine orange) at 32°C. When H^+-ATPase inhibitors were used (50 μM *N*-ethylmaleimide, 50 μM vanadate, and 5 μM oligomycin B), they were added to standard assay buffer, and the mixture was preincubated for 5 min at 32°C. After a stable baseline was established, the reaction was initiated by the addition of MgATP (2 mM final concentration). The acidification was reversed either by the addition of the protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (60 μM final concentration), which collapses the proton electrochemical gradient ($\Delta\mu H^+$), or by the addition of $(NH_4)_2SO_4$ (10 mM final concentration), which eliminates the pH gradient but not the transmembrane potential.

[^{3}H]GABA Uptake. [^{3}H]GABA uptake was measured as described (34) with minor modifications. A 100-μl bead suspension was preincubated for 3 min at 32°C. Then 50 μl of 3× uptake medium was added to the beads to obtain the following final concentrations: 320 mM sucrose, 4 mM $MgCl_2$, 4 mM KCl, 2 mM MgATP, 10 mM Mops-KOH (pH 7.3), 300 μM unlabeled GABA (present as a carrier molecule), 4 μCi (1 Ci = 37 GBq) of [^{3}H]GABA and either (*i*) no additions, (*ii*) 50 mM unlabeled GABA, or (*iii*) 60 μM FCCP. After a 5-min incubation at 32°C, the beads were sedimented by centrifugation for 30 sec at 5000 × g. The bead pellet was immediately resuspended in 500 μl of ice-cold 320 mM sucrose/10 mM Mops-KOH, pH 7.3. The beads were washed rapidly three times at 0°C by resuspension, vortexing, and centrifugation as described above. Scintillation fluid was added to the washed bead pellet, and ^{3}H content was determined by using a scintillation counter. The amount of [^{3}H]GABA that was recovered on the immunobead fraction in the presence of ATP plus FCCP was taken as a baseline value because FCCP completely dissipates $\Delta\mu H^+$, which drives GABA uptake into SVs. This baseline value represents either nonspecific binding to the immunoisolated vesicles or ATP-independent transport of GABA.

HPLC Determination of GABA Content of βTC3 and αTC9 Cells. βTC3 cells or αTC9 cells were grown in 175-mm flasks for 5 days until they were 70% confluent. Cells were detached from the flasks by incubation with PBS/10 mM EDTA, pelleted by centrifugation at 1000 × g for 10 min at room temperature, and resuspended in 150 mM NaCl/10 mM

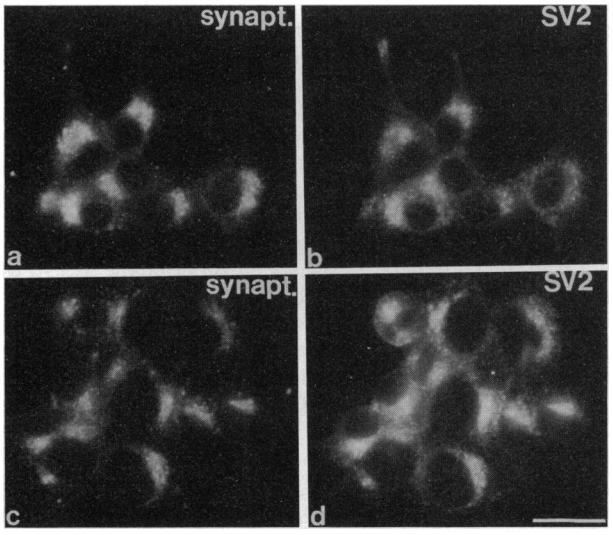

FIG. 1. Double-immunofluorescence micrographs showing colocalization of the SV proteins synaptophysin (synapt.) and SV2 in βTC3 (*a* and *b*) and αTC9 (*c* and *d*) cells. The two proteins have a similar distribution in both cell lines. Immunoreactivity is scattered throughout the cells but is concentrated at a paranuclear area, which corresponds to the Golgi complex area. (Bar = 17 μm.)

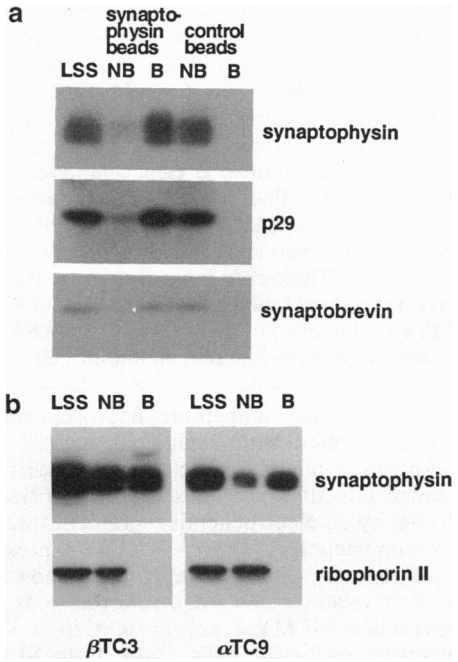

FIG. 2. Specificity of the SLMV immunoisolation procedure. (*a*) Recovery of synaptophysin, synaptobrevin, and protein p29 in organelles bound by anti-synaptophysin immunobeads and control immunobeads incubated with a crude extract (LSS fraction) of βTC3 cells. The starting material (LSS fraction), the resulting supernatant (NB, not bound material), and the immunoabsorbed material (B, bound material) were analyzed by gel electrophoresis followed by immunoblotting with antibodies directed against each of the three proteins. The immunoabsorbed material was resuspended to the original volume of the LSS, and equal volumes of LSS, NB, and B were loaded onto the gel. Therefore, the intensity of the labeled bands closely reflects the partition of the three proteins in the NB and B fractions. In this experiment an excess of immunobeads was used, and SLMV proteins were almost completely depleted from the starting LSS. None of SLMV proteins was recovered on control beads. (*b*) Western blot analysis of immunoisolation experiments performed on LSS fractions of α and β cells for the study of GABA uptake. In these experiments an amount of LSS was chosen to saturate the binding capacity of the beads and therefore to maximize recovery of synaptophysin-positive organelles. Organelle binding was still highly specific as shown by the lack of any detectable immunoreactivity for the endoplasmic reticulum marker ribophorin II in the bead-bound fraction.

Hepes, pH 7.3. A small aliquot was saved for protein determination. Perchloric acid was added to a final concentration of 1%, and the cells were solubilized by using a tip sonicator at 50% power for 3 min. Insoluble particles were removed by centrifugation, and the resulting supernatant was adjusted to pH 8.0 with 1 M NaOH and stored at −70°C until used for HPLC analysis. Determination of amino acid content was accomplished by precolumn derivatization with *o*-phthalaldehyde-3-mercaptopropionic acid followed by reverse-phase HPLC and fluorimetric detection (35).

Miscellaneous Procedures. Proteins were measured by the BCA assay (Pierce). SDS/PAGE was performed according to Laemmli (36) and Western blotting was according to Towbin *et al.* (37) using ^{125}I-labeled protein A (Amersham). Double immunofluorescence was performed as described (20). Electron microscopy of the immunobead fraction was performed as described (8, 32).

RESULTS

In this study, cell lines that are derived from pancreatic tumors of transgenic mice expressing the simian virus 40 large tumor antigen under the control of either the insulin (β-cell lines) or glucagon (α-cell lines) promoter (30, 31, 39) have been used. The β-cell line βTC3 expresses GAD and contains immunohistochemically detectable levels of GABA. However, levels of GAD and GABA in these cells are heterogeneous, and the relative abundance of GAD- and GABA-expressing cells varied in culture from different passages (20). The α-cell line αTC9 was derived from the αTC1 cell line (31), which is devoid of GAD and GABA immunoreactivity (20). The GABA content of a βTC3 extract, as determined by HPLC, was ≈4-fold higher (1.24 nmol/mg of protein) than that of an αTC9 extract (0.28 nmol/mg of protein). Since less than 20% of the βTC3 cells used for these experiments were positive for GAD and GABA by immunocytochemistry, the value obtained for βTC3 cells is clearly an underestimate of the value expected from an homogeneous population of GAD-expressing βTC3 cells. The amount of GABA measured by HPLC in αTC9 cells probably reflects nonvesicular metabolic levels of GABA since these cells do not have concentrated stores of GABA detectable by immunocytochemistry (20). The amount of GABA in isolated mouse islets was reported to be 3 nmol/mg of protein (40).

Both βTC3 and αTC9 cells were found to express membrane proteins characteristic of SVs and SLMVs, including synaptophysin and SV2 (Fig. 1) and synaptobrevin and protein p29 (data not shown). This agrees with previous observations made in intact islets (2, 20–22, 29). These proteins had a similar subcellular distribution in both β cells and α cells, as shown in Fig. 1 for SV2 and synaptophysin.

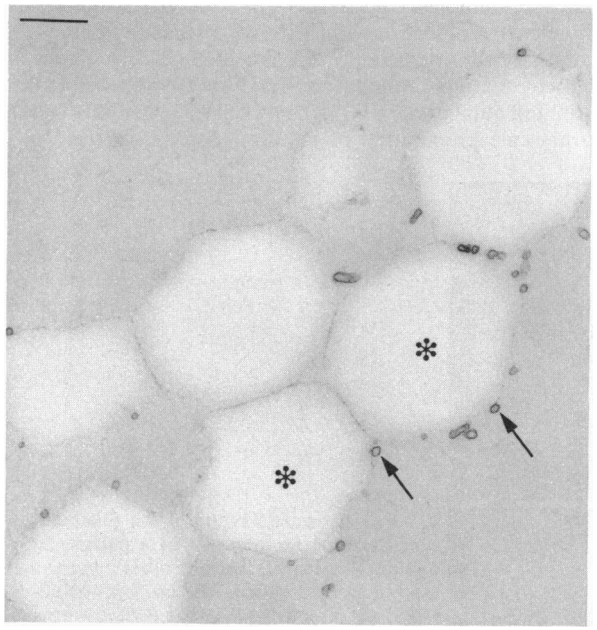

FIG. 3. Electron micrograph showing the morphology of organelles immunoisolated by anti-synaptophysin immunobeads from a crude extract (LSS fraction) of βTC3 cells. Bound organelles are represented by small round or elongated vesicles (arrows). The core of plastic beads is labeled by asterisks. Note the lack of secretory granules on the beads. (Bar = 300 nm.)

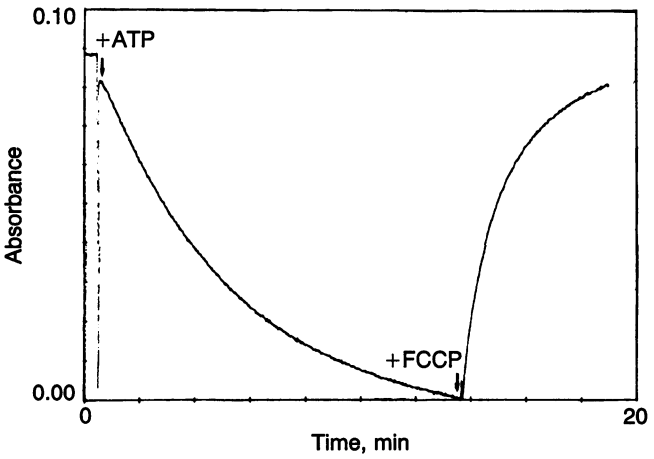

FIG. 4. ATP-dependent acidification monitored by acridine orange in SLMVs immunoisolated from βTC3 cells. A 100-μl bead suspension was incubated at 32°C with 900 μl of acidification buffer containing the pH-sensitive dye acridine orange. The absorbance was measured at 492 nm using 530 nm as a reference wavelength. Once a stable baseline had been established, MgATP was added (2 mM final concentration). The acidification of the vesicle is registered as a decrease in absorbance. Acidification could be reversed by the addition of the proton ionophore FCCP at 60 μM.

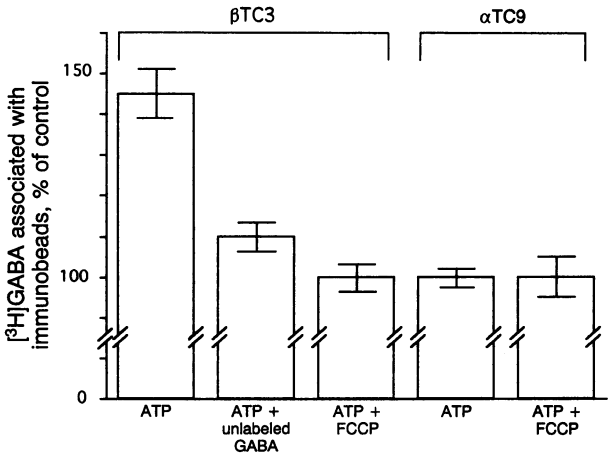

FIG. 5. [^{3}H]GABA uptake into SLMVs immunoisolated from βTC3 or αTC9 cells. Aliquots of immunobead fractions were incubated with [^{3}H]GABA with the additions indicated in the figure. [^{3}H]GABA uptake is expressed as percent increase of the radioactivity recovered in the immunobead fraction at the end of the incubation over the radioactivity recovered when FCCP (which dissipates $\Delta\mu H^+$) was present in the uptake medium. A FCCP-sensitive [^{3}H]GABA uptake, which could be blocked by competition with nonradioactive GABA, was observed only in SLMVs from βTC3 cells. Each incubation condition was performed in duplicate. Results represent the combined data of four independent experiments performed with βTC3 cells and two independent experiments performed with αTC9 cells. Standard error bars are indicated in the figure.

In the following experiments, methacrylate beads covalently conjugated to anti-synaptophysin mAbs were used to immunoisolate synaptophysin-containing organelles from a LSS fraction of a βTC3 homogenate. The enrichment of synaptophysin in the immunoabsorbed fraction paralleled the enrichment of p29 and synaptobrevin, which confirms the colocalization of these proteins on the same organelles (Fig. 2*a*). None of the proteins were recovered in control bead fractions prepared with nonimmune IgGs (Fig. 2*a*). The specificity of the immunoisolation procedure was further supported by the absence of the endoplasmic reticulum marker ribophorin II in the immunoabsorbed fraction (Fig. 2*b*).

An electron microscopic analysis of organelles immunoisolated from βTC3 cells is shown in Fig. 3. Organelles bound to the beads were represented by small vesicles and tubules in the same size range as SVs. They are similar to the synaptophysin-positive organelles of β-cells previously identified by *in situ* electron microscopy immunocytochemistry (20).

GABA uptake into SVs is dependent on a $\Delta\mu H^+$ generated by a V-ATPase (34). In each GABA-uptake experiment, aliquots of SLMVs, isolated from either βTC3 or αTC9 cells, were first tested for the presence of an active proton pump. Acidification of SLMVs was monitored photometrically using the pH-sensitive dye acridine orange as an indicator. In the presence of MgATP, the SLMVs acidified, as indicated by a decrease in absorbance of the dye (Fig. 4). The acidification was reversed by the addition of the protonophore FCCP, which confirms a proton-driven process (Fig. 4). Furthermore, acidification was completely abolished if SLMVs were preincubated with *N*-ethylmaleimide (inhibitor of V-ATPases) (ref. 41; data not shown), but not with either vanadate (inhibitor of the plasma membrane ATPase) (ref. 41; data not shown) or oligomycin B (inhibitor of the F_1F_0-ATPase) (ref. 41; data not shown), indicating that acidification of SLMVs is catalyzed by a vacuolar V-ATPase as observed in SVs.

For the measurement of GABA uptake, aliquots of SLMVs were incubated with [^{3}H]GABA in a buffer known to support GABA transport into SVs. In SLMVs isolated from βTC3 cells, GABA uptake that was dependent on ATP and sensitive to the protonophore FCCP was observed (Fig. 5). This result indicates that β-cell SLMVs contain a GABA transport system, similar to that present in SVs, which is dependent on $\Delta\mu H^+$ generated by a V-ATPase. [^{3}H]GABA uptake was reduced by competition by 50 mM unlabeled GABA, which demonstrates that GABA uptake is mediated by a saturable transporter. As observed for SVs, competition was incomplete, indicating the presence of a transporter with a low substrate affinity. In SLMVs isolated from αTC9 cells, no ATP-dependent uptake of GABA was detected (Fig. 5). Since SLMVs were isolated with equal efficiency from both β and α cells (Fig. 2*b*), the absence of GABA transport activity in organelles isolated from αTC9 cells is probably due to the absence of a GABA transporter.

A paracrine role of GABA in pancreatic islets was previously suggested by the detection of $GABA_A$ receptor-mediated responses and of $GABA_A$ receptor β-subunit immunoreactivity (mAb bd17) in glucagon-secreting and somatostatin-secreting cells (15–19). We have further investigated the localization of $GABA_A$ receptor immunoreactivity in the endocrine pancreas *in situ* by using two mAbs directed against the $\beta_2\beta_3$ isoforms of this receptor [mAb bd17 (23, 24) and mAb 62-3G1 (26, 27)]. In immunofluorescence experiments on rat pancreatic sections, both antibodies produced an identical staining pattern. Immunoreactivity was localized in peripheral islet cells and in a few scattered cells within the islet core (Fig. 6 *b* and *d*). Counterstaining of the same sections for either insulin (Fig. 6*a*) or GAD (Fig. 6*c*) demonstrated that virtually all $GABA_A$ receptor positive cells were non-β cells, although a few β cells that were also positive for $GABA_A$ receptor were occasionally observed (Fig. 6 and data not shown).

DISCUSSION

SLMVs of endocrine cells are biochemically similar to SVs in their membrane protein composition. It was previously hypothesized that SLMVs store and secrete neurotransmitter or neurotransmitter-like molecules (4, 20). To our knowledge, the present study provides the first demonstration that SLMVs are capable of transporting neurotransmitter molecules in a manner similar to SVs.

The function of SLMVs was investigated by using pancreatic β cells as model endocrine cells because they are known to contain high levels of the inhibitory neurotransmitter GABA (10–13) and, furthermore, a paracrine role for GABA in pancreatic islets has been proposed (15–19). Moreover, it was previously demonstrated that GABA and GAD immunoreactivity closely colocalize with SLMVs, suggesting that SLMVs are the storage organelle for GABA in β cells. A direct measurement of GABA content in SLMVs was not feasible due to the limited number of SLMVs that could be purified even from a large-scale preparation of cultured β cells, as used in this

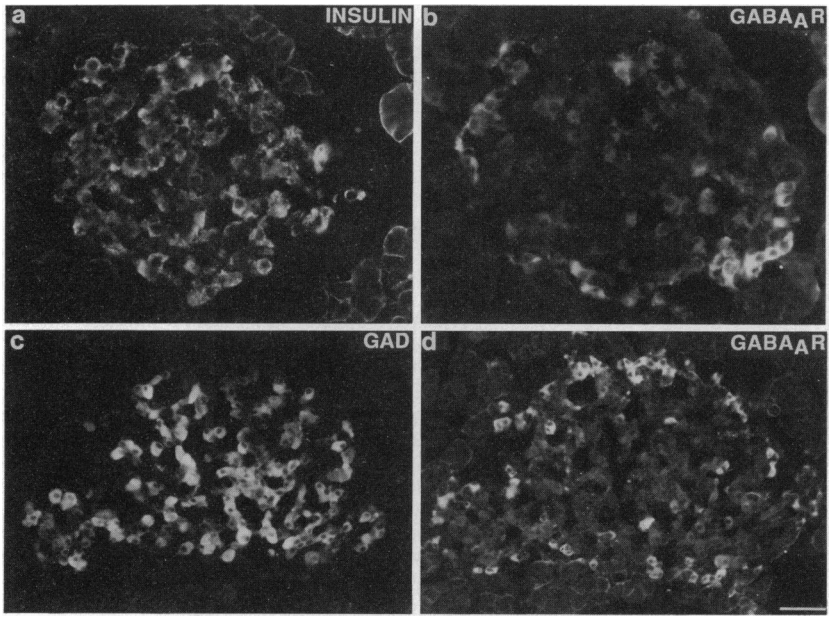

FIG. 6. Localization of the β subunit of the $GABA_A$ receptor in a pancreatic islet. Two frozen sections of rat pancreas were stained with mAbs bd17 (*b*) or 62-3G1 (*d*) directed against the $\beta_2\beta_3$ isoforms of the β subunit of the $GABA_A$ receptor ($GABA_AR$) and counterstained with antisera directed against insulin (*a*) or GAD (*c*). $GABA_A$ receptor immunoreactivity is present mainly in the peripheral cells of the islet, which are negative for GAD and for insulin. (Bar = 30 μm.)

study. However, a prerequisite for the storage of GABA within SLMVs is the presence of a GABA transporter in SLMVs capable of transporting GABA in a substrate-specific manner.

To discern if SLMVs contain a GABA transporter, a rapid immunoisolation procedure was used that yielded SLMVs with high purity and that was previously demonstrated to preserve SVs in a metabolically active form (32, 42). While SLMVs are present both in α and β cells of pancreatic islets, GABA transport activity was present only in β-cell SLMVs, which is consistent with the selective localization in β cells of GABA, GAD, and the GABA-metabolizing enzyme GABA transaminase (10–14). It will be of interest to determine the content of SLMVs of α cells and of other mantle cells of the islets.

A study of GABA secretion from βTC3 and its regulation was attempted by using HPLC to quantitate the amount of GABA released into the medium in response to various stimuli. An efflux of GABA was observed under depolarizing conditions (50 mM K^+). However, it was paralleled by the efflux of other amino acids, was Ca^{2+}-independent, and was probably due to nonvesicular release mediated by reversible plasmalemma amino acid transporters. No detectable peaks of GABA secretion were observed after treatments (high glucose, isobutylmethylxanthine and arginine, or phorbol ester and carbachol) that led to a substantial increase of insulin secretion into the medium. However, the levels of GABA in the medium were at the limit of detectability with a sensitive HPLC procedure, and no firm conclusions could be drawn from these studies (A.T.-R., M.J.D., and P.D.C., unpublished observations). Due to the absence of a high-affinity plasma membrane GABA transporter in βTC3 cells (A.T.-R. and P.D.C., unpublished observations), release could not be measured by preloading the cells with radioactive GABA, a procedure commonly used to study GABA release from nerve endings (43). A further investigation of GABA secretion and of its regulation in β cells and β-cell lines remains an important priority for future studies. β-cell lines that homogeneously express GABAergic features *in situ* will be very useful for this study.

Although a direct study of GABA secretion from βTC3 cells was not feasible, the presence of $GABA_A$ receptors in mantle cells [i.e., in the cells that are immediately adjacent to β cells and are downstream in the capillary blood flow (44)] strongly supports the hypothesis that GABA acts as a paracrine signal molecule in pancreatic islets. The presence of these receptors was previously suggested by experiments carried out *in vivo* as well as on freshly isolated pancreatic cells (15–19). GABA was found to produce a hyperpolarization of dissociated α cells and to inhibit glucagon secretion (18, 19). In addition, subpopulations of dissociated α and δ cells were immunoreactive with the mAb bd17 directed against the $\beta_2\beta_3$ subunits of the $GABA_A$ receptor (18). These data have been complemented by the present study, which demonstrates an identical staining of mantle cells *in situ* with two different antibodies that recognize the $\beta_2\beta_3$ subunits of the $GABA_A$ receptor. It is of interest to note that Zn^{2+} is stored together with insulin in secretory granules (25). Zn^{2+} is a modulator of $GABA_A$ receptor function in the central nervous system (38), and perhaps it also plays a modulatory role on pancreatic $GABA_A$ receptors. In conclusion, the present data strongly support the hypothesis that β-cell SLMVs are involved in GABA storage and secretion and more generally that SLMVs of endocrine cells may have functional similarity with neuronal SVs.

Note Added in Proof. After the submission of this manuscript for review, regulated GABA secretion from the amphicrine pancreatic cell line AR42J was reported (45).

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