Secretory Granule-mediated Co-secretion of L-Glutamate and Glucagon Triggers Glutamatergic Signal Transmission in Islets of Langerhans*

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1-L-Glutamate is believed to function as an intercellular transmitter in the islets of Langerhans. However, critical issues, i.e. where, when and how 1-glutamate appears, and what happens upon stimulation of glutamate receptors in the islets, remain unresolved. Vesicular glutamate transporter 2 (VGLUT2), an isoform of the vesicular glutamate transporter essential for neuronal storage of l-glutamate, is expressed in α cells (Hayashi, M., Otsuka, M., Morimoto, R., Hirota, S., Yatsushiro, S., Takeda, J., Yamamoto, A., and Moriyama, Y. (2001) J. Biol. Chem. 276, 43400–43406). Here we show that VGLUT2 is specifically localized in glucagon-containing secretory granules but not in synaptic-like microvesicles in αTC6 cells, clonal α cells, and islet α cells. VGLUT1, another VGLUT isofrom, is also expressed and localized in secretory granules in α cells. Low glucose conditions triggered co-secretion of stoichiometric amounts of l-glutamate and glucagon from αTC6 cells and isolated islets, which is dependent on temperature and Ca2+ and inhibited by phentolamine. Similar co-secretion of l-glutamate and glucagon from islets was observed upon stimulation of β-adrenergic receptors with isoproterenol. Under low glucose conditions, stimulation of glucamate receptors facilitates secretion of γ-aminobutyric acid from MIN6 m9, clonal β cells, and isolated islets. These results indicate that co-secretion of l-glutamate and glucagon from α cells under low glucose conditions triggers GABA secretion from β cells and defines the mode of action of l-glutamate as a regulatory molecule for the endocrine function. To our knowledge, this is the first example of secretory granule-mediated glutamatergic signal transmission.

1-L-Glutamate is the major excitatory neurotransmitter in the central nervous system and plays important roles in many neuronal processes such as fast synaptic transmission and neuronal plasticity (1, 2). To use l-glutamate as an intercellular signaling molecule, neuronal cells develop the glutamatergic system. Thus, l-glutamate is accumulated in synaptic vesicles through vesicular glutamate transporters (VGLUTs), and is secreted through exocytosis. The released l-glutamate binds to the receptor so as to transmit signals intercellularly. The excess amount of l-glutamate in synaptic cleft is sequestered through plasma membrane-type glutamate transporter.

Recent evidence has indicated that peripheral non-neuronal tissues also possess the glutamatergic system and use l-glutamate as an intercellular transmitter (3). The islet of Langerhans, a pancreatic miniature organ for the hormones regulating the blood glucose level, is composed of four major types of endocrine cells, i.e. insulin-secreting β cells, glucagon-secreting α cells, somatostatin-secreting δ cells, and pancreatic polypeptide-secreting F cells. These islet cells express functional glutamate receptors and plasma membrane-type glutamate transporter (4–11), suggesting that l-glutamate functions as an intercellular transmitter in islet. In fact, l-glutamate has been shown to affect secretion of insulin or glucagon from islet cells, isolated islets, or perfused pancreas (4–11). However, the role of l-glutamate as an intercellular chemical transmitter in the islets has been long controversial, mainly because critical issues, i.e. where, when, and how l-glutamate appears in the islets and what happens upon stimulation of glutamate receptors in the islets, remain unresolved.

Recent findings indicate that brain-specific Na+-dependent inorganic phosphate cotransporter (12) and its isoform, differentiation-associated Na+-dependent inorganic phosphate co-transporter (13), function as VGLUTs and are thus abbreviated as VGLUT1 and VGLUT2, respectively (14–21). These VGLUTs seem to be potential probes for the site of l-glutamate release in peripheral tissues as well as the central nervous system since these transporters are essential for l-glutamate signal output. We have shown that VGLUT2 is expressed in αTC6 cells, clonal α cells, and islet α cells, but not in β or δ cells (18). These results are consistent with the occurrence of Ca2+-dependent exocytosis of l-glutamate from αTC6 cells (22) and suggest that α cells are the sites of l-glutamate signal appearance.

During course of the study, we noticed that the expression and subcellular localization of VGLUTs are of extraordinary

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1 The abbreviations used are: VGLUT, vesicular glutamate transporter; MOFS, 4-morpholinopropanesulfonic acid; RT, reverse transcription; DMEM, Dulbecco’s modified Eagle’s medium; GABA, γ-aminobutyrate; AMPA, (R,S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; SLMV, synaptic-like microvesicles.
significance as to islet physiology. Here we show that both VGLUT1 and VGLUT2 are specifically localized with glucagon-containing secretory granules in αTC6 cells and islet α cells. Low glucose conditions triggers co-secretion of stoichiometric amounts of l-glutamate and glucagon. Stimulation of glutamate receptors in turn facilitates GABA secretion from β cells. These results solve, at least in part, where, when, and how l-glutamate appears in the islets and what happens upon stimulation of glutamate receptors in the islets. Since evidence for a role of GABA as a paracrine signal transmitter in the islet has been reported (23–26), these results also suggest the presence of l-glutamate- and GABA-mediated cross-talk between α and β cells in the islets of Langerhans.

EXPERIMENTAL PROCEDURES

Preparations—Islets of Langerhans were isolated from male Wistar rats at 7–8 postnatal weeks by the collagenase digestion method combined with discontinuous Ficoll gradient centrifugation (27). Islets were then handpicked and suspended in a bicarbonate-buffered Hanks’ solution supplemented with 0.2% bovine serum albumin. αTC6 cells were cultured as described (28). MIN6 m9 cells were cultured as described (29).

In some experiments, αTC6 cells (1.0 × 10^6) were washed with 20 mM MOPS-Tris (pH 7.0) containing 0.3 mM succinate, 5 mM EDTA, 5 μM/ml leupeptin, and 5 μM/ml pepstatin A and then extensively homogenized. The homogenate was centrifuged at 800 × g for 10 min, and the resultant supernatant was centrifuged at 100,000 × g for 30 min. The particulate fraction was stored in the above buffer applied to a continuous sucrose density gradient (0.4–1.4 m) and centrifuged at 75,000 × g for 3.5 h. Then, the supernatant was fractionated in 11 tubes from the bottom, and the glucagon content was determined (see Fig. 1C). Crude synaptic vesicles (LP2 fraction) were prepared as described previously (18). To prepare membrane fraction of islets, at least 500 islets were washed, suspended in 2 ml of 20 mM MOPS-Tris (pH 7.0) containing 0.3 mM succinate, 5 mM EDTA, 10 μM/ml leupeptin A and 10 μM/ml leupeptin, and homogenized by hand with a small glass homogenizer. The homogenate was centrifuged at 900 × g for 10 min, and the resultant supernatant was centrifuged at 266,000 × g for 30 min in a Beckman Optima TLX ultracentrifuge. The pellet was further treated in the same buffer and used for experiments.

Immunohistochemistry and Immunoelectronmicroscopy—Indirect immunofluorescence microscopy was performed as described previously, using an Olympus FV-300 confocal laser microscope (18). For immunoelectronmicroscopy, the LR White embedding immunogold method was used with a slight modification (30). The animals were anesthetized with ether and then perfused intracardially with saline followed by 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.4). Then each pancreas was cut into small pieces, washed with 0.1 M cacodylate buffer (pH 7.4), stained with uranyl acetate for 2 h, dehydrated, and then embedded in LR White for 2 days at −20 °C. Ultra-thin sections on nickel grids were incubated with phosphate-buffered saline containing 2% goat serum and 0.5% bovine serum albumin for 15 min and then treated with a mixture of antibodies against VGLUT2 (50-diluted serum), glucagon (1000-diluted), and insulin (200-diluted) or with a mixture of VGLUT2, glucagon, and somatostatin (25-diluted) for 30 min. In the experiments in Fig. 2D, a mixture of antibodies against VGLUT1 (50-diluted serum) and glucagon (1000-diluted) was used. Then the sections were washed and treated with the secondary antibodies conjugated with colloidal gold. The sections were then handpicked and suspended in a bicarbonate-buffered Hanks’ solution containing 1% SDS and 10% β-mercaptoethanol. Then Western analysis was conducted as described (18).

Assay for l-Glutamate Release—Isolated islets (20 pieces per assay) or cultured cells (4.0 × 10^6 cells/dish) were washed three times with DMEM and then incubated in Ringer’s solution containing 10 mM HEPES (pH 7.4), 0.2% bovine serum albumin and glucose at the specified concentrations for 1 h at 37 °C. Then, the islets or cultured cells were transferred to 350 μl of the above Ringer’s solution containing glucose at the specified concentrations. At the times indicated, samples (70 μl) were taken and the amount of l-glutamate was determined by high pressure liquid chromatography on a Resolve C18 column (3.9 × 150 mm; Waters Ltd.) and fluorescence detection as described previously (22). To determine the total l-glutamate concentration, islets (50 pieces per assay) were vigorously homogenized in a bicarbonate-buffered Hanks’ solution in the presence of 6% perchloric acid, sonicated for 5 min, and centrifuged at 25,000 × g for 15 min. Then, the supernatant was carefully taken, and its l-glutamate concentration was measured by HPLC, it being found to correspond to 102 ± 13 pmol/islet (n = 3).

Assay for GABA Release—Islets (20 pieces/assay) or cultured cells (4.0 × 10^6 cells/dish) were washed three times with DMEM and then incubated in a Ringer’s solution comprising 10 mM HEPES (pH 7.4), 0.2% bovine serum albumin and glucose at the specified concentrations for 1 h at 37 °C. Then, islets or cells were transferred to 350 μl of a Ringer’s solution containing glucose at the specified concentrations. After 30 min of incubation, samples (70 μl) were taken and the amount of GABA was determined by high pressure liquid chromatography on a CAPCELL PAK C18 column (2.0 × 250 mm; Sizuido Co., Ltd.) and amperometric detection (31). When necessary, l-glutamate, AMPA, or kainate at 0.5 mM each or CNQX at 50 μM was also included. In the experiments on MIN6 m9 cells, GABA release was also quantified by measuring radioactive GABA according to Refs. 32 and 33. Both procedures gave essentially the same results. For loading radiolabeled GABA, MIN6 m9 cells (4.0 × 10^6 cells/dish) were incubated in DMEM containing 1 μCi/ml [14C]GABA (3.9 × 150 mm; Waters Ltd.) for 2 h and then washed with a Ringer’s solution containing 16.7 mM glucose. Then the cells were incubated in a Ringer’s solution containing glucose at the specified concentrations for 1 h and transferred to 2 ml of the same solution for 30 min as described above. Then the radioactivity in the culture medium and cell lysate was counted.

Intracellular [Ca^{2+}] Measurement and Other Procedures—For the analysis of intracellular [Ca^{2+}], an Argus 20/2A ratio imaging system (Hamamatsu Photonics Co., Hamamatsu, Japan) was used (22). Cells were cultured on a thin glass coverslip precoated with poly-l-lysine (0.12 mm thick and 40 mm in diameter; 8.0 × 10^5 cells/coverslip). The cells were washed with 5 μM Fura-2 AM (Dianino Co., Kumamoto, Japan) for 50 min at 37 °C and washed twice with the same medium. The cells were perfused with the warmed Ringer’s solution or Ca^{2+}-Ringer’s solution. Images were continuously taken at 37 °C with a silicon-intensified camera (C2741–08; Hamamatsu...
RESULTS

Localization of VGLUT2 in Glucagon-containing Secretory Granules—αTC6 cells and islet α cells possess at least two kinds of secretory vesicles, glucagon-containing secretory granules and synaptic-like microvesicles (SLMVs) (18). Indirect immunofluorescence microscopy indicated that VGLUT2 is co-localized with glucagon, a marker of secretory granules, but not with synaptophysin, a marker of synaptic vesicles or SLMVs, in αTC6 cells (Fig. 1, A and B). Consistently, sucrose density gradient centrifugation of the particulate fraction of αTC6 cells separated glucagon (fractions 3–7) and synaptophysin (fractions 7–11), indicating the separation of glucagon-containing secretory granules and SLMVs (Fig. 1C). VGLUT2 is distributed similarly to glucagon but not to synaptophysin, which is in contrast with the co-localization of VGLUT2 and synaptophysin in neuronal synaptic vesicles (Fig. 1C). In the islets of Langerhans, triple labeling for immunoelectronmicroscopy indicated that 15 nm of gold particles for VGLUT2 were specifically associated with the membranes of secretory granules (Fig. 1, D and E, and insets, arrowheads) that had been labeled with 5 nm of gold particles for glucagon (Fig. 1, D and E, and insets, arrows). In contrast, essentially no gold particles for VGLUT2 were observed in any organelles, including secretory granules in β or δ cells in the islets (Fig. 1, D and E, white arrows). Quantitatively, the labeling densities for VGLUT2 in glucagon-containing secretory granules, cytoplasm, and nucleus of α cells were 21.13 ± 1.99, 0.35 ± 0.12, and 0.85 ± 0.21 (number of immunogold particles/μm², four independent experiments), respectively. The labeling densities less than 0.79 ± 0.13 (number of immunogold particles/μm², four independent experiments) were also observed in secretory granules and cytoplasm of β and δ cells. Neither control serum nor antiserum pre-absorbed with an antigenic peptide for VGLUT2 gave any specific labeling (data not shown). Taken together, these results demonstrated that VGLUT2 is associated with secretory granules in αTC6 cells and islet α cells.

Expression and Localization of VGLUT1 in Glucagon-containing Secretory Granules—To exclude the possibility of the presence of VGLUT1 in β and δ cells completely, we next examined the expression and localization of VGLUT1, another VGLUT isoform specifically expressed in neuron (14, 15).

Expression of VGLUT1 in αTC6 cells is under detection limit in our experimental systems (data not shown). However, unexpectedly, expression of the VGLUT1 gene in isolated islets was proven by RT-PCR using a specific DNA probe (Fig. 2A). An amplified product with the expected size and nucleotide and deduced amino acid sequences for VGLUT1 was obtained. Western blotting with specific antibodies for VGLUT1 detected a single islet polypeptide exhibiting similar migration to that of neuronal VGLUT1 (Fig. 2B) (14, 15). Immunohistochemistry on the frozen sections of islets revealed that VGLUT1 is co-localized with glucagon but not insulin or somatostatin (Fig. 2C). Together, these results indicate that VGLUT1 is also expressed in α cells but not in β or δ cells. Expression of VGLUT1 was somewhat heterogeneous among α cells, and some α cells showed intense immunoreactivity in contrast with ubiquitously intense expression of VGLUT2 (18).

Double labeling for immunoelectronmicroscopy indicated that gold particles for VGLUT1 were specifically associated with the
membranes of glucagon-containing secretory granules (Fig. 2D). Like localization of VGLUT2 shown in Fig. 1, D and E, essentially no gold particles for VGLUT1 were observed in any organelles, including secretory granules in β or δ cells in the islets (data not shown). Taken together, it is concluded that both VGLUT1 and VGLUT2 are expressed in islet α cells and only VGLUT2 is expressed in α/TC6 cells and that both VGLUTs are specifically localized with glucagon-containing secretory granules.

Co-secretion of L-Glutamate and Glucagon—The specific lo-
calization of VGLUTs with glucagon-containing secretory granules means that L-glutamate is co-stored and co-secreted with glucagon from α cells under low glucose conditions. Consistently, the L-glutamate immunoreactivity coincided with that of VGLUT2 in the islets (Fig. 3). The L-glutamate immunoreactivity decreased to the background level when the antibodies preabsorbed with L-glutamate conjugated with bovine serum albumin were used (Fig. 3D).

We then investigated whether or not the islets co-secrete L-glutamate with glucagon. To facilitate the secretion of glucagon, islets or αTC6 cells were incubated with 16.7 mM glucose and then transferred to low glucose conditions, i.e. 3.3 mM. It was found that appreciable amounts of L-glutamate as well as glucagon were secreted from the islets: 54.3 ± 2.9 pmol L-glutamate per islet at 30 min, corresponding to about 53% of total L-glutamate, was released (Fig. 4A), open circles). When the islets were transferred to the same glucose conditions at 16.7 mM, about 30% L-glutamate release was observed at 30 min (Fig. 4A, closed circles). Essentially the same basal level of L-glutamate release was observed when islets were first incubated with 3.3 mM glucose and then transferred to glucose solution at 16.7 mM (data not shown). Thus, L-glutamate release from the islets constitutes the low glucose-stimulated and glucose-independent one. The low glucose-stimulated L-glutamate release disappeared at below 20 °C or in the presence of EGTA, indicating that the low glucose-stimulated L-glutamate release was dependent on Ca2+ and temperature (Fig. 4, open squares). Phentolamine, which inhibits the exocytosis of glucagon via G2-dependent activation of calcineurin (34), inhibited the low glucose-stimulated and Ca2+-dependent secretion of L-glutamate and glucagon in a parallel manner (Fig. 4, open triangles). The ratio of the low glucose-stimulated and Ca2+-dependent secretion of L-glutamate and glucagon was always stoichiometric, being 1229 ± 105 (mol/mol, n = 4). Together, these results strongly suggest that the low glucose-stimulated L-glutamate release is due to exocytosis of glucagon-containing secretory granules. In contrast, the glucose-independent L-glutamate release was Ca2+-independent, suggesting that it is due to nonspecific leakage from islet cells.

Low glucose-stimulated and Ca2+-dependent secretion of L-glutamate and glucagon was also observed in αTC6 cells: 2.6 ± 0.3 nmol L-glutamate and 6.8 ± 0.7 ng glucagon per 10⁶ cells per hour (n = 6) were secreted. In this case, the L-glutamate released corresponded to about 23% of total L-glutamate, the stoichiometry being 1345 ± 208.

Next, we investigated whether or not isoproterenol at 1 μM triggers L-glutamate secretion from isolated islets since the compound is known to trigger glucagon secretion by way of β-adrenergic receptors on α cells irrespective of glucose conditions (35). As expected, even when the islets were incubated with 16.7 mM glucose condition, isoproterenol triggered the stoichiometric secretion of L-glutamate (35.9 ± 2.3 pmol/islet,

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**Fig. 2. Expression and localization of VGLUT1 in islets.** A, expression of the VGLUT1 gene in brain (lanes 1, 3) and isolated islets (lanes 2, 4) was detected by RT-PCR. No amplified products were obtained without the RT reaction (lanes 3 and 4). B, Western blotting indicates the presence of VGLUT1 in islets. Lanes 1 and 3, crude synaptic vesicles from rat brain (20 μg); lanes 2 and 4, islets (30 μg each). In lanes 3 and 4, antibodies preabsorbed with antigenic peptides (2 mg) were used. C, sections of pancreas were doubly immunostained with antibodies against VGLUT1 and glucagon, VGLUT1 and insulin, or VGLUT1 and somatostatin, and then observed under a confocal laser microscope. Merged pictures are also shown. Bar = 20 μm. D, double gold labeling immunoelectronmicroscopy of islets. Arrowheads and arrows indicate labeling for VGLUT1 (15 nm in diameter) and glucagon (5 nm in diameter), respectively. Bar = 500 nm.

**Fig. 3. Immunological co-localization of L-glutamate and VGLUT2 in the islet.** An islet was immunostained for L-glutamate (A) and VGLUT2 (B), and then observed under a confocal laser microscope. Merged picture is also shown as C. In D, antibodies against L-glutamate after incubation with 1 mg/ml bovine serum albumin-L-glutamate for overnight were used. Bar = 50 μm.
Low-glucose conditions trigger co-secretion of L-glutamate and glucagon from islets. Islets (20 pieces per assay) were incubated in a Ringer’s solution containing 16.7 mM glucose for 1 h. Then, the glucose concentration was changed to 3.3 mM (all open symbols) or 16.7 mM (closed circles). In open triangles, islets (20 pieces per assay) were incubated in a Ringer’s solution containing 16.7 mM glucose for 1 h. Then, the glucose concentration was changed to 3.3 mM in the presence of 100 μM phentolamine according to Ref. 34. In open squares, islets (20 pieces per assay) were incubated in a Ringer’s solution containing 16.7 mM glucose for 1 h. Then, the glucose concentration was changed to 3.3 mM in the presence of EGTA. Ca²⁺ in the medium was reduced to 40 nM. Then the medium was sampled at the times indicated, and the concentrations of L-glutamate (A) and glucagon (B) released from islets were measured. All the results are means ± S.E. (four independent experiments).

n = 4) and glucagon (0.091 ± 0.005 ng/islet, n = 4) at 30 min. The isoproterenol-evoked release of L-glutamate and glucagon was observed irrespective of glucose conditions employed, totally Ca²⁺-dependent, and inhibited by 91 ± 3 and 95 ± 3% (n = 4), respectively, by propranolol, a β blocker, at 10 μM. Thus, either the low glucose conditions or stimulation of β-adrenergic receptors triggers release of stoichiometric amounts of L-glutamate and glucagon from isolated islets and TC6 cells.

L-Glutamate Triggers GABA Secretion from β Cells under Low Glucose Conditions—What happens upon stimulation of glutamate receptors in the islets? β cells may receive the glutamate signal since they express glutamate receptors (6–8, 10, 11). It has been shown that β cells store GABA in SLMVs, but not insulin granules (36, 37), and secrete it through a Ca²⁺-dependent exocytotic pathway (32, 33). We investigated whether or not L-glutamate affects GABA secretion from β cells.

MIN6 m9 cells are subclonal MIN6 β cells that retain glucose-responsive insulin secretion capacity (29). Consistently, the cells could secrete insulin (205 ± 8 ng/10⁶ cells at 30 min, four independent experiments) when they were first incubated with 3.3 mM glucose and then transferred to 16.7 mM glucose (Fig. 5). Under other glucose conditions, e.g. the cells were first incubated with 16.7 mM glucose and then transferred to 16.7 mM or 3.3 mM glucose or the cells were incubated with 3.3 mM glucose throughout, a decreased level or only a background level of insulin secretion was observed (Fig. 5). MIN6 m9 cells also secrete GABA: on average, 0.62 ± 0.07 nmol of GABA/10⁶ cells at 30 min, which corresponds to about 6% of total GABA, was secreted when the cells were first incubated with 16.7 mM glucose and then transferred to 16.7 mM or 3.3 mM glucose, or the cells were incubated with 3.3 mM glucose throughout (Fig. 5). GABA secretion was stimulated about 1.2-fold, when they were first incubated with 3.3 mM glucose and then transferred to 16.7 mM glucose (Fig. 5). Thus, GABA secretion is not significantly dependent on the glucose conditions as compared with insulin secretion, confirming the presence of distinct secretory pathways for insulin and GABA, as shown by the published works (31–33).

It was found that AMPA and kainate as well as L-glutamate at 500 μM (glutamatergic stimulation) each stimulated GABA secretion about 1.5–1.9-fold when the cells were first incubated with 16.7 mM glucose and then transferred to 3.3 mM glucose (Fig. 5). Glutamate-stimulated GABA release was also observed when the cells were incubated with 3.3 mM glucose throughout. Such a stimulatory effect was not observed under high glucose conditions. The glutamatergic stimulation-evoked GABA secretion was blocked by CNQX, a specific antagonist for AMPA-type receptors. Taking 1.1 ± 0.1 nmol/10⁶ cells (n = 4) as a 100% control of kainate-stimulated GABA release, the omission of Ca²⁺ upon incubation of the cells with EGTA-AM at 50 μM inhibited the glutamatergic stimulation-evoked GABA release by 93 ± 4.4% (n = 4). Nifedipine, an L-type voltage-gated Ca²⁺ channel blocker, at 20 μM inhibited the kainate-evoked GABA release by 70 ± 11% (n = 4). When Na⁺ in the medium was replaced with N-methyl-D-glucamine, kainate-evoked GABA release was blocked by 80 ± 13% (n = 4). In parallel experiments, we measured intracellular [Ca²⁺] in MIN6 m9 cells under similar conditions to above. Intracellular [Ca²⁺] in the resting cells amounted to 93 ± 3 nM (n = 62), L-Glutamate, AMPA, and kainate at 500 μM each increased intracellular [Ca²⁺], which corresponded to 109 ± 9 (n = 6), 117 ± 16 (n = 7), and 132 ± 18 nM (n = 15), respectively. The kainate-evoked increase in intracellular [Ca²⁺] was blocked upon incubation with EGTA-AM at 50 μM or with nifedipine at 20 μM. Furthermore, replacement of Na⁺ in the medium with N-methyl-D-glucamine prevented the increase in intracellular [Ca²⁺] by 88 ± 13% (n = 20). Thus, the glutamatergic stimulation-evoked GABA secretion and increased intracellular [Ca²⁺] were well correlated, indicating that stimulation of AMPA receptors triggers Ca²⁺-dependent secretion of GABA under low glucose conditions.

Glutamatergic stimulation-evoked GABA secretion was also observed with the isolated islets (Fig. 6). The islets were first incubated under 16.7 mM glucose conditions and then transferred to 3.3 mM glucose conditions to facilitate the secretion of L-glutamate from α cells as described in the previous section. About 2-fold GABA was released from the islets as compared with the control level, i.e. the value obtained for islets with 16.7 mM glucose. The low glucose-responsive GABA release was sensitive to CNQX, suggesting that endogenous L-glutamate from α cells triggers GABA secretion. Exogenous L-glutamate and kainate stimulated the GABA release about 1.1–1.5-fold as compared with the absence of these compounds. CNQX decreased the L-glutamate- and kainate-evoked GABA release to the control levels. Consistent with the results for MIN6 m9 cells, under high glucose conditions, neither GABA secretion
nor insulin secretion was stimulated by the addition of either L-glutamate or kainate.

**DISCUSSION**

In this study, we presented evidence that \(\alpha\) cells are glutamatergic in nature and indicated the conditions when and how L-glutamate appears. L-Glutamate is co-stored with glucagon in secretory granules in \(\alpha\) cells. Then, the low glucose conditions facilitate secretion of L-glutamate and glucagon so as to trigger the glutamatergic signaling in the islet. In our previous study, we proposed that SLMVs in \(\alpha\)TC6 cells are responsible for storage and secretion of L-glutamate (22). However, combined immunohistochemistry and immunoelectronmicroscopy clearly indicate that glucagon-containing secretory granules, but not SLMVs, are the sites for L-glutamate storage in \(\alpha\)TC6 cells and \(\alpha\) cells. These results are consistent with observation by Tong et al., although they did not identify the VGLUT2-positive vesicles as glucagon-containing secretory granules in \(\alpha\) cell (11).

We showed that both VGLUT1 and VGLUT2 are specifically localized with glucagon-containing secretory granules in islet \(\alpha\) cells. L-Glutamate may be accumulated in glucagon-containing secretory granules by active transport through VGLUTs at the expense of an electrochemical gradient of protons across the membrane, which is established by vacuolar \(\text{H}^+\)-ATPase. Since apparent difference in transport properties between VGLUT1


and VGLUT2 has not been obtained yet (14–21), the VGLUT isoforms in α cells may be simply responsible for the storage of L-glutamate. Localization of VGLUTs with glucagon-containing secretory granules predicts the mode of L-glutamate signal output. In fact, we next showed that L-glutamate immunoreactivity is co-localized with VGLUT2 (Fig. 3). Moreover, we showed that either low glucose conditions or stimulation of β receptors on α cells actually triggers release of stoichiometric amount of L-glutamate and glucagon with similar Ca^{2+}- and temperature dependence and drug sensitivities. Overall, we concluded that L-glutamate is co-stored and co-secreted from α cells under low glucose conditions. α cells may be regarded as L-glutamate-secreting endocrine cells.

Several works have reported that L-glutamate or agonists of glutamate receptor stimulate to some extent the secretion of insulin under high glucose conditions in cultured cells, isolated islets, and perfused pancreas (4, 6, 7, 9). Consistently, we observed that L-glutamate slightly stimulates (~10%) insulin secretion from MIN6 m9 cells (Fig. 5B). However, the L-glutamate-stimulated insulin secretion might not occur under physiological conditions, since the high glucose conditions do not trigger the L-glutamate signaling (Fig. 4), and therefore, L-glutamate is not expected to become an intercellular transmitter in islets under high glucose conditions.

Another significant finding obtained in the present study is that the L-glutamate signaling triggers GABAergic response in clonal β cells and isolated islets. The released L-glutamate may bind to the corresponding receptors on the islet cells, causing a paracrine or autocrine response (3–11). Islet β cells contain GABA in SLMVs at concentrations comparable level to the central nervous systems (36, 37). Upon depolarization, β cell secretes GABA through Ca^{2+}-dependent exocytosis (32, 33), and the released GABA becomes a paracrine or autocrine transmitter (24–26). However, the mode of action of GABA as an intercellular chemical transmitter, especially the timing for its appearance with the receptor-accessible manner in islet, was less understood. Previous studies on GABA release have been performed after a long incubation period, around a day (31, 38, 39). Under such conditions, amounts of GABA release may reflect the metabolic state of β cells but not directly reflect the rate of GABA secretion through exocytosis of SLMVs. In contrast, depolarization-evoked exocytosis of GABA-containing SLMVs seems to be completed within around 10–20 min (32, 33). We found that L-glutamate, AMPA or kainate stimulates GABA release from clonal β cells and isolated islets. The properties of the GABA secretion, e.g. time course, temperature, and Ca^{2+}-dependence and sensitivities to Ca^{2+} channel blockers are similar to those of GABA secretion through depolarization-evoked exocytosis of SLMVs (32, 33). Thus, it is concluded that the L-glutamate triggers GABA secretion through enhanced exocytosis of GABA-containing SLMVs. It is noteworthy that our results indicate for the first time that α cells and β cells communicate together by way of L-glutamate- and GABA-mediated signaling. The glutamatergic signaling and resultant GABAergic signaling cease when the blood glucose concentration increases because of voltage-dependent inhibition of Na^{+} channel due to closure of the K’^-ATP channel of α cells (40). Then, the exocytosis of insulin is facilitated through KATP channel-mediated depolarization of β cells (40).

The glutamatergic stimulation-evoked GABA secretion exhibits some unique features as to the mode of L-glutamate signal reception. At first, the glutamatergic signals become effective when the glucose concentration in the medium decreases. This suggests that the ability of glutamate signal input of β cells changes with glucose concentration, and then β cells can receive L-glutamate signals only when α cells secrete L-glutamate. Although we cannot explain the molecular mechanism underlying the glucose-dependent change on L-glutamate signal reception at present, one plausible explanation is that AMPA receptors on β cells take on agonist-accessive and -in-accessive forms depending on the glucose concentrations. Another important feature is that the glutamatergic stimulation selectively triggers GABA secretion and does not facilitate exocytosis of insulin granules. Although the exocytosis of GABA containing SLMVs and insulin granules requires an increase in intracellular [Ca^{2+}] (this study and Refs. 32 and 41), the present results strongly suggest that the exocytosis of these two kinds of secretory vesicles is differently regulated. Recently, synaptotagmins were reported to form a hierarchy of exocytotic Ca^{2+} sensors with distinct Ca^{2+} affinities (42). Furthermore, β cells express various kinds of synaptotagmin isoforms involved or not involved in insulin exocytosis (43–45). It is possible that synaptotagmins and/or the related proteins associated with SLMVs may cause a secretory response distinct to that of insulin granules. Both possibilities are now under investigation in our laboratory.

As to the physiological significance of the glutamatergic and GABAergic signaling in the islets, we propose that these signaling pathways may be involved in negative regulatory mechanism on glucagon secretion, since the secreted GABA in turn binds to GABA receptors on α cells, causing inhibition of glucagon secretion (25, 26, 46). Consistent with the idea, stimulation of metabotropic glutamate receptor type 8 (mGluR8), a class III receptor, on α cells, strongly inhibited glucagon secretion under the low glucose condition (11). In this case, L-glutamate may function as an autocrine-type chemical transmitter. We predict that L-glutamate also triggers somatostatin secretion since δ cells express AMPA type receptors (8), and somatostatin inhibits glucagon secretion by way of the somatostatin receptors on α cells (47). We are now investigating this possibility in more details.

In conclusion, we solved critical issues, at least partly, i.e. where, when, and how L-glutamate appears, and what happen upon stimulation of glutamate receptors in the islets: the low glucose conditions and β adrenergic stimulation trigger L-glutamate secretion, and the released L-glutamate in turn triggers GABA secretion in the isolated islets. We presented the direct evidence that α cells and β cells mutually interact by way of L-glutamate- and GABA-signaling. Although the results obtained in vitro assay conditions may not necessary apply to native islet of Langerhans, it is probable that in vivo changes of blood glucose concentration directly regulate the glutamatergic signal transmission in the islets.

To our knowledge, this is the first example of secretory granule-mediated glutamatergic chemical transduction. Recently, we showed that α-aspartate is accumulated in secretory granules and secreted from PC12 cells (48). Thus, co-secretion of excitatory amino acids with hormones might be a common feature in endocrine signal transmission.

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