Ca^{2+}-Dependent Exocytosis of L-Glutamate by αTC6, Clonal Mouse Pancreatic α-Cells

Hiroshi Yamada,[1] Masato Otsuka,[1] Mitsuko Hayashi,[1] Shuuichi Nakatsuka,[1] Kazuyuki Hamaguchi,[2] Akitsugu Yamamoto,[3,4] and Yoshinori Moriyama[1,4]

Pancreatic islet cells express receptors and transporters for L-glutamate and are thus believed to use L-glutamate as an intercellular signaling molecule. However, the mechanism by which L-glutamate appears in the islets is unknown. In the present study, we investigated whether L-glutamate is secreted through exocytosis by αTC6 cells (clonal mouse pancreatic α-cells). An appreciable amount of L-glutamate was released from cultured cells after the addition of KCl or A23187 in the presence of Ca^{2+} and 10 mmol/l glucose in the medium. The KCl-induced glutamate release was significantly reduced when assayed in the absence of Ca^{2+} or when the cells were pretreated with EGTA-AM. The KCl-induced Ca^{2+}-dependent glutamate release was inhibited ~40% by voltage-gated Ca^{2+} channel blockers, such as nifedipine at 20 μmol/l. The degree of KCl-induced Ca^{2+}-dependent glutamate release was correlated with an increase in intracellular $[Ca^{2+}]$, as monitored by fura-2 fluorescence. Botulinum neurotoxin type E inhibited 55% of the KCl-induced Ca^{2+}-dependent glutamate release, followed by specific cleavage of 25 kDa synaptosomal-associated protein. Furthermore, bafilomycin A_1, a specific inhibitor of vacuolar H^+-ATPase, inhibited 40% of the KCl-induced Ca^{2+}-dependent glutamate release. Immunoelectronmicroscopy with antibodies against synaptophysin, a marker for neuronal synaptic vesicles and endocrine synaptic-like microvesicles, revealed a large number of synaptophysin-positive clear vesicles in cells. Digitonin-permeabilized cells took up L-glutamate only in the presence of MgATP, which is sensitive to bafilomycin A_1 or 3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile (a proton conductor) but insensitive to either oligomycin or vanadate. From these results, it was concluded that αTC6 cells accumulate L-glutamate in the synaptophysin-containing vesicles in an ATP-dependent manner and secrete it through a Ca^{2+}-dependent exocytic mechanism. The Ca^{2+}-dependent glutamate release was also triggered when cells were transferred in the medium containing 1 mmol/l glucose, suggesting that low glucose treatment stimulates the release of glutamate. Our results are consistent with the idea that L-glutamate is secreted by α-cells through Ca^{2+}-dependent regulated exocytosis. *Diabetes* 50:1012–1020, 2001

From the [1]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama; the [2]Faculty of Medicine, Oita Medical University, Oita; the [3]Department of Physiology, Kansai Medical University, Moriguchi; and the [4]CREST, Japan Science and Technology Corporation, Osaka, Japan.

Address correspondence and reprint requests to Dr. Y. Moriyama, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan. E-mail: moriyama@pheasant.pharm.okayama-u.ac.jp.

H.Y., M.O., and M.H. contributed equally to this work. H.Y.'s current address is the Department of Biochemistry, Faculty of Medicine, Okayama University, Okayama, Japan.

Received for publication 12 June 2000 and accepted in revised form 19 January 2001.

AM, acetoxymethylester; AMPA, (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BoNT/E, botulinum neurotoxin type E; DMEM, Dulbecco's modified Eagle medium; GABA, γ-aminobutyrate; HPLC, high-performance liquid chromatography; SF6847, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile; SNAP25, 25 kDa synaptosomal-associated protein.

In the mammalian central nervous system, L-glutamate is the major excitatory neurotransmitter 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 glutamatergic systems comprising glutamate exocytosis (signal output), glutamate receptors (signal input), and glutamate reuptake systems (signal termination). Recent evidence has indicated that peripheral endocrine cells also develop glutamatergic systems: mammalian pinealocytes (endocrine cells for melatonin) use L-glutamate as an inhibitory chemical mediator of melatonin synthesis (3).

The Langerhans' islet, a pancreatic endocrine miniature organ, is composed of four major types of endocrine cells, i.e., insulin-secreting β-cells, glucagon-secreting α-cells, polypeptide-secreting cells, and somatostatin-secreting δ-cells. The Langerhans' islet is another example of the presence of peripheral glutamatergic systems (4). A clonal β-cell line, MIN6, expresses functional ionotropic glutamate receptors (5). Functional (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptors have been identified in α- and β-cells and the polypeptide-secreting cells of islets using reverse-transcriptase–polymerase chain reactions, immunohistochemistry, and electrophysiology (6–8). The kainate receptor has also been identified in islet α-cells (7,8). After stimulation of islets with AMPA or kainate, intracellular $[Ca^{2+}]$ increased by way of activation of voltage-gated Ca^{2+} channels (6,7), resulting in an elevated level of insulin secretion through increased exocytosis of insulin granules in β-cells (9). In α-cells, AMPA treatment increased intracellular $[Ca^{2+}]$ and triggered exocytosis of glucagon-containing secretory granules, resulting in increased secretion of glucagon (10). Moreover, the Na^+-dependent glutamate transporter sequesters extracellular glutamate in the α-cell–rich islet mantle (11).

Before a conclusion can be made regarding the functional operation of the glutamatergic system in the islets, there is an important issue remaining to be solved: How does L-glutamate appear in the extracellular space of the

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

islets? At least two possibilities have been proposed. One is that L-glutamate is secreted by presumptive glutamatergic output neurons innervating the islets. However, no morphological evidence has been obtained as yet regarding this possibility. The other is that L-glutamate is secreted by neighboring islets cells, such as α-cells, through a paracrine-like chemical transduction mechanism (4). This possibility is more likely because it is known that α-cells possess phosphate-activated glutaminase (12), a peculiar enzyme for glutamate-releasing nerve endings in the central nervous system (13).

To elucidate the mechanism by which L-glutamate appears in the extracellular space of the islets, we examined whether a clonal α-cell line, αTC6, secretes L-glutamate through an exocytic mechanism. The use of clonal cells may exclude the possible participation of other islet cells, including β-cells. It was found that αTC6 cells secrete L-glutamate in a Ca^{2+}-dependent manner. Furthermore, we identified synaptophysin-positive vesicles as putative storage organelles for L-glutamate in αTC6 cells. The present results are fully consistent with the idea that α-cells store L-glutamate in synaptophysin-containing vesicles and secrete it through a paracrine-like exocytic mechanism.

RESEARCH DESIGN AND METHODS

Cell cultures. αTC6 (14), HeLa, and COS cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal calf serum, 55 μg/ml sodium pyruvate, 4.5 g/l glucose, 0.1 mg/l streptomycin, 100 U/ml penicillin G, and 0.25 mg/l fungizone and incubated at 37°C under 5% CO_2. The αTC6 cells showed morphologic features similar to those in the original report (14) (Fig. 4), and like the cells in the original report, they did not produce detectable levels of insulin and contained 64 ng glucagon per 10^5 cells (14), suggesting that the cells maintained the original properties. MIN6 cells, a clonal β-cell line (15), were maintained in DMEM supplemented with 15% fetal calf serum. PC 12 cells were cultured in DMEM containing 5% fetal calf serum, 5% horse serum, and 25 ng/ml nerve growth factor (16). The dispersed cells were washed three times with the above medium then placed in a 35-mm culture dish coated with poly-L-lysine (5.0×10^5 cells per dish) and cultured in the above medium at 37°C under 5% CO_2. For experimental procedures, cells were maintained for 5 days, washed with culture medium, further cultured for 1 h, then used for experiments.

Assay of glutamate release. Cultured cells (2.5×10^6 cells per dish) were washed three times with Ringer's solution comprising 128 mmol/l NaCl, 1.9 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 2.4 mmol/l $CaCl_2$, 1.3 mmol/l $MgSO_4$, 26 mmol/l $NaHCO_3$, 10 mmol/l glucose, and 10 mmol/l HEPES (pH 7.4), or they were washed with low-concentration Ca^{2+}-Ringer's solution comprising 128 mmol/l NaCl, 1.9 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 0.2 mmol/l $CaCl_2$, 1 mmol/l EGTA, 3.8 mmol/l $MgSO_4$, 26 mmol/l $NaHCO_3$, 10 mmol/l glucose, and 10 mmol/l HEPES (pH 7.4). After cells had been incubated in 2 ml of either of the above mediums at 37°C, the release of L-glutamate was stimulated by the addition of 50 mmol/l KCl (17), as described previously. When necessary, various antagonists for voltage-gated Ca^{2+} channels were included in the incubation medium. Aliquots (10 μl) were taken at time intervals, and the amount of extracellular glutamate was determined by high-performance liquid chromatography (HPLC) with precolumn O-phthalaldehyde derivatization, separation on a reverse-phase Resolve C18 column (3.9 × 150 mm) (Waters), and fluorescence detection (18).

Treatment with botulinum neurotoxin type E. The intoxication of α-cells with botulinum neurotoxin type E (BoNT/E) was performed by a procedure similar to that previously described (19,20). α-Cells (2.5×10^6 cells per dish) were incubated at 37°C for 24 h in a low ionic strength buffer consisting of 5 mmol/l NaCl, 4.8 mmol/l KCl, 2.2 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 20 mmol/l HEPES-NaOH, 10 mmol/l glucose, 220 mmol/l sucrose, and 0.5% bovine serum albumin (pH 7.4) in the presence or absence of 10 or 50 nmol/l BoNT/E. Then, the cells were washed with fresh culture medium and incubated for an additional 12 h at 37°C. Finally, KCl-evoked glutamate release was measured as described above.

Measurement of intracellular [Ca^{2+}]. For the analysis of intracellular [Ca^{2+}], an Argus 20/CA ratio imaging system (Hamamatsu Photonics, Shizuoka, Japan) was used. Cells were cultured for 3 days on a thin glass coverslip precoated with poly-L-lysine (0.12 mm thick and 40 mm in diameter, 8.0×10^5 cells per coverslip). After exchanging the old medium for fresh culture medium, the cells were treated with 5 μmol/l Fura 2-acetoxymethylester (AM) (Dojindo, Kumamoto, Japan) for 50 min at 37°C and then washed twice with the same medium. The cells were perfused with the warmed Ringer's solution or the low-concentration Ca^{2+}-Ringer's solution. Images were continuously taken at 37°C with a silicon-intensified camera (C2741–08, Hamamatsu Photonics). The velocity of data acquisition for F334 by F380 images was 4 s at a resolution of 256 × 256 pixels per image. A personal computer with appropriate software (U4469, Hamamatsu Photonics) was used to control the optical equipment, then to record and analyze the data. The software enabled subtraction of background fluorescence, pixel-to-pixel division of F334 by F380 images, fitting of the F334-to-F380 ratios to a [Ca^{2+}] calibration curve prepared separately, and digital averaging of the Ca^{2+} concentration in multiple cells (21).

Immunoblotting. αTC6 cells or a membrane fraction of rat brain prepared as described (20) was denatured with SDS sample buffer containing 1% SDS and 10% β-mercaptoethanol and then electrophoresed on a 12% polyacrylamide gel in the presence of SDS (22). After electrotransfer at 0.3 amperes for 2 h, the nitrocellulose filters were blocked in a buffer consisting of 20 mmol/l Tris-Cl (pH 7.6), 5 mmol/l EDTA, 0.1 mol/l NaCl, and 0.5% bovine serum albumin for 4 h and then probed with 50-μg antibodies in the above buffer. The filters were washed with 20 mmol/l Tris-Cl buffer (pH 7.6) containing 5 mmol/l EDTA, 0.1 mol/l NaCl, and 0.1% Tween 20, treated with peroxidase-labeled anti-rabbit IgG or anti-mouse IgG at a dilution of 1:2000 for 30 min, washed again with the same buffer, and then subjected to enhanced chemiluminescence amplification according to the manufacturer's manual (Amersham).

Immunoelectronmicroscopy. The pre-embedding silver enhancement immunogold method described by Mandai et al. (23) was used with a slight modification (24,25). Cells on poly-L-lysine-coated plastic coverslips were fixed in 4% paraformaldehyde in 0.1 mol/l sodium phosphate buffer for 30 min. After the cells were washed three times for 5 min, they were cryo-protected in buffer containing 35% sucrose and 14% glycerol for 15 s, frozen in liquid nitrogen, and thawed at room temperature. The cells were then incubated in buffer containing 0.005% saponin, 10% bovine serum albumin, 10% normal goat serum, and 0.1% cold water fish skin gelatin (Sigma) for 30 min and reacted with anti-synaptophysin antibodies (10 μg/ml) in the above buffer overnight at 4°C. The cells were then washed five times in the same buffer containing 0.005% saponin and 1% bovine serum albumin and incubated with goat anti-rabbit IgG conjugated to colloidal gold (1.4 nm in diameter) (Nanogold; Nanoprobes) in the same buffer containing 0.005% saponin and 1% bovine serum albumin for 2 h. The cells were then washed five times with the buffer for 10 min and fixed with 1% glutaraldehyde for 10 min. After washing, the gold labeling was intensified using a silver enhancement kit (HQ silver; Nanoprobes) for 6 min at room temperature in the dark. After washing in distilled water, the cells were postfixed with 0.5% OsO_4 for 90 min at 4°C, washed in distilled water, dehydrated with a graded series of ethanol, and embedded in epoxy resin. Ultra-thin sections were doubly stained with uranyl acetate and lead citrate and observed under a Hitachi H7000 electron microscope. Dr. M. Takahashi (Mitsubishi Life Science Institute, Tokyo) provided mAbBR05 (monoclonal antibodies against 25 kDa synaptosomal-associated protein [SNAP25]), and mAb171b5 (monoclonal antibodies against synaptophysin) (26).

Glutamate uptake by intracellular organelles in digitonin-permeabilized cells. αTC6 cells were rinsed with 1 ml of the buffer comprising 20 mmol/l MOPS-Tris (pH 7.0), 0.3 mol/l sucrose, 2 mmol/l Mg-acetate, and 4 mmol/l KCl. The cells were then permeabilized for 10 min at 37°C in 0.5 ml of the buffer containing 10 μmol/l digitonin (27). The medium was then replaced with fresh buffer containing Tris-ATP at 2 mmol/l in the absence of digitonin. In some experiments, bafilomycin A_1 at 1 μmol/l was also included in the medium. Then, glutamate uptake was immediately started by the addition of radioactive glutamate (2.5 μCi, 0.1 mmol/l) at 37°C, as described previously (28,29). After 10 min incubation, uptake was terminated by washing the cells twice with 1 ml of ice-cold 20 mmol/l MOPS-Tris (pH 7.0) containing 0.3 mol/l sucrose. Then, the cells were lysed with 1 ml of 1% SDS, and the radioactivity was counted with a liquid scintillation counter.

Other procedures. Indirect immunofluorescence microscopy was performed as described previously (24). Content of glucagon and insulin were determined by enzyme-linked immunoassay according to the manufacturer's manual (Amersham). Protein concentrations were determined with a BioRad Protein Assay Kit with bovine serum albumin as a standard.

Other chemicals. The L-[2,3-^{3}H]-glutamate (9.25 MBq) was obtained from NEN Life Science Products (Boston, MA). Digitonin was purchased from Wako Chemical (Osaka, Japan). BoNT/E was provided by Dr. S. Kozaki (Osaka Prefecture University). Other chemicals were of the highest grade commercially available.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

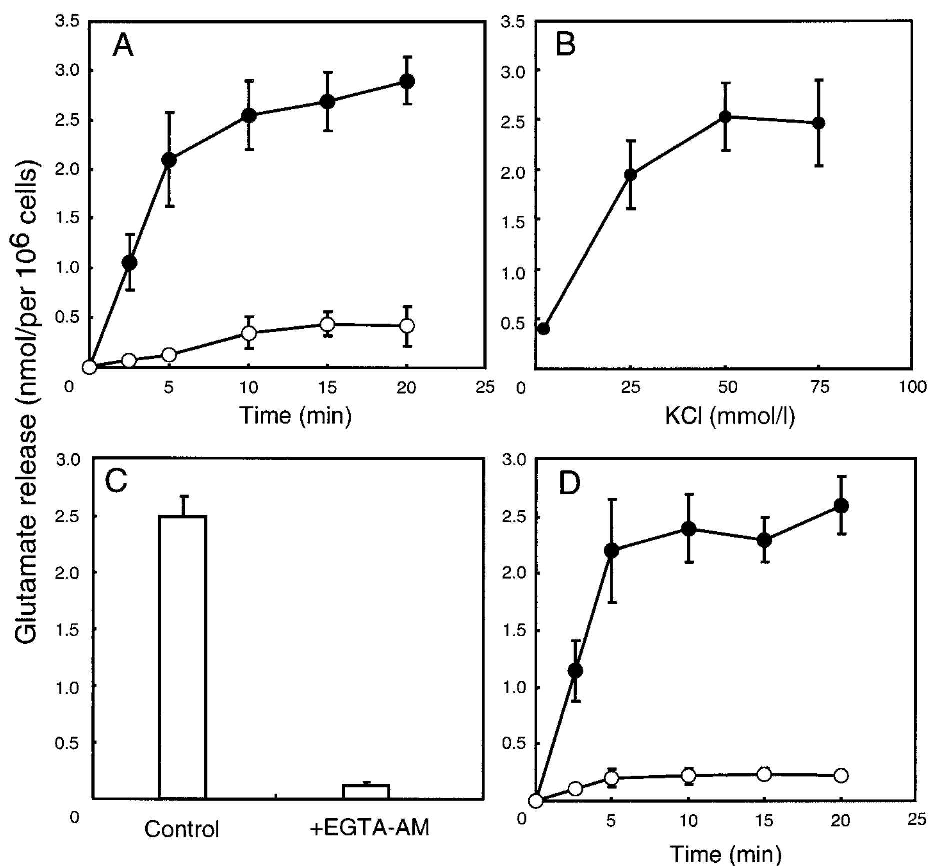

FIG. 1. Ca^{2+}-dependent glutamate release by αTC6 cells. *A*: Time course of the KCl-induced glutamate release. The cells were incubated in the high-concentration Ca^{2+}-Ringer's solution (●) or low-concentration Ca^{2+}-Ringer's solution (○) containing 10 mmol/l glucose, and then KCl at 50 mmol/l was applied. After the times indicated, the medium was carefully taken, and the glutamate concentration was determined. *B*: The KCl-dose dependence of the Ca^{2+}-dependent glutamate release was measured at 10 min after the addition of the indicated concentrations of KCl. *C*: αTC6 cells (2.5 × 10^6 cells) were incubated in the absence (control) or presence of EGTA-AM at 50 μmol/l for 30 min and then washed with Ringer's solution. Then, the KCl-induced Ca^{2+}-dependent glutamate release was measured as previously described. *D*: The time course of A23187-evoked glutamate release was measured in the high-concentration Ca^{2+}-Ringer's solution (●) or low-concentration Ca^{2+}-Ringer's solution (○). At time 0, A23187 at 5 μmol/l was added to the medium. After incubation for the times indicated, the glutamate concentration in the medium was measured. A23187 was dissolved in DMSO. The solvent itself does not affect the glutamate release at all. All the results in the figure are means ± SE (four independent experiments).

RESULTS

Ca^{2+}-dependent glutamate release. As the first step of the study, we examined whether αTC6 cells release L-glutamate through an exocytic mechanism. In analogy to the glutamate exocytosis by neurons and pinealocytes (17,30,31), the glutamate concentration in the medium of cultured αTC6 cells was measured by HPLC after stimulation of the cells with KCl. An appreciable amount of L-glutamate (2.52 ± 0.4 nmol per 10^6 cells, which corresponds to 23% total free glutamate, 50 determinations) has been released by αTC6 cells at 10 min after the addition of KCl in the presence of Ca^{2+}. The amount of released glutamate was dependent on time (Fig. 1*A*) and the concentration of KCl (Fig. 1*B*): the glutamate concentration was saturated at 10 min and with 50 mmol/l KCl, respectively. In the absence of $CaCl_2$, little glutamate was released by the cells with the addition of KCl. When cells were treated with EGTA-AM to remove intracellular free Ca^{2+}, the cells lost the ability to release L-glutamate in the presence of KCl (Fig. 1*C*). The Ca^{2+}-dependent fraction of KCl-induced glutamate release is designated as the KCl-induced Ca^{2+}-dependent glutamate release in this report. Similarly, A23187, a Ca^{2+} ionophore, caused rapid glutamate release by the cells only in the presence of Ca^{2+} (Fig. 1*D*). These results indicate that L-glutamate is released by cultured cells in a manner dependent on Ca^{2+}. It is possible that entry of Ca^{2+} from the extracellular space is necessary for the glutamate release. No KCl-induced Ca^{2+}-dependent glutamate release (<0.04 ± 0.01 nmol per 10^6 cells at 10 min after the addition of KCl) was observed when COS, HeLa, MIN6, and PC 12 cells were assayed.

We then examined the effects of voltage-gated Ca^{2+} channel blockers on the KCl-induced Ca^{2+}-dependent glutamate release because the requirement of Ca^{2+} suggests the participation of Ca^{2+} channel(s) in this process. Table 1 indicates that the cadmium ion, a nonspecific inhibitor of voltage-gated Ca^{2+} channels (32), inhibited the KCl-induced Ca^{2+}-dependent glutamate release; the concentration for 50% inhibition was 100 μmol/l. Furthermore, it was found that at 20 μmol/l each, the L-type Ca^{2+} channel blockers

TABLE 1
The effects of Ca^{2+}-channel antagonists on the KCl-induced Ca^{2+}-dependent glutamate release and intracellular [Ca^{2+}] in αTC6 cells

Agent	Concentration (μmol/l)	Glutamate release (% of control)	Peak of $[Ca^{2+}]_i$ (nmol/l)
None	—	13.1 ± 3.6 (4)	98 ± 12 (300)
KCl (50 mmol/l)	—	100 ± 15.9 (50)	548 ± 73 (450)
$-Ca^{2+}$	—	13.9 ± 6.8 (4)	84 ± 14 (106)
$+Cd^{2+}$	200	37.2 ± 8.0 (4)	188 ± 24 (136)
+Nifedipine	20	60.0 ± 8.3 (4)	227 ± 22 (80)
+Nitrendipine	20	40.2 ± 8.9 (4)	160 ± 17 (73)
+Diltiazem	20	36.2 ± 4.3 (4)	143 ± 7 (97)
+ω-Conotoxin GIVA	10	97.0 ± 10.0 (4)	536 ± 47 (52)
+ω-Conotoxin MVIIC	10	105.0 ± 4.2 (4)	574 ± 66 (85)
+ω-Agatoxin IVA	10	104.5 ± 8.5 (4)	541 ± 59 (96)

The 100% value for glutamate release corresponds to 2.52 ± 0.4 nmol/10^6 cells (*n* of independent experiments). The peaks of intracellular [Ca^{2+}] are expressed as means ± SEM (*n* of α-cells examined). The KCl-induced Ca^{2+}-dependent glutamate release at 10 min and intracellular [Ca^{2+}] in Fura-2–loaded αTC6 cells in the presence of the listed compounds was measured as described in RESEARCH DESIGN AND METHODS. +, with; –, without.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

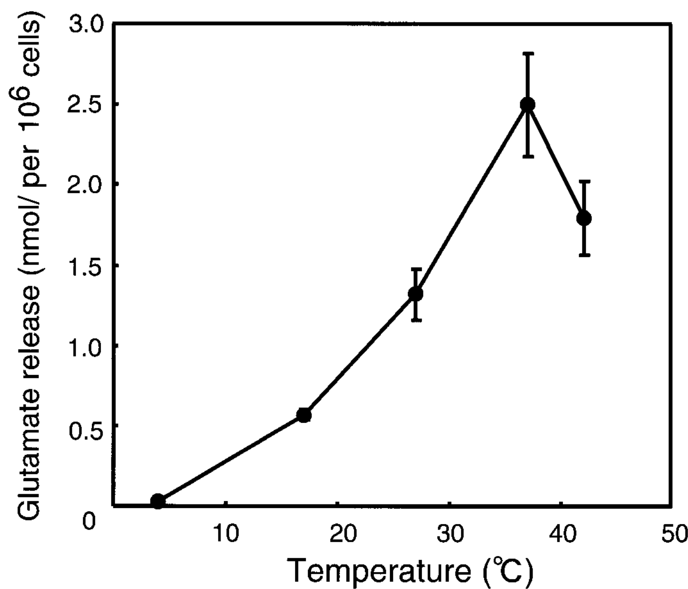

FIG. 2. The effect of incubation temperature on KCl-induced Ca^{2+}-dependent glutamate release. αTC6 cells (2.5 × 10^6 cells) were incubated at the indicated temperatures for 15 min, and then the KCl-induced Ca^{2+}-dependent glutamate release by αTC6 cells was measured at 10 min. The results are presented as means ± SE (four independent experiments).

nifedipine, nitrendipine, and diltiazem inhibited 40, 60, and 64% of the KCl-induced Ca^{2+}-dependent glutamate release, respectively (Table 1). On the other hand, the KCl-induced Ca^{2+}-dependent glutamate release was not affected by ω-conotoxin GIVA (an N-type Ca^{2+} channel blocker) (33), ω-conotoxin MVIIC (a P/Q-type Ca^{2+} channel blocker) (34), or ω-agatoxin IVA (a P-type Ca^{2+} channel blocker) (35,36) (Table 1). These results suggest that L-type Ca^{2+} channels are at least partly involved in the KCl-induced Ca^{2+}-dependent glutamate release by αTC6 cells.

Intracellular [Ca^{2+}] change. As mentioned above, Ca^{2+} entry through voltage-gated Ca^{2+} channels may facilitate the KCl-induced Ca^{2+}-dependent glutamate release. To confirm that intracellular [Ca^{2+}] actually increases after KCl treatment, intracellular [Ca^{2+}] was measured in fura-2–loaded cells. Under the experimental conditions used, almost all cells were labeled with Fura-2-AM. The intracellular [Ca^{2+}] level in the resting cells was 98 ± 12 nmol/l (300 determinations) (Table 1). The addition of KCl at 50 mmol/l increased the intracellular [Ca^{2+}] level to 548 ± 73 nmol/l in the presence of Ca^{2+} in the medium. In contrast, essentially no increase in intracellular [Ca^{2+}] was observed in the absence of Ca^{2+} (Table 1) or when the cells were pretreated with EGTA-AM at 50 μmol/l (104 ± 9 nmol/l, 62 determinations). Furthermore, nifedipine, nitrendipine, and diltiazem inhibited 71, 86, and 90% of the KCl-evoked increase in intracellular [Ca^{2+}], respectively (Table 1). ω-Conotoxin GIVA, ω-conotoxin MVIIC, and ω-agatoxin IVA (antagonists for N- or P/Q-type channels) did not affect the KCl-evoked [Ca^{2+}] increase in these cells (Table 1). Thus, degrees of the KCl- and Ca^{2+}-dependent intracellular [Ca^{2+}] change and glutamate release were well correlated to each other. Taking all the results together, it was concluded that the KCl-induced Ca^{2+}-dependent glutamate release is triggered by the entry of Ca^{2+} through voltage-gated Ca^{2+} channels in cultured αTC6 cells.

Evidence for exocytosis of glutamate. We further characterized KCl-induced Ca^{2+}-dependent glutamate release by αTC6 cells with regard to the effects of temperature. As shown in Fig. 2, KCl-induced Ca^{2+}-dependent glutamate release was affected by the temperature: change was not observed at 4°C, but an increase in glutamate release appeared gradually with increasing temperature and was maximum at 37°C. Furthermore, once glutamate had been secreted, for a time the αTC6 cells lost the ability to release Ca^{2+}-dependent glutamate in the presence of KCl. Although a first stimulation of glutamate release with KCl decreased the cell glutamate content by only 20%, a second stimulation applied within 1 h was ineffective. Its efficiency was gradually restored with incubation and had recovered completely after 12 h, suggesting that charged and discharged processes are involved in the KCl-induced Ca^{2+}-dependent glutamate release. These properties are similar to those of exocytosis of glutamate by synaptic vesicles and synaptic-like microvesicles (17,30,31), supporting the idea that glutamate is secreted through exocytosis.

The sensitivity to BoNT/E constitutes evidence of Ca^{2+}-dependent regulated exocytosis because this neurotoxin is a protease that is specific to SNAP25, splitting the SNAP25 and resulting in inhibition of exocytosis via secretory granules, synaptic vesicles, and synaptic-like microvesicles (17,19,37–39). BoNT/E cleaved SNAP25, yielding a low-molecular fragment that migrated faster on a polyacrylamide gel during electrophoresis (Fig. 3*A*, asterisk). In contrast, BoNT/E did not affect vesicle-associated membrane protein 2, syntaxin-1, synaptotagmin, synaptophysin, *N*-ethylmaleimide-sensitive fusion protein, β-SNAP, or V-ATPase subunit A or E (data not shown). Under the same assay conditions, BoNT/E inhibited the KCl-induced Ca^{2+}-dependent glutamate release (Fig. 3*B*). The inhibitory potency of BoNT/E was essentially the same as that

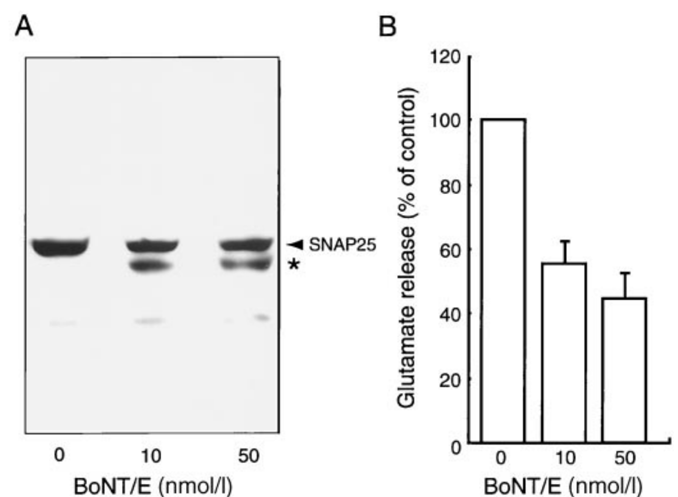

FIG. 3. BoNT/E-evoked cleavage of SNAP25 and inhibition of KCl- and Ca^{2+}-dependent glutamate release. *A*: Cells were treated with BoNT/E at the indicated concentrations, washed with phosphate-buffered saline containing 0.01% DNase and 10 μg/ml each of leupeptin and pepstatin A, and then dissolved in the sample buffer containing 10% SDS and β-mercaptoethanol. After the dissociation of proteins, samples were applied to a 12.5% polyacrylamide gel containing SDS then electrophoresed and analyzed by immunoblotting with antibodies against SNAP25. *B*: After the same BoNT/E treatment described above, the KCl-induced Ca^{2+}-dependent release at 10 min was measured as described in RESEARCH DESIGN AND METHODS. The results are presented as means ± SE (four independent experiments); 100% corresponds to 2.48 ± 0.39 nmol per 10^6 cells.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

observed in the exocytosis of various neurotransmitters (17,19,37–39). The BoNT/E treatment did not affect the KCl-induced [Ca^{2+}] increase in these cells (550 ± 31 nmol/l, 120 determinations). These results indicated that SNAP25 is involved in the KCl-induced Ca^{2+}-dependent release of glutamate by cultured cells.

It is known that bafilomycin A1, a specific inhibitor of V-ATPase in vivo and in vitro (40–42), effectively inhibits the exocytosis of L-glutamate (43,44) because the compound dissipates an electrochemical proton gradient necessary for glutamate uptake into vesicles (45,46). As expected, bafilomycin A_1 at 1 μmol/l inhibited 40% of the KCl-induced Ca^{2+}-dependent glutamate release without affecting the KCl-evoked [Ca^{2+}] increase in these cells (553 ± 21 nmol/l, 100 determinations). The results suggest that an electrochemical proton gradient for vesicular glutamate uptake is necessary at least in part for the KCl- and Ca^{2+}-dependent glutamate release by αTC6 cells. From all the results shown above, it was concluded that glutamate is released by cultured αTC6 cells through a soluble NSF attachment protein protein-dependent exocytic mechanism.

Mechanism of storage of L-glutamate. Before exocytosis, L-glutamate must be accumulated in vesicular structures, such as synaptic vesicles or synaptic-like microvesicles (3, 47,48). We attempted to identify the presumptive secretory vesicles for L-glutamate in αTC6 cells using immunoelectronmicroscopy with antibodies against synaptophysin because synaptophysin-containing vesicles are responsible for the storage of L-glutamate in neurons and pinealocytes (28,29,45,46). As shown in Fig. 4*A*, immunogold for synaptophysin is primarily associated with clear vesicles with diameters of 50–200 nm. The synaptophysin-positive vesicles are distributed throughout the cells and are especially abundant in peripheral regions (Fig. 4*B*). A lower amount of labeling was also observed in the region of the Golgi apparatus (Fig. 4*C*, arrows). Only the background level of labeling was observed in other areas, including the nucleus, mitochondria, lysosomes (Fig. 4*A*–C), and dense-cored vesicles (Fig. 4*D*). These results suggest that synaptophysin-containing vesicles are distinct from these organelles.

As in the case of glutamate uptake by synaptic vesicles and synaptic-like microvesicles (28,29,45,46), the glutamate transporter energetically coupled with V-ATPase may be responsible for the accumulation of L-glutamate in synaptophysin-positive vesicles. As shown in Fig. 5, radiolabeled L-glutamate was taken up by digitonin-permeabilized cells in a manner dependent on the presence of ATP. The omission of Mg^{2+} drastically reduced the ATP-dependent glutamate uptake. Consistent with glutamate exocytosis by αTC6 cells, bafilomycin A_1 at 1 μmol/l inhibited ATP-dependent glutamate uptake (Fig. 5). Furthermore, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile (SF6847), a proton conductor that dissipates an electrochemical proton gradient, also inhibited the ATP-dependent glutamate uptake, whereas neither oligomycin at 5 μmol/l (an inhibitor for mitochondrial ATPase) nor vanadate at 1 mmol/l (an inhibitor for P-type ATPase) affected the uptake (Fig. 5). These results constitute evidence that the glutamate transporter energetically coupled with V-ATPase is responsible for the storage of L-glutamate in vesicles in αTC6 cells.

The effect of glucose on L-glutamate release. In the final part of the study, we investigated whether αTC6 cells secrete glutamate by physiological stimulation. At first, to define the sensitivity of αTC6 cells to glucose, the cultured cells were further incubated for 2 h in the medium containing either 10 or 1 mmol/l glucose, then glucagon concentrations in the medium were determined according to the procedure previously described (49). In the presence of 1 mmol/l glucose, glucagon (13.3 ± 0.7 ng per 10^5 cells, $n = 4$) was released by αTC6 cells. However, in the presence of 10 mmol/l glucose, glucagon release was reduced to 40 ± 4.4% ($n = 4$) of that found in the presence of 1 mmol/l glucose. These results were consistent with previous observations (49). When the glucose concentration was lowered as described above, intracellular [Ca^{2+}] increased to 573 ± 64 nmol/l ($n = 11$), and an appreciable amount of glutamate (1.70 ± 0.22 nmol per 10^6 cells at 10 min, n = 5) was released from the cells. We observed that <0.38 ± 0.02 nmol glutamate per 10^6 cells ($n = 4$) was released when cells were incubated in the presence of 10 mmol/l glucose. When the low glucose treatment was conducted in the absence of Ca^{2+}, neither an increase in intracellular [Ca^{2+}] nor an increase in glutamate release was observed. These results indicate that low glucose treatment stimulates secretion of glutamate as well as glucagon by αTC6 cells.

DISCUSSION

Recent biological and biochemical cell studies have indicated that endocrine cells may secrete classical neurotransmitters through an exocytic mechanism. Endocrine cells contain synaptophysin-containing vesicles in which classical neurotransmitters are accumulated (3,50,51). Synaptophysin-containing vesicles contain V-ATPase and vesicular transporters specific to the neurotransmitters and accumulate the corresponding neurotransmitters in a manner dependent on MgATP. For instance, synaptophysin-containing vesicles in pancreatic β-cells accumulate γ-aminobutyrate (GABA) through a vesicular GABA transporter (52). GABA that has accumulated in the vesicles is exocytized through a Ca^{2+}-dependent regulated secretion pathway (53,54). Upon secretion, GABA may bind to the GABAA receptors in neighboring α-cells and inhibit glucagon secretion through decreased exocytosis of glucagon-containing secretory granules (55); however, there is also evidence against a role of GABA in the inhibition of glucagon secretion (56). We hypothesized that α-cells accumulate L-glutamate through a mechanism similar to that observed in β-cells and secrete the L-glutamate through regulated exocytosis. In the present study, we obtained compelling evidence that αTC6 cells accumulate L-glutamate in synaptophysin-containing vesicles through a glutamate transporter and secrete it through Ca^{2+}-dependent exocytosis.

Most regulated exocytosis is triggered by an increase in Ca^{2+} through voltage-gated Ca^{2+} channels after depolarization. In the first line of evidence, we showed that αTC6 cells secrete L-glutamate in a manner dependent on Ca^{2+}. Both the KCl-induced Ca^{2+}-dependent glutamate release and the increase in intracellular [Ca^{2+}] were sensitive to antagonists for voltage-gated Ca^{2+} channels, especially L-type Ca^{2+} channels, but not N-type or P/Q-type channels,

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

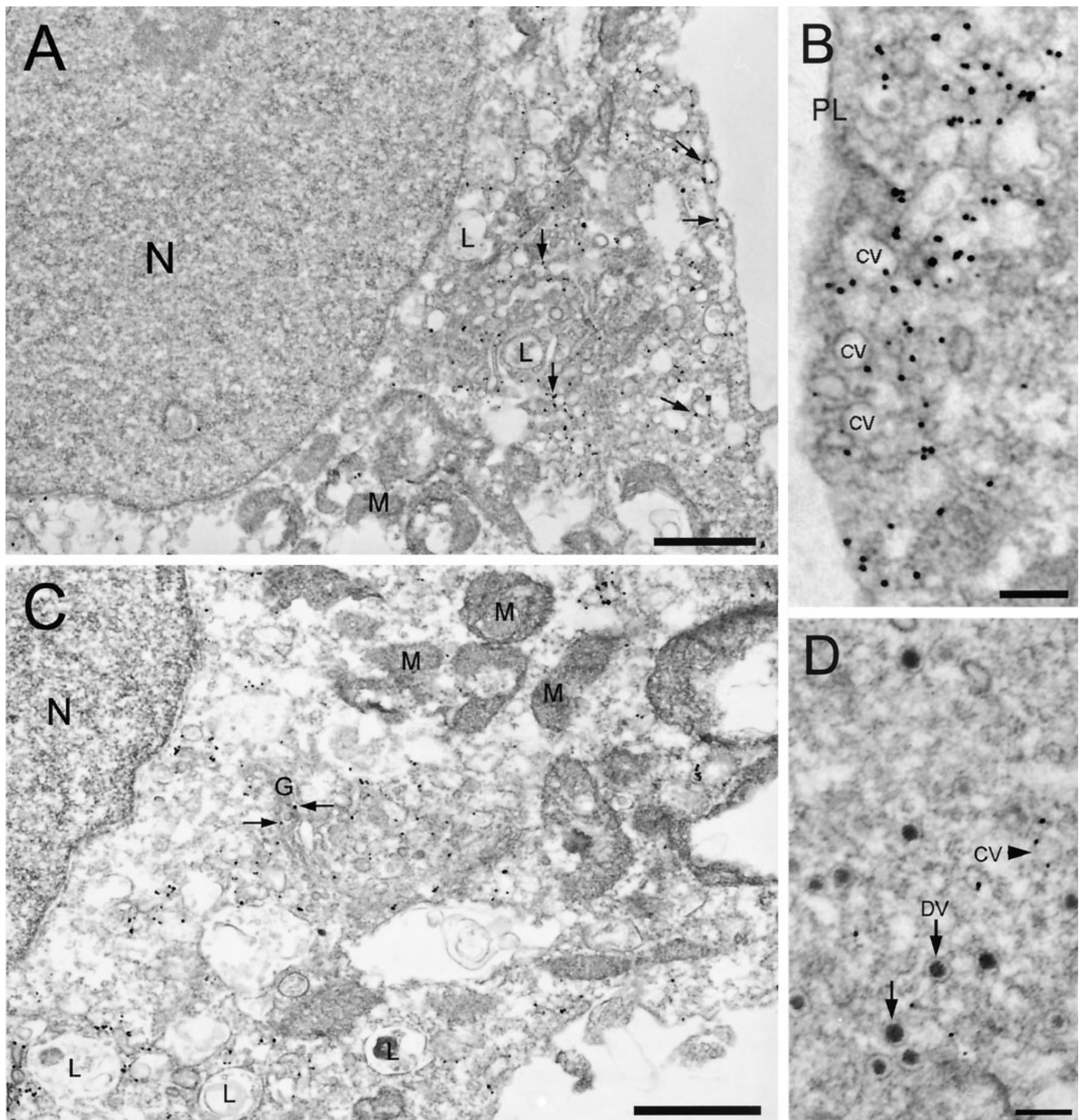

FIG. 4. Immunoelectronmicroscopy revealing synaptophysin-containing vesicles. *A*: Gold particle for synaptophysin is associated with clear vesicles, as indicated by arrows. *B*: Synaptophysin-containing vesicles are abundant in the peripheral region. *C*: Gold particle for synaptophysin is also associated with the Golgi apparatus, as indicated by arrows. *D*: Gold particles are absent in dense-cored vesicles (arrows) but present in clear vesicles (arrowhead). CV, clear vesicles; DV, dense-cored vesicles; G, Golgi apparatus; L, lysosome; M, mitochondrion; N, nucleus; PL, plasma membrane. Bar: *A* and *C*, 1 μm; *B* and *D*, 200 nm.

suggesting that L-type Ca^{2+} channels are involved in KCl-induced Ca^{2+}-dependent glutamate release. The presence of L-type Ca^{2+} channels in αTC6 cells was also evident by indirect immunofluorescence microscopy with antibodies against the L-type Ca^{2+} channel subunit (M.H. and Y.M., unpublished observations).

The second line of evidence is the dependence on temperature and the requirement of an appropriate duration of response after the second stimulation. These properties may reflect complex membrane dynamics, including the charging and discharging of neurotransmitters. Similar phenomena were observed for Ca^{2+}-dependent exocytosis found in various endocrine cells and neuronal cells (17,19, 30,31). The relatively slow rate of glutamate release (Fig. 1*A*) is also similar to that of GABA release by β-cells and glutamate release by rat pinealocytes (17,19,53,54).

The third line of evidence is sensitivity to BoNT/E and bafilomycin A_1. It is well established that BoNT/E is a pro-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

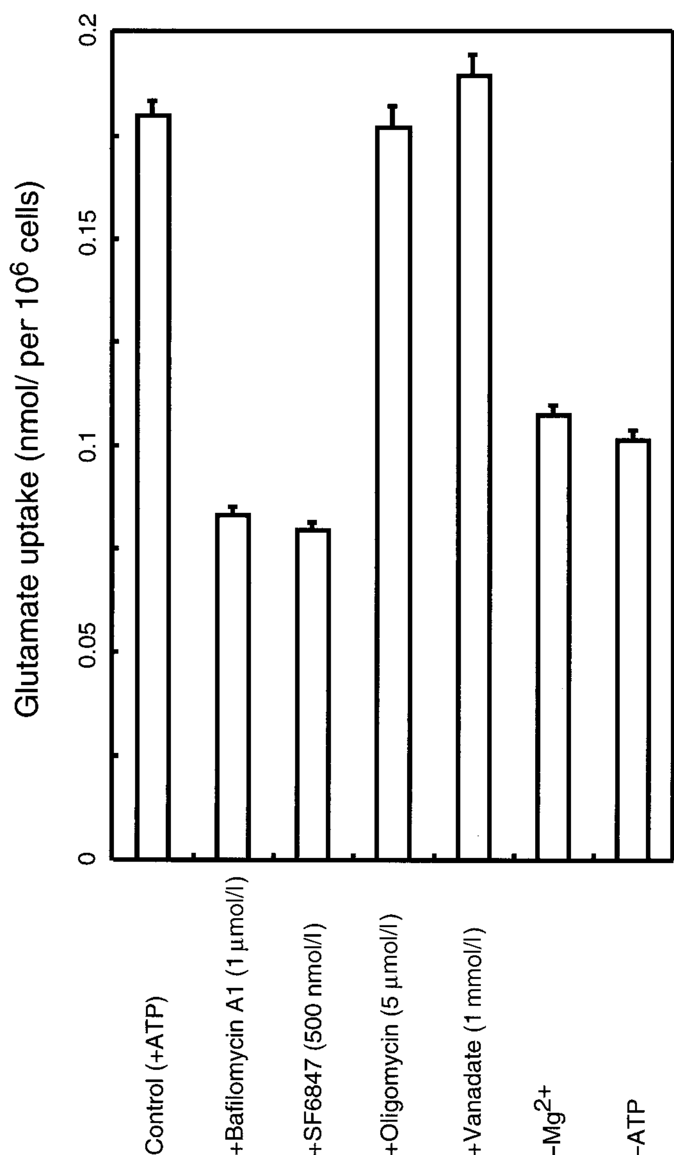

FIG. 5. ATP-dependent uptake of radiolabeled L-glutamate by digitonin-permeabilized cells. Glutamate uptake by permeabilized cells at 10 min was carried out as described in RESEARCH DESIGN AND METHODS, in the presence or absence of the listed compounds. In some experiments, Mg-acetate (–Mg^{2+}) or ATP (–ATP) was omitted. The results are the means ± SE of four independent experiments.

tease specific to SNAP25. Once activated in sensitive cells, BoNT/E cleaves SNAP25 into low molecular forms, causing inhibition of exocytosis of synaptic vesicles and secretory granules (17,19,37–39). The sensitivity to BoNT/E is thus considered one of the criteria for regulated exocytosis. Bafilomycin A_1 is a relatively hydrophobic macrolide antibiotic that specifically inhibits V-ATPase–linked secondary transport after the dissipation of an electrochemical proton gradient in the vesicles (45,46). It can deplete L-glutamate in secretory vesicles, resulting in a reduced level of exocytosis by neurons and astrocytes (43,44). Because bafilomycin A_1 inhibits the glutamate release from αTC6 cells, glutamate may accumulate in vesicular structures before being released by αTC6 cells in the same manner it is released in other secretory vesicles.

We then looked for the organelles responsible for the storage of glutamate. Vesicles containing synaptophysin are candidates for such an organelle because two kinds of synaptophysin-containing organelles, synaptic vesicles in glutamatergic neurons and synaptic-like microvesicles in pinealocytes, are known to be responsible for the storage and exocytosis of glutamate (28,29,45,46). Immunoelectronmicroscopy revealed synaptophysin-containing vesicles in αTC6 cells (Fig. 4). Synaptophysin-containing vesicles are different from dense-cored vesicles because the latter are devoid of both synaptophysin (Fig. 4*D*) and glucagon, as revealed with indirect immunofluorescence microscopy (data not shown). Furthermore, we showed that αTC6 cells accumulated L-glutamate in an ATP-dependent manner sensitive to bafilomycin A_1 and proton conductors (Fig. 5). This strongly suggests that a vesicular glutamate transporter energized by V-ATPase is responsible for the accumulation of glutamate. These results suggest that the synaptophysin-containing vesicles are counterparts to endocrine synaptic-like microvesicles and that they are responsible for storage and secretion of L-glutamate in αTC6 cells.

After stimulation, the glutamate-containing vesicles may secrete internal L-glutamate through Ca^{2+}-dependent exocytic processes. The released glutamate may bind to the glutamate receptor in neighboring islet cells, after which the receptor transmits glutamate signals (4). These mechanisms are essentially similar to the accumulation and release of L-glutamate in glutamatergic synaptic terminals (48) and endocrine pinealocytes (3). It is noteworthy that low glucose treatment stimulates the release of glutamate as well as glucagon from αTC6 cells. Furthermore, exogenous glutamate stimulates the release of glucagon from rat pancreatic islets (10). These results suggest a regulatory role of L-glutamate in the endocrine function of αTC6 cells.

In conclusion, αTC6 cells possess machinery for glutamate signal output. αTC6 cells may constitute a suitable experimental system for the peripheral glutamatergic mechanism. Our present study may provide an insight into the origin of L-glutamate in the extracellular space of islets, as stated above, and it raises the following questions: *1*) Does the regulated exocytosis of L-glutamate occur in α-cells in islets? and *2*) If so, what is the in vivo stimulation that triggers glutamate exocytosis? Further studies are necessary to answer these questions and to determine the entire features of the glutamatergic system in islets.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

ACKNOWLEDGMENTS

This work was supported in part by grants from the Japanese Ministry of Education, Science and Culture; CREST; the Japan Science and Technology Corporation; and the Japan Securities Scholarship Foundation. M.H. was supported by the Hayashi Memorial Foundation for Female Natural Scientists. H.Y. is a fellow at the Venture Business Laboratory of Okayama University.

We thank Prof. M. Takahashi of Mitsubishi Life Science Institute for the monoclonal antibodies against synaptophysin and Prof. S. Kozaki of the University of Osaka Prefecture for supplying the botulinum neurotoxin type E. We are also grateful to M. Kinoshita for her excellent technical assistance.

REFERENCES

1. Foster A, Fagg G: Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. *Brain Res* 319:103–164, 1984

2. Mayer ML, Westbrook G: The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog Neurobiol* 28:197–276, 1987
3. Moriyama Y, Hayashi M, Yamada H, Yatsushiro S, Ishio S, Yamamoto A: Synaptic-like microvesicles, synaptic vesicle counterparts in endocrine cells, are involved in a novel regulatory mechanism for hormonal synthesis and secretion. *J Exp Biol* 203:117–125, 2000
4. Satin LS, Kinard TA: Neurotransmitters and their receptors in the islets of Langerhans of the pancreas: what messages do acetylcholine, glutamate, and GABA transmit? (Review) *Endocrine* 8:213–223, 1998
5. Gonoi T, Mizuno N, Inagaki N, Kuromi H, Seino Y, Miyazaki J, Seino S: Functional neuronal ionotropic glutamate receptors are expressed in the non-neuronal cell line MIN6. *J Biol Chem* 269:16989–16992, 1994
6. Inagaki N, Kuromi H, Gonoi T, Okamoto Y, Ishida H, Seino Y, Kaneko T, Iwanaga T, Seino S: Expression and role of ionotropic glutamate receptors in pancreatic islet cells. *FASEB J* 9:686–691, 1995
7. Weaver CD, Partridge JG, Yao TL, Moates JM, Magnuson MA, Verdoorn TA: Activation of glycine and glutamate receptors increases intracellular calcium in cells derived from the endocrine pancreas. *Br J Pharmacol* 54:639–646, 1999
8. Weaver CD, Yao TL, Powers AC, Verdoon TA: Differential expression of glutamate receptor subtypes in rat pancreatic islets. *J Biol Chem* 271: 12977–12984, 1996
9. Bertrand G, Gross R, Puech R, Loubatieres-Mariani MM, Bockaert J: Evidence for a glutamate receptor of the AMPA subtype which mediates insulin release from rat perfused pancreas. *Br J Pharmacol* 106:354–359, 1992
10. Bertrand G, Gross R, Puech R, Loubatieres-Mariani MM, Bockaert J: Glutamate stimulates glucagon secretion via an excitatory amino acid receptor of the AMPA subtype in rat pancreas. *Eur J Pharmacol* 237:45–50, 1993
11. Weaver CD, Gundersen V, Verdoorn TA: A high affinity glutamate/aspartate transport system in pancreatic islets of Langerhans modulates glucose-stimulated insulin secretion. *J Biol Chem* 273:1647–1653, 1998
12. Michalik M, Nelson J, Erecinska M: Glutamate production in islets of Langerhans: properties of phosphate-activated glutaminase. *Metabolism* 41:1319–1326, 1991
13. Akiyama H, Kaneko T, Mizuno N, McGeer PL: Distribution of phosphate-activated glutaminase in the human cerebral cortex. *J Comp Neurol* 297:239–252, 1990
14. Hamaguchi K, Leiter EH: Comparison of cytokine effects on mouse pancreatic α-cell and β-cell lines: viability, secretory function, and MHC antigen expression. *Diabetes* 39:415–425, 1990
15. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K: Establishment of a pancreatic β-cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
16. Liu Y, Schweitzer ES, Nirenberg MJ, Pickel VM, Evans CJ, Edwards RH: Preferential localization of vesicular monoamine transporter to dense core vesicles in PC 12 cells. *J Cell Biol* 127:1419–1433, 1994
17. Yamada H, Yamamoto A, Yodozawa S, Kozaki S, Takahashi M, Michibata H, Morita M, Furuichi T, Mikoshiba K, Moriyama Y: Microvesicle-mediated exocytosis of glutamate is a novel paracrine-like chemical transduction mechanism and inhibits melatonin secretion in pinealocytes. *J Pineal Res* 21:175–191, 1996
18. Godel H, Graser T, Foldi P, Pfaender P, Fuerst P: Measurement of free amino acids in human biological fluids by high-performance liquid chromatography. *J Chromatography* 297:49–61, 1984
19. Yatsushiro S, Yamada H, Kozaki S, Kumon H, Michibata H, Yamamoto A, Moriyama Y: L-Aspartate but not the D form is secreted through microvesicle-mediated exocytosis and is sequestered through Na^+-dependent transporter in rat pinealocytes. *J Neurochem* 69:340–347, 1997
20. Yamada H, Ogura A, Koizumi S, Yamaguchi A, Moriyama Y: Acetylcholine triggers L-glutamate exocytosis via nicotinic receptors and inhibits melatonin synthesis in rat pinealocytes. *J Neurosci* 18:4946–4952, 1998
21. Ogura A, Myojo Y, Higashida H: Bradykinin-evoked acetylcholine release via inositol triphosphate-dependent elevation in free calcium in neuroblastoma x glioma hybrid NG108–15 cells. *J Biol Chem* 265:3577–3584, 1990
22. Moriyama Y, Nelson N: Purification and properties of a vanadate- and N-ethylmaleimide-sensitive ATPase from chromaffin granule membranes. *J Biol Chem* 263:8521–8527, 1988
23. Mandai K, Nakanishi H, Satoh A, Obaishi H, Wada M, Nishioka H, Ito M, Mizoguchi A, Aoki T, Fujimoto T, Matsuda Y, Tsukita S, Takai Y: Afadin: a novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction. *J Cell Biol* 139:517–528, 1997
24. Hayashi M, Yamamoto A, Yatsushiro S, Yamada H, Futai M, Yamaguchi A, Moriyama Y: Synaptic vesicle protein SV2B, but not SV2A, is predominantly expressed and associated with microvesicles in rat pinealocytes. *J Neurochem* 71:356–365, 1998
25. Mrini A, Moukhles H, Jacomy H, Posler O, Doucet G: Efficient immunodetection of various protein antigens in glutaraldehyde-fixed brain tissue. *J Histochem Cytochem* 43:1285–1291, 1995
26. Obata K, Nishiya H, Fujita SC, Shirao T, Inoue H, Uchizono K: Identification of a synaptic vesicle-specific 38,000-dalton protein by monoclonal antibodies. *Brain Res* 375:37–45, 1986
27. Erickson JD, Eiden LE, Hoffman BJ: Expression cloning of a reserpine-sensitive vesicular monoamine transporter. *Proc Natl Sci Acad U S A* 89:10993–10997, 1992
28. Moriyama Y, Yamamoto A: Microvesicles isolated from bovine pineal gland specifically accumulate L-glutamate. *FEBS Lett* 367:233–236, 1995
29. Moriyama Y, Yamamoto A: Vesicular L-glutamate transporter in microvesicles from bovine pineal glands: driving force, mechanism of chloride anion activation, and substrate specificity. *J Biol Chem* 270:22314–22320, 1995
30. Nicholls DG: The release of glutamate, aspartate, and GABA from isolated nerve terminals. *J Neurochem* 52:331–341, 1989
31. Heidelberger R, Heinemann C, Nehr E, Matthews G: Calcium dependence of the rate of exocytosis in a synaptic terminal. *Nature* 371:513–515, 1994
32. Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP: Multiple types of neuronal calcium channels and their selective modulation (Review). *Trends Neurosci* 11:431–438, 1988
33. Olivera BM, Mclintosh JM, Cruz LJ, Luque FA, Gray WR: Purification and sequence of a presynaptic peptide toxin from *Conus geographus* venom. *Biochemistry* 23:5087–5090, 1984
34. Hillyard DR, Monje VD, Mintz IM, Bean BP, Nadasdi L, Ramachandran J, Miljanich G, Azimi-Zoonooz A, Mclintosh JM, Cruz LJ, Imperial JS, Olivera BM: A new conus peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron* 9:69–77, 1992
35. Mintz IM, Venema VJ, Swiderek KM, Lee TD, Bean BP, Adams ME: P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature* 355:827–829, 1992
36. Turner TJ, Adams ME, Dunlap K: Calcium channels coupled to glutamate release identified by ω-Aga-IVA. *Science* 258:310–313, 1992
37. Binz T, Blasi J, Yamasaki S, Baumeister A, Link E, Südhof TC, Jahn R, Niemann H: Proteolysis of SNAP-25 by type E and A botulinum neurotoxins. *J Biol Chem* 269:1617–1620, 1994
38. Schiavo G, Santucci A, DasGupta BR, Mehta PP, Jontes J, Benfenati F, Wilson MC, Montecucco C: Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett* 335:99–103, 1993
39. Sadoul K, Lang J, Montecucco C, Weller U, Regazzi R, Catsicas S, Wollheim CB, Halban PA: SNAP-25 is expressed in islets of Langerhans and is involved in insulin release. *J Cell Biol* 128:1019–1028, 1995
40. Bowman EJ, Siebers A, Altendorf K: Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. *Proc Natl Acad Sci U S A* 85:7972–7976, 1988
41. Umata T, Moriyama Y, Futai M, Mekada E: The cytotoxic action of diphtheria toxin and its degradation in intact Vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase. *J Biol Chem* 265:21940–21945, 1990
42. Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y: Bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* 261:17707–17712, 1991
43. Cousin MA, Nicholls DG, Pocock JM: Modulation of ion gradients and glutamate release in cultured cerebellar granule cells by ouabain. *J Neurochem* 64:2097–2104, 1995
44. Araque A, Li N, Doyle RT, Haydon PG: SNARE protein-dependent glutamate release from astrocytes. *J Neurosci* 20:666–673, 1999
45. Maycox PR, Deckwerth T, Hell JW, Jahn R: Glutamate uptake by brain synaptic vesicles: energy dependence of transport and functional reconstitution in proteoliposomes. *J Biol Chem* 263:15423–15428, 1988
46. Moriyama Y, Maeda M, Futai M: Energy coupling of L-glutamate transport and vacuolar H^+-ATPase in brain synaptic vesicles. *J Biochem* 108:689–693, 1990
47. Maycox PR, Hell JW, Jahn R: Amino acid neurotransmission: spotlight on synaptic vesicles. *Trends Neurosci* 13:83–87, 1990
48. Nicholls D, Attwell D: The release and uptake of excitatory amino acids. *Trends Pharmacol Sci* 11:462–468, 1990
49. Gaskins HR, Baldeon ME, Selassie L, Beverly JL: Glucose modulates γ-aminobutyric acid release from the pancreatic βTC6 cell line. *J Biol Chem* 270:30286–30289, 1995
50. Moriyama Y, Yamamoto A, Yamada H, Tashiro Y, Futai M: Role of endocrine cell microvesicles in intercellular chemical transduction. *Biol Chem* 377:155–165, 1996
51. Thomas-Reetz A, De Camilli P: A role for synaptic vesicles in non-neuronal

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025

cells: clues from pancreatic β-cells and from chromaffin cells. *FASEB J* 8:209–216, 1994

52. Thomas-Reetz A, Hell JW, During MJ, Walch-Solimena C, Jahn R, De Camilli P: A γ-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic β-cells. *Proc Natl Acad Sci U S A* 90:5317–5321, 1993
53. Ahnert-Hilger G, Stadtbäumer A, Strübing C, Scherübl H, Schultz G, Riecken E-O, Wiedenmann B: γ-Aminobuytric acid secretion from pancreatic neuroendocrine cells. *Gastroenterology* 110:1595–1604, 1996
54. Ahnert-Hilger G, Wiedenman B: The amphicrine pancreatic cell line, AR42, secretes GABA and amylase by separate regulated pathways. *FEBS Lett* 314:41–44, 1992
55. Rorsman P, Berggren PO, Bokvist K, Ericson H, Mohler H, Ostenson CG, Smith PA: Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels. *Nature* 341:233–236, 1989
56. Gilon P, Bertrand G, Loubatieres-Mariani MM, Remacle C, Henquin JC: The Influence of γ-aminobutyric acid on hormone release by the mouse and rat endocrine pancreas. *Endocrinology* 129:2521–2529, 1991

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/50/5/1012/368670/1012.pdf by guest on 28 April 2025