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Increase in cellular glutamate levels stimulates exocytosis in pancreatic β-cells

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Abstract **Glutamate has been implicated as an intracellular messenger in the regulation of insulin secretion in response to glucose. Here we demonstrate by measurements of cell capacitance in rat pancreatic β-cells that glutamate (1 mM) enhanced Ca^{2+}-dependent exocytosis. Glutamate (1 mM) also stimulated insulin secretion from permeabilized rat β-cells. The effect was dose-dependent (half-maximum at 5.1 mM) and maximal at 10 mM glutamate. Glutamate-induced exocytosis was stronger in rat β-cells and clonal INS-1E cells compared to β-cells isolated from mice and in parental INS-1 cells, which correlated with the expressed levels of glutamate dehydrogenase. Glutamate-induced exocytosis was inhibited by the protonophores FCCP and SF6847, by the vacuolar-type H^+-ATPase inhibitor bafilomycin A_1 and by the glutamate transport inhibitor Evans Blue. Our data provide evidence that exocytosis in β-cells can be modulated by physiological increases in cellular glutamate levels. The results suggest that stimulation of exocytosis is associated with accumulation of glutamate in the secretory granules, a process that is dependent on the transgranular proton gradient.**
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Key words: Glutamate; Exocytosis; Insulin; Beta cell

1. Introduction

In addition to closing the ATP-regulated K^+-channels, glucose also promotes insulin secretion under conditions of clamped cytoplasmic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) [1]. This secretory response requires one or several additive messengers that are distinct from ATP and generated by mitochondrial metabolism. A putative factor was recently identified as glutamate [2]. In the pancreatic β-cell, glutamate can be formed in the mitochondria from α-ketoglutarate, a tricarboxylic-acid (TCA) cycle intermediate, by glutamate dehydrogenase (GDH). Stimulation of insulin secretion by glutamate is glucose-dependent since the amino acid is produced from glucose [3]. In addition, glutamate does not initiate insulin secretion but potentiates secretion induced by a rise in $[Ca^{2+}]_i$ [2]. The increase in glutamate levels occurs 5–10 min after cell stimulation with glucose [2] and coincides with the second sustained phase of the biphasic glucose-induced insulin secretion [4].

In the present study, we have measured the exocytotic response to glutamate in primary rat and mouse β-cells and INS-1 insulinoma cells. We provide evidence that the glutamate uptake mechanism in the insulin-containing granules is related to the mechanisms initially reported for synaptic vesicles and depends on the transgranular H^+ gradient.

2. Materials and methods

2.1. Isolation and culture of cells

Islets were isolated from male Wistar rats (200–250 g) by collagenase digestion of excised pancreata. The animals were obtained from Bomholtgård (Ry, Denmark) or in-house breeding (CMU-Zootechnie, Geneva, Switzerland). Mouse islets were obtained from female NMRI mice (22–25 g; Bomholtgård). The islets were dispersed into single cells by shaking in a Ca^{2+}-free medium. The cells were cultured in a humidified atmosphere with 5% CO_2 for up to 3 days in RPMI-1640 medium (Gibco BRL, Life Technologies, UK) supplemented with 10% heat-inactivated fetal calf serum, 100 i.u./ml penicillin and 100 μg streptomycin. Parental INS-1 and INS-1E cells, cloned from the parental INS-1 cell line [5], were cultured as described in [2].

2.2. Electrophysiology

Exocytosis was monitored as increases in cell membrane capacitance using the standard whole-cell configuration, an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lamprecht/Pfalz, Germany) and the Pulse software (version 8.01; HEKA Elektronik). The interval between two successive points was 0.2 s and the measurements of cell capacitance were initiated < 5 s following establishment of the whole-cell configuration. The extracellular medium consisted of (in mM) 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4 with NaOH) and 5 D-glucose. The electrode solution consisted of (in mM) 135 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 3 Mg-ATP, 10 EGTA (ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 7 or 9 $CaCl_2$ (pH 7.15 with KOH). In some experiments equimolar concentrations of KCl were exchanged for potassium-L-glutamate. The free Ca^{2+} concentrations of the resulting buffers were 0.50 and 1.96 μM using the binding constants of Martell and Smith [6]. Bafilomycin A_1 was obtained from Calbiochem (La Jolla, CA, USA). All other chemicals were purchased from Sigma.

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Abbreviations: $[Ca^{2+}]_i$, cytoplasmic-free Ca^{2+} concentration; GDH, glutamate dehydrogenase; TCA, tricarboxylic acid

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2.3. Insulin secretion from permeabilized β-cells

Rat β-cells cultured for 2 days were permeabilized with *Staphylococcus* α-toxin as previously described in [7], then perfused at 500 nM free Ca^{2+} (in mM: 140 KCl, 5 NaCl, 7 $MgSO_4$, 1 ATP, 20 HEPES, 10.2 EGTA, 6.67 $CaCl_2$, pH 7.0, 0.1% bovine serum albumin) and the effluent was collected. Insulin was detected by radioimmunoassay using rat insulin as standard [2].

2.4. Immunoblotting

Western blot analysis was performed as described previously [4].

2.5. Statistical analysis

Results are presented as mean values ± S.E.M. for indicated numbers of experiments. The exocytotic rate is presented as increases in cell capacitance during the first 60 s following establishment of the whole-cell configuration, excluding any rapid changes during the initial 10 s required for equilibration of the pipette solution with cytosol. Statistical significance was evaluated using Student's *t*-test (Figs. 1 and 3) or Dunnett's test for multiple comparisons (Fig. 2A–D).

3. Results

Exocytosis was elicited by intracellular dialysis with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 500 nM through the recording pipette. Inclusion of 1 mM glutamate in the pipette solution stimulated exocytosis (Fig. 1A). On average (Fig. 1B), glutamate accelerated exocytosis by 43% ($P < 0.05$; $n = 5$) when measured over the first 60 s after establishment of the whole-cell configuration. This is consistent with the observation that insulin secretion was stimulated by 1 mM glutamate in permeabilized rat β-cells perfused with 1 mM ATP and 500 nM free Ca^{2+} (Fig. 1C).

The effect of glutamate on exocytosis was dose-dependent (Fig. 2A, insert). In this series of experiments exocytosis was elicited by infusion of a maximal $[Ca^{2+}]_i$ of 2 μM. Exocytosis was stimulated already at 1 mM glutamate and maximal enhancement of secretion was observed at 10 mM (Fig. 2A). Half-maximal stimulation was observed at 5.1 mM glutamate. The observed effects are specific for L-glutamate as D-glutamate (10 mM) did not produce significant changes of exocytosis and L- and D-aspartate (10 mM) were inactive (data not shown).

It has previously been reported that the expression of GDH is lower in mouse than in rat islets. This is paralleled by a clear biphasic glucose-induced insulin secretion in the perfused rat pancreas, whereas in the mouse glucose essentially elicits transient monophasic insulin release [4]. Interestingly, the effect of glutamate on high (2 μM) Ca^{2+}-induced exocytosis was stronger in rat than mouse β-cells (Fig. 2A,B). In mouse β-cells, a maximal glutamate concentration of 10 mM only stimulated exocytosis by 35%. This should be compared to 56% stimulation in rat β-cells (Fig. 2A). This results mainly from a lower rate of exocytosis in the absence of glutamate in rat β-cells (11.0 ± 0.4 fF/s; $n = 5$) compared to mouse β-cells (13.4 ± 1.5 fF/s; $n = 10$), whereas the maximal secretion rates were similar. Half-maximal stimulation was observed at 5.9 mM glutamate in mouse β-cells.

Glutamate (10 mM) also elicited a more pronounced stimulation of exocytosis in clonal INS-1E cells (84% stimulation) as compared to parental INS-1 cells (34% enhancement; Fig. 2C,D). Again, this difference mainly reflects a lower rate of exocytosis in the absence of glutamate in INS-1E cells. Half-maximal stimulation was observed at 2.4 mM glutamate in INS-1E cells and 3.3 mM in parental INS-1 cells, respectively. Interestingly, the stronger stimulation of exocytosis by glutamate in INS-1E cells was associated with a 34% ($P < 0.005$; $n = 3$) higher expression of GDH as revealed by quantitative immunoblotting analysis (parental INS-1 cells: 0.40 ± 0.09 ng GDH/μg total cellular protein vs. 0.54 ± 0.08 ng GDH/μg total cellular protein in INS-1E cells; Fig. 2E).

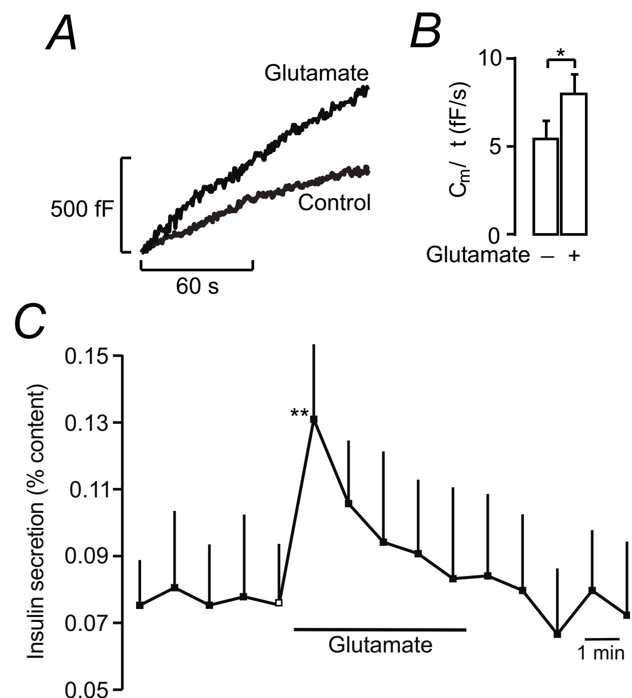

Fig. 1. Glutamate stimulates insulin exocytosis in rat pancreatic β-cells. A: Increases in cell capacitance observed during the first 2 min after establishment of the standard whole-cell configuration elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 500 nM in the absence (Control) or presence of 1 mM L-glutamate in the pipette solution. Throughout the recording, the cell was clamped at −70 mV in order to avoid activation of the voltage-dependent Ca^{2+}-channels that would otherwise interfere with the capacitance measurements. B: Histogram depicting mean rates of increase in cell capacitance ($\Delta C_m/\Delta t$) in the absence and presence of 1 mM glutamate during the first 60 s after establishment of the whole-cell configuration. Data are mean values of five individual experiments under both conditions. *$P < 0.05$ vs. control. C: Effect of L-glutamate (1 mM) on insulin secretion in α-toxin permeabilized rat pancreatic islet cells. The results are means ± S.E.M. of six individual experiments. **$P < 0.01$ vs. the time point just preceding stimulation (open square).

How might glutamate promote exocytosis? The insulin-containing granules are acidic inside. This pH gradient is generated by a vacuolar-type H^+-ATPase and is believed to drive glutamate uptake into the granules via an as yet unidentified transporter. This is consistent with the finding that dissipation of the proton gradient by the protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 0.1 μM) inhibited glutamate (10 mM)-induced exocytosis in rat β-cells (Fig. 3A,B). FCCP also reduced basal secretion by 44%. Oligomycin (1 μg/ml) did not affect basal and glutamate-evoked exocytosis (Fig. 3A,B), suggesting that inhibition of exocytosis observed in the presence of FCCP does not interfere with mitochondria function. A similar inhibition of glutamate-induced exocytosis was observed in the presence of the protonophore SF6847 (3,5-di-tert-butyl-4-hxdroxybenzylidenermalononitrile; 0.5 μM). It is important to emphasize that all experiments were performed in the presence of 3 mM Mg-

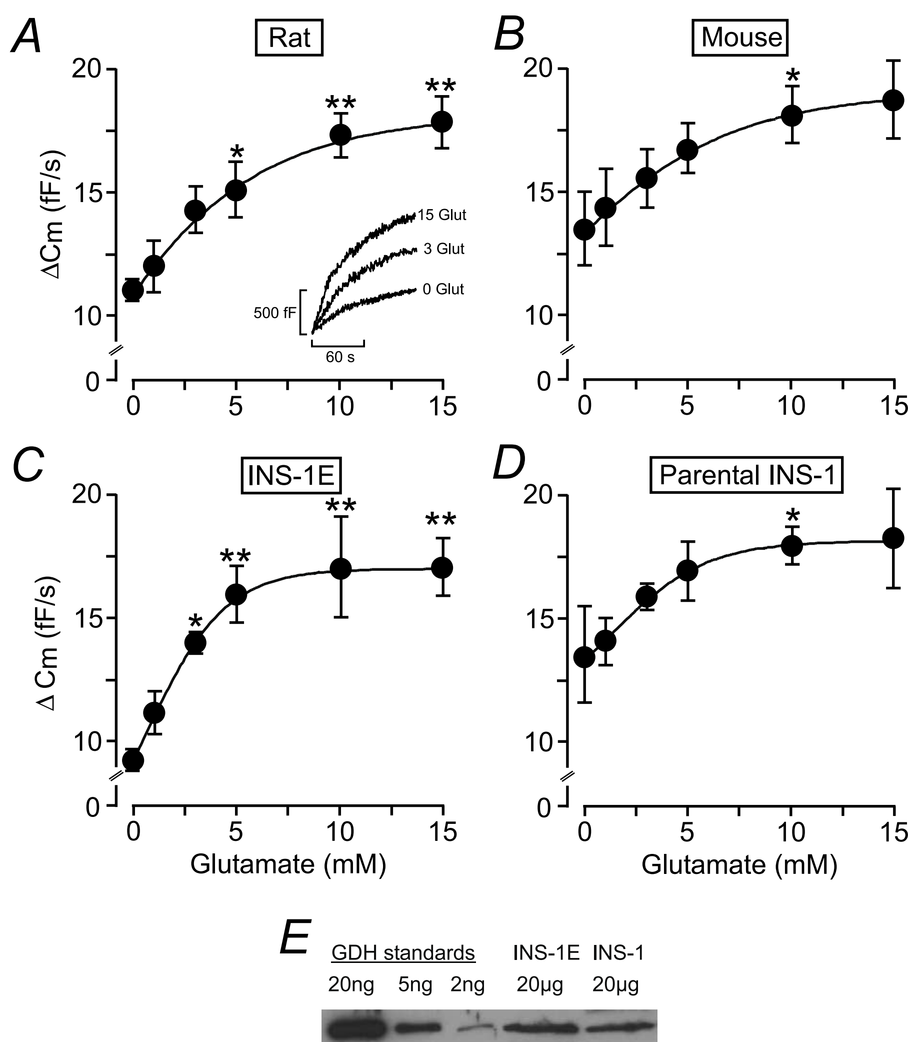

Fig. 2. Dose–response relationship for glutamate-induced exocytosis in (A) rat β-cells, (B) mouse β-cells, (C) INS-1E cells and (D) parental INS-1 cells. Increases in cell capacitance (ΔC_m) were elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+}-concentration of 2 μM. The insert in panel A shows representative traces of increases in cell capacitance with 0, 3 and 15 mM glutamate in the pipette-filling solution dialyzing the cell. The lines are the best fit of the mean data to the Hill equation. The data are mean values ± S.E.M. of 4–11 experiments. E: Immunoblotting for GDH. Immunoblotting was performed after SDS–PAGE using 20 μg proteins of INS-1E and parental INS-1 cell extract per lane or standard of GDH. The data are representative of three independent cell preparations. *$P<0.05$; **$P<0.01$ compared to control (0 mM glutamate).

ATP in the pipette-filling solution. Accordingly, the use of these inhibitors affecting mitochondria-dependent energy metabolism on intact cells would result in impaired insulin secretion.

The effect of glutamate on exocytosis was also abolished by inhibition of the vacuolar-type H^+-ATPase with bafilomycin A_1 (100 nM; Fig. 3C,D). In contrast, vanadate (1 mM), an inhibitor of P-type ion-transporting ATPases, did not affect glutamate-induced exocytosis (data not shown). It has been suggested that glutamate might stimulate insulin secretion by inhibition of β-cell serine-threonine protein phosphatases [8]. However, this is unlikely to be the case since glutamate (10 mM) stimulated exocytosis in cells treated with the protein phosphatase inhibitor okadaic acid (0.5 μM) to an extent (63% stimulation) similar to that observed under control conditions (56% stimulation; data not shown). These data suggest that the abrogation of glutamate-induced exocytosis observed with FCCP, SF6847 and bafilomycin A_1 is probably secondary to inhibition of glutamate uptake by the secretory granules. This is supported by the observation that Evans Blue (2 μM), a competitive inhibitor of the vesicular glutamate transporter, abolished glutamate-induced exocytosis without affecting basal secretion (Fig. 3E,F). Recently, it has been convincingly demonstrated that chloride ions play an important role in priming of insulin-containing granules. Chloride is taken up by the granules through ClC3 chloride channels [9]. Our findings extend these observations since inclusion of the chloride channel inhibitor DIDS (100 μM) in the pipette-filling solution completely inhibited exocytosis in the absence of glutamate but did not affect glutamate-induced exocytosis (Fig. 3G,H).

4. Discussion

Glucose stimulation elicits a biphasic secretory response consisting of an initial transient phase and a second, sustained component. The first phase of insulin secretion is thought to reflect the rapid release of a readily releasable pool of gran-

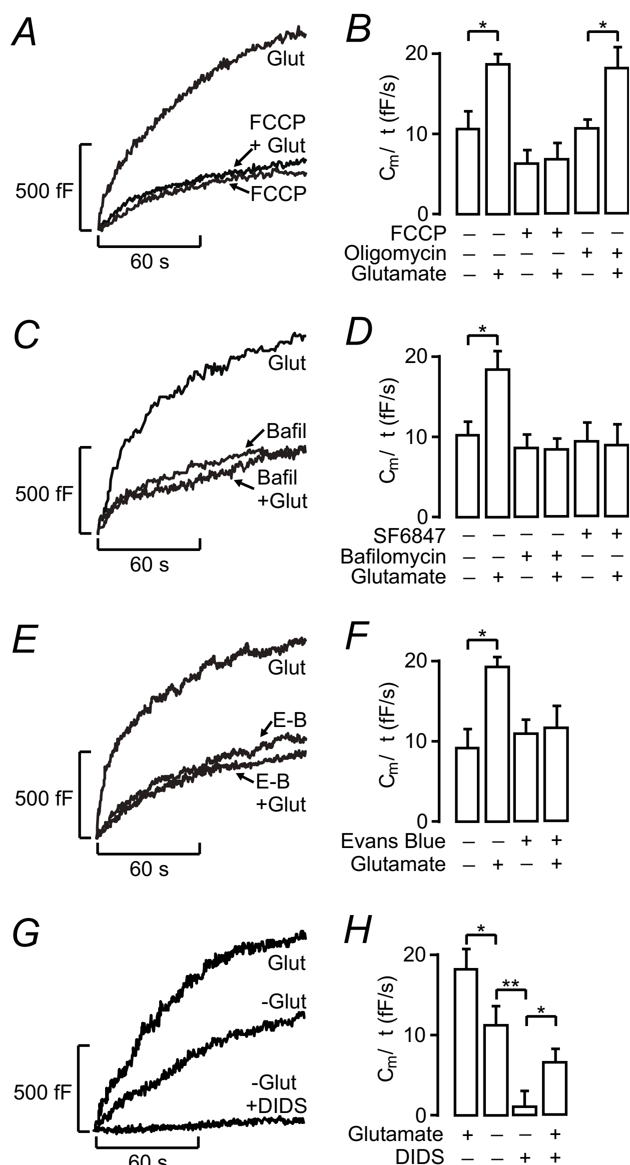

Fig. 3. Glutamate-induced exocytosis depends on the transgranular proton gradient. A: Changes in cell capacitance elicited by 2 μM $[Ca^{2+}]_i$ in the presence of 10 mM glutamate (Glut), 0.1 μM FCCP and in the combined presence of glutamate and FCCP. B: The histogram depicts the mean rates of cell capacitance increase ($\Delta C_m/\Delta t$) measured over the first 60 s after establishment of the whole-cell configuration in the absence and presence of 10 mM glutamate, in the presence of FCCP and in the simultaneous presence of glutamate and FCCP. The histogram also depicts the average rates of capacitance increase in the presence of oligomycin (1 μg/ml) and in the simultaneous presence of oligomycin and glutamate. C: As in panel A, except FCCP was replaced with 0.1 μM bafilomycin A_1 (Bafil). D: Average rates of capacitance increase in the absence and presence of bafilomycin A_1 or SF6847 (0.5 μM) and in the simultaneous presence of glutamate and bafilomaycin A_1 or SF6847. E,F: As in panel A, except FCCP was replaced with 2 μM Evans Blue. Mean increase in cell capacitance in the absence and presence of glutamate and Evans Blue. G:As in panel A, except that exocytosis was measured in the absence and presence of 10 mM glutamate and in the absence and presence of 0.1 μM DIDS. H: Mean increase in cell capacitance in the absence and presence of glutamate and DIDS. The data are mean values ± S.E.M. of five to seven different cells. $^{*}P<0.05$; $^{**}P<0.01$.

ules. The second, slower phase reflects the time-dependent replenishment of this pool by priming of granules originated from the reserve pool [10]. Glutamate has been implicated in the control of the second sustained phase of insulin secretion [4]. Here we extend these observations by demonstrating that glutamate primes secretory granules and thereby accelerates Ca^{2+}-dependent exocytosis in primary rat and mouse β-cells as well as in INS-1 cells using capacitance measurements.

Glucose increases the cellular glutamate content in human islets [2], mouse and rat islets [11] and in the rat β-cell lines INS-1 and BRIN-D11 [2,3]. These data support the idea that glutamate plays a role in glucose-induced insulin secretion although this view has recently been challenged [11,12]. It is noteworthy that these two papers are internally conflicting since there was no change in islet glutamate levels in one [12] and a marked increase during glucose stimulation in the other [11]. In the latter study glutamate changes correlated with secretory responses upon glucose stimulation, but not in the presence of glutamine given as glutamate precursor. Under these experimental conditions, actual cytosolic glutamate levels may become saturating, as demonstrated here for the enhancement of Ca^{2+}-induced exocytosis. Glutamate is formed at elevated glucose concentrations in the mitochondria [2,13], mainly from the TCA-cycle intermediate α-ketoglutarate by GDH [3]. Subsequently, glutamate is transferred to the cytosol, where it is probably taken up by the insulin-containing granules.

Since mitochondria and possibly secretory granules contain glutamate, measurements of glutamate in whole cells do not yield information on changes in the cytosol, the critical compartment for glutamate as the putative second messenger. In the present study we have overcome this problem by infusion of glutamate directly to the cytosol through the recording pipette. Indeed, we demonstrate that exocytosis is stimulated by low millimolar glutamate concentrations and with a half-maximal stimulatory effect at 2–6 mM glutamate. Interestingly, the rates of exocytosis observed in the absence of added glutamate to the cytosol in different cell types display inverse correlation with the GDH expression levels. Cells with lower GDH expression (parental INS-1 and mouse β-cells) exhibit higher exocytosis rates in the absence of glutamate compared to that in cells with higher GDH levels (INS-1E and rat β-cells). However, inclusion of glutamate in the cytosol equalized the exocytotic responses in all cell types. It is noteworthy that GDH expression in human islets is comparable to rat islet levels [4]. These data suggest that cell types with higher GDH expression levels are more dependent on glutamate to elicit their full exocytotic response, whereas in the cells with lower GDH levels, glutamate is less important for maintaining the high exocytosis rate.

Priming depends in part on ATP hydrolysis by the H^+-ATPase that electrogenically pumps protons into the granular interior [9]. The pump will generate a large proton electrochemical gradient and in the absence of a counter-ion, the large membrane potential that develops (positive inside) will prevent further uptake of protons and thus acidification of the granules. The activity of the pump can be used to drive the uptake of negatively charged molecules, like glutamate, into the secretory granules. This will allow a larger pH gradient to develop across the granule membrane. Acidification of the secretory granules is essential for glutamate-activated priming. This is suggested by the present measurements of exocytosis

by cell capacitance and previous observations in permeabilized cells [2] that dissipation of the H^+ gradient by the protonophores FCCP and SF6847 abolished the action of glutamate on exocytosis. Furthermore, the inhibitor of H^+-type ATPase bafilomycin A_1 prevented glutamate-induced exocytosis. And finally, blockade of glutamate uptake into the granules with Evans Blue abolished glutamate-evoked exocytosis.

The nature of the glutamate transporter in insulin-containing granules is unknown but it is tempting to speculate that they have similarities with the recently identified vesicular glutamate transporters VGLUT1/BNPI [14,15] and VGLUT2/DNPI [16,17]. The present study demonstrates that glutamate is not the only counter-ion required for priming since large capacitance increases were recorded even in the complete absence of this mitochondrial factor. In agreement with previous observations [9], our data suggest that the capacitance increase observed in the absence of glutamate is likely to result from chloride influx into the granules. Blockade of granular ClC3 chloride channels with DIDS was shown to inhibit granular acidification and priming [9].

Glutamate accumulated inside granules is getting secreted during stimulation of β-cell exocytosis and may serve as an extracellular signalling molecule in a manner similar to that in the central nervous system. Ionotropic and metabotropic glutamate receptors have been identified on the plasma membrane in all islet cell types [18]. Thus, glutamate may act both in autocrine and paracrine manners in the complex regulation of hormone release from the different cell types within an islet. Interestingly, it has recently been reported that αTC6 glucagonoma cells accumulate glutamate in their secretory granules similar to that reported in the present study and secrete glutamate through exocytosis, the mechanism resembling that in neurons and pinealocytes [19].

In conclusion, our data suggest that glutamate at physiological levels primes β-cell secretory granules for release. This process is dependent on glutamate uptake into the granules, a process fuelled by a proton electrochemical gradient across the secretory granule membrane. Finally, the rate of glutamate-stimulated exocytosis correlates with the expression levels of GDH, with the highest level observed in rat β-cells and INS-1E cells. This suggests that INS-1E cells may provide a good model to explore further the mechanism by which glutamate primes granules and its relationship to the GDH expression level.

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