Exocytosis of d-Aspartate from INS-1E Clonal β Cells

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d-Aspartate is present in the central nervous system and various endocrine organs, and modulates their neuroendocrine function. In islets of Langerhans, α and β cells contain d-aspartate. Here we show that INS-1E clonal β cells contain the highest amount of d-aspartate. Immunohistochemical analysis with specific antibodies against d-aspartate indicated that d-aspartate is co-localized with insulin. Upon the addition of K+, both d-aspartate and insulin are secreted from the cells in a Ca2+-dependent manner. A Ca2+-ionophore, A23187, also triggers the release of d-aspartate and insulin in the presence of Ca2+. Bafilomycin A1, a specific inhibitor of V-ATPase and V-ATPase-linked secondary transport, inhibits the secretion of d-aspartate. These results support the idea that d-aspartate is present in insulin-containing secretory granules and co-secreted with insulin through exocytosis.

Key words d-aspartate; islet of Langerhans; α cell; β cell; insulin

Substantial levels of d-amino acids are present in mammals, and they have various physiological roles.1,2 d-Aspartate is present in the central nervous system and various neuroendocrine cells, which include a subset of satellite and basket cells in the cerebellum, adrenal chromaffin cells, pituitary gland cells, Leydig cells and pinealocytes.2–10) d-Aspartate is believed to modulate neuroendocrine functions in either an intercellular or intracellular manner. In pinealocytes, d-aspartate is synthesized de novo, localized in the cytoplasm, and released through a Na+-dependent glutamate/aspartate transporter at the plasma membrane.6,10) Then, d-aspartate may act as an intracellular messenger and inhibit melatonin synthesis in a receptor-mediated manner.10) Pheochromocytoma PC12 cells also synthesize d-aspartate de novo, store it in dopamin-containing secretory granules, and secrete it through exocytosis.11) In Leydig cells, d-aspartate is present in the cytoplasm and stimulates testosterone synthesis through modulation of steroidogenesis.3,12) In pituitary cells, d-aspartate is metabolically converted to N-methyl-d-aspartate (NMDA), which in turn stimulates the secretion of hypothalamic releasing hormones.9) Thus, the modes of action of d-aspartate in neuroendocrine cells seem to be diverse in nature but remain poorly understood.

Recently, using immunohistochemical approaches with d-aspartate specific antibodies, we found that the islet of Langerhans, a miniature endocrine organ for blood glucose-regulating hormones, also contains d-aspartate.13) More precisely, the highest amount of d-aspartate is present in glucagon-secreting α cells, there being a lower amount in insulin-secreting β cells. It would be interesting to determine whether islet cells secrete d-aspartate. In the present study, we investigated this issue using clonal islet β cells.

MATERIALS AND METHODS

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.14) Islets were then handpicked and suspended in a bicarbonate-buffered Hank’s solution supplemented with 0.2% bovine serum albumin (BSA). INS-1E cells were cultured in 10 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4, 25 mM sodium bicarbonate, 100 units/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 mg/ml fungizone, and then incubated at 37 °C under 5% CO2. The dispersed cells were washed three times with the above medium, placed in 100-mm culture dishes to give 2×106 cells, and then cultured in the above medium at 37 °C under 5% CO2. These cells were maintained for 5 d, washed with the culture medium, cultured further for 1 h, and then used for the experiments.

Assays d-Aspartate was quantified as previously described.10,11) In brief, cultured cells (×103 cells) were washed three times with a Ringer’s solution comprising 10 mM HEPES, pH 7.4, 0.2% BSA, 4.7 mM KCl and 2.5 mM CaCl2 (+Ca2+-Ringer) or 10 mM HEPES, pH 7.4, 0.2% BSA, 4.7 mM KCl and 2.5 mM CaCl2 (−Ca2+-Ringer), and then incubated in the same Ringer’s solution at 37 °C. Upon stimulation with KCl, +Ca2+-Ringer containing 50 mM KCl was used. The times indicated, samples (100 μl) were taken, and the amounts of d-aspartate and l-aspartate were determined by HPLC on a COSMOSIL 5C18-AR column (4.6×150 mm; Nacalai Tesque Ltd.) and fluorescence detection. Insulin was quantified with an enzyme immunoassay (EIA) kit obtained from Yanaihara Inc., according to the manufacturer’s manual (Yanaihara Inc., Shizuoka, Japan).

Immunohistochemical Analysis Immunohistochemical analysis was performed as described11 with a slight modification. Cells were incubated in phosphate-buffered saline (PBS) containing 1% BSA and 2% goat serum and 0.2% saponin to permeabilize the cellular and organelle membranes for 15 min. Then, specimens were reacted with 0.7 μg/ml antibodies in PBS containing 0.5% BSA for 1 h at room temperature. Specimens were washed four times with PBS and reacted with the second antibodies for 1 h at room temperature, and then washed seven times with PBS. The second antibodies used were Alexa Fluor 568-labeled antimouse IgG, 2 μg/ml, or Alexa Fluor488-labeled anti-rabbit IgG, 2 μg/ml. These second antibodies were obtained from Molecular Probes. Finally, immunoreactivity was examined under an Olympus FV300 confocal laser microscope.

Antibodies Antibodies against d-aspartate were prepared according to Schell et al. as described previously.2,6,11)
The antiserum was further purified by affinity chromatography on CNBr-activated Sepharose 4B conjugated with D-aspartate. The purified antibodies were divided into small portions and frozen at −80 °C.11) The specificity of the purified antibodies was examined by enzyme-linked immunosorbent assaying. The antibodies did not cross react with L-aspartate or L-glutamate, or other related amino acids, the details being given previously.11) Monoclonal antibodies against insulin were obtained from Cymbus Biotechnology Ltd.

RESULTS AND DISCUSSION

Using immunohistochemical approaches, we have revealed that islets of Langerhans contain D-aspartate predominantly in α and F cells, and less in β cells.13) Quantitatively, the content of D-aspartate was determined to be 0.13 pmol/islet, which is about 8% of total aspartate in the islet (Table 1). INS-1E cells also contain D-aspartate, 1.50±0.48 nmol/10^7 cells, which accounts for about 2% of the total cellular free aspartate in the cells (Table 1). This cellular density of D-aspartate is the highest compared with in Y79 cells and PC12 cells.6,7,11) Immunohistochemistry indicated that all INS-1E cells exhibited positive immunoreactivity as to D-aspartate. The immunoreactivity was distributed punctately throughout the cells, and in some of them it was co-distributed with insulin (Fig. 1). The immunoreactivity was not observed when preabsorbed antibodies were used (data not shown). These results suggest that INS-1E cells contain D-aspartate in insulin-containing secretory granules and are a useful model system for measurement of D-aspartate exocytosis from islet cells.

Then, we measured the secretion of D-aspartate from INS-1E cells. Since clonal cells are less sensitive to D-glucose, the secretion of D-aspartate and also insulin was initiated by means of KCl-evoked depolarization. As shown in Figs. 2A and 2B, KCl stimulates the secretion of D-aspartate. About 20% of the total cellular D-aspartate had been released K^+-dependently at 10 min. In the absence of Ca^{2+}, this secretion was reduced to about 54% of the control level, indicating the

![Fig. 1. Immunohistochemical Detection of D-Aspartate in INS-1E Cells](image1)

Cells were doubly immunostained with antibodies against D-aspartate (A) and insulin (B), and then observed under a confocal microscope. A merged picture is also shown (C). Bar=10 μm.

Table 1. Contents of D-Aspartate and L-Aspartate in Islets of Langerhans and INS-1E Cells

<table>
<thead>
<tr>
<th></th>
<th>D-Aspartate</th>
<th>L-Aspartate</th>
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<tbody>
<tr>
<td>Islets</td>
<td>0.13 pmol/islet</td>
<td>1.55 pmol/islet</td>
</tr>
<tr>
<td>INS-1E</td>
<td>1.50±0.48 nmol/10^7 cells</td>
<td>91.6±16.8 nmol/10^7 cells</td>
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![Fig. 2. KCl-Evoked Secretion of D-Aspartate and Insulin from INS-1E Cells](image2)

(A) Time course. Cells were incubated in the +Ca^{2+}Ringer’s solution or the −Ca^{2+}Ringer’s solution, and then the release of D-aspartate was measured at the times indicated. Assays were initiated by the addition of 50 mM KCl. (B) D-Aspartate (left) and insulin (right) released upon stimulation with KCl at 10 min in the +Ca^{2+}Ringer’s solution or −Ca^{2+}Ringer’s solution is shown.

K^+-evoked secretion of D-aspartate is dependent on extracellular Ca^{2+}. Essentially the same tendency was observed for the secretion of insulin (Fig. 2B, right): 6% insulin was secreted with KCl treatment. Then, we examined whether A23187, a Ca^{2+} ionophore, stimulates the secretion of D-aspartate. The addition of A23187 stimulated the secretion of D-aspartate, which was not observed in the absence of Ca^{2+} (Fig. 3, left). A similar tendency was observed for insulin secretion, although the degree of stimulation was not significant (Fig. 3, right). In addition, the KCl-dependent secretion
followed by regulation of blood glucose: L-glutamate is an intercellular messenger to regulate the secretion of glucagon, being secreted under the control condition and stored in glucagon-containing secretory granules in a subpopulation of F cells. treatment with the calcium ionophore A23187 at 5 °C. Furthermore, the KCl-evoked D-aspartate secretion from islet cells, including those containing secretory granules in a subpopulation of F cells. A23187-Evoked Secretion of D-Aspartate and Insulin from INS 1E Cells Cells were incubated in the + Ca2+ Ringer's solution or the −Ca2+ Ringer's solution, and then the release of D-aspartate (left) and insulin (right) was assayed upon the addition of A23187 at 5 μM.

REFERENCES

Acknowledgements We thank Dr. P. Maecheller (Centre Medical Universitaire, Switzerland) for kind supplying the INS-1E cells. MH was supported by a research fellowship grant from the Japan Society for Promotion of Science for Young Scientists. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.