D-Aspartate Is Stored in Secretory Granules and Released through a Ca\textsuperscript{2+}-dependent Pathway in a Subset of Rat Pheochromocytoma PC12 Cells*

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D-Aspartate in mammalian neuronal and neuroendocrine cells is suggested to play a regulatory role(s) in the neuroendocrine function. Although D-aspartate is known to be released from neuroendocrine cells, the mechanism underlying the release is less understood. Rat pheochromocytoma PC12 cells contain an appreciable amount of D-aspartate (257 ± 31 pmol/10\textsuperscript{7} cells). Indirect immunofluorescence microscopy with specific antibodies against D-aspartate indicated that the amino acid is present within a particulate structure, which is co-localized with dopamine and chromogranin A, markers for secretory granules, but not with synaptophysin, a marker for synaptic-like microvesicles. After sucrose density gradient centrifugation of the postnuclear particulate fraction, about 80% of the D-aspartate was recovered in the secretory granule fraction. Upon the addition of KCl, an appreciable amount of D-aspartate (about 40 pmol/10\textsuperscript{7} cells at 10 min) was released from cultured cells on incubation in the presence of Ca\textsuperscript{2+} in the medium. The addition of A23187 also triggered D-aspartate release. Botulinum neurotoxin type E inhibited about 40% of KCl- and Ca\textsuperscript{2+}-dependent D-aspartate release followed by specific cleavage of 25-kDa synaptosomal-associated protein. α-Latrotoxin increased the intracellular [Ca\textsuperscript{2+}] and caused the Ca\textsuperscript{2+}-dependent D-aspartate release. Bafilomycin A1 dissipated the intracellular acidic regions and inhibited 40% of the Ca\textsuperscript{2+}-dependent D-aspartate release. These properties are similar to those of the exocytosis of dopamine. Furthermore, digitonin-permeabilized cells took up radiolabeled D-aspartate depending on MgATP, which is sensitive to bafilomycin A1 or 3,5-di-tet-butyl-4-hydroxybenzylidene-malononitrile. Taken together, these results strongly suggest that D-aspartate is stored in secretory granules and then secreted through a Ca\textsuperscript{2+}-dependent exocytotic mechanism. Exocytosis of D-aspartate further supports the role(s) of D-aspartate as a chemical transmitter in neuroendocrine cells.

Although the L-enantiomers of amino acids are predominant in living organisms, substantial levels of free D-amino acids have recently been detected in mammals. Among these D-amino acids, D-serine and D-aspartate are of special interest because neuronal and endocrine cells contain high levels of these amino acids (1–3; for review, see Ref. 4). D-Serine was found to potentiate the N-methyl-D-aspartate receptor through binding to the glycine site on the receptor, suggesting that D-serine might be a modulator of the N-methyl-D-aspartate receptor (5). Consistently, indirect immunofluorescence microscopy with specific antibodies against D-serine demonstrated the localization of D-serine in astrocytes (6). Specific antibodies against D-aspartate revealed the localization of D-aspartate in a subset of stellate and basket cells of the cerebellum, adrenal chromaffin cells, pituitaries, and pinealocytes (7). In the pineal gland, it has been shown that D-aspartate is present in pinealocytes, endocrine cells for melatonin (8, 9). Upon incubation of pinealocytes with exogenous D-aspartate, melatonin synthesis is strongly inhibited through the inhibition of N-acetyltransferase activity (9, 10). Thus, D-aspartate seems to be a modulator of melatonin synthesis. Furthermore, exogenous D-aspartate stimulates the release of luteinizing hormone and growth hormone in the anterior pituitary (11).

As chemical transmitters, D-serine and D-aspartate should be secreted from neuroendocrine cells. However, the mechanism by which these amino acids are secreted from neuroendocrine cells is less understood. D-Serine is released from astrocytes upon stimulation by glutamate (5). Because D-serine is present in the cytoplasm, reversed D-serine transport through a Na\textsuperscript{+}-dependent serine transporter at the plasma membrane was proposed (5). Similarly, D-aspartate is present in the cytoplasm of pinealocytes and is released from the cells (8, 9). Pinealocytes express the Na\textsuperscript{+}-dependent glutamate transporter, which recognizes D-aspartate as a substrate, and its inhibition by various antagonists decreases release of D-aspartate (9, 10), suggesting that the Na\textsuperscript{+}-dependent glutamate transporter is involved in the release of D-aspartate in pinealocytes.

Here we present another type of mechanism of secretion of D-aspartate in neuroendocrine cells. A subset of rat pheochromocytoma PC12 cells contains an appreciable amount of D-aspartate (12). We have extensively investigated the localization and release of D-aspartate in PC12 cells and found that PC12 cells store D-aspartate in secretory granules and secrete it through a Ca\textsuperscript{2+}-dependent exocytotic mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells were cultured in 20 ml of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum, 5% horse serum, 55 μg/ml sodium pyruvate, 4.5 g/liter

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glucose, 0.1 mg/liter streptomycin, 100 units/ml penicillin G, and 0.25 mg/liter Fungizone and incubated at 37 °C under 5% CO₂ (13). When necessary, 20 ng/ml nerve growth factor was included in the medium. The dispersed cells were washed three times with the above medium, placed in a 100-mm culture dish coated with poly-L-lysine to give 1 × 10⁵ cells/dish, and cultured in the above medium at 37 °C under 5% CO₂. These cells were maintained for 5 days, washed with culture medium, cultured further for 1 h, and then used for experiments.

Subcellular Fractionation—Subcellular fractionation of PC12 cells was performed according to a published procedure (14) with slight modifications. In brief, cultured PC12 cells (1.0 × 10⁹ cells) were extensively washed with PBS¹ containing 5 μg/ml leupeptin and 5 μg/ml pepstatin A and homogenized by passage through a 25-gauge needle. The homogenate was then centrifuged at 800 g for 10 min, and the pellets (P1) and the supernatant (S1) were obtained. Then the S1 was centrifuged at 100,000 g for 1 h, and the supernatant (S2) and the pellet (P2) were obtained. The P2 was suspended in 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose, 5 mM EDTA, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A (SME buffer), applied to a continuous sucrose gradient (0.6–1.6 M), and then centrifuged at 76,000 g for 3.5 h. Then samples were collected in nine tubes.

Assay of α-Aspartate Release— Cultured cells (1 × 10⁶ cells/dish) were washed three times with a +Ca²⁺-Ringer’s solution comprising 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH₂PO₄, 2.4 mM CaCl₂, 1.3 mM MgSO₄, 26 mM NaHCO₃, 10 mM glucose, and 10 mM HEPES (pH 7.4) (standard assay solution) or a −Ca²⁺-Ringer’s solution comprising 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH₂PO₄, 0.2 mM CaCl₂, 1 mM EGTA, 3.8 mM MgSO₄, 26 mM NaHCO₃, 10 mM glucose, and 10 mM HEPES (pH 7.4). After cells had been incubated in 5 ml of the above medium at 37 °C, the release of α-aspartate was stimulated by the addition of 50 mM KCl (15) as specified. When necessary, various compounds were added to the incubation medium. Aliquots (10 μl) were taken at time intervals, and the amount of α-aspartate was determined by HPLC according to published procedures (16, 17). Simultaneously, their dopamine contents were measured by HPLC combined with amperometric detection as described previously (18).

Treatment with BoNT/E—The intoxication of PC12 cells with BoNT/E was performed using a procedure similar to that previously described (15). The cultured cells (1 × 10⁶ cells/dish) were incubated at 37 °C for 24 h in a low ionic strength buffer consisting of 5 mM NaCl, 4.8 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES-NaOH, 10 mM glucose, 220 mM sucrose, and 0.5% bovine serum albumin (pH 7.4) in the presence or absence of 10 or 50 nM BoNT/E. Then the cells were washed with fresh culture medium and incubated for an additional 12 h at 37 °C. Finally, the KCl-evoked release of α-aspartate and dopamine was measured in +Ca²⁺-Ringer’s solution as described above.

Measurement of Intracellular [Ca²⁺]—For the determination of intracellular [Ca²⁺], an Argus 20/CA ratio imaging system (Hamamatsu Photonics Co., Hamamatsu, Japan) was used. Cells were cultured for 3 days on a thin glass coverslip precoated with (poly)-lysine (0.12 mm thick and 40 mm in diameter; 2.5 × 10⁶ cells/coverslip). After exchange with fresh culture medium, the cells were treated with 10 μM Fura-2/AM (Dojindo Co., Kumamoto, Japan) plus 0.1% Pluronic F-127 ( Molecular Probes) for 2 h at 37 °C and then washed twice with the same medium. The cells were perfused with the warmed +Ca²⁺-Ringer’s solution or −Ca²⁺-Ringer’s solution. Images were continuously taken at 37 °C with a silicon-intensified camera (C2741–08; Hamamatsu Photonics Co.). The velocity of data acquisition for F₃₄₀/F₅₀₀ images was 4 s at a resolution of 256 × 256 pixels/image. A personal computer with appropriate software (U4469; Hamamatsu Photonics Co.) was used to control the optical equipment, recording, and data analysis. The software enabled subtraction of background fluorescence, pixel-to-pixel division of F₃₄₀/F₅₀₀ images, fitting of the F₃₄₀/F₅₀₀ ratios to a [Ca²⁺] calibration curve prepared separately, and digital averaging of the Ca²⁺ concentration in multiple cells (15).

Antibodies Specific to α-Aspartate—Antibodies against α-aspartate were prepared according to Schell et al. (7) and Lee et al. (19). The antisera were further purified by affinity chromatography with CNBr-activated Sepharose 4B conjugated with α-aspartate. The purified antibodies against α-aspartate were divided into small portions and frozen at −85 °C until use. These antibodies are active for at least 1 year after preparation. The specificity of the purified antibodies was examined by enzyme-linked immunosorbent assay as follows. Various amino acids and peptides (10 μmol each) were coupled to a SUMILON MS-3696F microplate with glutaraldehyde. After blocking with PBS containing 10% goat serum, the plates were incubated with the purified antibodies

¹ The abbreviations used are: PBS, phosphate-buffered saline; BoNT/E, botulinum neurotoxin type E; AM, acetoxyxymethylster; SNAP25, 25-kDa synaptosomal-associated protein; MOPS, 4-morpholino-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid.
P1, P2, and S2 fractions were prepared as described under "Experimental Procedures." The results were expressed as percentage of d-aspartate and dopamine found in whole cells, which corresponded to 250 ± 31 pmol of d-aspartate/10^7 cells and 32.75 ± 6.10 nmol of dopamine/10^7 cells. In a separate experiment, PC12 cells were incubated with 10 μM 2',7'-bis-(2-carboxylethyl)-5,6-carboxyfluorescein acetoxymethylester (BCECF-AM) for 30 min and washed with PBS. Then the cells trapped with BCECF (a cytosolic marker) were fractionated as described above. Major BCECF fluorescence (93%) was recovered in the S2 fraction, whereas only 5% was found in the P2 fraction, indicating that contamination of cytoplasm into the P2 fraction is scarce.

| Fraction | d-Aspartate (pmol/10^7 cells) | Dopamine (nmol/10^7 cells) |
|----------|------------------------------|---------------------------|
| Whole cells | 100 ± 12 | 100 ± 19 |
| P1 | 16 ± 4 | 13 ± 3 |
| P2 | 74 ± 5 | 62 ± 4 |
| S2 | 15 ± 1 | 19 ± 2 |

(10 to 1250 dilutions) for 2 h at room temperature and then washed four times with PBS containing 0.1% Tween 20. Then the immunoreactivities were visualized with peroxidase secondary antibodies and quantified using a microplate reader (Bio-Rad, Model 550). The antibodies gave immunoreactivities of less than 1.0% (taking those of d-aspartate to be 100%) to the following compounds: t-aspartate, t-glutamate, t-glutamate, t-asparagine, d-asparagine, t-serine, d-serine, glycine, β-alanine, taurine, carnosine, Asp-Ala, Asp-Asp, Asp-Glu, serine-O-sulfate, Glu-Asp, and Gly-Asp. Furthermore, the purified antibodies stained some chromaffin cells in the section of rat adrenal glands and almost all pinealocytes in the primary culture, which is consistent with previous reports (7, 9, 19).

**Immunoblotting—**A membrane fraction of PC12 cells 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 (20). Following electrophoresis at 0.3 A for 2 h, the nitrocellulose filters were blocked in a buffer consisting of 20 mM Tris-ATP at 2 mM in the absence of digitonin. After the cells trapped with BCECF (a cytosolic marker) were fractionated as described above. Major BCECF fluorescence (93%) was recovered in the S2 fraction, whereas only 5% was found in the P2 fraction, indicating that contamination of cytoplasm into the P2 fraction is scarce.

**Immunohistochemistry—**The published procedure was used (21). In brief, PC12 cells on (poly)lysine-coated glass coverslips were washed three times with PBS and incubated in 80 mM PIPES-KOH buffer (pH 6.8) containing 1 μg/ml digitonin, 5 mM EDTA, and 1 mM MgCl2, for 5 min at room temperature. Then the cells were fixed with 4% paraformaldehyde in PBS for 30 min. After washing with PBS again, the cells were further permeabilized with 50 μg/ml digitonin in PBS for 5 min. Then ammonium chloride at 50 mM was added to the solution followed by incubation for 10 min after which the cells were washed with 1% gelatin in PBS for 5 min. Finally, the fixed cells were incubated for 1 h with antibodies to 10 μg/ml diluted in PBS containing 0.1% gelatin. The samples were washed three times with PBS containing 0.1% gelatin and then incubated with the secondary antibodies conjugated with either fluorescein or Texas Red. After washing the cells with PBS containing 0.1% gelatin, immunoreactivity was observed under an Olympus confocal laser microscope (FLUOVIEW).

**d-Aspartate Uptake by Digitonin-permeabilized Cells—**PC12 cells were rinsed with 1 ml of buffer composed of 20 mM MOPS-Tris (pH 7.0), 0.3 mM sucrose, 2 mM magnesium acetate, and 4 mM KCl. The cells were then permeabilized for 10 min at 37 °C in 0.5 ml of the buffer containing 10 μM digitonin (22, 23). The medium was then replaced with fresh buffer containing Tris-ATP at 2 mM in the absence of digitonin. After incubation for 10 min, uptake of d-aspartate was immediately started by the addition of radioactive d-aspartate (2.5 μCi, 0.1 mM) at 37 °C. Uptake was terminated by washing the cells twice with 1 ml of ice-cold 20 mM MOPS-Tris (pH 7.0) containing 0.3 mM sucrose. The cells were lysed with 1 ml of 1% SDS, and the radioactivity was counted with a liquid scintillation counter.

**Other Procedures, Preparations, and Chemicals—**Vital staining with acridine orange was performed as described previously (24). BoNT/E was purified as described previously (25). Polyclonal antibodies against chromogranin A were raised by injecting chromogranin A electrophoretically purified from bovine chromaffin granules. The following antibodies were obtained commercially: monoclonal antibodies against SNAPP25 (mAbBR05) (Wako Chemical Co.), monoclonal antibodies against synaptophysin (SY38) and chromogranin A (Progen), monoclonal antibodies against dopamine from Biogenesis. d-[2,3-3H]Aspartate (18 Ci/mmol) was obtained from PerkinElmer Life Sciences and ICN Biomedicals, Inc. (Irvine, CA). α-Latrotoxin was purchased from Alomone Labs, Ltd. (Jerusalem, Israel). Other chemicals were of the highest grade commercially available.

**RESULTS**

**Subcellular Localization of d-Aspartate—**PC12 cells contain an appreciable amount of d-aspartate (257 ± 31 pmol/10^7 cells). To reveal the subcellular localization of d-aspartate in PC12 cells, indirect immunofluorescence microscopy was performed with specific antibodies against d-aspartate. Fig. 1A shows that the antibodies stained vesicular structures. The d-aspartate immunoreactivity was co-localized with dopamine (Fig. 1, A–C) and chromogranin A (Fig. 1, D–F), markers for secretory granules.
D-aspartate immunoreactivity is not present in preimmune serum did not stain the cells (Fig. 1, A, time course. After the times indicated, the medium was carefully taken, and the d-aspartate concentrations were determined. B, the KCl dose dependence was measured at 10 min after the addition of the indicated concentrations of KCl. C, the time course of A23187-evoked d-aspartate. At time 0, A23187 at 5 μM was added to the medium. D, PC12 cells (2.5 × 10⁷ cells) were incubated in the absence (Control) or presence of EGTA-AM at 50 μM for 30 min and then washed with +Ca²⁺-Ringer’s solution. Then the KCl-evoked d-aspartate release was measured as described above. The dopamine contents in the same samples as described above (A–D) were simultaneously measured and expressed as shown in E–H, respectively. All the results in the figure are means ± S.E. (three independent experiments).

Subcellular fractionation was conducted to confirm the localization of d-aspartate in secretory granules. d-Aspartate immunoreactivity is not present in all PC12 cells: about 10% of cells exhibited d-aspartate immunoreactivity among the more than 10⁶ cells examined, although more than 95% of the cells exhibited synaptophysin and chromogranin immunoreactivities. The percentage of d-aspartate-positive cells did not change during culture in the presence or absence of nerve growth factor (data not shown).

Subcellular fractionation was conducted to confirm the localization of d-aspartate in secretory granules. After homogenization of PC12 cells (1 × 10⁶ cells), 74 ± 5% of d-aspartate and 62 ± 4% of dopamine were recovered in the P2 fraction that contains secretory granules (Table I). Then secretory granules were separated from the P2 fraction by sucrose density gradient centrifugation. As shown in Fig. 2, secretory granules were recovered in fractions 1–7 (peak fraction, 2), whereas synaptic-like microvesicles were recovered in fractions 2–9 (peak fractions, 6–8) as revealed by the distribution of marker proteins. Consistent with the distribution of marker proteins, about 80% of each of d-aspartate and dopamine in the P2 fraction was recovered in the secretory granule fraction (Fig. 2B). Together with the immunohistochemical localization of d-aspartate shown above, it is concluded that d-aspartate is present in secretory granules.

Ca²⁺-dependent d-Aspartate Release—The localization of d-aspartate in secretory granules suggests secretion of d-aspartate through regulated exocytosis. In fact, when PC12 cells were cultured under the standard culture conditions, an appreciable amount of d-aspartate appeared in the medium, suggesting the presence of a d-aspartate-releasing system(s) (12). To obtain evidence of d-aspartate exocytosis, we measured the d-aspartate concentration in the medium upon stimulation of the PC12 cells with KCl, which is known to trigger exocytosis of secretory granules following depolarization of plasma membrane.

An appreciable amount of d-aspartate (40.0 ± 7.1 pmol/10⁷ cells, which corresponds to 16% of the total d-aspartate; four determinations) was released by 10 min upon the addition of KCl in the presence of Ca²⁺ (Fig. 3A). Essentially no l-aspartate (<0.1 pmol/10⁷ cells) was released under these conditions. The amount of released d-aspartate depended on time (Fig. 3A) and the concentration of KCl (Fig. 3B): the d-aspartate concentration was saturated at 10 min and increased at least by 50 mm KCl. In the absence of CaCl₂, KCl-evoked d-aspartate release was significantly reduced (Fig. 3A, A and B). A23187, a Ca²⁺ ionophore, caused rapid release of d-aspartate by the cells only in the presence of Ca²⁺ (Fig. 3C). Furthermore, upon treatment with EGTA-AM to remove intracellular free Ca²⁺, the cells lost the ability of KCl-evoked release of d-aspartate (Fig. 3D). Likewise dopamine was released from PC12 cells with similar kinetics and Ca²⁺ dependence (Fig. 3, E–H). These results indicate that d-aspartate is released from PC12 cells depending on Ca²⁺ and suggested that the entry of extracellular Ca²⁺ following depolarization is necessary for the d-aspartate release as in the case of dopamine.

Intracellular [Ca²⁺]—To support the importance of the entry of extracellular Ca²⁺ in d-aspartate release, we measured the intracellular [Ca²⁺] of Fura-2-loaded PC12 cells. The intracellular [Ca²⁺] in PC12 cells at the resting state is 101 ± 1 nM (n = 74) in +Ca²⁺-Ringer’s solution or −Ca²⁺-Ringer’s solution. Treatment with KCl at 50 mM increased it to 375 ± 14 nM (n = 33) in the presence of Ca²⁺ in the medium. The KCl-evoked increase of the intracellular [Ca²⁺] was not observed when the cells were incubated in −Ca²⁺-Ringer’s solu-
tion or when cells were treated with EGTA-AM. Furthermore, the cadmium ion, a nonspecific inhibitor of voltage-gated Ca\(^{2+}\) channels (26), at 10 \(\mu\)M inhibited 60% of the KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release and blocked 75% of the KCl-evoked increase in intracellular [Ca\(^{2+}\)]. These results suggested that voltage-gated Ca\(^{2+}\) channels, at least in part, are involved in the KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release by PC12 cells.

Evidence of Exocytosis of \(\delta\)-Aspartate—We further characterized the K\(^+\)- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release by PC12 cells by examining the effect of temperature. The KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release was affected by the temperature: it was not observed at 4°C but appeared gradually with increasing temperature and reached the maximum at 37°C. Furthermore, once \(\delta\)-aspartate had been secreted the successive stimulation by KCl within 1 h was not effective. The KCl-evoked release of \(\delta\)-aspartate was gradually restored upon incubation and had recovered completely by incubation for 12 h, suggesting that charged and discharged processes are involved in the KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release. These properties are similar to those of the exocytosis of dopamine, supporting that \(\delta\)-aspartate is secreted through exocytosis.

The sensitivity to BoNT/E constitutes evidence of Ca\(^{2+}\)-dependent regulated exocytosis because this neurotoxin splits SNAP25 and inhibits the late postdocking steps, resulting in inhibition of the exocytosis of secretory granules in PC12 cells (27–29). As shown in Fig. 4A, BoNT/E cleaved SNAP25, yielding a low molecular weight fragment (Fig. 4A, asterisk) and inhibited the KCl- and Ca\(^{2+}\)-dependent release of \(\delta\)-aspartate (Fig. 4B) and dopamine (Fig. 4C). The inhibitory potency of BoNT/E was essentially the same as that in the exocytosis of dopamine. Under the same assay conditions, the addition of K\(^+\) increased intracellular [Ca\(^{2+}\)] in BoNT/E-treated cells to an extent similar to that in control cells (data not shown). These results indicated that SNAP25 is involved in the KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release.

\(\alpha\)-Latrotoxin, a component of black widow spider venom, triggers Ca\(^{2+}\)-dependent exocytosis from neurons and neuroendocrine cells (30). The neurotoxin, therefore, stimulates dopamine exocytosis in PC12 cells (31). As shown in Fig. 5A, \(\alpha\)-latrotoxin at 1 nM increased intracellular [Ca\(^{2+}\)] to 405 ± 13 nM (n = 54) in the presence of Ca\(^{2+}\). In the absence of Ca\(^{2+}\), increase of intracellular [Ca\(^{2+}\)] was not observed. Under these conditions, \(\alpha\)-latrotoxin caused the release of \(\delta\)-aspartate and dopamine to a similar extent in the presence of Ca\(^{2+}\) (Fig. 5, B and C). In the absence of Ca\(^{2+}\), however, neither \(\delta\)-aspartate nor dopamine was released on the addition of \(\alpha\)-latrotoxin (Fig. 5, B and C). These results indicated that the \(\alpha\)-latrotoxin-evoked stimulation of the release of \(\delta\)-aspartate is Ca\(^{2+}\)-dependent as in the case of dopamine.

It is known that bafilomycin A1, a specific inhibitor of vacuolar H\(^{-}\)-ATPase (32), effectively inhibits the exocytosis of neurotransmitters (33, 34) because the compound dissipates the electrochemical proton gradient necessary for neurotransmitter uptake into vesicles. As expected, bafilomycin A1 at 1 \(\mu\)M dissipated the transmembrane pH gradient of acidic organelles in the cells as revealed on acridine orange vital staining (Fig. 6, A and B). Under similar assay conditions, bafilomycin A1 inhibited 40% of the KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release (Fig. 6C) and 55% of the dopamine release (Fig. 6D). The results suggest that an electrochemical proton gradient is necessary at least in part for the KCl- and Ca\(^{2+}\)-dependent \(\delta\)-aspartate release by PC12 cells as in the case of dopamine.

Uptake of \(\delta\)-Aspartate—Sensitivities to bafilomycin A1 suggest the presence of systems for the accumulation of \(\delta\)-aspartate in secretory granules. In the final part of the study, we investigated whether or not such a transport system is present in the cells. As shown in Fig. 7, radiolabeled \(\delta\)-aspartate was taken up by the digitonin-permeabilized cells depending on ATP. The omission of Mg\(^{2+}\) reduced the ATP-dependent \(\delta\)-aspartate uptake to the control level. Consistent with the Ca\(^{2+}\)-dependent release of \(\delta\)-aspartate, bafilomycin A1 at 1 \(\mu\)M inhibited the ATP-dependent \(\delta\)-aspartate uptake. SF6847 (3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile), a proton conductor that dissipates an electrochemical proton gradient, also inhibited the ATP-dependent \(\delta\)-aspartate uptake. These results suggest that the \(\delta\)-aspartate transporter energetically coupled with vacuolar H\(^{-}\)-ATPase is responsible for the storage and release of \(\delta\)-aspartate in PC12 cells.

**DISCUSSION**

PC12 cells develop two different kinds of secretory machinery: one is secretory granules, which are responsible for the secretion of monoamines such as dopamine, and the other is synaptic-like microvesicles, counterparts of neuronal synaptic vesicles that contain acetylcholine (14). Here we showed that \(\delta\)-aspartate is stored in secretory granules but not in synaptic-like microvesicles. Furthermore, \(\delta\)-aspartate is secreted
Thus, it is possible that a novel vesicular D-aspartate transporter is now in progress in our laboratory. The absence of BNPI in secretory granules raises an important issue of the origin of D-aspartate in PC12 cells. The D-aspartate content in the culture medium was below the detection limit (<1 fmol). The D-aspartate content in the cells did not change with the duration of culture or during 5–10 passages (12). Furthermore, PC12 cells do not express a plasma membrane-type Na\(^+\)-dependent glutamate transporter that recognizes D-aspartate as a substrate. Thus, it is likely that D-aspartate is not of exogenous origin but is de novo synthesized by D-aspartate synthase in the cells. In this respect, it is noteworthy that D-aspartate racemase-like activity was detected in rat pinealocytes (9) and adrenal glands (38).

Several criteria are known for regulated exocytosis. We found that the properties of the release of D-aspartate in PC12 cells satisfied the criteria. At first, PC12 cells secrete the amino acid depending on Ca\(^{2+}\)-dependent exocytosis found in various endocrine cells and neurons (15, 42–44). The relatively slow rate of D-aspartate release and blocked the KCl-evoked increase in intracellular Ca\(^{2+}\), which suggests the involvement of voltage-gated Ca\(^{2+}\) channels in the process. Our preliminary experiments indicated that antagonists for voltage-gated L-type Ca\(^{2+}\) channels such as nifedipine inhibited the Ca\(^{2+}\)-dependent D-aspartate release and blocked the KCl-evoked increase in intracellular Ca\(^{2+}\), but antagonists for N-type or P/O-type channels did not have such effects. These results suggest that L-type Ca\(^{2+}\) channels are responsible for the entry of Ca\(^{2+}\) from the extracellular space into PC12 cells. Consistently, the presence of L-type Ca\(^{2+}\) channels in PC12 cells has been demonstrated (39–41).

The second line of evidence is the temperature sensitivity and the requirement of an appropriate duration of the response for a 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 (15, 42–44). The relatively slow rate of D-aspartate release (minute order as shown in Fig. 3A) is also similar to that of the release of dopamine by PC12 cells and glutamate by rat pinealocytes (15). The third line of evidence is the sensitivity to \(\alpha\)-latrotoxin, BoNT/E, and bafilomycin A1. The sensitivity to these compounds strongly suggests the involvement of the \(\alpha\)-latrotoxin receptor, SNAP receptor complex, and vacuolar H\(^+\)-ATPase, which are important components of Ca\(^{2+}\)-dependent regulated exocytosis, in the secretion of D-aspartate.
Ca\(^{2+}\)-dependent exocytosis is a novel mechanism for the release of D-aspartate from neuroendocrine cells. Although it is unknown whether or not Ca\(^{2+}\)-dependent exocytosis of D-aspartate occurs under physiological conditions, Snyder and his colleagues (7) observed that the injection of potassium ion into the cerebral cortex of mice caused a pronounced increase in D-aspartate staining in the choroid plexus. This suggests that depolarization elicited by potassium ion injection releases D-aspartate and that the exocytotic mechanism for D-aspartate secretion operates in the choroid plexus. Very recently it was reported that depolarization by the addition of K\(^+\) or acetylcholine released D-aspartate from sliced adrenal gland (38). Upon exocytosis, D-aspartate may interact with target cells, which is very consistent with the neuroendocrine role of D-aspartate in neuroendocrine cells as suggested by Snyder and his colleagues (7).

In this study, we showed that PC12 cells accumulate D-aspartate in secretory granules and secrete it through exocytosis. PC12 cells may constitute a suitable experimental system for studies on the mode of action of D-aspartate in neuroendocrine cells. Our present results may provide an insight into the mode of action of D-aspartate in neuroendocrine cells. Further studies are necessary to determine which type of cell secretes D-aspartate through the Ca\(^{2+}\)-dependent exocytotic pathway in vivo.

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