Vesicular glutamate transporter is present in neuronal synaptic vesicles and endocrine synaptic-like microvesicles and is responsible for vesicular storage of L-glutamate. A brain-specific Na\(^+\)-dependent inorganic phosphate cotransporter (BNPI) functions as a vesicular glutamate transporter in synaptic vesicles, and the expression of this BNPI defines the glutamatergic phenotype in the central nervous system (Bellocchio, E. E., Reimer, R. J., Fremeau, R. T., Jr., and Edwards, R. H. (2000) Science 289, 957–960; Takamori, S., Rhee, J. S., Rosenmund, C., and Jahn, R. (2000) Nature 407, 189–194). However, since not all glutamatergic neurons contain BNPI, an additional transporter(s) responsible for vesicular glutamate uptake has been postulated. Here we report that differentiation-associated Na\(^+\)-dependent inorganic phosphate cotransporter (DNPI), an isoform of BNPI (Aihara, Y., Mashima, H., Onda, H., Hisano, S., Kasuya, H., Hori, T., Yamada, S., Tomura, H., Yamada, Y., Inoue, I., Kojima, I., and Takeda, J. (2000) J. Neurochem. 74, 2622–2625), also transports L-glutamate at the expense of an electrochemical gradient of protons established by the vacuolar proton pump when expressed in COS7 cells. Molecular, biological, and immunohistochemical studies have indicated that besides its presence in neuronal cells DNPI is preferentially expressed in mammalian pinealocytes, αTC6 cells, clonal pancreatic α cells, and α cells of Langerhans islets, these cells being proven to secrete L-glutamate through Ca\(^{2+}\)-dependent regulated exocytosis followed by its vesicular storage. Pancreatic polypeptide-secreting F cells of Langerhans islets also expressed DNPI. These results constitute evidence that DNPI functions as another vesicular transporter in glutamatergic endocrine cells as well as in neurons.

l-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and plays important roles in many neuronal processes such as fast synaptic transmission and neuronal plasticity (1, 2). To use l-glutamate as an intercellular signaling molecule, neuronal cells develop glutamatergic systems comprising the storage of glutamate in synaptic vesicles and its 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 (3, 4). For instance, mammalian pinealocytes, endocrine cells for melatonin, secrete l-glutamate through Ca\(^{2+}\)-dependent regulated exocytosis and use it as a paracrine- or autocrine-like chemical transmitter to inhibit melatonin synthesis (3, 4).

Vesicular glutamate transporter plays its primary role in the storage of l-glutamate in neurons (5, 6) and endocrine cells (3, 4) through the transport of l-glutamate at the expense of an electrochemical gradient of protons that is established by vacuolar H\(^+\)-ATPase. Although vesicular glutamate transporter has been characterized to some extent, its protein nature has not been known long. Very recently brain-specific Na\(^+\)-dependent inorganic phosphate cotransporter (BNPI),\(^1\) representing a family of proteins that use the inwardly directed Na\(^+\) gradient across the membrane and transport inorganic phosphate (7), has been identified as the vesicular glutamate transporter in synaptic vesicles (8, 9). Upon expression in either PC12 or BON6 cells, BNPI becomes associated with secretory vesicles and accumulates l-glutamate (8, 9). BNPI is associated with synaptic vesicles in various glutamatergic neurons (8–11). However, it is not present in all glutamatergic neurons (8–11), suggesting that another vesicular glutamate transporter(s) may function in the neurons lacking BNPI (12).

Differentiation-associated Na\(^+\)-dependent inorganic phosphate cotransporter (DNPI), a homologue of BNPI isolated from AR42J cells differentiating into neuroendocrine cells, shows 82% amino acid identity and 92% similarity to human BNPI (13). In human and rat, the DNPI gene as well as the DNPI protein was shown to be expressed in neurons in various regions, especially in the encephalon, its expression patterns being somewhat different from that of BNPI (14–16). Furthermore, DNPI was shown to be located in synaptic vesicles in the neocortex (16). One can expect that DNPI is another vesicular glutamate transporter.

In the present study, we tested this hypothesis and found that DNPI shows ATP-dependent glutamate transport activity
when expressed in COS7 cells. We also showed that besides its presence in neurons DNPI is also present in pinealocytes, αT6 cells, and α and pancreatic polypeptide-secreting F cells in Langerhans islets, which contain a gluta
tamaticergic system.

MATERIALS AND METHODS

Organ and Cell Cultures—Pineal glands and Langerhans islets were isolated from male Wistar rats at postnatal week 6. COS7 cells and αT6 cells, a clonal α cell line (17), were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 50 μg/ml sodium pyruvate, 4.5 mM glucose, 0.1 M digitonin, 0.1% tryp
topen, 100 U/ml penicillin, and 0.5% streptomycin. The cultured cells were maintained for 5 days with culture medium, washed further for 1 h, and then used for experiments.

Expression of DNPI—Rat DNPI cDNA, as previously described (13), was subcloned into the EcoRI site of expression vector pcDNA3.1 (In
tigon, San Diego, CA). The resultant construct, DNPI-pcDNA3.1, was used to transfect COS7 cells by lipofection using Trans IT REagent (Mirus, WI). COS7 cells were cultivated on 

Site-specific polyclonal antibodies against rat DNPI were generated polypeptide was included during antibody treatment and used for western blot analysis. The monoclonal antibodies against glucagon and insulin (MABI) were from Sigma and Cymbus Biotechnology Ltd., respectively. The rat monoclonal antibodies against somatostatin were from Chemicon. Guinea pig polyclonal antisera against rat pancreatic polypeptide were from Linco Research, Inc.

Immunoblotting—Membrane fractions (particulate fractions) of rat brain, pineal gland, Langerhans islets, and cultured cells prepared as described previously (18, 20) were denatured with SDS sample buffer containing 1% SDS and 10% b-mercaptoethanol and then electrophore
d on a 12% polyacrylamide gel in the presence of SDS. Following electrotransfer at 0.3 A for 2 h, the nitrocellulose filters were blocked in a 

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis—Total RNA extracted from isolated glands (1 μg) was transcribed into cDNA in a final volume of 20 μl of a reaction buffer containing 0.5 mM of each dNTP, 10 mM dithiothreitol, 25 pmol of 

RESULTS

DNPI as a Vesicular Glutamate Transporter in Endocrine Cells

DNPI was a Vesicular Glutamate Transporter in Endocrine Cells. As shown in Fig. 1A, anti-DNPI antibodies recognized a major broad protein band corresponding to an apparent molecular mass of ~65 kDa when DNPI-pcDNA3.1, a DNPI-expressing vector, was transfected to COS7 cells. The molecular mass corresponding to the DNPI immunoreactivity is similar to that expected from its primary alpha amino acid sequence and DNPI from the brain (Fig. 1A). Two additional protein bands with apparent molecular masses of ~72 and ~42 kDa were observed. Neither the control vector nor COS7 cells transfected with a control vector expressed any DNPI gene, as revealed on RT-PCR analysis (data not shown), or the immunoreactive polypeptide (Fig. 1A). The DNPI immunoreactivity disappeared when the anti
genomic polypeptide was included during antibody treatment (Fig. 1A). These results indicated that DNPI is expressed in COS7 cells.
Immunohistochemical analysis indicated that DNPI is co-localized with EEA1 or Rab 5, early endosomal markers (Fig. 1C). DNPI is partially co-localized with GM130, a marker protein of the cis Golgi apparatus but not with protein disulfide isomerase, a marker protein of endoplasmic reticulum (Fig. 1C). These results suggest that DNPI is mainly associated with early endosomes in the cells.

We examined whether or not DNPI shows vesicular glutamate transport activity. As shown in Fig. 1D, digitonin-permeabilized DNPI-expressing cells took up radiolabeled L-glutamate depending on ATP. Neither untransfected control cells nor cells transfected with a control vector showed ATP-dependent glutamate uptake activity. The omission of Mg$^{2+}$ reduced the ATP-dependent L-glutamate uptake to the control level. Bafilomycin A1, a specific inhibitor of vacuolar H$^+$/H$_2$PO$_4^-$-ATPase (24), at 1 $\mu$M inhibited the ATP-dependent L-glutamate uptake. SF6847, a proton conductor that dissipates an electrochemical proton gradient, also inhibited the ATP-dependent L-glutamate uptake. In contrast, vanadate (1 mM), an inhibitor of P-type ion-transporting ATPases, did not affect the ATP-dependent glutamate uptake. These results indicated that the glutamate uptake is driven by an electrochemical gradient of protons.

**Fig. 1.** DNPI expressed in COS7 cells functions as a vesicular glutamate transporter. A, expression of DNPI in COS7 cells. Membrane fractions prepared from brain (lanes 1 and 4), control (vector alone) cells (lane 2), and DNPI-pcDNA3.1-transfected cells (lanes 3 and 5) (100 $\mu$g of protein) were solubilized with SDS sample buffer and then subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting using anti-DNPI antibodies. The immunoreactivity was visualized with ECL. For lanes 4 and 5, the nitrocellulose sheet was incubated with 1 mg of antigenic peptide during the antibody treatment. The positions of the molecular markers are shown. B, immunohistochemical detection of DNPI expressed in COS7 cells. Control (vector alone) cells and DNPI-pcDNA3.1-transfected cells were immunostained with anti-DNPI antibodies (1:1000) and then observed under a fluorescence microscope. Bar = 20 $\mu$m. C, subcellular localization of DNPI expressed in COS7 cells was investigated. DNPI-pcDNA3.1-transfected cells were doubly immunostained with antibodies against DNPI (green) and EEA1 (red) (1), DNPI (green) and Rab 5 (red) (2), DNPI (green) and GM130 (red) (3), or DNPI (green) and protein disulfide isomerase (PDI) (red) (4) and then observed under a confocal microscope. Dilution of the antibodies is as follows: EEA1, 1:100; Rab 5, 1:100; GM130, 1:200; and protein disulfide isomerase (PDI), 1:50. The superposition (merge) of the two images is also shown. Bar = 10 $\mu$m. D, the ATP-dependent uptake of L-glutamate by digitonin-permeabilized DNPI-pcDNA3.1-transfected cells. Glutamate uptake by permeabilized cells was monitored as described under “Materials and Methods” in the presence or absence of the listed compounds: 1 $\mu$M bafilomycin A1, 0.5 $\mu$M SF6847, 1.0 mM sodium vanadate, 1.0 mM L-aspartate, and 1.0 mM D-aspartate. The ATP-dependent D-aspartate uptake was also measured (lower panel). In some experiments, magnesium acetate (–Mg$^{2+}$) or ATP (–ATP) was omitted. Control (vector alone) cells or untransfected cells were also permeabilized with digitonin, and their glutamate uptakes under the standard condition were measured. The results are the means ± S.E. of four independent experiments.
FIG. 2. Expression and localization of DNPI in pinealocytes. A, RT-PCR detection of gene expression of DNPI in pineal gland and cultured cells. Transcripts of DNPI for brain (lanes 2 and 5), pineal gland (lanes 3 and 6), and cultured pineal cells (lanes 4 and 7) are shown. The PCR product was not detected if reverse transcriptase was omitted from the reaction mixture (lanes 5–7). The apparent molecular mass is also shown (lane 1). B, expression of mRNA for DNPI was measured by Northern blotting. The amplified PCR products were hybridized with total RNA from brain (lane 1), pineal gland (lane 2), liver (lane 3), or PC12 cells (lane 4), and the resultant hybridization was visualized with a BAS2000 imaging analyzer. The positions of 18 and 28 S RNA were shown. The lower panel shows expression of glyceraldehyde-3-phosphate dehydrogenase as a control. C, DNPI protein was detected by Western blotting. Membrane fractions prepared from pineal gland (lanes 1 and 4), cultured pineal cells (lanes 2 and 5) (100 μg of protein), and brain (lanes 3 and 6) (50 μg of protein) were solubilized, electrophoresed, and then subjected to Western blotting with anti-DNPI antibodies as described in the legend to Fig. 1A. For lanes 4–6, the nitrocellulose sheet was incubated with 1 μg of antigenic peptide during the antibody treatment. The positions of the molecular markers are shown. D, immunohistochemical localization of DNPI in pineal gland. Sections of a pineal gland were doubly immunostained with antibodies against DNPI (green) and synaptophysin (red) (1), DNPI (green) and glial fibrillary acidic protein (GFAP) (red) (2), DNPI (green) and OX42 (red) (3), or DNPI (green) and vimentin (red) (4) and then observed under a confocal microscope. The superposition (merge) of the two images is also shown. Bar = 10 μm. E, immunohistochemical localization of DNPI in cultured pinealocytes. Cultured pinealocytes were doubly immunostained with antibodies against DNPI (green) and synaptophysin (red) and then observed under a confocal microscope. Arrows indicate the process terminal that contains DNPI and synaptophysin. Arrowheads indicate the process terminal lacking DNPI. Dilution of the antibodies is as follows: DNPI, ×1000; synaptophysin, ×50; OX42, ×800; glial fibrillary acidic protein (GFAP), ×100; and vimentin, ×10. Bar = 10 μm. kb, kilobases.
accumulate L-glutamate in SLMVs, counterparts of synaptic glutamatergic systems (3, 4). Mammalian pinealocytes in the central nervous system, peripheral endocrine tissues possess the neurotransmitter pancreatic polypeptide (PP), somatostatin, glucagon, and insulin (1000; somatostatin, 1000; glucagon, 1000). Besides DNPI, CL2 cells express various types of ionotropic glutamate receptors and reuptake systems (36–38). Clonal pancreatic αTC6 cells store and secrete L-glutamate through exocytosis, the mechanism acting in a subclass of pineal cells as well as brain used. The nucleotide and deduced amino acid sequences of the amplified products exactly matched that of the DNPI gene. Northern blot analysis with the amplified RT-PCR products further demonstrated the expression of mRNA for DNPI in pineal glands: two major bands (~3.2 and 4.1 kilobases) for pineal glands and brain mRNA were detected (Fig. 2B). Western blot analysis indicated that the anti-DNPI antibodies recognized a single polypeptide of ~65 kDa in pineal glands, cultured pinealocytes, and brain membranes (Fig. 2C). The DNPI immunoreactivity was blocked when the nitrocellulose sheet was treated with an antigenic peptide during the immunodecoration (Fig. 2C). Overall it is concluded that DNPI is expressed in pineal glands and cultured pinealocytes.

Immunohistochemistry with frozen-sectioned pineal gland revealed the localization of DNPI at the cellular level. We used the following cell markers to classify DNPI-positive cells: synaptophysin for pinealocytes (30, 31), glial fibrillary acidic protein for astrocytes (32), OX42 for microglia (33), and vimentin for interstitial cells (34). The antibodies against these marker proteins immunostained the corresponding populations of pineal cells with a similar morphology as reported previously (33, 35) (Fig. 2D). The DNPI-positive cells coincided with synaptophysin but not with any of the above-mentioned cell markers, indicating that pinealocytes contain DNPI (Fig. 2D). Essentially the same results were obtained for cultured pineal cells (data not shown). DNPI and synaptophysin are enriched in the process terminal regions, the site for glutamate exocytosis (3, 4) (Fig. 2B). These results strongly suggested that DNPI is associated with SLMVs in pinealocytes.

DNPI in αTC6 Cells of Langerhans Islets—Langerhans islets express various types of ionotropic glutamate receptors and reuptake systems (36–38). Clonal pancreatic αTC6 cells store and secrete L-glutamate through exocytosis, the mechanism being similar to those in neurons and pinealocytes (20). Thus, it is possible that Langerhans islets are another example of an endocrine glutamatergic system and that DNPI is responsible for the storage of L-glutamate in the islets. To examine this possibility, the expression of DNPI in αTC6 cells and islets was
measured. RT-PCR analysis indicated the presence of DNPI mRNA in αTC6 cells (Fig. 3A). Western blotting and immunohistochemistry indicated the presence of DNPI in αTC6 cells (Fig. 3, B and C).

Langerhans islets are composed of four major types of endocrine cells, i.e., insulin-secreting β cells, glucagon-secreting α cells, pancreatic polypeptide-secreting F cells, and somatostatin-secreting δ cells. Western blotting clearly indicated the presence of DNPI in the islets (Fig. 3B). DNPI was co-localized with glucagon but not with insulin or somatostatin in horizontal sections of the islets, indicating the presence of DNPI in δ cells but not in β or δ cells (Fig. 3C). DNPI is also co-localized with pancreatic polypeptides. These results suggested that DNPI is mainly present in δ cells and partially in pancreatic polypeptide-secreting F cells (Fig. 3C).

**DISCUSSION**

Vesicular glutamate transporter is responsible for the glutamatergic characteristics of neurons and was originally identified in synapsin I-associated synaptic vesicles (25, 39). In the earlier stage of studies, vesicular glutamate transporter was defined as the ATP-dependent proton conductor-sensitive glutamate transport activity in synaptic vesicles, but little was known about the protein nature of the transporter at the molecular level (25–27, 39). In the last year, two groups have independently reported that BNPI is vesicular glutamate transporter itself and that BNPI is a potential tool for substantial studies on vesicular glutamate transporter. DNPI is a potential candidate for another vesicular glutamate transporter since DNPI is distributed throughout the brain, being especially abundant in the nerve endings of glutamatergic neurons where BNPI is scarce (15, 16). Here we showed that DNPI actually functions as a vesicular glutamate transporter when expressed in COS7 cells.

DNPI expressed in COS7 cells seems to be mainly localized in endosomes. Since endosomes contain vacuolar H−-ATPase (40, 41), the active transport of glutamate into the organelles should be expected upon the addition of ATP in digitonin-permeabilized cells. In fact, digitonin-permeabilized cells took up l-glutamate depending on MgATP, and the properties of the uptake are consistent with those of vesicular glutamate transporter (25–27, 39), indicating that DNPI expressed functions as a vesicular glutamate transporter.

The fact that DNPI exhibits vesicular glutamate transport activity is not surprising since the amino acid identity of the core portions of DNPI and BNPI excluding their hydrophilic N- and C-terminal regions is over 90% with 12 putative transmembrane helices (7, 13). In the original studies on the expression and functions of BNPI and DNPI, both proteins were found to facilitate the transport of inorganic phosphate depending on extracellular Na+ (7, 13). On the other hand, vesicular glutamate transporters use a proton as a coupling ion and only recognize l-glutamate and a few cyclic glutamate analogues as substrates (25–27, 39). Thus, DNPI and BNPI are versatile in the active transport of glutamate into the organelles of various stages of studies, vesicular glutamate transporter was defined as the ATP-dependent proton conductor-sensitive glutamate transport activity in synaptic vesicles, but little was known about the protein nature of the transporter at the molecular level (25–27, 39), indicating that DNPI expressed functions as a vesicular glutamate transporter.

**REFERENCES**

1. Foster, A., and Fagg, G. (1984) *Brain Res.* **319**, 103–164
2. Mayer, M. L., and Westbrook, G. (1997) *Prog. Neurobiol.* **57**, 197–276
3. Moriyama, Y., Yamamoto, A., Yamada, H., Tashiro, Y., and Futah, M. (1996) *Bioc. Chem.* **377**, 155–165
4. Moriyama, Y., Hayashi, M., Yamada, H., Yataushiro, S., Ishio, S., and Yamamoto, A. (2000) *J. Exp. Biol.** **203**, 117–125
5. Liu, Y., and Edwards, R. H. (1997) *Annu. Rev. Neurosci.* **20**, 125–156
6. Maycox, P. R., Hell, J. W., and Jahn, R. (1999) *Trends Neurosci.* **19**, 83–87
7. Ni, B., Restock, P. R., Jr., Nadi, N. S., and Paul, S. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5607–5611
8. Bellocco, E. E., Reimer, R. J., Fremeau, R. T., Jr., and Edwards, R. H. (2000) *Science* **289**, 857–860
9. Takahashi, S., Sato, R. S., Rosenblum, C., and Jahn, R. (2000) *Nature* **407**, 189–194
10. Ni, B., Wu, X., Yan, G.-M., Wang, J., and Paul, S. M. (1996) *J. Neurosci.* **15**, 5789–5799
11. Bellocco, E. E., Hu, H., Pohorille, A., Chan, J., Pickel, V. M., and Edwards, R. H. (1998) *J. Neurosci.* **18**, 8648–8659
12. Otis, T. S. (2001) *Neuron* **29**, 143–149
13. Aihara, Y., Mashima, H., Onda, H., Hisano, S., Kasuya, H., Hori, T., Yamada, S., Tomura, H., Yamada, Y., Inoue, I., Kojima, I., and Takeda, J. (2000) *J. Neurochem.* **74**, 2622–2625
14. Hisano, S., Hoshi, K., Ikeda, Y., Maruyama, D., Kanemoto, M., Ichijo, H., Kojima, I., Takeda, J., and Nogami, H. (2000) *Mol. Brain Res.* **83**, 34–43
15. Sakata-Haga, H., Kanemoto, M., Maruyama, D., Hoshi, K., Mogi, K., Narita, M., Okado, N., Ikeda, Y., Nogami, H., Fukui, Y., Kojima, I., Takeda, J., and Hisano, S. (2001) *Brain Res.* **902**, 143–155
16. Fujisawa, F., Furuta, T., and Kaneko, T. (2001) *J. Comp. Neurol.* **435**, 379–387
17. Hamaguchi, K., and Leiter, E. H. (1990) *J. Neurochem.* **53**, 165–176
18. Yamada, H., Yataushiro, S., Hayashi, M., Nishi, T., Yamamoto, A., Futai, M., Yamaguchi, A., and Moriyama, Y. (1998) *J. Neurosci.*, **18**, 2056–2062
19. Erickson, J. D., Eiden, L. E., and Hoffman, B. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10993–10997.
20. Yamada, H., Otuska, M., Hayashi, M., Nakatsuka, S., Hamaguchi, K., Yamamoto, A., and Moriyama, Y. (1998) *J. Neurochem.* **71**, 356–365
21. Hayashi, M., Yamamoto, A., Yataushiro, S., Yamada, H., Futai, M., Yamaguchi, A., and Moriyama, Y. (1998) *J. Neurochem.* **71**, 356–365
22. Hayashi, M., Haga, M., Yataushiro, S., Yamamoto, A., and Moriyama, Y. (1998) *J. Neurochem.* **71**, 356–365
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
