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Rat Brain Synaptic Vesicles are Devoid of Mg^{2+}-ATPase Activity and Contain β-Amyloid Precursor Protein

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Rat brain synaptic vesicles (SVs) isolated by gel filtration on Sephacryl S-500 had little $Mg^{2+}(H^+)$-ATPase activity, though it was identified by Western blots with antibodies against the H^+-ATPase A-subunit and other vesicle proteins. In contrast, tyrosine hydroxylase and dopa decarboxylase activities in the SVs were substantial, suggesting that the absence of $Mg^{2+}(H^+)$-ATPase activity was not due to inactivation during isolation but rather to the nature of the SVs. The vesicle component reactive to H^+-ATPase antibody was also identified in the synaptosomal cytosol, so the antibody for the A-subunit seemed unnecessary to detect H^+-ATPase. The SVs contained β-amyloid precursor protein of ~100 kDa. Based on these observations, SVs without $Mg^{2+}(H^+)$-ATPase seemed to play a role(s) in the delivery of cytoplasmic and plasma membrane proteins to nerve terminals as well as in neurotransmission.

Key words: β-amyloid precursor protein, dopa decarboxylase, H^+-ATPase, synaptic vesicles, tyrosine hydroxylase.

INTRODUCTION

Synaptic vesicles (SVs) arise from the Golgi apparatus in the cell body of nerve cells (*1*), some of which contain enzymes for catecholamine biosynthesis (*2–4*), and they are transported by the rapid axonal flow to the nerve terminals along with microtubules (*5, 6*). On the other hand, classical non-peptide neurotransmitters such as norepinephrine and dopamine are synthesized mainly in the nerve terminals (*7, 8*), and are immediately stored in the SVs via the transmembrane electrochemical proton gradient ($\triangle\mu H^+$) generated by H^+-ATPase (Mg^{2+}-dependent H^+-translocating ATPase) in the SV membranes (*9, 10*) They are then released through fusion of the SVs with the pre-synaptic membranes (*11*). However, the molecular mechanism of the exocytotic neurotransmitter release remains largely elusive. Furthermore recent studies, providing new insights into the physiological functions of SVs, have shown that β-amyloid precursor proteins (βAPPs) are transported in neurosecretory vesicles by fast axonal transport to the nerve terminals, where they are released in a Ca^{2+}-dependent manner (*12–14*).

Previously I attempted to isolate rat brain SVs from the following sources by gel filtration on Sephacryl S-500 (*15, 16*): (i) crude SV fraction prepared by salting out the synaptosomal hypo-osmotic lysate and (ii) iso-osmotic lysate obtained by freeze-thawing synaptosomes. Both SV preparations contained little Mg^{2+}-ATPase (H^+-ATPase) [EC 3.6.1.3] activity, although the vesicles were identified by Western blotting with antibodies against H^+-ATPase, vesicular ACh transporter and other vesicle proteins. To determine whether the enzyme was inactivated during the vesicle preparation, the present study tested whether tyrosine hydroxylase (TH) [EC 1.14.16.2] and dopa decarboxylase (DDC) [EC 4.1.1.28] present in the SVs are inactivated during isolation. These enzymes were substantially active after preparation, indicating that the absence of Mg^{2+}-ATPase activity was not due to inactivation during isolation. Also I examined the subcellular distribution of the component (antigen) that is reactive to the antibody for H^+-ATPase A-subunit, a 72 kDa-constituent of the catalytic domain V1 of H^+-ATPase (*17, 18*). Since the vesicle component was also found in the synaptosomal cytosol, it was not apparently specific to the SVs and this procedure is invalid for the study of H^+-ATPase localization. Interestingly, βAPP(s) was found in the SVs. These results suggested that SVs without $Mg^{2+}(H^+)$-ATPase activity participate in the axonal transport of cytoplasmic and plasma membrane proteins to the nerve terminals.

MATERIALS AND METHODS

Experimental Animals and Chemicals—Male Wistar rats (4 weeks old) were decapitated and then brain was quickly removed and placed in ice-cold 0.32 M sucrose. Anti-H^+-ATPase A-subunit antibodies [rabbit IgG raised against a synthetic 17 aa (amino acids) peptide corresponding to H^+-ATPase A-subunit of bovine adrenal chromaffin granules] were purchased from Wako Chem. Co., Osaka. Anti-dopamine transporter (anti-DAT) antibodies (rabbit anti-serum raised against the N-terminal 18 aa peptide of rat brain DAT) were purchased from Alpha Diagnostic International, USA. Anti-vesicular monoamine transporter 2 (anti-VMAT 2) antibodies (guinea pig whole anti-serum raised against a synthetic 20 aa peptide corresponding to rat VMAT 2), anti-synaptotagmin 1 (anti-Syt 1) antibodies (rabbit IgG

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raised against a synthetic 17 aa peptide corresponding to the N-terminus of rat brain Syt 1), anti- DDC antibodies (mouse monoclonal IgG2b raised against a synthetic 24 aa peptide corresponding to the N-terminal of human DDC) and anti-APP antibodies (rabbit IgG raised against a synthetic peptide corresponding to the C-terminal 20 aa of human APP695) were purchased from Sigma-Aldrich Inc., USA. Sephacryl S-500 HR (average exclusion size, 200 nm) was purchased from Amersham Pharmacia Biotech, Uppsala. Fine particles were decanted from the degassed resin before use. The resin (700 ml) was packed into a glass column (2.9×110 cm packed gel height) and equilibrated with 0.1 M Tris-HCl-0.1 M KCl, pH 7.6. Carboxymethyl cellulose, CM 52, from Whatman International Ltd., England, was packed into a glass tube (2×7 cm gel height) and equilibrated with 20 mM Na-phosphate, pH 6.0. Sequi-Blot^{TM} PVDF (polyvinillidene difluoride) membranes (0.2 μm pore) were from Nippon Bio-Rad Lab, Japan. L-[U-^{14}C]tyrosine and [2,8-^{3}H]ATP were from Moravek Biochem. Inc., USA and (6R)-5,6,7,8-tetrahydro-L-biopterin-2HCl and activated alumina (300 mesh, H^+ form) were from Wako Chem. Co. Amberlite CG-50 (100-200 mesh, H^+ form), pyruvate kinase, phosphoenolpyruvate and pyridoxal 5′-phosphate were from Sigma-Aldrich Inc. The molecular weight (MW) standards were rainbow markers (Amersham Pharmacia Biotech), horse heart cytochrome c, bovine serum albumin and blue dextran (Sigma-Aldrich).

Isolation of Synaptosomes and the Hypo-osmotic Lysate—Synaptosomal P2 fraction was isolated at 0-4°C essentially according to Gray and Whittaker (*19*). Twenty whole brains including the cerebella were homogenized in 9 vol of 0.32 M sucrose with a Teflon-glass homogenizer at 300 rpm. The homogenate was centrifuged at $1,300 \times g$ for 15 min to remove cell debris. The supernatant was centrifuged at $15,000 \times g$ for 30 min, then the pellets (P2 fraction) were hypo-osmotically disrupted in 10 vol of ice-cold de-ionized water using the homogenizer at 100 rpm. The lysate was centrifuged at $22,000 \times g$ for 30 min to remove synaptosomal plasma membranes, and buffered with 50 mM Tris-HCl, pH 7.6.

Isolation of Synaptic Vesicles by Gel Filtration on Sephacryl S-500—Solid ammonium sulphate was added to 600 ml of hypo-osmotic lysate to bring to 50% saturation (313 g/l) and centrifuged at $15,000 \times g$ for 30 min. The precipitate containing SVs was dissolved in 20–30 ml of 0.1 M Tris-HCl-0.1 M KCl, pH 7.6, and dialysed against 2.5 l of the same medium for 8 h to remove any remaining ammonium sulphate. The dialysate was centrifuged at $40,000 \times g$ for 30 min to remove insoluble material. The supernatant (about 60 ml; 3–4 mg protein/ml) was rich in SVs. An aliquot of the vesicle suspension (20–25 ml) was applied to the Sephacryl column, eluted with the same medium at a flow rate of 70 ml/h, and 7 ml fractions were collected (Fig. 1). Peak I at the void volume contained large membranous vesicles and a few coated vesicles and SVs. Peak II consisted exclusively of SVs and was eluted before the blue dextran marker at an average MW of 2×10^6, followed by peak III of soluble proteins. The results of SDS–PAGE (Sodium dodecyl sulphate–polyacrylaminde

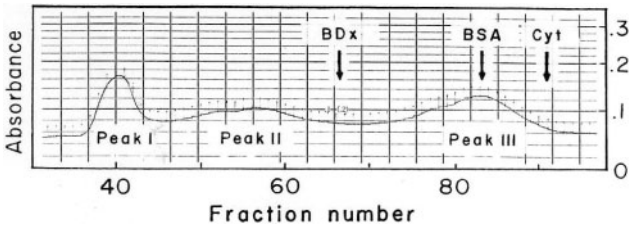

Fig. 1. **Typical elution profile of crude SV suspention (~80 mg proteins in 20–25 ml) from Sephacryl S-500 (exclusion size, 200 nm), monitored photometrically at 280 nm.** Peak I at void volume was turbid and comprised large membranous vesicles, coated vesicles and SVs. Peak II consisted exclusively of SVs, eluted before blue dextran. Peak III comprised soluble proteins after blue dextran. Elution positions of MW standards are shown by arrows; BDx (blue dextran, average MW, 2×10^6), BSA (bovine serum albumin, 66,000), and Cyt (cytochrome c, 12,000).

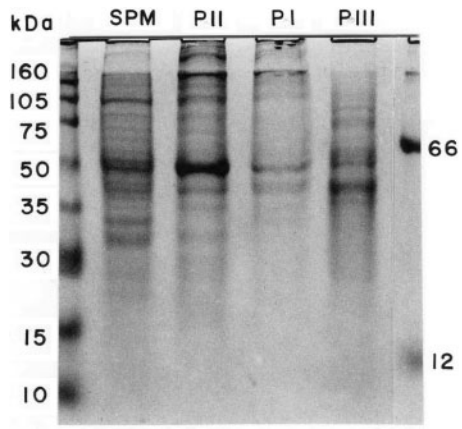

Fig. 2. **SDS–PAGE of peaks I–III (PI–PIII) and synaptosomal plasma membranes (SPM).** Sample proteins (20.0 μg per well) were resolved by electrophoresis on 9% polyacrylaminde gels and stained with Coomassie brilliant blue G. MW standards were Amersham Rainbow Markers (10–160 kDa), BSA (66 kDa) and cytochrome c (12 kDa).

gel electrophoresis) analysis of the fractions are shown in Fig. 2, and their electron micrographs have been published elsewhere (*15, 16*).

Conventioal Isolation of Synaptic Vesicles—According to Kadota and Kadota (*20*), synaptosomal P2-fraction was hypo-osmotically processed and then centrifuged at $47,000 \times g$ for 30 min to remove synaptosomal plasma membranes. The supernatant rich in SVs was centrifuged at $105,000 \times g$ for 90 min and the pellets were homogenized in a small volume of 0.32 M sucrose -50 mM Tris-HCl, pH 7.6. A milky white suspension of ~10 ml (~10 mg protein/ml) was obtained from 10 rats. Their electronmicrographs have been published elsewhere (*21, 22*).

Preparation of Synaptosomal Plasma Membranes—The synaptosomal membrane fraction obtained above was washed 2–3 times in 0.32 M sucrose by repeated homogenization and centrifugation at $15,000 \times g$ for 15 min. The pellets were used as the plasma membrane preparation. One of the pellets was fixed with 1% glutaldehyde and post-fixed with 1% OsO_4 for 1 h at 0°C. Thin sections on grids were stained with uranyl acetate and lead

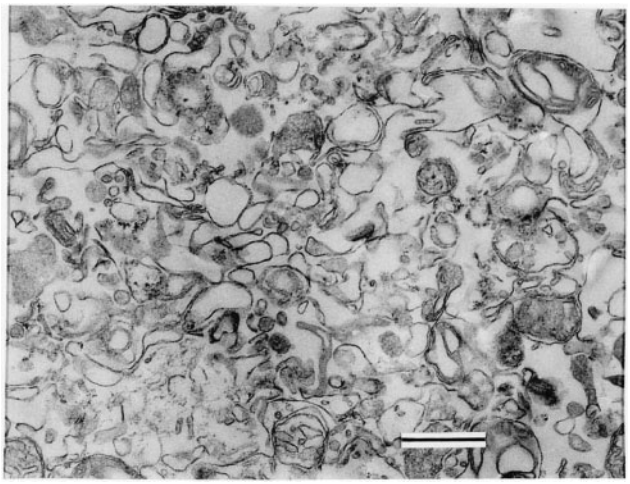

Fig. 3. **Electronmicrograph of the SPM preparation.** Some SVs are enclosed in ruptured synaptosomes (×29,000). Scale bar, 500 nm.

acetate, and examined with a JEM 1200EX electron microscope (Nippon Denshi Co., Japan). Figure 3 shows an electron micrograph of the sample.

SDS–PAGE and Western Blot Analysis—SVs and synaptosomal plasma membranes (~2 mg protein/ml) were heated with 1% SDS and 5% β-mercaptoethanol at 100°C for 3 min, and then resolved by electrophoresis in 9% polyacrylamide slab gels containing 0.1% SDS and 0.18 M Tris-HCl, pH 8.7, at 40–50 V, 40–50 mA at 25°C for 7 h. Half of the gel was stained for protein with Coomassie brilliant blue G. Proteins in the other half of the gel were transferred to PVDF membranes and blotted with antibodies. Components that reacted with the antibodies were visualized using the ECL western blotting detection system (Amersham Pharmacia Biotech., Sweden).

Assays of Enzymatic Activities—DDC [EC 4.1.1.28] activity was measured according to Ichinose *et al.* (*23*). The reaction mixture of 2.2 ml contained 30 mM Na-phosphate, pH 7.2, 1 mM L-dopa, 0.1 mM pyridoxal 5′-phosphate, 0.1 mM pargyline-HCl, 0.17 mM ascorbic acid, 0.3 mM EDTA and 200–400μg of protein samples. The reaction proceeded for 30 min at 35°C and was stopped with 1 ml of 10% trichloroacetic acid (TCA). After the precipitate was removed by centrifugation, the pH of the supernatant was adjusted to 7.0 by adding 1 N NaOH dropwise, under stirring. The produced dopamine was adsorbed on 0.6 ml Amberlite CG-50 (Na^+ form) packed in a 0.4 × 5 cm glass tube and eluted with 1.4 ml of 0.8 N HCl. The amount of dopamine was determined fluorometrically at 530 nm, excited at 418 nm, after condensation with ethylenediamine at pH 10.5, essentially according to Weil-Malherbe and Bone (*24*). TH [EC 1.14.16.2] activity was measured according to Nagatsu *et al.* (*25*) in a 2.2 ml reaction mixture comprised of 0.1 M Na-phosphate, pH 6.5, 3 mM L-tyrosine, trace amounts of L-[^{14}C]tyrosine, 5 mM tetrahydrobiopterin (THBP), 0.1 mM $FeSO_4$, 3 mM β-mercaptoethanol and 300–500 μg of protein samples. The reaction proceeded for 30 min at 35°C and was stopped by adding 1 ml of 10% TCA. After centrifugation, the pH of the supernatant was brought to 7.0 by adding 1 N NaOH dropwise. The pH was further adjusted to 8.4 with 0.8 M Tris-HCl, pH 8.8. The dopa generated was adsorbed on 0.6 ml alumina (activated) packed in a 0.4 × 5 cm glass tube and eluted with 1.4 ml of 0.8 N HCl. Adenylate cyclase [EC 4.6.1.1] was measured according to Alvarez and Daniels (*26*). The 0.5 ml reaction mixture contained 1 mM ATP, 0.16 μM (1.7 μCi) [2,8-^{3}H]ATP, 4 mM $MgCl_2$, 0.1 mM adenine, 0.1 mM GTP, 21 mM phosphoenolpyruvate, 6 units of pyruvate kinase, 0.1 M Tris-HCl, pH 7.4 and 150–250 μg of protein samples. The reaction proceeded for 30 min at 35°C and was stopped by the addition of 1 ml of 0.005 N HCl containing 0.1 mM cAMP. The solution was eluted through an alumina column (1 × 2 cm alumina height) and washed with 4 ml of 0.005 N HCl. The [^{3}H]cAMP produced was eluted with 3 ml of 0.1 M ammonium acetate. Radioactivity of [^{3}H]cAMP and [^{14}C]dopa was measured in Bray's scintillator (*27*) using an Aloka LSC-3000 scintillation counter (Tokyo, Japan). Mg^{2+}-ATPase [EC 3.6.1.3] was measured as reported (*15, 16*) and 5′-nucleotidase [EC 3.1.3.5] was assayed according to Aronson, Jr and Touster (*28*) in a 2.2 ml reaction mixture containing 0.1 M Tris-HCl, pH 7.6, 5 mM AMP, 6 mM $MgCl_2$ and 50–200 μg of protein samples at 35°C. The latter reaction was terminated with 1 ml of 10% TCA in 20 min. Inorganic phosphate liberated from ATP or AMP was determined according to Martin and Doty (*29*). Acetylcholine esterase [EC 3.1.1.7] activity was assayed as described by Ellman *et al.* (*30*) in a 3.3 ml reaction mixture containing 0.1 M Na-phosphate, pH 8.0, 3.5 mM acetylthiocholine, 1.2 mM dithiobisnitrobenzoic acid and 100–200 μg of protein samples, and monitored photometrically at 412 nm and 25°C.

Protein Concentration—Proteins were determined by the method of Lowry *et al.* (*31*) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Enzymatic Activities of the Synaptic Vesicles—SVs isolated by gel filtration showed little $Mg^{2+}(H^+)$-ATPase activity, although they were identified by the antibodies against H^+-ATPase A-subunit and other vesicle-specific proteins. To test whether other enzymes in the SVs were inactivated during isolation, the catalytic activities of TH and DDC in peak II were compared with those of peaks I, III, SV (SVs prepared by the conventional method) and SPM (synaptosomal plasma membranes) as shown in Table 1.

The specific activity of Mg^{2+}-ATPase in peak I seemed consistent with its protein content comprising miscellaneous large membranes. On the other hand, the specific activity of peak II, consisted exclusively of SVs (which are known to contain H^+-ATPase), was as low as 1/30 of that of peak I and of SV, which was in good agreement with previous findings (*15, 16, 32*) The Mg^{2+}-ATPase activity in the SPM was increased up to 170–200% of control by adding 100 mM NaCl and 5 mM KCl due to

Table 1. **Enzymes in preparations.**

	Mg^{2+}-ATPase +(Na,K)*		Tyrosine hydroxylase –(THBP,Fe)**		Dopa decarboxylase
Peak I	12.4	13.0	125.5	7.0	
Peak II	0.41	0.4	121.0	7.0	10–12
Peak III	n.d.	n.d.	204.0	10.0	18–20
SV	17.8	18.8	184.0	8.5	9.0
SPM	32.6	56.1			

ATPase was assayed using 150 μg protein of peak I, 625 μg peak II, 144 μg SV and 100 μg SPM in 2.2 ml reaction mixtures containing 0.1 M Tris-HCl, pH 7.6, 5 mM ATP and 6 mM $MgCl_2$. *0.1 M NaCl and 5 mM KCl were added; n.d., not detected; activity, μmol ATP hydrolyzed/mg protein/h at 35°C. TH was assayed in 2.2 ml of 0.12 M Na-phosphate, pH 6.5, containing 300–500 μg sample proteins, 3 mM L-tyrosine, trace amounts of L-[^{14}C]tyrosine, 10 mM THBP, 0.1 mM $FeSO_4$, and 5 mM β-mercaptoethanol. **THBP and Fe were omitted. Activity, nmol dopa produced/mg protein/h at 35°C. DDC was assayed in 2.2 ml of 30 mM Na-phosphate, pH 7.2, containing 1 ml L-dopa, 0.1 mM pyridoxal 5′-phosphate, 0.1 mM pargyline-HCl, 0.17 mM ascorbic acid, 0.3 mM EDTA and 200–400 μg protein of samples. Activity, nmol DA produced/mg protein/h at 35°C.

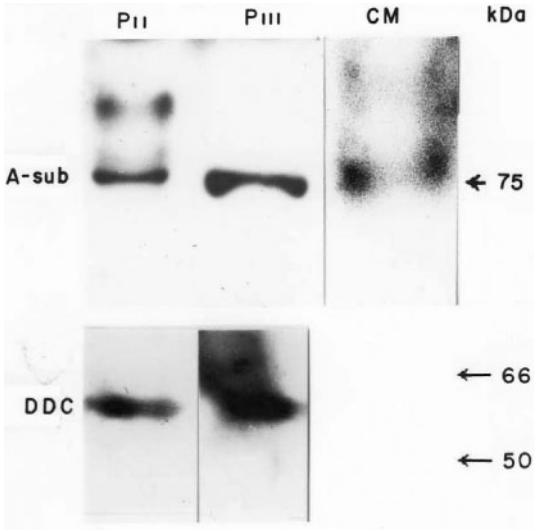

Fig. 4. **Western blots using antibodies for H^+-ATPase A-subunit (A-sub), 73 kDa and DDC, 55 kDa.** Protein component reacted with H^+-ATPase antibody in cytosolic fraction of peak III and in SVs of peak II. This component was adsorbed on CM-cellulose (CM) at pH 6. DDC was also detected in both fractions (PII and PIII), which was consistent with distribution of catalytic activity shown in Table 1.

the Na^+, K^+-ATPase present in the sample, whereas the Mg^{2+}-ATPase activity of peak II was hardly affected by the salts, indicating that SPM contamination in the fraction was negligible. Peak III contained no ATPase activity.

TH moves down in amine granules to the nerve endings (*4*) and exists as a tetramer *in vivo* with an apparent MW of $250–280 \times 10^3$ (*33*). Figure 1 shows that peaks I and II were eluted before the blue dextran marker with an average MW of 2×10^6, followed by peak III of soluble proteins after the MW marker. The TH activity in all peaks was comparable to that of the conventional SV. This implies that TH tetramers of $250–280 \times 10^3$ MW can be recovered in peak III in a soluble form while the enzymes in peaks I and II are enclosed in the SVs. The subcellular distribution of TH in both soluble and granular fractions has been observed with the bovine brain (*34*). TH has been immunochemically detected in the SVs of peak II (*16*), and the enzymatic activity of TH, as shown in Table 1, was reduced to ~1/20 by omitting THBP and ferrous ions. Thus, the TH molecules in the SVs of peak II remained unchanged after isolation by gel filtration, and its catalytic activity was comparable to that of the conventional SVs.

DDC or L-aromatic amino acid decarboxylase is abundant in the cytosol of many tissues (*23, 35*). In nerve cells, DDC is transported in neurosecretory granules to the nerve terminals (*2*) like TH (*4*). Table 1 shows that DDC activity was measurable in SVs isolated by gel filtration and by the conventional method in addition to the cytosolic fraction (peak III). This finding was consistent with the immunoblotting result as shown in Fig. 4 indicating the presence of DDC of 55 kDa in both peaks II and III.

Thus, the present SVs contained TH and DDC with activities comparable to those of the conventional SVs. These results indicate that the lack of Mg^{2+}-ATPase activity in the SVs is not derived from its inactivation during isolation but is rather due to the nature of the SVs.

According to the current theory of exocytotic neurotransmitter release, SVs take up neurotransmitters through exchanging them with the intravesicular protons accumulated by H^+-ATPase associated with the vesicle membrane (*11–13*). If so, SVs without $Mg^{2+}(H^+)$-ATPase could not be involved in the exocytotic release of neurotransmitters. An alternative physiological role of such inactive SVs might be to transport enzymes for the biosynthesis of neurotransmitters and other cytoplasmic proteins, because enzymes such as TH, DDC, and DβH are axonally transported in neurosecretory vesicles to the nerve terminals (*2–4*).

β-*Amyloid Precursor Protein(s) in Synaptic Vesicles*—Adrenal chromaffin cells have long been used as a model system of exocytotic neurotransmitter release. Recent studies, however, have found that βAPPs are located in chromaffin granules and they are released dependently upon Ca^{2+} (*36–38*). βAPPs are plasma membrane proteins that are widely distributed in many tissues and the proteolytic cleavage product, a β-amyloid peptide of ~5 kDa, is the main constituent of amyloid deposits in Alzheimer's disease. Rat brain slices and synaptosomes also similarly secrete βAPP (*12–14*). Like the chromaffin granules, βAPP of ~100 kDa (full-length) was detected by Western blot using the antibody against the C-terminal of human βAPP695 from the SVs in peak II (and in conventional SV) in addition to the SPM as shown in Fig. 5. The intensity of the βAPP-immunoreaction of peak II and SPM was comparable. In contrast, the specific activities of Mg^{2+}-ATPase and Na^+, K^+-ATPase (marker enzyme for plasma membranes) of the SVs in peak II were as low as ~3% of those of the SPM,

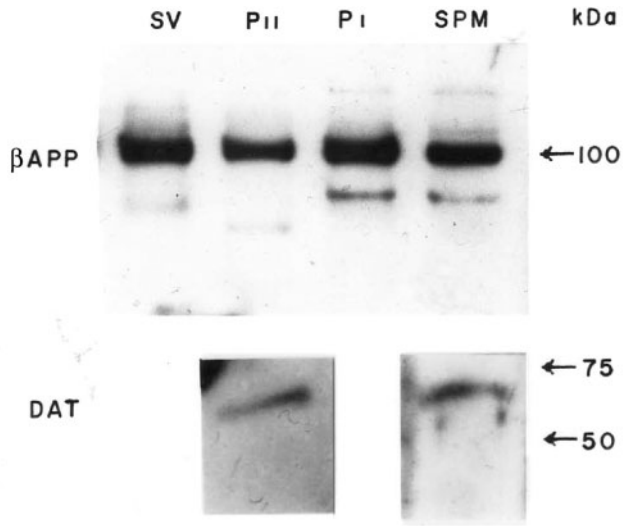

Fig. 5. **Western blots of SVs in peaks I, II and SV with antibodies against C-terminal of human βAPP695 and DAT.** Protein components corresponding to βAPP (100 kDa) and DAT (70 kDa) indicated SPM-specific proteins in SVs of peak II, as well as in SPM. SV, conventional one.

Table 2. **Marker enzymes of plasma membrane in preparations.**

	ACh esterase	Adenylate cyclase	5′-Nucleotidase
Peak II	0.14–0.18	5.0–8.0	0.03–0.04
SPM	3.59–4.13	103–117	0.81–1.07

ACh esterase was assayed using 80 and 240 μg proteins of SPM and SVs in peak II, respectively. Activity, μmol acetylthiocholine hydrolyzed/mg protein/h at 25°C. Adenylate cyclase was assayed using 200 and 400 μg proteins of SPM and SVs, respectively. Activity, nmol cAMP produced/mg protein/h at 35°C. 5′-Nucleotidase was assayed using 70 and 280 μg proteins of SPM and SVs, respectively. Activity, μmol AMP hydrolyzed/mg protein/h at 33°C.

indicating negligible SPM contamination in the preparation as shown in Table 1. To further confirm this, other marker enzymes for the plasma membrane, ACh esterase, adenylate cyclase and 5′-nucleotidase, were examined (Table 2). The activities of these enzymes in peak II were around 5% of those of the SPM, which was consistent with the above results. The results thus indicated that the βAPP-immunoreactivity in peak II originated from the SVs but not from the SPM, even if contaminated. The βAPP in the SVs is a full-length molecule of ~100 kDa that has a single membrane-spanning domain. However, whether the molecule spans the SV membrane or exists inside the vesicles remains obscure. That the βAPP is simply held by electric charges on the outer surface of the vesicle membrane is highly unlikely, because the SVs were prepared in medium with an ionic strength of ~0.3.

DAT is also an SPM-specific protein of ~70 kDa that terminates dopaminergic neurotransmission by Na^+-dependent re-uptake of DA (*39–40*). However, the component corresponding to DAT was also detected in SVs of peak II (Fig. 5), in which little SPM was detected

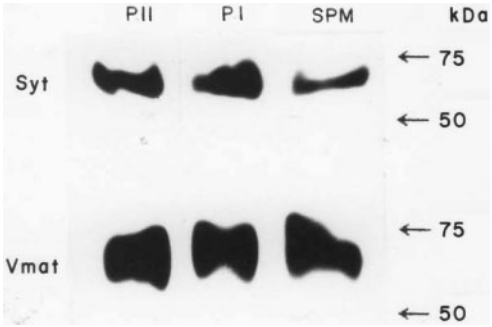

Fig. 6. **Western blot of SPM using antibodies for synaptotagmin 1 (Syt) of 65 kDa and vesicular monoamine transporter 2 (Vmat) of ~70 kDa.** SV -specific proteins were detected in SPM preparation, as well as in peaks I and II.

as noted above. All of these results suggest that SVs without $Mg^{2+}(H^+)$-ATPase are involved in the delivery of plasma membrane proteins (*12–14*) and cytoplasmic enzymes such as TH, DDC and DβH to the nerve terminals (*2–4*).

Synaptic Vesicle Proteins in Synaptosomal Plasma Membranes (SPM)—In contrast to the above, some SV proteins are located in the plasma membranes. Morel *et al.* (*41*) identified the membrane domain V0 of H^+-ATPase of Torpedo SVs in pre-synaptic plasma membranes (mediatophore functioning in the ACh release), and Dittman and Kaplan (*42*) found abundant synaptobrevin (vesicle-associated membrane protein, VAMP; ~18 kDa) in the neural plasma membrane of a nematode worm. These vesicle-specific proteins are thought to be introduced into the plasma membrane on the exocytotic release of neurotransmitters through the complete or partial fusion of SVs with SPM. Jones *et al.* (*43*) also identified glycosaminoglycan specific to Torpedo SVs in pre-synaptic plasma membranes.

In the present study, synaptotagmin 1 (Syt 1), an SV protein of ~65 kDa involved in docking SVs to the SPM (*44, 45*), and vesicular monoamine transporter 2 (VMAT 2), another vesicle protein of ~70 kDa involved in catecholamine uptake (*46, 47*), were substantially recovered in the SPM (Fig. 6). Much of this immunological positivity would be derived from these vesicle proteins being introduced into the SPM via exocytosis because only a trace amount of free SVs were found in the large amount of SPM in this fraction (Fig. 2). Therefore, the present results support the findings (*41, 42*) that some SVs proteins are introduced into the pre-synaptic plasma membranes by exocytosis. Transporters such as VMAT 2 in SPM might play a role in the transporter-mediated release of catecholamines.

Subcellular Distribution of the Component Reactive to the H^+-ATPase A-subunit Antibody—The present SVs had little $Mg^{2+}(H^+)$-ATPase activity despite the presence of the component reactive to antibodies for the H^+-ATPase A-subunit, a 72 kDa constituent of the catalytic domain V1 of H^+-ATPase (*17, 18*). We thus further examined the SVs in peak II using antibodies for Syt 1 and VMAT 2, and both antigenic components were detected in the vesicles (Fig. 6). The subcellular

distribution of the component reactive to the H^+-ATPase antibody was also examined. The component was detected even in the cytosolic fraction of peak III in addition to the SVs in peak II (Fig. 4). The component in peak III adsorbed to CM-cellulose at pH 6.0, indicating that it is a basic protein (lane, CM). A similar MW component(s) was observed in the non-adherent fraction to the CM cellulose, probably neutral protein(s), but it did not react with the H^+-ATPase antibody (data not shown). Thus, the component reactive to the H^+-ATPase antibody was apparently not specific to SVs and invalid for detecting H^+-ATPase. Morel *et al.* (*48*) have reported that the V1 and V0 domains of Torpedo H^+-ATPase are transported separately in the axon, suggesting that detached V1 domains are located in nerve endings that no longer have ATPase activity. In the present study, the component that reacted with the H^+-ATPase antibody in peak III might be derived from detached V1 domains because peak III had no ATPase activity. SVs in peak II might have the V1 domains considering that the A-subunit antibody detected them. Nevertheless, they had little $Mg^{2+}(H^+)$-ATPase activity. The most plausible explanation for this discrepancy is that the antigenic component was not specific to SVs.

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