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# Microvesicles isolated from bovine pineal gland specifically accumulate L-glutamate

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**Abstract** **Pinealocytes, endocrine cells that synthesize and secrete melatonin, possess a large number of synaptic-like microvesicles (MVs) containing synaptophysin. By monitoring cross-reactivity with anti-synaptophysin antibody, the MVs were highly purified from bovine pineal glands. The purified MVs were morphologically similar to but distinct from neuronal synaptic vesicles by their lack of synapsin I. Immunological study indicated that the MVs contained vacuolar $H^+$-ATPase, synaptotagmin and synaptobrevin 2 (VAMP2). The MVs accumulated L-glutamate at the expense of ATP hydrolysis by vacuolar $H^+$-ATPase. No uptakes of melatonin, serotonin, noradrenaline, γ-aminobutyrate or acetylcholine were observed. These results indicated that the MVs are organelles for storage of L-glutamate in pinealocytes and suggested a possibility that pinealocytes transmit glutamate signals by MVs-mediated exocytosis.**

*Key words:* Pinealocyte; Microvesicle; Synaptic vesicle; Glutamate; Glutaminergic neuron; Vacuolar $H^+$-ATPase; Chemical transduction

## 1. Introduction

Pinealocytes are endocrine cells that synthesize and secrete melatonin into blood [1–3]. This process is controlled by sympathetic neurons: upon binding of noradrenaline from the nerve endings to its receptor at plasma membrane of pinealocytes, *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase, key enzymes for melatonin synthesis, are stimulated, resulting in increased synthesis and secretion of melatonin [1–3]. Recently, L-glutamate was shown to inhibit the noradrenaline-stimulated melatonin synthesis [4–6]. This process may be mediated by a signal transduction pathway via glutamate receptor [7,8]. However, projection of glutaminergic neurons into pineal gland is not known and the mechanism by which glutamate transmits signal into pinealocytes remains unclear.

Pinealocytes are known to contain relatively high concentration of glutamate [9] and a large number of synaptic-like microvesicles (MVs) [10]. Recent evidence indicated that MVs from other endocrine cells show ability for accumulation of neurotransmitters such as γ-aminobutyrate or noradrenaline by similar mechanism to synaptic vesicles [11–13]. Therefore, it is possible that, like glutaminergic neurons, pinealocytes accumulate glutamate inside MVs and evoke glutamate signals by MVs-mediated exocytosis. To test the possibility, we developed as a first time a procedure for isolation of MVs from bovine pineal glands. It was found that the purified MVs accumulate L-glutamate through vesicular glutamate transporter, which is energized by V-ATPase.

## 2. Experimental

### 2.1. Preparation of MVs from bovine pineal glands

Thirty bovine pineal glands were kept in ice and used within a few hours after isolation at local slaughter house. After removing connective tissues and veins, the glands were washed, cut into small pieces with scissors, suspended in 50 ml of SME buffer and homogenized in a Dounce homogenizer. The homogenate was centrifuged at $900 \times g$ ($R_{max}$) for 10 min and the postnuclear supernatant was centrifuged at $11{,}000 \times g$ ($R_{max}$) for 20 min. The supernatant was centrifuged at $139{,}000 \times g$ for 40 min. The resultant pellet (microsome fraction) was washed once with the same buffer and applied to a discontinuous sucrose density gradient (0.7 ml of 1.8, 1.2, 1.0, 0.8, 0.6, and 0.4 M sucrose containing 5 μg/ml pepstatin A and 5 μg/ml leupeptin). After centrifugation at $207{,}700 \times g$ ($R_{max}$) for 3 h, four distinct bands were obtained. The top broad white band was collected, diluted 5-fold with SME buffer and centrifuged at $139{,}000 \times g$ for 40 min. The pellet (purified MVs fraction) was suspended in SME buffer and kept in ice bath or frozen at −85°C until use.

### 2.2. Preparations

Rat brain synaptic vesicles were prepared as described in [14] without last permeation chromatography. Monoclonal antibodies against synaptophysin [15] and synaptotagmin [16], site specific polyclonal antibody against VAMP2, and polyclonal antibody against purified synapsin Ia from bovine brain [17] were kindly provided by Dr. M. Takahashi (Mitsubishi Life Science Institute) and Dr. K. Sobue (Osaka University). Polyclonal antibodies against bovine chromaffin granule V-ATPase subunits A and E were raised by injecting individual proteins into albino rabbits. Polyclonal antibody against glutamate was obtained from Chemicon Internatinal Inc.

### 2.3. Analytical procedures

Conventional and immunoelectron microscopy were carried out according to De Camilli et al. [18]. ATP-dependent uptake of L-glutamate by purified MVs was measured in 0.5 ml of 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose, 4 mM KCl, 2 mM Mg-acetate, 2 mM ATP-Tris, and 50 μg purified MVs. Assay was started by the addition of [$^3$H]L-glutamate (2.5 μCi, 0.1 mM) at 30°C and aliquots (100 μl) were taken at intervals and filtered through 0.45 μm Millipore filters (type HA). The filters were washed once with 8 ml of 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose and 4 mM KCl and the radioactivity remaining on the filters was counted [19]. Uptakes of other radiolabeled transmitters (0. 1 mM each) were assayed essentially by the same procedure as described above except that KCl concentration was increased to 0.1 M.

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*Abbreviations:* MOPS, morpholinopropane sulfonic acid; MVs, synaptic-like microvesicles; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; SME buffer, 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; VAMP2, vesicle-associated membrane protein 2, also termed synaptobrevin 2; V-ATPase, vacuolar $H^+$-ATPase.


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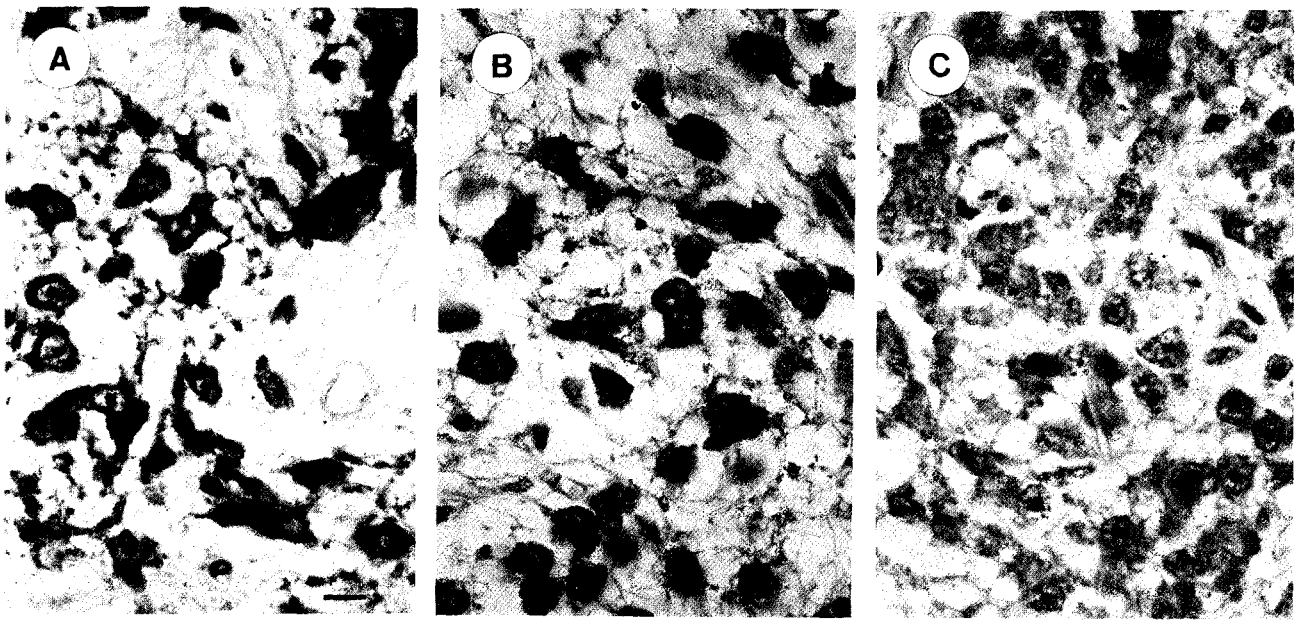


Fig. 1. Bovine pinealocytes are rich in synaptophysin, V-ATPase and glutamate. Paraffin sections of bovine pineal gland were immunostained by antibodies against (A) synaptophysin; (B) V-ATPase subunit E; (C) glutamate using biotin-avidin horseradish peroxidase method. Bar = 10 μm.

## 3. Results and discussion

MVs, morphologically similar organelles to neuronal synaptic vesicles, are present in various endocrine cells and contain synaptophysin as a common component [11–13,20,21]. As shown in Fig. 1A, anti-synaptophysin antibody immunostained pinealocytes strongly, confirming presence of MVs in pinealocytes. We prepared MVs from bovine pineal glands by combined differential and sucrose density gradient centrifugation as monitored with cross-reactivity with anti-synaptophysin antibody. The final preparation showed 14-fold enrichment of MVs over the homogenate with about 20% of recovery. Purified MVs fraction was composed of a large number of relatively clear vesicles with average diameter of 50 nm (Fig. 2A). The small vesicles contained synaptophysin as shown by immunoelectron microscopy with anti-synaptophysin antibody (Fig. 2B), demonstrating that the small vesicles corresponded to MVs. Only limited number of larger membrane vesicles, which were not recognized by anti-synaptophysin antibody, were contaminated in this fraction (Fig. 2). The purified MVs fraction contained synaptotagmin and VAMP2 (Fig. 3), proteins necessary for vesicular transport and fusion [21,22], suggesting that

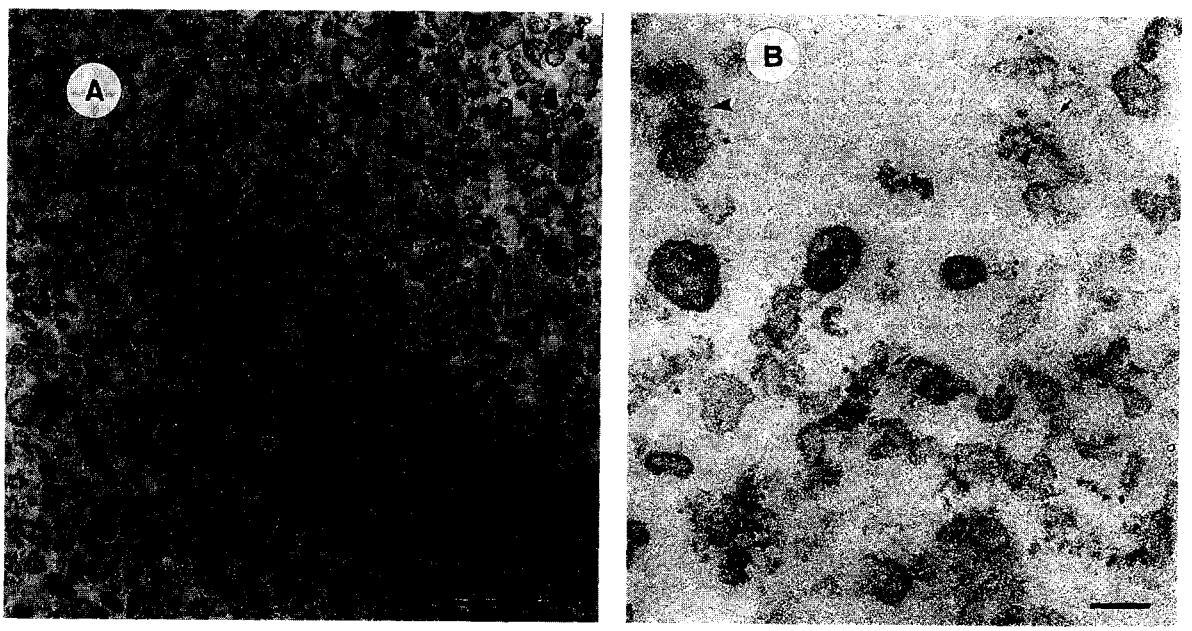


Fig. 2. Electron micrographs of MVs. (A) Electron micrograph of an ultra-thin section of the purified MVs fraction. Some larger membrane vesicles shown by an asterisk were contaminated organelles (see text). Bar = 500 nm. (B) Immunoelectron microscopy of the purified MVs fraction double-labeled by immunogold for synaptophysin (10 nm, arrows) and V-ATPase subunit E (5 nm, arrowheads). Bar = 100 nm.

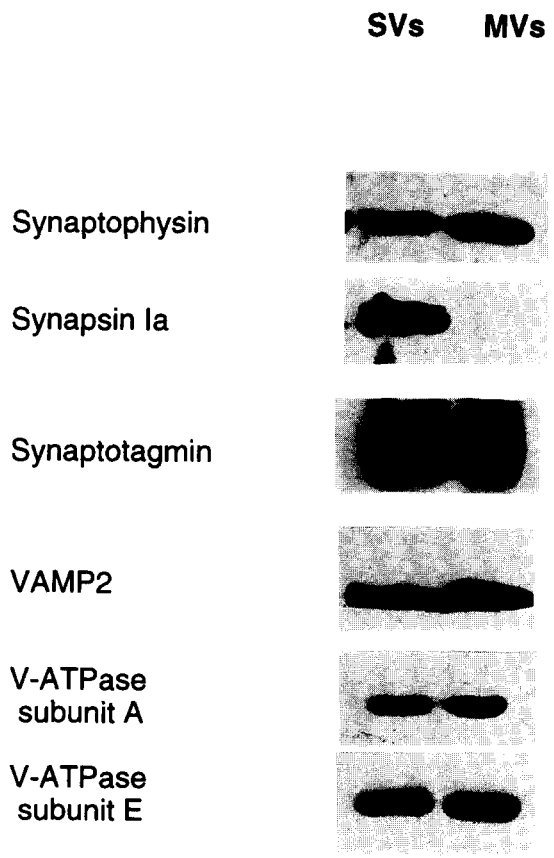


Fig. 3. Immunological comparison of membrane components of pineal MVs (right lane) and rat brain synaptic vesicles (SVs) (left lane). Samples (20 μg protein) were denatured by SDS sample buffer and were electrophoresed on 12.5% polyacrylamide gel as described in [13]. Following electrotransfer, the nitrocellulose paper was blocked, decorated with the listed antibodies with 1000–2000-fold dilutions, and the cross-reactivity was visualized with ECL detection kit (Amersham) [13].

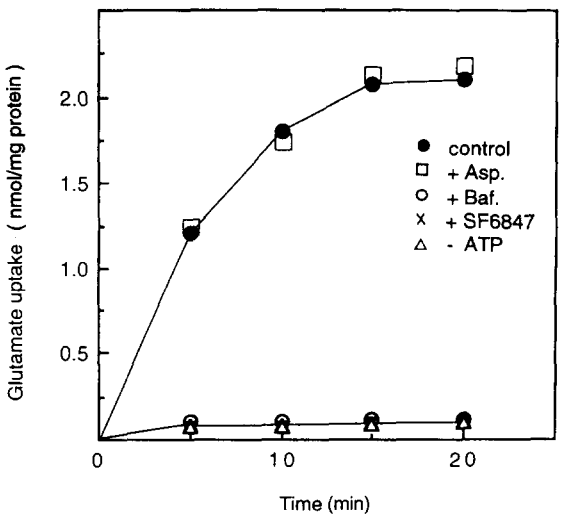


Fig. 4. ATP-dependent uptake of L-glutamate into pineal MVs. Time course for ATP-dependent uptake of [$^3$H]L-glutamate by purified MVs (50 μg) was measured in the presence of the listed compounds. Glutamate uptake in the absence of ATP was also shown. Additions: L-aspartate (5 mM), SF6847 (0.5 μM), bafilomycin A1 (20 nM).

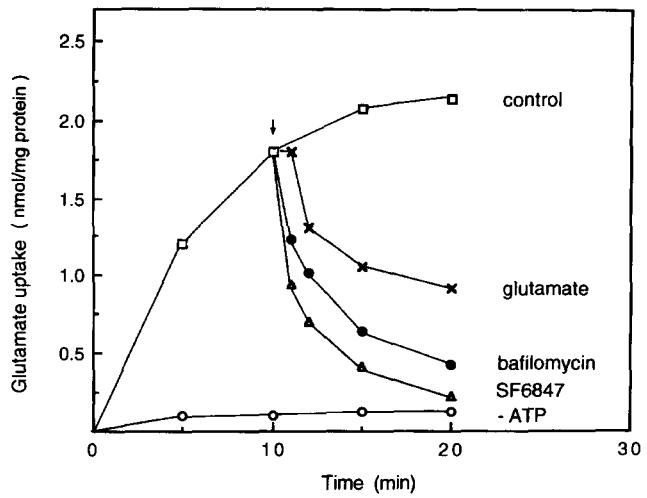


Fig. 5. Release of [$^3$H]L-glutamate from MVs. MVs were incubated with ATP and [$^3$H]L-glutamate as described in Fig. 4. At 10 min indicated by an arrow, SF6847 (0.5 μM), bafilomycin A1 (20 nM) or L-glutamate (1 mM) were added and [$^3$H]L-glutamate inside vesicles was assayed by filtration method. Time course of intravesicular [$^3$H]L-glutamate in the absence of ATP was also shown to indicate the background level.

MVs are involved in exocytosis. MVs were distinct from neuronal synaptic vesicles by their lack of synapsin I (Fig. 3), confirming previous histochemical result [10]. The lack of synapsin I in the MVs ruled out a possibility that synaptic vesicles derived from nerve endings projected into pineal gland are contaminated into the MVs fraction.

Pinealocytes were also rich in V-ATPase (Fig. 1B) and glutamate (Fig. 1C). As in the case of synaptophysin, V-ATPase and glutamate were especially abundant within particle-like structures in the process terminals. Immunoelectron microscopy indicated that V-ATPase and synaptophysin were present in the same MVs (Fig. 2B), presumably with similar density to brain synaptic vesicles (Fig. 3). Anti-V-ATPase antibody did not recognize larger membrane vesicles, indicating absence of V-ATPase in the contaminated vesicles. Since V-ATPase is the primary proton pump for storage of neurotransmitters in synaptic vesicles [23], these results suggested that glutamate is stored in MVs by the mechanism similar to synaptic vesicles. Actually, the MVs accumulated L-glutamate upon addition of ATP (Fig. 4). The ATP-dependent glutamate uptake was inhibited by bafilomycin A1, a V-ATPase inhibitor [24], or a proton conductor SF6847, indicating that the uptake was driven by an electrochemical proton gradient established by V-ATPase. L-Aspartate (5 mM), a substrate for $Na^+$-dependent glutamate transporter at plasma membrane [25], did not affect the glutamate uptake, indicating that the glutamate transporter is not plasma membrane-type. Maximum velocity of the glutamate uptake was 8.7 nmol/min/mg protein with 1.3 mM of $K_m$ value. These kinetic parameters were comparable to those of synaptic vesicle glutamate transporter [19,26,27]. The intravesicular glutamate was rapidly released from the vesicles by collapse of an electrochemical proton gradient by either bafilomycin A1 or SF6847, indicating that energization of MVs by V-ATPase is also necessary to maintain glutamate inside vesicles (Fig. 5). Addition of cold L-glutamate induced release of intravesicular radioactive glutamate, showing counterflow phenomenon (Fig. 5). From

these results, we concluded that vesicular glutamate transporter similar, if not identical, to that in synaptic vesicles [19,26,27] is operating in pineal MVs. To our knowledge this is the first example of vesicular glutamate transporter outside neuronal cells. No other transmitters including melatonin, serotonin, adrenaline, noradrenaline, $\gamma$-aminobutyrate, or acetylcholine were taken up by pineal MVs, suggesting that transporters for these transmitters are absent in pineal MVs.

Here, we showed evidence that pineal MVs accumulate L-glutamate by vesicular glutamate transporter energetically coupled with V-ATPase. Since MVs contain at least parts of protein components necessary for exocytosis (Fig. 3), MVs may store glutamate and extrude it by exocytosis. Process terminals and synaptic ribbon-region seem to be the sites of exocytosis of glutamate, because MVs are condensed in these areas [10]. Although further studies such as detection of the glutamate-driven intercellular signal transfer and precise localization of glutamate receptor will be necessary, the putative MVs-mediated signal transduction system may be important for understanding of the mechanism of regulation of melatonin secretion.

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