**Vesicular L-Glutamate Transporter in Microvesicles from Bovine Pineal Glands**

**DRIVING FORCE, MECHANISM OF CHLORIDE ANION ACTIVATION, AND SUBSTRATE SPECIFICITY***

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Pinealocytes, endocrine cells that synthesize and secrete melatonin, possess a large number of synaptic-like microvesicles (MVs) containing the L-glutamate transporter (Moriyama, Y., and Yamamoto, A. (1995) FEBS Lett., 367, 233–236). In this study, the L-glutamate transporter in MVs isolated from bovine pineal glands was characterized as to its driving force, requirement of anions, and substrate specificity. Upon the addition of ATP, the MVs accumulated L-glutamate. The uptake was significantly dependent on the extravesicular Cl⁻ concentration, being negligible in the absence of Cl⁻ and maximum at 2—5 mM and decreasing gradually at 20—100 mM. The membrane potential (inside positive) was maximum at 0—10 mM Cl⁻ and then decreased gradually depending on the Cl⁻ concentration, whereas a pH gradient was practically absent without Cl⁻ and increased gradually up to 100 mM Cl⁻. Ammonium acetate or nigericin plus K⁺, a dissipator of a pH gradient, had little effect on or was slightly stimulatory toward the uptake, whereas valinomycin plus K⁺ inhibited both formation of the membrane potential and the glutamate uptake to similar extents. The ATP- and Cl⁻-dependent glutamate uptake was inhibited by fluoride, iodide, or thiocyanate, without vacuolar H⁺-ATPase being affected. An anion channel blocker, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, similarly inhibited the glutamate uptake in a Cl⁻ protectable manner. Furthermore, ATP- and glutamate-dependent acidification of MVs was observed when 4 mM Cl⁻ was present. Among more than 50 kinds of glutamate analogues tested, only a few compounds, including 1-aminocyclohexane-trans-1,3-dicarboxylic acid, caused similar acidification. A good correlation was observed between the acidification and the inhibition of glutamate uptake by glutamate analogues. These results indicated that 1) the major driving force of the glutamate uptake is the membrane potential, 2) Cl⁻ regulates the glutamate uptake, probably via anion-binding site(s) on the transporter, and 3) the transporter shows strict substrate specificity. Hence, the overall properties of the vesicular glutamate transporter in the MVs well matched those of the synaptic vesicle glutamate transporter. We concluded that the vesicular glutamate transporter, being similar if not identical to the neuronal counterpart, operates in endocrine cells.

Pinealocytes are parenchymal endocrine cells of pineal glands that synthesize and secrete melatonin into the blood (1—3). At least two kinds of secretory vesicle-like organelles have been identified in pinealocytes: dense core granules, which are speculated to be involved in the storage and secretion of melatonin and some neuropeptides such as arginine vasotocin (3, 4), and a large number of synaptic-like microvesicles (MVs) containing synaptophysin (5). Although histochemical evidence indicated that MVs are distinct from neuronal synaptic vesicles in their lack of synapsin I, the possible participation of MVs in some secretory pathways in pinealocytes was also speculated (5).

Very recently, we established a procedure for isolating MVs from bovine pineal glands and found that these vesicles were actually devoid of synapsin I but possess synaptotagmin and synaptobrevin 2, proteins necessary for vesicular transport (6). Furthermore, the MVs specifically accumulated L-glutamate in an energy-dependent manner (6). These results suggested that MVs are the organelles in pinealocytes that store and secrete L-glutamate. Therefore, it seems likely that pinealocytes possess novel glutamate-evoked signal transducing systems.

To reveal the entire features of the putative MV-mediated signal transduction systems in pinealocytes, at first we focused on the glutamate transporter in MVs. So far, the ATP-dependent vesicular glutamate transporter has been identified in brain synaptic vesicles (7—13). Because the glutamate transporter in pineal MVs is the first example of a vesicular glutamate transporter outside neuronal cells, it is of interest to determine the mechanistic difference or similarity between glutamate transporters of the neuronal and endocrine origins. In this study, we characterized the glutamate transporter in pineal MVs and obtained evidence that it is quite similar to that in brain synaptic vesicles.

**EXPERIMENTAL PROCEDURES**

Isolation of MVs from Pineal Glands—The details are given in Ref. 6. In brief, bovine pineal glands (about 50 glands for one preparation) obtained from a local slaughterhouse within a few hours after killing were washed, cut into small pieces with scissors, suspended in 80 ml of SME buffer (20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose, 5 mM EDTA, 5 μM pepstatin A, and 5 μM leupeptin), and then homogenized in a Dounce homogenizer. The homogenate was centrifuged at 900 × g (R_max) for 10 min, and then the resultant supernatant was centrifuged at 11,000 × g (R_max) for 20 min. The resultant supernatant was centrifuged at 130,000 × g for 40 min. The resultant pellet was washed once, suspended in 2.5 ml of SME buffer (crude MV fraction), and then applied to a discontinuous sucrose density gradient (0.7 ml of

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§ The abbreviations used are: MV, synaptic-like microvesicle; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; ΔpH, transmembrane pH gradient; Δψmax, transmembrane potential difference; V-ATPase, vacuolar H⁺-ATPase; MOPS, 4-morpholinopropanesulfonic acid; oxonol-V, bis-3-phenyl-5-oxo-oxazaiso-4-yl)peptamethine oxonol.
1.8, 1.2, 1.0, 0.8, 0.6, and 0.4 mM sucrose containing 5 μg/ml peptatin A and 5 μg/ml leupeptin). After centrifugation at 207,700 × g (Rmax) for 3 h, four distinct bands were obtained. The top broad white band was collected, diluted 5-fold with SME buffer, and then centrifuged at 139,000 × g for 40 min. The pellet (purified MVs) was suspended in SME buffer and stored at −85°C until use. More than 95% of the vesicles in the final preparation were MVs (relatively clear small vesicles with an average diameter of 50 nm containing synaptophysin and V-ATPase), as revealed on conventional and immunoelectron microscopy using an anti-synaptophysin monoclonal antibody (6). The MVs were distinct from synaptic vesicles in their lack of synapsin I (6).

**Transport Assay**—Unless otherwise specified, the uptake of radioactive L-glutamate by MVs was assayed in 0.5 ml of 20 mM MOPS-Tris (pH 7.0) containing 4 mM KCl, 0.3 mM sucrose, 2 mM magnesium acetate, 2 mM ATP (Tris salt, pH 7.0), and MVs (about 50 μg of protein) (standard assay conditions). The assay was started at 30°C by the addition of radioactive glutamate (2.5 μCi, 0.1 mM). Aliquots (100 μl) were taken at intervals and filtered through 0.45 mM Millipore filters (type HA). After extensive washing, the remaining radioactivity on the filters was counted with a liquid scintillation counter.

The formation of ΔpH (inside acidic) was measured by means of acridine orange fluorescence quenching as described previously, the excitation and emission wavelength pair being 492 nm and 540 nm, respectively (11). Δψ (inside positive) was measured by means of oxonol-V fluorescence quenching (excitation, 580 nm; emission, 620 nm) (11).

Chemicals—L-[2,3-3H]Glutamate (638.3 GBq/mmol) was purchased from Amersham Corp. The glutamate analogues listed in Tables III and IV were from Tocris Neuramin, except for methyleneglutamate, γ-ethylglutamate, methylglutamate, aspartate, and γ-aminobutyrate, which were from Sigma. ATP (Tris salt) was from Sigma. DIDS was obtained from Dojindo Laboratories (Japan). Other chemicals were of the highest grade commercially available.

**RESULTS**

**Requirement of Cl⁻ for L-Glutamate Uptake by Pineal MVs—**MV purified from bovine pineal glands took up L-glutamate, which was coupled with an electrochemical proton gradient established by V-ATPase (6). During the assaying glutamate uptake by pineal MVs, we noticed that a low concentration of Cl⁻ significantly enhanced the L-glutamate uptake, as shown in Fig. 1A. Cation species were not responsible for the activation (data not shown). The Cl⁻ concentration required for the maximum glutamate uptake was 2–5 mM, with the uptake decreasing gradually at higher concentrations (Fig. 1B). Only 6% of the maximum activity was observed in the absence of Cl⁻ (Fig. 1B). Chloride and bromide anions were equally effective for the activation (Fig. 1C). The nitrate, iodide, and gluconate ions were slightly effective, whereas the acetate and bicarbonate ions were not effective, and the fluoride and thiocyanate ions were inhibitory (Fig. 1C). These results indicated that the ATP-dependent glutamate uptake by pineal MVs is anion-sensitive and requires Cl⁻.

The requirement of a low concentration of Cl⁻ may be one of the significant properties of the glutamate uptake. The following alternative mechanisms may possibly explain the activation: one is direct interaction of Cl⁻ with the glutamate transporter and another is the necessity of Cl⁻ for the formation of an electrochemical proton gradient, because V-ATPase is an anion-sensitive proton pump (14–16). To clarify the role of Cl⁻ in the glutamate uptake, we next analyzed the effects of anions on ATP-dependent membrane energization.

**Characteristics of ATP-dependent Membrane Energization of Pineal MVs—**Fig. 2 shows the Cl⁻ dependence of ATP-dependent formation of ΔpH (inside acidic) and Δψ (inside positive) in MVs, as measured with fluorescent indicators. A proton conductor, 3,5-di-tet-butyl-4-hydroxybenzylidene malononitrile (SF6847) (0.5 μM), or a V-ATPase inhibitor, bafilomycin A1 (50 nM), inhibited the formation of both ΔpH and Δψ, confirming that V-ATPase formed an electrochemical proton gradient across the MV membrane (Fig. 2, A and B). The formation of ΔpH was dependent on the exogenous Cl⁻ concentration; a little ΔpH was generated in the absence of Cl⁻, but it increased linearly up to 20 mM and then gradually to 100 mM Cl⁻ (Fig. 2C). On the other hand, Δψ was highest at 0–10 mM Cl⁻ and then decreased gradually depending on the Cl⁻ concentration (Fig. 2C). These results indicated that the formation of Δψ prevented the formation of ΔpH electrically in the absence of Cl⁻, and that the collapse of Δψ due to Cl⁻ uptake, possibly through an anion channel or anion transporter, induced further acidification, as in the case of other endomembrane systems (16–18). This was confirmed by the observation that Br⁻,
another substrate of the endomembrane anion channel (transporter) (19), also induced the ATP-dependent acidification, whereas other anions, such as sulfate or acetate, which are not substrates for the anion channel, did not (Fig. 2A).

Energetics of ATP-dependent and Cl⁻-activated Glutamate Uptake—The dose-dependent effects of Cl⁻ on the formation of ΔpH and Δψ differed from those of the glutamate uptake (Fig. 1C), indicating clearly that ΔpH and Δψ are not simply linked to the Cl⁻-activated glutamate uptake. Two alternative possibilities as to the role of Cl⁻ can be considered. One is that Δψ is the driving force and Cl⁻ acts as a co-factor or a coupling anion for the uptake. The other possibility is that Δψ is a major driving force but that the small ΔpH present at a low concentration of Cl⁻ is also necessary for the uptake.

Then, we compared the effects of ionophores and the ammonium ion on the glutamate uptake, ΔpH and Δψ (Table I). To control the magnitude of ΔpH and Δψ due to ionophores, 0.1M potassium acetate was included in the assay medium as a K⁺ source, because this salt did not affect the Cl⁻-stimulated glutamate uptake. Nigericin or ammonium acetate dissipated ΔpH without affecting or slightly increasing Δψ. Under these conditions, glutamate uptake was not affected or slightly stimulated. Valinomycin, on the other hand, partially abolished Δψ and inhibited glutamate uptake to similar extents. The combination of valinomycin and nigericin or ammonium acetate dissipated both ΔpH and Δψ, resulting in almost complete inhibition of the glutamate uptake. These results indicated that the magnitude of glutamate uptake into MVs is correlated with the magnitude of Δψ but not that of ΔpH. Thus, Δψ seems to be the driving force for glutamate uptake in pineal MVs, although we

buffer (pH 7.0) containing 0.3M sucrose, 2mM magnesium acetate, 2μM acridine orange, and 20μg of protein of purified MVs in the presence of the indicated salts (0.1M), if otherwise stated. In an experiment shown in K-acetate + KCl (4 mM) and KCl + K-acetate, potassium acetate (0.1M) plus KCl (4 mM) and KCl plus potassium acetate (0.1M each) were included in the assay mixture, respectively. The increase in the acridine orange fluorescence on the addition of ATP was omitted from the figure because it is an artifact due to the interaction of the dye and ATP (46). B, oxonol-V fluorescence quenching was measured in the same buffer as in A except that 5μM oxonol-V was used instead of acridine orange. C, acridine orange (closed circles) and oxonol-V (open circles) fluorescence quenching were measured in the presence of the indicated concentrations of KCl and expressed as relative values, taking maximum quenching as 100%. Other additions: 2μM valinomycin, 2μM nigericin.

### Table I

| Reagents | Uptake | Δψ | ΔpH |
|----------|--------|----|-----|
| None     | 100    | 100| 100 |
| Valinomycin | 67 | 63 | 100 |
| Nigericin | 109 | 110| 0   |
| NH₄-acetate (2 mM) | 116 | 130| 0   |
| NH₄-acetate (10 mM) | 121 | 149| 0   |
| Valinomycin + NH₄-acetate (2 mM) | 44 | 30| 0   |
| Valinomycin + nigericin | 0 | 0| 0 |

*As shown in Fig. 2A, the magnitude of ΔpH under the standard condition is 5% of that in the presence of 0.1M KCl.

![Figure 2](image-url)
could not completely rule out the participation of a small ΔpH in the glutamate transport.

Characteristics of a Putative Anion-binding Site in the Glutamate Transporter—The stimulatory effect of Cl$^-$, therefore, suggests the presence of a Cl$^-$-binding site in the glutamate transporter. To reveal the properties of this putative Cl$^-$-binding site, we investigated the effects of various anions on the Cl$^-$-stimulated glutamate uptake. As shown in Fig. 3A, the fluoride, iodide, thiocyanate, and nitrate ions were all inhibitory, whereas other anions had little effect. The inhibitory effect of the nitrate ion was due to a decrease in the magnitude of the driving force (loss of Δψ) due to inhibition of V-ATPase (Fig. 3B). On the other hand, the fluoride and thiocyanate ions under the concentrations used in this experiment had little effect on Δψ (Fig. 3B), suggesting that the inhibition was due to direct interaction with the glutamate transporter. The iodide ion had a similar effect (not shown). The inhibition was reversible, and full activity was recovered after washing of the membranes (not shown).

To find compounds that interact with the anion-binding site(s) irreversibly or that are more potent than thiocyanate, we tested several kinds of anion channel blockers. DIDS was found to be a strong inhibitor of the glutamate uptake (Table II). DIDS also inhibited V-ATPase (19, 20), the ID$_{50}$ value being 3.8 μM. However, the ID$_{50}$ value on inhibition of the glutamate uptake was only 0.3 μM. Furthermore, inhibition of the glutamate uptake by DIDS was prevented by Cl$^-$ but not by acetate (Table II). Taken together, these results suggested that the Cl$^-$-binding site(s) in the glutamate transporter can be occupied by F$^-$, thiocyanate, or DIDS. The chloride-binding site(s) may be important for regulation of the uptake activity.

Substrate Specificity and Inhibitors of the Cl$^-$-activated Glutamate Transporter—It is difficult to elucidate the substrate specificity of the glutamate transporter, because only a limited number of radioactive glutamate analogues is available commercially. Even in the case of the synaptic vesicle glutamate transporter, the substrate specificity was evaluated indirectly by measuring the spectrum of inhibition of glutamate uptake in the presence of glutamate analogues (7, 22). As in the case of the brain synaptic vesicle glutamate transporter (9–11), we noticed that the combined addition of glutamate and ATP caused acidification in MVs when 4 mM Cl$^-$ was present in the assay medium (Fig. 4). The acidification was sensitive to bafilob-
mycin A1 (50 nM). Furthermore, the rate of acidification became saturated depending on the glutamate concentration, the apparent \( K_m \) value being 3 mM, which is comparable with the \( K_m \) value (1.3 mM) obtained using the radioisotopes (6). Some glutamate analogues, such as 1-aminocyclohexane-trans-1,3-dicarboxylic acid, showed similar ATP and Cl\(^{-}\) dependent acidification (Fig. 4d). These results indicated that the Cl\(^{-}\) and glutamate-dependent acidification reflected glutamate uptake by MVs; the uptake of the glutamate anion through the glutamate transporter dissipated \( \Delta \psi \), which in turn accelerated the formation of \( \Delta \phi \). Therefore, we can estimate the kinetic parameters for the uptake of glutamate analogues by measuring the rate of analogue-evoked acidification.

Table III shows the degree of acidification and the \( K_m \) values obtained on glutamate analogue-induced acidification as described above. Aspartate, a substrate for the Na\(^{+}\)-dependent glutamate transporter in plasma membranes (23), was not effective. Again, cyclic glutamate analogues were relatively good substrates, with higher affinity than glutamate. The cis forms of these cyclic compounds were all ineffective. Four other compounds (D-glutamate, \( \alpha \)-methyl-D,L-glutamate, \( \gamma \)-methylene-D,L-glutamate, and D,L-2-amino-4-phosphonobutyrinic acid) caused low acidification, suggesting that these compounds act as less efficient substrates.

Inhibition of glutamate uptake by the same compounds was also examined (Table IV). Consistent with the acidification, cyclic glutamate analogues (trans form) strongly inhibited the glutamate uptake. Compounds causing acidification always inhibited glutamate uptake with a similar order of effectiveness. Other compounds, including sulfur-containing amino acids, which inhibited glutamate uptake in synaptic vesicles (22, 24), neither affected the uptake nor caused acidification (Table III). At more than 5 mM, these sulfur-containing amino acids partially inhibited the glutamate uptake, but the inhibition was due to the decreased driving force upon inhibition of V-ATPase (data not shown). The following glutamate analogues also neither affected glutamate uptake nor caused acidification: L-glutamate amide, D,L-hydroxyglutaric acid, 2,4-dinitrophenyl-L-glutamate, N-phthaloyl-D,L-glutamic acid, N-carboxymethylglutamic acid, N-acetyl-L-glutamic acid, N-phenacetyl-D,L-glutamic acid, N-methylpyro-D,L-glutamic acid, N-isopropylpyro-D,L-glutamic acid, ethyl-D-3-carboxy-3-pyrrolidinyl-5-carboxylate, N-trimethyl-D,L-\( \gamma \)-glutamate, butyl-D,L-pyroglutamate, N-dimethyl-D,L-hydroxyglutamate, N-caproyl glutamate, \( \beta \)-oxo glutamate, \( \beta \)-hydroxyglutamate, N-benzoylcarbamoylglutamate, N-toluene sulfonyl-L-glutamate, and S-carboxymethyl-L-cysteine. 1-Amino-trans-3-phosphopyrophosphate carboxylic acid inhibited about 40% of the glutamate uptake without acidification (Table III), suggesting that this compound may bind to the transporter but not be transported. Taken together, these results suggested that the glutamate transporter in pineal MVs shows strict substrate recognition; a compound on the replacement of an amino group and a change in the carbon...
Glutamate, an excitatory neurotransmitter, is stored in synaptic vesicles, is extruded into the synaptic cleft upon stimulation, and binds to its receptors present in postsynaptic membranes so as to transmit signals intercellularly (25, 26). The vesicular glutamate transporter in synaptic vesicles is responsible for the storage of glutamate in neurons (27–30). Like other neurotransmitter transporters, the glutamate transporter is energetically coupled with V-ATPase (27–30) but uses $\Delta \psi$ as the major driving force (9–11, 31, 32). The chloride anion is suggested to regulate the transport activity via anion-binding site(s) (21). However, the properties of this transporter at the molecular level remain obscure because neither purification of the transporter nor cloning of its cDNA have been successful. One of the aims of this study was to find a new approach for this fascinating transporter, because a comparative study will be possible if the vesicular glutamate transporter in pineal MVs resembles the synaptic vesicle counterpart.

As summarized, the glutamate transporter in pineal MVs is driven by $\Delta \psi$ (positive inside) (Fig. 2 and Table I). The spectrum of the requirement of anions for the uptake activity was essentially the same as that of the synaptic vesicle counterpart (7). The activation by Cl$^-$ or Br$^-$ could be attributed to their direct interaction with the transporter, presumably via an anion-binding site (Fig. 3 and Table II). At present, it is difficult to prove that Cl$^-$ acts as a counter ion for chloride and glutamate co-transport, because we cannot demonstrate Cl$^-$ movement through either the glutamate transporter or the Cl$^-$ channel in MVs. Furthermore, we showed that the substrate specificity of the vesicular glutamate transporter was quite similar to that of the synaptic vesicle counterpart, as judged from the inhibition of glutamate uptake by glutamate analogues (7, 22). The overall properties of the glutamate transporters in synaptic vesicles and pineal MVs are similar to each other. We concluded that a glutamate transporter that is similar, if not identical, to the synaptic vesicle counterpart operates in MVs. Thus, pineal MVs constitute another experimental system for studies on the vesicular glutamate transporter.

Pineal MVs contain synaptobrevin and synaptotagmin, proteins important for vesicular transport (6). Recently, we found that pinealocytes express relatively high concentrations of N-ethylmaleimide-sensitive fusion protein, Ca$^{2+}$-channel protein, and smg25A (a small GTP-binding protein). Some of N-ethylmaleimide-sensitive fusion protein and smg25A was found to be associated with MVs. These results further support that MVs are the organelles that store glutamate and are involved in the glutamate-evoked signal transduction system. Released glutamate may bind to the glutamate receptor identified in pinealocytes (33, 34) and may inhibit noradrenaline-stimulated N-acetylaspartase and hydroxyindole-O-methyltransferase activities, resulting in inhibition of melatonin synthesis (35–37).

Finally, we should point out the similarity between pinealocytes and retinal photoreceptor cells. Pinealocytes accumulate glutamate inside MVs and may extrude it through exocytosis, possibly at process terminals and/or the synaptic ribbon region (Ref. 6 and this paper). Similarly, retinal photoreceptor cells use glutamate as a transmitter possibly via synaptic vesicle-mediated exocytosis at the ribbon synapse (38, 39). Both types of cells lack of synapsins, suggesting that pineal MVs and retinal synaptic vesicles move to their release sites though a novel mechanism (40). Furthermore, pinealocytes, especially from lower vertebrates, can receive photosignals via photoreceptive molecules such as pinopsin (41). These profound mechanistic similarities will be useful for understanding the mechanism underlying the signal transduction in pineal glands and retinal cells.

MVs from pancreatic $\beta$ cells (42), adrenal chromaffin cells (43), PC12 cells (44), and posterior pituitaries (45) have been shown to accumulate $\gamma$-aminobutyrate, noradrenaline, acetylcholine, and noradrenaline, respectively. Upon stimulation, these transmitters may be extruded from the cells and then transmit signals intercellularly. Therefore, the presence of V-ATPase and the neurotransmitter transporter in MVs may be one of the usual features of endocrine cells.
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