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# Artificially imposed electrical potentials drive L-glutamate uptake into synaptic vesicles of bovine cerebral cortex

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L-Glutamate is a major excitatory neurotransmitter in the central nervous system. MgATP-dependent glutamate uptake and $H^+$-pumping ATPase activity were reported in highly purified synaptic vesicles [Naito & Ueda (1983) J. Biol. Chem. **258**, 696–699; Shioi, Naito & Ueda (1989) Biochem. J. **258**, 499–504], and it is hypothesized that an electrochemical $H^+$ gradient across the vesicle membrane, the so-called protonmotive force, elicits the neurotransmitter uptake. An inside-positive diffusion potential across the vesicle membrane was established with valinomycin plus $Rb^+$. This artificial electrical potential promoted the uptake of glutamate, but not aspartate, in the synaptic vesicles prepared from bovine cerebral cortex. The uptake was inhibited by the protonmotive-force dissipators carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or nigericin, and was enhanced by concomitant imposition of a pH jump (alkalinization) in the external medium. Subcellular and subvesicular distributions showed the uptake system to be predominantly associated with small synaptic vesicles. The results support the hypothesis that glutamate uptake into synaptic vesicles is coupled with a $H^+$ efflux down the electrochemical potential gradient, which is generated by $H^+$-pumping ATPase.

## INTRODUCTION

Glutamate is ubiquitously distributed in brain tissue, where it is present in high concentrations as compared with other amino acids. Glutamate has now been accepted as a major excitatory transmitter in the brain (for reviews see Fonnum, 1984; Cotman *et al.*, 1987; Robinson & Coyle, 1987; Takeuchi, 1987).

Naito & Ueda (1983, 1985) have isolated small, spherical synaptic vesicles from cerebral cortex, by using antibodies to a synaptic-vesicle-specific protein (Naito & Ueda, 1981), and have demonstrated that glutamate is taken up by these vesicles in an ATP-dependent manner. This glutamate-uptake system *in vitro* has been shown in synaptic vesicles derived from glutamatergic neurons, but not from $\gamma$-aminobutyric acid (GABA)-ergic neurons (Fischer-Bovenkerk *et al.*, 1988). Immunocytochemical studies have implicated glutamate to be concentrated in vesicles of nerve terminals (Storm-Mathisen *et al.*, 1983; Riveros *et al.*, 1986), and physiological studies identified an exocytotic pool of glutamate in synaptosomes capable of rapid $Ca^{2+}$-dependent release (Nicholls & Sihra, 1986; Nicholls *et al.*, 1987; Sanchez-Prieto *et al.*, 1987). Therefore there is a growing body of evidence for the hypothesis that glutamate is released by exocytosis during synaptic transmission.

In addition, Naito & Ueda (1985) reported that the vesicular uptake of glutamate is inhibited by $H^+$-gradient dissipaters. They suggested that the uptake is driven by an electrochemical potential difference of $H^+$ across the membrane, the so-called 'protonmotive force', that is generated by a vesicular $H^+$-pumping ATPase. Although the concept of $H^+$-coupled biogenic-amine transport into storage organelles, such as chromaffin granules, is well established (Njus *et al.*, 1986), little is known about the mechanism of uptake of neuroactive amino acids by storage vesicles in the central nervous system.

In order to demonstrate chemi-osmotic coupling between ATP hydrolysis and glutamate transport into the synaptic vesicles, it is essential to satisfy three criteria: (1) agents capable of dissipating the protonmotive force inhibit ATP-dependent transport; (2) ATP hydrolysis generates protonmotive force; and (3) protonmotive force imposed by means other than ATP also induces glutamate transport. Naito & Ueda (1985) provided evidence satisfying the first criterion as mentioned above (see also reports by Maycox *et al.*, 1988; Carlson *et al.*, 1989; Fykse *et al.*, 1989; Cidon & Sihra, 1989). Maycox *et al.* (1988) and Cidon & Sihra (1989) reported a large $\Delta$pH across the synaptic-vesicle membrane generated by ATP at high $Cl^-$ concentrations (100–150 mM). They also observed a small $\Delta$pH but a large membrane potential ($\Delta\psi$), and a maximal uptake of glutamate at low $Cl^-$ concentrations (4–10 mM, a range of the physiological cytoplasmic concentrations). Independently, we have reported on the MgATP-dependent generation of a protonmotive force, with $\Delta\psi$ as the major component, in synaptic vesicles, and on the quantitative correlation of $\Delta\psi$ and ATP-dependent glutamate uptake in a variety of conditions (Shioi *et al.*, 1989). These findings substantiate the second criterion. The present study specifically addresses the third criterion. Using valinomycin and $Rb^+$, we demonstrate the uptake of glutamate to be driven by an artificially imposed $\Delta\psi$ across the synaptic-vesicle membrane. This valinomycin/$Rb^+$-dependent uptake is sensitive to $H^+$-gradient dissipaters and is enhanced by a concomitant pH jump (alkalinization) in the external medium. The results support the hypothesis that glutamate uptake into synaptic vesicles is coupled with $H^+$ efflux down an electrochemical potential gradient across the membrane through a putative $H^+$/glutamate transporter.

## EXPERIMENTAL

### Materials

L- and D-glutamic acids, L-aspartic acid, ATP (Tris salt), $RbNO_3$, valinomycin and *NN'*-dicyclohexylcarbodi-imide (DCCD) were purchased from Sigma (St. Louis, MO, U.S.A.). RbCl was obtained from Alpha Products (Danvers, MA, U.S.A.). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and nigericin were generously provided by Dr. R. Holz, Department of Pharmacology, University of Michigan.

Abbreviations used: protonmotive force, the transmembrane electrochemical potential difference of proton; $\Delta$pH, $\Delta\psi$, the transmembrane pH and electrical potential difference respectively; GABA, $\gamma$-aminobutyric acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *NN'*-dicyclohexylcarbodi-imide.

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L-[G-$^3$H]Glutamic acid (39 Ci/mmol) and L-[2,3-$^3$H]aspartic acid (16.3 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, U.S.A.).

#### Preparation of synaptic vesicles and other subfractions

Preparation of synaptosomes from bovine cerebral cortex and the subsequent preparation of synaptic vesicles by osmolysis and sucrose-density-gradient centrifugation were described in our previous report (Shioi *et al.*, 1989). However, the immunoprecipitation step was omitted (see the Results and discussion section).

Vesicle subfractions were obtained after the discontinuous sucrose-density-gradient centrifugation of the synaptosome lysate, through which the synaptic vesicles were also collected (Shioi *et al.*, 1989). The band in each layer was diluted with cold deionized water to give a final sucrose concentration of 0.25 M, and spun at 150000 $g_{av.}$ for 50 min. The pellets were resuspended in 0.32 M-sucrose containing 1 mM-$NaHCO_3$ and stored at −70 °C, as for the synaptic-vesicle preparation (Shioi *et al.*, 1989).

#### Assay of vesicular glutamate uptake

The ATP-dependent uptake of glutamate into synaptic vesicles and other vesicle subfractions was assayed essentially as described previously (Naito & Ueda, 1985). Briefly, vesicles (20 $\mu$g of protein) were incubated with 0.25 M-sucrose, 5 mM-Tris/maleate, pH 7.4, 4 mM-KCl, 50 $\mu$M-[$^3$H]glutamate (sodium salt, 0.13 Ci/mmol) and 4 mM-$MgSO_4$ in the absence or presence of 2 mM-ATP for 1.5 min at 30 °C. For $Na^+$-dependent uptake, the uptake medium (final volume 100 $\mu$l) contained 0.25 M-sucrose, 5 mM-Tris/maleate, pH 7.4, and synaptic vesicles (20 $\mu$g of protein). The mixture of vesicles and buffer (80 $\mu$l) was preincubated for 5 min at 30 °C. Then L-[$^3$H]glutamate or L-[$^3$H]aspartate (10 $\mu$l of potassium salt solution; final concn. 50 $\mu$M; 0.13 Ci/mmol) and NaCl (10 $\mu$l of 1 M solution; final concn. 100 mM) were added, and the mixture was incubated further for 1.5 min. Control experiments were done without NaCl. For valinomycin/$Rb^+$-dependent uptake, the medium (final volume 100 $\mu$l) contained 0.25 M-sucrose, 5 mM-Tris/maleate, pH 7.4, 0.5 $\mu$M-valinomycin and synaptic vesicles (generally 100 $\mu$g of protein). After preincubation of the mixture in 80 $\mu$l for 5 min at 30 °C, L-[$^3$H]glutamate or L-[$^3$H]aspartate (10 $\mu$l of sodium salt solution; final concn. 10 $\mu$M; 3 Ci/mmol) was added and the mixture incubated for 15 s. RbCl or one of the other salts (10 $\mu$l of 1 M solution; final concn. 100 mM) was then added and the mixture incubated for 20 s. Control experiments were done without valinomycin. The uptake increased linearly with increased vesicle concentrations up to 125 $\mu$g of protein per 100 $\mu$l. The glutamate uptakes were stopped by the addition of 2 ml of ice-cold 0.15 M-KCl solution and immediate filtration through a Millipore HAWP filter (25 mm diam., 0.45 $\mu$m pore size). The test tube (10 mm × 75 mm) was washed with 3 × 2 ml of KCl solution, the filter was washed a further three times with the same solution, and radioactivity retained in the filter was determined. At the same time, non-specific binding of glutamate or aspartate was determined in each experiment by filtering vesicles first, then [$^3$H]glutamate or aspartate second, and by measuring the radioactivity retained on the filter as described previously (Naito & Ueda, 1983). The uptake was calibrated for this binding. Inhibitors such as FCCP and nigericin were added during the preincubation period. ATP-dependent glutamate uptake was generally performed in duplicate, and $Na^+$-dependent and valinomycin/$Rb^+$-dependent uptake in triplicate, and the results were expressed as means ± S.E.M. (or range). Valinomycin, FCCP and nigericin were dissolved in ethanol. In experiments in which these reagents were used, the final concentration of ethanol was 0.15 % (v/v) or less. The control assay mixture contained the corresponding amount of ethanol.

#### Lineweaver–Burk analysis of valinomycin/$Rb^+$-dependent glutamate uptake

Since 5 s or 10 s uptakes were too small to produce reliable and reproducible values, we could not obtain the accurate initial velocity of the uptakes. Alternatively, we utilized 20 s time points to approximate to the uptake velocities. The uptakes at various glutamate concentrations and the corresponding glutamate concentrations were double-reciprocally plotted to obtain an approximate $K_m$.

## RESULTS AND DISCUSSION

#### Valinomycin/$Rb^+$-dependent uptake of glutamate into synaptic vesicles

In the presence of $K^+$ or $Rb^+$, valinomycin gives vesicles a $K^+$- or $Rb^+$-diffusion potential, $\Delta\psi$. It is expressed as below:

$$\Delta\psi = -(RT/F)\ln([C^+]_{in}/[C^+]_{out})$$

where $R$ is the gas constant, $T$ the absolute temperature, $F$ the Faraday constant, $[C^+]_{in}$ and $[C^+]_{out}$, intra- and extra-vesicular activities (approximated by concentrations) of $K^+$ or $Rb^+$ respectively. An inside-positive electrical potential across the membrane can be created by addition of valinomycin and $Rb^+$. Therefore, valinomycin/$Rb^+$-dependent glutamate uptake is predicted from the hypothesis that glutamate uptake is driven by the protonmotive force. The time courses of ATP-dependent L-glutamate uptake and of valinomycin/$Rb^+$-dependent L-glutamate uptake by synaptic vesicles are shown in Fig. 1. Both experiments were carried out with the same vesicle preparation. Synaptic vesicles showed some basal uptake in the absence of valinomycin and RbCl (Fig. 1*a*). Although most of the endogenous glutamate was lost during the preparation process (J. Shioi & T. Ueda, unpublished work), it is probable that the

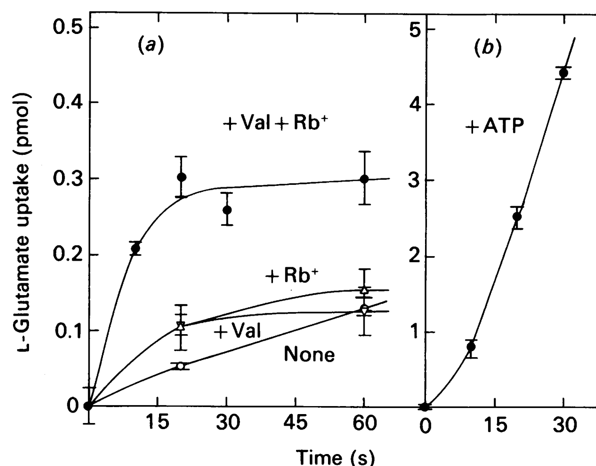



**Fig. 1. Valinomycin/$Rb^+$-dependent and ATP-dependent uptakes of L-glutamate into synaptic vesicles**

(*a*) The synaptic vesicles (50 $\mu$g of protein) were incubated at 30 °C in 10 $\mu$M-L-[$^3$H]glutamate with both 100 mM-RbCl and 0.5 $\mu$M-valinomycin (●), 100 mM-RbCl alone (△), 0.5 $\mu$M-valinomycin alone (▽) or no addition (○) for the times indicated, and the amount of glutamate retained in the vesicles was determined (see the Experimental section). Experiments were done in triplicate and expressed as means ± S.E.M. (*b*) The synaptic vesicles (50 $\mu$g of protein) were incubated with 2 mM-ATP, 4 mM-$MgSO_4$ and 10 $\mu$M-L-[$^3$H]glutamate for the times indicated. Experiments were done in duplicate and are expressed as means ± range.

**Table 1. Cation and substrate specificity for valinomycin-dependent uptake system**

Synaptic vesicles (100 $\mu$g of protein) were first preincubated in the absence or presence of 0.5 $\mu$M-valinomycin in 0.25 M-sucrose/5 mM-Tris/maleate (pH 7.4) for 5 min at 30 °C. Then L-[$^3$H]glutamate or L-[$^3$H]aspartate (final concn. 10 $\mu$M; 3 Ci/mmol) was added. After 15 s incubation, uptake was initiated by the addition of chloride salt (final concn. 100 mM) of the indicated cation and was allowed to continue for 20 s. Values are means ± S.E.M. of three determinations.

| Uptake of: | Cation | Uptake (pmol) | | |
|---|---|---|---|---|
| | | No valinomycin | + Valinomycin | Difference |
| L-[$^3$H]Glutamate | Control ($H_2O$) | 0.073 ± 0.005 | 0.096 ± 0.023 | 0.023 ± 0.028 |
| | $Li^+$ | 0.096 ± 0.026 | 0.181 ± 0.011 | 0.085 ± 0.034 |
| | $Na^+$ | 0.221 ± 0.053 | 0.261 ± 0.016 | 0.040 ± 0.068 |
| | $K^+$ | 0.063 ± 0.018 | 0.348 ± 0.038 | 0.285 ± 0.052 |
| | $Rb^+$ | 0.069 ± 0.005 | 0.515 ± 0.036 | 0.446 ± 0.044 |
| | $Cs^+$ | 0.050 ± 0.009 | 0.360 ± 0.015 | 0.310 ± 0.021 |
| | Choline$^+$ | 0.045 ± 0.008 | 0.011 ± 0.005 | −0.034 ± 0.011 |
| L-[$^3$H]Aspartate | $Rb^+$ | 0.026 ± 0.021 | 0.044 ± 0.005 | 0.018 ± 0.026 |

radioactive exogenous glutamate exchanges with the minor amount of the endogenous glutamate inside the vesicles. Only the combination of valinomycin and $Rb^+$ caused a significantly enhanced uptake of L-glutamate into synaptic vesicles. Although ATP-dependent glutamate uptake increased linearly for 1.5 min after ATP addition (Naito & Ueda, 1983), valinomycin/$Rb^+$-dependent glutamate uptake reached a steady state at about 20 s after the addition of RbCl, presumably as a result of a rapid dissipation of the imposed $\Delta\psi$.

### Cation specificity for valinomycin-dependent glutamate uptake

As shown in Table 1, $Rb^+$, $K^+$ and $Cs^+$ gave significant uptakes in a valinomycin-dependent manner; however, $Na^+$, $Li^+$ and choline ion did not (see the far right column in Table 1). The observed cation-species dependency was exactly as expected from the reported cation specificity of valinomycin with regard to its ionophoretic activity in a membrane (Reed, 1979). Since $K^+$ and $Cs^+$ also gave increased uptake, it is not likely that intravesicular $Rb^+$ itself activated the glutamate-uptake system. A logarithmic increase in $Rb^+$ concentration gave a linear increase in the valinomycin-dependent glutamate uptake (Fig. 2), consistent with a logarithmic concentration-dependency of $Rb^+$ diffusion potential expressed in the formula shown above. The results indicate $\Delta\psi$ to be the driving force for the uptake.

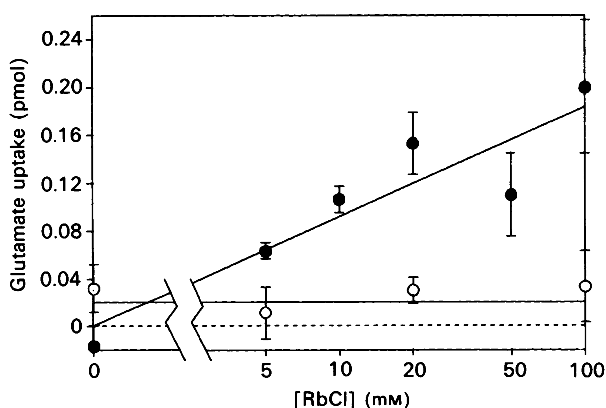



**Fig. 2. Valinomycin/$Rb^+$-dependent glutamate uptake at various concentrations of RbCl**

L-Glutamate uptake into synaptic vesicles (100 $\mu$g) was measured at 20 s at 30 °C in the presence of various concentrations of RbCl and 50 $\mu$M-L-[$^3$H]glutamate without (○) or with (●) 0.5 $\mu$M-valinomycin. The vesicular uptake was plotted as a function of RbCl concentration in a logarithmic scale.

### Carrier-mediated glutamate uptake

Radioactive L-aspartate was not transported in the synaptic vesicles in a valinomycin/$Rb^+$-dependent manner (Table 1), excluding the possibility of a simple binding of the substrate by its electric charge. Glutamate concentration-dependency showed typical saturation kinetics. The Lineweaver–Burk analysis yielded an approximate $K_m$ of 1.7 mM-L-glutamate for the valinomycin/$Rb^+$-dependent uptake system (results not shown), implying a specific carrier with an affinity to L-glutamate. The approximate $K_m$ value is in agreement with the $K_m$ of the ATP-dependent glutamate uptake (1.6 mM; Naito & Ueda, 1983), but quite different from that of $Na^+$-dependent glutamate uptake (1.9 $\mu$M, Bennett *et al.*, 1974; or 3.0 $\mu$M, Kanner & Sharon, 1978).

An attempt to show valinomycin/$Rb^+$-dependent uptake of glutamate into antibody-purified synaptic vesicles (Naito & Ueda, 1983) was unsuccessful. Antibody-aggregated synaptic vesicles blocked the filter membrane at the high concentration (100 $\mu$g of vesicle protein/100 $\mu$l) of vesicles employed in this assay system. To ensure that this valinomycin/$Rb^+$-dependent uptake system was specifically associated with synaptic vesicles, subcellular and subvesicular distributions were studied. The uptake activities in different subfractions from the sucrose gradient of the osmolysed synaptosome preparation are shown in Fig. 3. The boundary of 0.32 M-/0.4 M-sucrose and the 0.4 M-sucrose layer are known to be rich in synaptic vesicles; the boundary of 0.4 M-/0.6 M-sucrose has mainly large synaptic vesicles; both 0.6 M- and 0.8 M-sucrose layers contain myelin and synaptic plasma membrane: mitochondria precipitate at the bottom (Whittaker & Barker, 1972; Ueda *et al.*, 1979). ATP-dependent L-glutamate uptake was highest in a combined fraction of the 0.32 M-/0.4 M-sucrose boundary and the 0.4 M-sucrose layer. The valinomycin/$Rb^+$-dependent uptake system had a similar distribution in the sucrose density gradient to the ATP-dependent system, but differed from the $Na^+$-dependent system (Fig. 3). Synaptosomes did not show any valinomycin/$Rb^+$-dependent glutamate uptake, but showed an inhibition of $Na^+$-dependent glutamate uptake by valinomycin in the presence of $Rb^+$ (results not shown). The inhibition is consistent with the electrogenic uptake of glutamate in plasma membranes (see below). These results indicate that the valinomycin/$Rb^+$-depen-

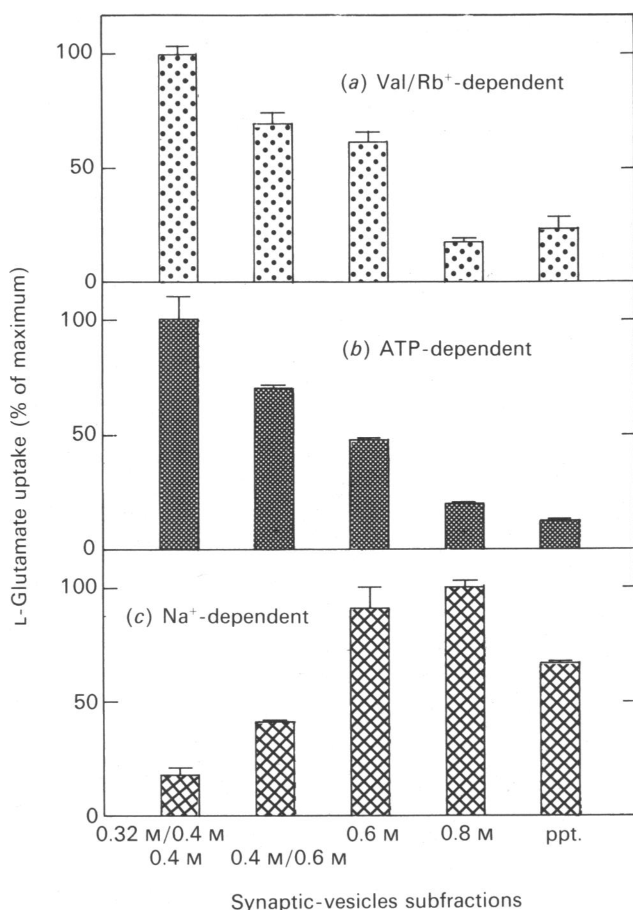



**Fig. 3. $Na^+$-dependent, ATP-dependent and valinomycin/$Rb^+$-dependent L-glutamate uptakes in synaptic-vesicle subfractions**

Vesicles were subfractionated by sucrose-density-gradient centrifugation of the osmolysed synaptosome preparation and were assayed for their uptake activities as described in the Experimental section. Valinomycin (Val)/$Rb^+$-dependent uptake (*a*): the differences between the uptakes in the presence and absence of valinomycin were calculated and normalized with the highest value ($0.506 \pm 0.018$ pmol) obtained from a combined fraction from the 0.32 M-/0.4 M-sucrose boundary and the 0.4 M-sucrose layer. Experiments were done in triplicate. ATP-dependent uptake (*b*): the differences between the uptakes in the presence and absence of ATP are shown normalized with the value ($9.99 \pm 0.97$ pmol) obtained from the combined fraction of the 0.32 M-/0.4 M-sucrose boundary and the 0.4 M-sucrose layer. Experiments were done in duplicate. $Na^+$-dependent uptake (*c*): the differences between the uptakes in the presence and absence of NaCl are shown normalized with the value ($7.48 \pm 0.03$ pmol) obtained from a fraction of a 0.8 M-sucrose layer. Experiments were done in triplicate. Values on the abscissa are sucrose concentrations.

dent glutamate-uptake system studied was associated with synaptic vesicles, but not with plasma membranes or mitochondria.

It is known that a synaptic plasma membrane takes up L-glutamate and L- and D-aspartate through the same high-affinity uptake system in a $Na^+$-dependent manner (Logan & Synder, 1972; Bennett *et al.*, 1974). It was confirmed that $Na^+$-dependent L-aspartate uptake was high in a fraction of the 0.8 M-sucrose layer (i.e. synaptic plasma-membrane fraction), but not in a synaptic-vesicle fraction (results not shown). Consistently, $Na^+$-dependent L-glutamate uptake was highest in the 0.8 M-sucrose layer (Fig. 3). The results argue against the possibility of a non-specific binding or permeation of negatively charged glutamate by an inside-positive electrical potential across the vesicle membrane, but support the concept of a carrier-mediated transport.

#### Involvement of $H^+$ in the valinomycin/$Rb^+$-dependent glutamate uptake

Imposition of an extravesicular pH jump from 5.9 to 7.7 enhanced the valinomycin/$Rb^+$-dependent uptake of glutamate from $0.173 \pm 0.030$ to $0.731 \pm 0.021$ pmol/20 s per 100 $\mu$g of protein. In a control experiment, the same magnitude of pH jump without valinomycin/RbCl did not produce a significant uptake. FCCP is a potent $H^+$ conductor which dissipates the protonmotive force. This reagent inhibited valinomycin/$Rb^+$-dependent glutamate uptake as well as ATP-dependent uptake (Table 2), but not $Na^+$-dependent uptake (results not shown). Nigericin is known to dissipate the chemical potential difference of $H^+$ across the membrane (but not the electrical potential difference) by exchanging $H^+$ with $K^+$ or $Rb^+$ across the membrane (Reed, 1979). Nigericin strongly inhibited valinomycin/$Rb^+$-dependent glutamate uptake (Table 2). DCCD, which generally inhibits the $H^+$-transport system including a $H^+$/catecholamine antiporter (Schuldiner *et al.*, 1978; Apps *et al.*, 1980), significantly inhibited the valinomycin/$Rb^+$-dependent glutamate uptake of synaptic vesicles (Table 2). These results are consistent with the idea that a protonmotive force is the driving force for the vesicular uptake of glutamate. According to this model, glutamate influx is coupled with a $H^+$ efflux down the electrochemical potential gradient across the membrane through a putative $H^+$/glutamate antiporter (see below).

**Table 2. Effects of protonmotive-force inhibitors on L-glutamate uptakes into synaptic vesicles**

In the ATP-dependent uptake experiments, synaptic vesicles (20 $\mu$g of protein) were first preincubated in the presence of the test agent in 0.25 M-sucrose/5 mM-Tris/maleate (pH 7.4)/4 mM-$MgSO_4$/4 mM-KCl with (Expt. 2) or without (Expt. 1) 100 mM-RbCl for 5 min at 30 °C. Uptake was then initiated by addition of a mixture of ATP (final concn. 2 mM) and L-[$^3$H]glutamate (final concn. 50 $\mu$M; 0.14 Ci/mmol) and allowed to continue for 1.5 min. The difference between the uptake in the presence and absence of ATP was normalized with the control values (7.20 and 3.77 pmol for Expts. 1 and 2 respectively). In the valinomycin/$Rb^+$-dependent uptake experiments, synaptic vesicles (100 $\mu$g of protein) were first incubated in the presence of the agent in 0.25 M-sucrose/5 mM-Tris/maleate (pH 7.4)/0.5 $\mu$M-valinomycin for 5 min at 30 °C, after which L-[$^3$H]glutamate (final concn. 10 $\mu$M; 3 Ci/mmol) was added. After 15 s incubation, RbCl (final concn. 100 mM) was added and incubation continued for another 20 s. The difference between the uptake in the presence and absence of valinomycin was normalized with the control values (0.184 and 0.397 pmol for Expts. 1 and 2 respectively).

| Reagent | Concn. ($\mu$M) | L-Glutamate uptake (%) ATP-dependent | Valinomycin/$Rb^+$-dependent |
|---|---|---|---|
| Expt. 1 | | | |
| Control | | $100 \pm 6.5$ | $100 \pm 18.7$ |
| FCCP | 1 | $46.6 \pm 3.6$ | $28.4 \pm 5.4$ |
| | 4 | $13.7 \pm 0.6$ | $18.6 \pm 9.4$ |
| DCCD | 100 | $78.8 \pm 6.7$ | $30.6 \pm 8.6$ |
| Expt. 2 | | | |
| Control | | $100 \pm 4.1$ | $100 \pm 8.2$ |
| Nigericin | 2 | $20.5 \pm 5.1$ | $0.0 \pm 2.4$ |
| | 4 | $18.5 \pm 0.9$ | $6.6 \pm 2.8$ |

### Other characteristics of the valinomycin/Rb$^+$-dependent glutamate-uptake system in synaptic vesicles

Non-radioactive D-glutamate at 5 mM inhibited valinomycin/Rb$^+$-dependent L-[$^3$H]glutamate (50 $\mu$M) uptake by $86 \pm 13$%, but neither glutamine nor GABA at 5 mM inhibited significantly. Although L-aspartate itself could not be taken up into the synaptic vesicles in a valinomycin/Rb$^+$-dependent manner (as shown in Table 1), L-aspartate (potassium salt) at 5 mM inhibited the valinomycin/Rb$^+$-dependent glutamate uptake by $53 \pm 5$% (9 experiments). As mentioned above, the Na$^+$-dependent glutamate-uptake system does not distinguish between glutamate and aspartate (Logan & Snyder, 1972; Davies & Johnston, 1976), and ATP-dependent glutamate uptake is strictly specific for glutamate and is not inhibited by L-aspartate (Naito & Ueda, 1985). Thus the valinomycin/Rb$^+$-dependent uptake system apparently manifested an intermediate substrate specificity between those of ATP-dependent and Na$^+$-dependent uptake systems; the valinomycin/Rb$^+$-dependent uptake system seemed to bind L-aspartate on the surface of the vesicle membrane, but not to transport it into the vesicle lumen.

Naito & Ueda (1985) reported that the ATP-dependent L-glutamate uptake is remarkedly enhanced by 4 mM-Cl$^-$ but not by 100 mM-Cl$^-$ or 4 mM-$NO_3^-$ (see also Maycox *et al.*, 1988). A minor effect was observed with 4 mM-Cl$^-$ on ATPase, $\Delta$pH and $\Delta\psi$, which suggested that Cl$^-$ may be involved in the transport system rather than H$^+$-pumping ATPase (Shioi *et al.*, 1989). However, 4 mM-RbCl (together with 96 mM-$RbNO_3$) did not show any significant enhancement of valinomycin-dependent glutamate uptake over that with 100 mM-$RbNO_3$ (results not shown). The possibility cannot be excluded that an intravesicular Cl$^-$ itself or a Cl$^-$ efflux enhances the presumed H$^+$/glutamate antiport. It may be noteworthy that the Cl$^-$-channel inhibitor 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) is one of the most potent inhibitors for ATP-dependent glutamate uptake (Shioi *et al.*, 1989); it is probable that SITS inhibits H$^+$/glutamate transport as well as ATP-driven H$^+$-pump. Recent reports showed little or minor potentiation by Cl$^-$ for ATP-dependent uptakes of other neurotransmitters, such as GABA and glycine, by synaptic vesicles (Kish *et al.*, 1989; Fykse *et al.*, 1989).

### Models of the transport mechanism

Maycox *et al.* (1988) reported that ATP-dependent L-glutamate uptake is qualitatively correlated with $\Delta\psi$, but not with $\Delta$pH. In addition, L-glutamate specifically induces acidification of the vesicle interior with a minor change in $\Delta\psi$. From these results, they proposed a uniport model (Fig. 4*a*) in which negatively charged glutamate is transported electrophoretically through the specific carrier. Cidon & Sihra (1989) also proposed the same model. Their model agrees with our previous results (Shioi *et al.*, 1989) on the quantitative correlation between ATP-dependent glutamate uptake and $\Delta\psi$. The present study, however, implicates an involvement of H$^+$ in the transport system itself. We propose an alternative model involving a H$^+$/glutamate antiporter (Fig. 4*b*). In this model, one H$^+$ and one neutral glutamate molecule (undissociated or zwitterionic form of glutamic acid) are exchanged through the antiporter across the polarized membrane; $\Delta\psi$ could be a major driving force for the transport. Transported glutamic acid will dissociate and liberate H$^+$ ions inside the vesicles, thus facilitating a further influx of neutral glutamate. It should be noted that the transport in this model is electrogenic and that the net changes are glutamate accumulation and acidification of intravesicular space, as previously demonstrated by Maycox *et al.* (1988). However, acidification by both H$^+$ pumping and glutamate accumulation will be inhibitory of

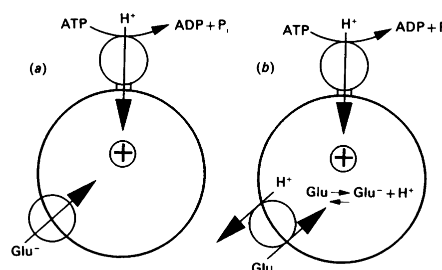
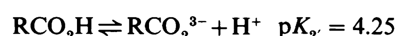



**Fig. 4. Generation of $\Delta\psi$ by H$^+$-pumping ATPase and possible mechanisms of L-glutamate uptake**

(*a*) A uniport model; (*b*) an antiport model. See the text for details.

transport of the undissociated glutamate, because of a back-shift toward the undissociated form in the dissociation equilibrium of glutamic acid:



$$RCO_2H \rightleftharpoons RCO_2^{3-} + H^+ \quad pK_{2'} = 4.25$$

It is therefore necessary to postulate an auxiliary system, such as a H$^+$/Cl$^-$ symporter, which would remove H$^+$ from the interior of vesicles by an electroneutral process (an alternative is a H$^+$/cation antiporter, as suggested by Maycox *et al.*, 1988). The present model is speculative, particularly regarding the form of transported glutamate (neutral or negatively charged) and the role of Cl$^-$. Further work is needed central to this working hypothesis utilizing this valuable valinomycin/Rb$^+$-dependent uptake system in order to ascertain the precise mechanism.

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