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Active Transport of L-Glutamate by Membrane Vesicles Isolated from Rat Brain†

Baruch I. Kanner* and Ilana Sharon

ABSTRACT: Membrane vesicles, isolated after osmotic shock of synaptosomal rat brain fractions, actively accumulate L-glutamate. This process requires the presence of external sodium ions and internal potassium ions and is driven by artificially imposed ion gradients as the sole energy source. Either an Na^+ gradient (out > in) or a K^+ gradient (in > out) or both can be utilized to concentrate L-glutamate inside the vesicles. Transport is enhanced by valinomycin or by external thiocyanate ions and is about 50% inhibited by the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone. This transport thus appears to be stimulated by a membrane potential (interior negative). The glutamate transporter, the K_m of which has been determined to be 3 μM, is specific for L-glutamate. The transport process is unaffected by ouabain but is strongly inhibited by *p*-hydroxymercuribenzoate as well as by nigericin, which collapses the energizing ion gradients across this membrane. Unlike the sodium dependent, but potassium independent active accumulation of γ-aminobutyric acid in these vesicles (Kanner, B. I. (1978) *Biochemistry 17,* 1207) active L-glutamate uptake is not dependent on the presence of small monovalent anions in the external medium. The results provide direct evidence for Na^+-coupled electrogenic active L-glutamate transport by rat brain membrane vesicles. The dependence on internal potassium ions is discussed.

High affinity, sodium dependent, uptake systems for a variety of established and putative transmitters have been detected in brain preparations, such as synaptosomes (Iversen, 1971, 1973; Kuhar, 1973; Bennett et al., 1974). These uptake systems have been implicated in the termination of transmitter action on postsynaptic receptors (Iversen, 1971) as well as in maintaining constant levels of transmitters in the neurons (Hedgvist & Stjarne, 1969). This transport is inhibited by conditions interfering with intracellular ATP synthesis (Iverson & Neal, 1968; White & Keen, 1970), as well as by the inhibitor of the $(Na^+ + K^+)$-ATPase[1] ouabain (Iversen & Neal, 1968; Balcar & Johnston, 1972a). It has been suggested that the participation of the $(Na^+ + K^+)$-ATPase in transport is in-

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[1] Abbreviations used: ATPase, adenosine triphosphatase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tris, tris(hydroxymethyl)-aminomethane; Tricine, *N*-tris[hydroxymethyl]methylglycine; Mes, 2-[*N*-morpholino]ethanesulfonic acid; Gaba, γ-aminobutyric acid.

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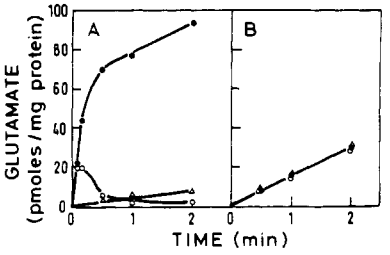

FIGURE 1: Glutamate uptake by rat brain membrane vesicles. Membrane vesicles were loaded with 0.1 M potassium phosphate + 1 mM $MgSO_4$, pH 6.8 (Figure 1A) or with 0.1 M Tris phosphate +1 mM $MgSO_4$, pH 6.8 (Figure 1B). Transport assays were performed as described in Methods. The external solution contained 0.1 M NaCl (●—●), 0.1 M KCl (△—△), or 0.1 M NaCl + 5 μM nigericin (○—○).

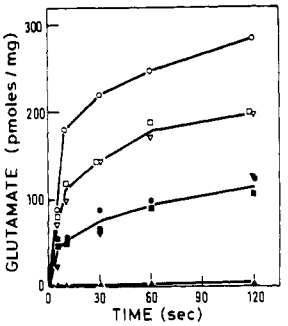

FIGURE 2: The effect of external ions on glutamate transport. Membrane vesicles were loaded and transport assays were made as described in Methods. The external medium contained: 100 mM NaCl (▽—▽), 100 mM $NaNO_3$ (○—○); 100 mM NaSCN (□—□); 100 mM sodium glucuronate (▼—▼); 50 mM Na_2SO_4 + 50 mM sucrose (●—●); 67 mM sodium phosphate + 33 mM sucrose (■—■); 100 mM of LiCl (▲—▲).

direct and that in fact the sodium ion and also potassium ion gradients across the membranes of presynaptic nerve terminals and/or glial cells may represent the immediate driving forces for active neurotransmitter uptake (Bogdanski et al., 1968; Martin & Smith, 1972; Martin, 1973; Holtz & Coyle, 1974). This is in accordance with general hypotheses that solute accumulation can be achieved by cotransport with ions, which move down their electrochemical gradient into the cell or cell organelle (Riggs et al., 1958; Crane, 1965; Mitchell, 1963).

For neurotransmitter transport in brain this concept has recently received strong support by studies on active Gaba transport into membrane vesicles derived from rat brain in which artificially imposed sodium gradients (out > in) and also chloride gradients (out > in) were able to drive this process (Kanner, 1978). Surprisingly this Gaba transport was absolutely dependent on the presence of external small monovalent anions of which chloride was the most effective (Kanner, 1978). It was of interest therefore to examine if this negative charge required for Gaba transport would also be required for the transport of the putative transmitter glutamate, which is negatively charged at neutral pH. A sodium dependent uptake system for this compound has been identified in brain slices (Balcar & Johnston, 1972b).

This study describes the active transport of L-glutamate into the membrane vesicle preparation obtained after osmotic shock of rat brain synaptosomes. This process was measured in the absence of any exogenous energy source, except for ion gradients. It appears that glutamate transport does not require chloride ions, but requires in addition to external sodium also internal potassium ions. This dependence of glutamate transport on a potassium ion gradient (in > out) cannot be merely explained as a necessity to create a membrane potential of the appropriate sign to drive this active transport.

TABLE I: The Effect of Various Inhibitors on Glutamate Transport.[a]

inhibitor[b]	initial rate (% of control)	extent (% of control)
ouabain (100)	105	103
CCCP (5)	100	48
gramicidin (50)	16	18
p-hydroxymercuribenzoate (100)	0	0
triphenyltin chloride (5)	57	45

[a] Loading of membrane vesicles and transport assays were performed as described in Methods. The external solution contained the indicated final concentrations of compounds. In the case of gramicidin and *p*-hydroxymercuribenzoate, the membrane vesicles were also preincubated on ice with the same concentration of inhibitors used during the assay. For initial rate transport, values of 10 s and for extent those at 1 min were considered. [b] μM concentration given in parentheses.

Experimental Procedure

Methods

Preparation of Membrane Vesicles. Membrane vesicles from 14-day-old female rats were prepared and stored as described (Kanner, 1978). Under these conditions the transport abilities of the membrane vesicles were stable for at least 2 months. Transport values were very similar using the same preparation, but some variability was noted when comparing various preparations. Prior to the transport assays the membrane vesicles were preloaded as described (Kanner, 1978), using 0.1 M potassium phosphate + 1 mM $MgSO_4$, pH 6.8, unless indicated otherwise in the legends to the figures. Protein was determined according to the Lowry method (Lowry et al., 1951).

Transport Assays. Transport assays were performed as previously described (Kanner, 1978). Briefly, 20 μL of preloaded membrane vesicles (2–3 mg/mL) were diluted at room temperature into 180 μL of external solution. This consisted of 0.1 M NaCl containing 0.2 μM L-[G-^{3}H]glutamic acid (1.25 Ci/mmol), unless indicated otherwise in the legends to the figures and tables. At the indicated times, reactions were stopped by addition of 2 mL of an ice cold 0.15 M NaCl solution. After filtration and washing with the stopping solution, radioactivity was determined using liquid scintillation spectrometry. When the reactions were performed at 0 °C, no transport was detected.

Materials

L-[G-^{3}H]glutamic acid was obtained from Amersham. Valinomycin, gramicidin, and CCCP were purchased from Sigma Chemical Co. Nigericin was a generous gift of Dr. R. J. Hosley from Eli Lilly. All other materials were of the highest purity commercially available.

Results

The data presented in Figure 1A illustrate the uptake of L-[G-^{3}H]glutamate into membrane vesicles derived from rat brain. An artificial sodium ion gradient is created by diluting (at time zero) the membrane vesicles loaded with potassium phosphate tenfold into a NaCl solution containing L-[G-^{3}H]glutamate. In this experiment approximately 75 pmol of glutamate/mg of protein have been taken up. The accumulated radioactivity in fact represents unmodified L-glutamate, since

TABLE II: The Effect of Internal Anions on L-Glutamate Transport[a]

loading solution	initial rate (pmol/(min·mg of protein))	extent (pmol/mg of protein)
100 mM K phosphate	372 ± 60	106
100 mM K Mes	516 ± 40	113
50 mM K Mes + 50 mM K arsenate	348 ± 13	67
100 mM K Tricine	180 ± 25	98

[a] Transport was measured as described in Methods. Membrane vesicles were loaded by incubating with the indicated loading solutions (all adjusted to pH 6.8). For initial rates of transport, values were obtained from the 5-s time points. Extents were obtained from the 1-min time points.

L-glutamate accumulated in the vesicles (released with acid) was found to cochromatograph with authentic L-glutamate using thin-layer chromatography.[2]

In the presence of ionophores, such as nigericin (Figure 1A) or of gramicidin (Table I), able to collapse Na^+ ion gradients, uptake of L-glutamate is strongly inhibited. This uptake is sodium dependent, similar to L-glutamate transport reported in brain slices (Balcar & Johnston, 1972b).

No glutamate uptake is observed when external sodium ions are replaced by potassium ions (Figure 1A) or by Li^+ (Figure 2). Similar lack of glutamate uptake is noted with external $Tris^+$ or NH_4^+ ions (data not shown). When internal potassium ions are replaced by $Tris^+$ ions, a very slow uptake process is observed, which is neither sodium dependent nor sensitive to nigericin (Figure 1B). The initial rate of this slow process is 10% or less than the uptake with internal potassium ions present. Also with internal Na^+, Li^+, or NH_4^+ ions, results, similar to those obtained in the presence of internal $Tris^+$ ions, are obtained (data not shown). The nature of this slow, sodium-independent uptake of glutamate is unknown at present and will not be considered here. In this communication the fast uptake process, requiring external sodium ions and internal potassium ions, will be analyzed.

In contrast to active, sodium-dependent Gaba accumulation in this vesicle preparation (Kanner, 1978), no absolute dependence on small external monovalent anions is noted (Figure 2). The nature of the internal anion is apparently not critical since, when internal phosphate is replaced by Tricine, Mes, or arsenate, about 50% or more of the uptake rate of the phosphate loaded vesicles is observed (Table II).

The extent of L-glutamate uptake is sensitive to the medium osmolarity (Figure 3) providing additional evidence that L-glutamate is in fact transported across the vesicle's membrane. The stimulation by low sucrose concentration is possibly due to the slight hypotonicity of the uptake medium as compared with the "loading" solution. Moreover, when the membranes are washed with water rather than with isotonic salt solution, most of the accumulated radioactivity is lost (data not shown). The extent of uptake (1-min time points) is dependent on the batch of membrane vesicles and ranges from 75 to 200 pmol/mg of protein. Using the determined value of the total intravesicular space as 7.4 μL/mg (Kanner, 1978), the internal glutamate concentration ranges from 10 to 27 μM. This is a minimal estimate, since possibly not all vesicles in the preparation have the ability to accumulate glutamate. Thus a concentration gradient of at least 50–135-fold is reached.

[2] The solvent system, chloroform 40% (v/v)–methanol 40% (v/v)–ammonia 20% (v/v), separates L-glutamate from Gaba, glutamine, and α-ketoglutaric acid.

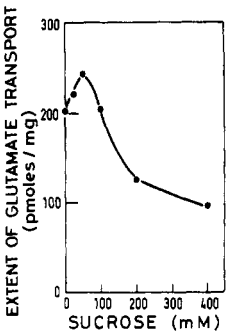

FIGURE 3: The effect of external osmolarity on the extent of glutamate transport. Transport assays were performed as described in Methods. The external (NaCl) solution also contained the indicated amounts of sucrose, given as final concentrations. The average values of transport at 1 and 2 min are taken as a measure of the extent of sodium dependent glutamate transport.

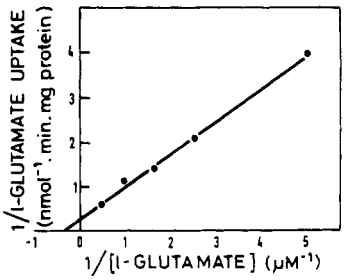

FIGURE 4: The kinetics of L-glutamate uptake. Membrane vesicles were loaded and transport assays were performed as described in Methods. Increasing concentrations of L-glutamate at constant specific radioactivity (1.25 Ci/mmol) were used. In order to determine the initial velocity, transport was measured at time zero and at 5 s.

In addition to nigericin also the sulfhydryl reagent *p*-hydroxymercuribenzoate appears to be a potent inhibitor of L-glutamate uptake. The latter observation is consistent with results obtained with brain slices (Balcar & Johnston, 1972a). The uncoupler CCCP is only partially inhibitory (Table I). The resistance of this sodium-dependent L-glutamate uptake to ouabain (Table I) supports the view that the $(Na^+ + K^+)$-ATPase is not directly involved in neurotransmitter transport (Bogdanski et al., 1968; Martin, 1973; Holtz & Coyle, 1974). The resistance of the process to arsenate also indicates that the possibility of indirect involvement of the $(Na^+ + K^+)$-ATPase to utilize the artificially created sodium and potassium ion gradients for ATP synthesis (for energization) is unlikely. It is of interest to note that triphenyltin chloride, an extremely potent inhibitor of Gaba transport (Kanner, 1978) is only partly effective under these conditions of L-glutamate transport (Table I).

Transport of L-glutamate exhibits saturation kinetics. From Lineweaver–Burk plots a K_m of 3.0 μM and a V_{max} of 4.2 nmol/(min·mg) have been determined (Figure 4). This K_m value is in excellent agreement with that reported in the literature for intact synaptosomal preparations (Bennett et al., 1974).

Efflux of L-[G-^{3}H]glutamate, previously accumulated by the vesicles is observed with either nigericin or with an excess of unlabeled L-glutamate (Figure 5). The specificity of the carrier is illustrated by the fact that both D-glutamate and Gaba are unable to cause efflux of L-[G-^{3}H]glutamate under these conditions. Moreover, also L-aspartate is able to cause efflux (data not shown).

Glutamate uptake is apparently electrogenic. Conditions expected to generate a membrane potential (interior negative),

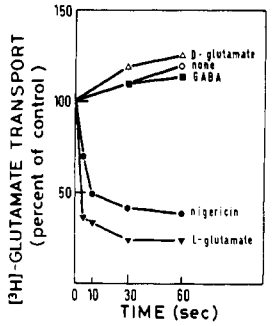

FIGURE 5: Influx and subsequent efflux of L-[G-^{3}H]glutamate from membrane vesicles. Transport assays were made as indicated in Methods, except that the external (NaCl) solution contained 5 μM L-[G-^{3}H]glutamate (0.2 Ci/mmol). Additions on the time scale of the figure at time zero were made after 1 min of transport. At the indicated times after the additions, the amount of L-[G-^{3}H]glutamate retained by the vesicles was determined. Additions: none (O—O); nigericin, 5 μM (●—●); L-glutamate, 200 μM (▼—▼); GABA, 200 μM (■—■); D-glutamate, 200 μM (Δ—Δ).

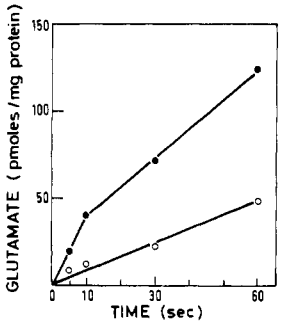

FIGURE 6: The effect of valinomycin on glutamate uptake. Membrane vesicles were loaded with 40 mM potassium phosphate + 10 mM potassium thiocyanate + 50 mM Tris phosphate, pH 6.8. Transport assays were made as described in Methods. Additions to the external medium: none (O—O) or valinomycin, 2.5 μM (●—●).

such as the presence of external thiocyanate, enhance the initial rate of glutamate transport. In the present of a Na^+ concentration gradient, external thiocyanate (0.1 M) increases the rate of transport by 50 to 100%. Conversely, internal thiocyanate (10 mM), which would be expected to make the membrane potential less negative, inhibits the initial rate of transport by about 60% (control 242 pmol/(min·mg of protein); with internal SCN^- (96 pmol/(min·mg of protein)). Increasing the potassium permeability of the membrane with valinomycin results in a 2.5-fold stimulation of the inhibited rate (Figure 6). A similar stimulation by valinomycin (to 195% of the control rate), albeit less pronounced, is observed in the absence of internal thiocyanate ions. Observations on intact nerve preparations (Baker et al., 1962) suggest that the permeability of the membrane to potassium ions is sufficient that the potassium gradient (in > out) may generate a membrane potential also in the absence of valinomycin. This potential may thus contribute to L-glutamate accumulation. Consistent with this is the partial inhibition of accumulation observed with the proton ionophore CCCP (Table I). This ionophore would be expected to collapse any existing membrane potential.

Artificially imposed ion gradients provide a driving force for active L-glutamate transport. The simultaneous presence of two gradients across the membrane appears to be required for high rates of L-glutamate transport: (1) Na^+ concentration gradient $[Na^+]_{out} > [Na^+]_{in}$ and (2) a K^+ concentration gradient $[K^+]_{in} > [K^+]_{out}$ (Figure 7). When either of these two gradients alone is present, significant but much reduced glu-

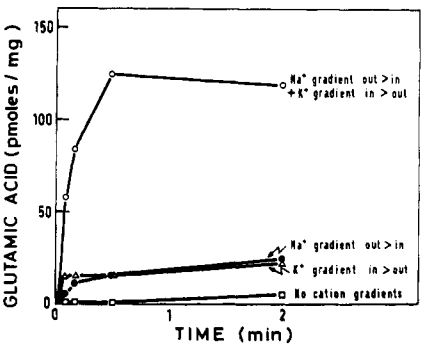

FIGURE 7: Dependency of glutamate transport on transmembranous sodium and potassium ion gradients. Assays were performed as described in Methods. $Na^+ + K^+$ gradients (O—O): vesicles loaded with 50 mM potassium phosphate + 50 mM Tris phosphate, pH 6.8, diluted into 75 mM NaCl + 75 mM Tris-Cl. Na^+ gradient (●—●): vesicles loaded with 50 mM potassium phosphate + 50 mM Tris phosphate, pH 6.8, diluted into 75 mM NaCl + 75 mM KCl K^+ gradient (Δ—Δ): vesicles loaded with 50 mM sodium phosphate + 50 mM potassium phosphate, pH 6.8, diluted into 75 mM NaCl + 75 mM Tris-Cl. No gradients (□—□): vesicles loaded with 50 mM sodium phosphate + 50 mM potassium phosphate, pH 6.8, diluted into 75 mM NaCl + 75 mM KCl.

tamate transport is observed. No uptake at all occurs in the absence of both types of gradients (Figure 7). The requirement for the potassium ion gradient (in > out) cannot be explained by the mere requirement of a membrane potential (inside negative). We may draw this conclusion because of the observation that, when internal potassium ions are replaced by $Tris^+$ ions, with thiocyanate present in the external medium (a condition also expected to create a membrane potential with the correct polarity, inside negative), no sodium dependent glutamate transport can be detected (data not shown).

Discussion

Glutamate transport in membrane vesicles derived from rat brain has many similar features to that measured in less fractionated preparations, such as brain slices and synaptosomes. These features include sodium ion dependence (Balcar & Johnston, 1972b), sensitivity to sulfhydryl reagents (Balcar & Johnston, 1972a) as well as substrate and stereospecificity (Balcar & Johnston, 1972a,b) and also affinity (Bennett et al., 1974). The sole source of energy input under our experimental conditions during glutamate transport into these membrane vesicles is that of artificially imposed transmembranous ion gradients, notably sodium (out > in) and potassium (in > out) (Figures 1 and 7). This transport is not inhibited by ouabain (Table I) in contrast to that in the more intact preparations. In the latter, energy is supplied in the form of glucose. Apparently, the ATP formed upon glucose catabolism is used by the $(Na^+ + K^+)$-ATPase to create the above described ion gradients, which appear to be the major driving forces for glutamate uptake.

The membrane vesicles under study here probably originate from the synaptic plasma membrane. The procedure of preparation of purified synaptosomes is very similar to those described by various laboratories (Abdel-Latif, 1966; Haldar, 1971; Cotman, 1974). The purity of the synaptosomes prepared by such procedures is estimated from criteria including electron microscopy and marker enzymes, and about 70% of the membranes are synaptosomal (Cotman et al., 1971). Moreover, the method of preparation of the membrane vesicles from synaptosomes is similar to other methods used to prepare "synaptic plasma membrane vesicles" (Cotman, 1974). In addition when the various gradient fractions obtained during the preparation of the purified synaptosomes are subjected to

osmotic shock and glutamate transport is subsequently measured, it appears that the typical mitochondrial fractions (the lower two fractions) are completely devoid of this activity (data not shown).

Recently Gaba transport in the same vesicle preparation has been measured (Kanner, 1978). The two transport processes apparently proceed via different translocators. A large excess of unlabeled Gaba does not cause the efflux of previously accumulated L-glutamate, in contrast to a large excess of unlabeled L-glutamate (Figure 5). Second, the inhibition of Gaba transport by triphenyltin chloride is total (Kanner, 1978) while under the same conditions this compound inhibits L-glutamate transport only partially. Moreover, although the active transport of both neurotransmitters is driven by a sodium ion gradient (out > in) and a membrane potential (interior negative), there is a marked difference in the ion dependence of the two transport processes. Whereas Gaba transport shows an absolute dependence on the presence of small monovalent external anions, of which chloride is the most effective, this requirement is not apparent in the case of L-glutamate transport (Figure 2). This latter process, however, shows an absolute dependency on internal potassium ions.

The role of external chloride or internal potassium ions in Gaba or glutamate transport respectively is not only their mere presence but in fact a transmembranous gradient of these ions is required for driving the transport of these neurotransmitters (Kanner, 1978; Figure 7). Moreover, just as is the case for the chloride ion gradient (out > in) with Gaba transport, also with L-glutamate transport the function of the potassium ion gradient (in > out) is not merely to create the membrane potential of the appropriate polarity (interior negative). Also for L-glutamate transport two possibilities come to mind. One is the requirement for internal potassium ions to bring the carrier in the right conformation for L-glutamate transport, together with its ability to impose a membrane potential (interior negative). The other possibility is that glutamate is translocated inward, simultaneously with inward transport of sodium ions while potassium ions are translocated outward as part of the translocation cycle.

We do not know which form of glutamate is actually translocated by the carrier. If this is the negatively charged species, the predominant one at neutral pH, and potassium ions indeed participate in the translocation cycle, one must make a minimal requirement for the translocation stoichiometry $n\mathrm{Na}^+$:$m\mathrm{K}^+$:glutamate$^-$, namely, $n > m + 1$, in order to explain the electrogenic phenomena. If the translocated glutamate species is the neutral one, then according to the same considerations it follows that $n > m$.

Several sodium dependent transport systems have recently been identified in membrane vesicles which require an additional ion. In addition to the Gaba and glutamate transport systems identified in membrane vesicles derived from brain, sodium dependent serotonin transport in platelet membrane vesicles (Rudnick, 1977) also requires external chloride ions. Other examples of sodium transport systems requiring additional ions have been noted several years ago. Thus chloride is also needed for optimal glycine uptake in the avian red blood cell (Vidaver, 1964) and also a role for internal potassium ions has been considered (Riggs et al., 1958; Baker & Potashner, 1971). However, in some cases the requirement for these ions might be explained by effects not directly involving the translocators; for instance to set up a membrane potential of the right polarity required for transport. In the case of neurotransmitter transport, one of the intriguing questions is to distinguish between the two distinct possibilities (outlined above) for the involvement of these additional ions in the translocation of these solutes. Direct flux measurements are required to decide the issue. These might be possible upon insertion of the detergent solubilized, purified carriers into artificial lipid vesicles. Attempts to reconstitute these carriers from brain are currently in progress.

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