Binding Order of Substrates to the Sodium and Potassium Ion Coupled L-Glutamic Acid Transporter from Rat Brain†

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ABSTRACT: Efflux of L-glutamic acid from synaptic plasma membrane vesicles requires external potassium. This requirement is saturated by concentrations of about 15 mequiv/L potassium. In the absence of potassium, L-glutamic acid can be released from the vesicles in the presence of external L-glutamic acid. This stimulation does not require external sodium but is dependent on the external concentration of L-glutamic acid. Half-maximal effects are obtained by concentrations of about 1 μM which are very similar to the apparent K_m for L-glutamic acid influx. Efflux of labeled glutamate driven by external sodium plus glutamate requires internal sodium. These findings suggest that the transporter displays an asymmetric behavior toward sodium. This ion dissociates much more slowly than L-glutamic acid on the external surface of the membrane but not on the internal surface. Furthermore, it appears that the transporter translocates potassium in a step distinct from the L-glutamic acid translocation step. The simplest explanation is that upon translocation of sodium and L-glutamic acid and their release to the inside, potassium binds to the transporter, enabling it to return to the outside to allow initiation of a new transport cycle.

The use of membrane vesicles derived from the synaptic plasma membrane from rat brain cortex (Kanner, 1980) has contributed significantly to our understanding of the mechanism of transport of neurotransmitters such as GABA[1] (Kanner, 1978; Kanner & Kifer, 1981) and L-glutamic acid (Kanner & Sharon, 1978; Kanner & Marva, 1982). These two transport systems appear to be distinct units. It appears that GABA transport occurs via cotransport with two sodium ions and one chloride ion per GABA molecule (Kanner, 1978; Kanner & Kifer, 1981; Radian & Kanner, 1983). On the other hand, it appears that L-glutamic acid transport is catalyzed by its translocator by cotransport with probably more than one sodiuum ion, while it simultaneously catalyzes transport of potassium in the opposite direction. This is supported by various experimental observations: (a) glutamic acid influx requires the simultaneous presence of external sodium and internal potassium, (b) the level of L-glutamic acid transport is determined by the gradients of sodium (out > in) and potassium (in > out), (c) although transport appears to be electrogenic, the ion dependence is not a result of charge compensation (Kanner & Sharon, 1978), and (d) internal sodium and external potassium are required for the efflux of L-glutamic acid (Kanner & Marva, 1982).

Recent studies with membrane vesicles from other sources indicate that there are also several other transport systems that function by cotransport with sodium as well as additional ions. Examples are the transport of serotonin in platelet membranes (Rudnick, 1977; Nelson & Rudnick, 1979; Keyes & Rudnick, 1982) and glutamate transport in renal brush border vesicles (Burckhardt et al., 1980; Schneider & Sacktor, 1980) and in membrane vesicles from rat liver (Sips et al., 1982).

Study of the ion dependence of influx and efflux of solutes provides information on the question of which ions are cotransported with the solute. Furthermore, the ability of a solute to transstimulate efflux of solute through a transport system, as a function of the ion composition of the medium, can provide information on the binding order of the solute and the ions to the transporter. In this communication we describe a study where this principle is applied to ($Na^+ + K^+$)-dependent L-glutamic acid transport from rat brain synaptic plasma membrane.

Experimental Procedures

Methods. (1) Preparation of Membrane Vesicles. Membrane vesicles from 14-day-old female rats were prepared and stored as described (Kanner, 1978). Protein was determined according to the Lowry method (Lowry et al., 1951).

(2) Influx and Efflux Experiments. Influx of L-glutamic acid was measured as described (Kanner & Sharon, 1978). Efflux of L-glutamic acid from actively or passively loaded vesicles was performed as published previously (Kanner & Marva, 1982). In all cases reactions were terminated by the addition of 2 mL of ice-cold 0.15 M NaCl and rapid filtration of the mixture through nitrocellulose filters (Schleicher & Schuell, 0.45-μm pore size, 25-mm diameter). Subsequently, the filters were washed with another 2 mL of the NaCl solution. The filters were dried and counted by liquid scintillation spectrometry. The absolute values, when experiments were repeated, were quite variable from day to day, but qualitatively always the same result was obtained. This was verified by repeating experiments 3 or 4 times. In view of this, the data of representative experiments are shown.

Materials. L-[3,4-$^3H_2$]Glutamic acid was obtained from New England Nuclear. Nigericin was a generous gift of Dr. R. J. Hosley from Eli Lilly.

Results

Net Efflux of L-Glutamic Acid. In the experiment depicted in Figure 1, membrane vesicles loaded with potassium phosphate have been diluted into sodium chloride containing L-[3,4-$^3H_2$]glutamic acid. This results in accumulation of this solute against its concentration gradient. When, after 1 min of accumulation (Figure 1), the vesicles are diluted 20-fold into a lithium phosphate medium, a very slow efflux is observed. On the other hand, dilution into a potassium phosphate medium results in a very fast efflux (Figure 1) which is not

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[1] Abbreviation: GABA, γ-aminobutyric acid.

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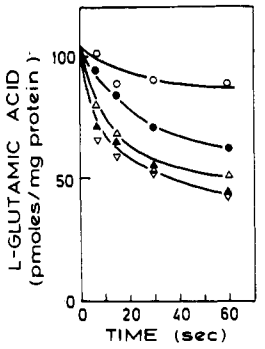

FIGURE 1: Effect of external potassium on L-glutamic acid efflux from actively loaded vesicles. Membrane vesicles [5 μL; 33.5 μg of protein was diluted into 45 μL of 0.1 M NaCl containing 0.5 μCi of L-[3,4-$^3H_2$]glutamic acid (42.3 Ci/mmol). After 1 min of influx, the vesicles were diluted (t = 0 on abscissa) by adding 1 mL of efflux solution of the following composition, all at pH 6.8]: (○) 100 mM LiP_i, (●) 5 mM KP_i plus 95 mM LiP_i, (△) 15 mM KP_i plus 85 mM LiP_i, (▲) 30 mM KP_i plus 70 mM LiP_i, and (▽) 100 mM KP_i.

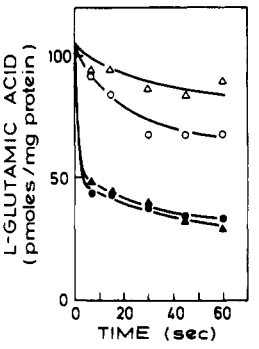

FIGURE 2: Transstimulation of L-glutamic acid efflux from actively loaded vesicles. Influx (1 min) was performed as above by using 25 μg of protein. The composition of the efflux medium (pH 6.8) was (○) 100 mM LiP_i, (●) the same plus 20 μM L-glutamic acid, (△) 100 mM NaP_i, and (▲) the same plus 20 μM L-glutamic acid.

affected by the presence of proton ionophores (Kanner & Marva, 1982). Thus, efflux of L-glutamic acid requires a specific interaction of external potassium with the transporter (Kanner & Marva, 1982) just as influx displays a specific requirement for internal potassium (Kanner & Sharon, 1978). It is of interest to note that the requirement for external potassium is saturated by rather low concentrations; at about 15 mequiv of K^+/L the effect is already maximal, while half-maximal effects are achieved by concentrations of about 5 mequiv of K^+/L (Figure 1). Efflux of L-glutamic acid also requires internal sodium (Kanner & Marva, 1982). Thus, one possibility is that efflux occurs by simultaneous movement of sodium ions and L-glutamic acid outward and of potassium ions inward, followed by reorientation of the binding sites so that a new cycle can start. On the other hand, the process may be an ordered one: for instance, (1) movement of sodium and L-glutamic acid outward via the transporter, (2) their release on the outside, (3) binding of external potassium to the transporter, and (4) return of the potassium loaded transporter to the inside. If this latter possibility were indeed occurring, then sodium and L-glutamic acid, both external, would be able to release the labeled L-glutamic acid from the vesicles (exchange of L-glutamic acid).

Exchange of L-*Glutamic Acid. (1) Effect on the Composition of the External Medium.* Figure 2 shows a typical experiment in which dilution of membrane vesicles—actively loaded with L-[3,4-$^3H_2$]glutamic acid—into a medium containing sodium phosphate and 20 μM unlabeled L-glutamic acid results in a very fast release of the radioactive L-glutamic

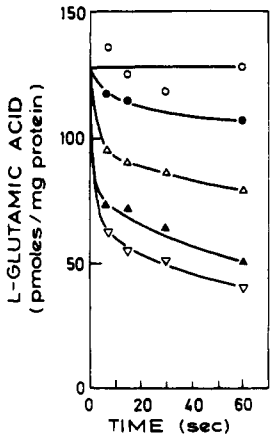

FIGURE 3: Concentration dependence of the transstimulation of L-glutamic acid efflux. Influx was performed by active loading using 34 μg of protein. The composition of the efflux medium (pH 6.8) was (○) 100 mM NaP_i, and the same plus either (●) 0.2, (△) 1, (▲) 6, or (▽) 20 μM L-glutamic acid.

Table I: Effect of External Sodium and Internal Potassium on the Kinetic Constants of L-Glutamic Acid Influx[a]

condition			
out	in	K_m (μM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]
100 mM NaCl	100 mM KP_i	0.79 ± 0.12	5.50 ± 0.84
25 mM NaCl + 75 mM LiCl	100 mM KP_i	2.05 ± 0.52	2.42 ± 0.50
100 mM NaCl	25 mM KP_i + 75 mM LiP_i	2.17 ± 0.38	1.98 ± 0.38

[a] Membrane vesicles were loaded (all media had a pH of 6.8) and transport assays were described under Experimental Procedures. The reactions were terminated after 5 s. The data were plotted according to Lineweaver-Burk and the appropriate constants were calculated.

acid. This release is absolutely dependent on the presence of the external L-glutamic acid since, in its absence, none was observed (Figure 2). It is of interest that this release also proceeds very rapidly in the absence of sodium ions, i.e., in a lithium phosphate containing medium (Figure 2). A similar result is obtained in the presence of choline as the external cation (data not shown). It is of interest to note again that influx of L-glutamic acid has an absolute requirement for sodium ions (Kanner & Sharon, 1978).

Although L-glutamic acid exchange does not require external sodium, the following observations indicate that the external L-glutamic acid is exerting its effect via the L-glutamic acid transporter. The stimulation by L-glutamic acid is concentration dependent (Figure 3). The release is very fast and therefore accurate estimates of the initial rate cannot be made. Although this prohibits determination of the K_m of the effect of external L-glutamic acid, its half-maximal effect (about 1 μM) is in good agreement with the K_m for L-glutamic acid influx (Table I). Furthermore, the action of glutamic acid is stereospecific since D-glutamic acid has a much smaller effect than L-glutamic acid (Figure 4A). This is to be expected since D-glutamic acid blocks influx of L-[3,4-$^3H_2$]glutamic acid very poorly (Figure 4B). Moreover, GABA which is not a substrate for the L-glutamic acid transporter (Figure 4B) does not induce much release of L-[3,4-$^3H_2$]glutamic acid. On the other hand, L-aspartic acid which effectively competes with L-glutamic acid for influx (Figure 4B) is able to release internal L-glutamic acid (Figure 4A). Thus, release of L-glutamic acid by external L-glutamic acid occurs via the transporter and is independent of external sodium. It is of interest to note also that L-aspartic

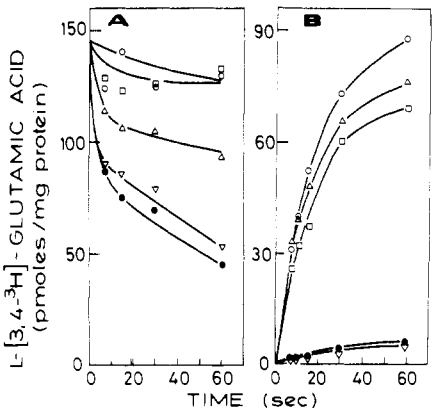

FIGURE 4: Effect of various ligands of efflux and influx of L-glutamic acid. (A) Efflux: upon active loading by using 17.5 μg of protein, the vesicles were diluted into a medium containing 0.1 M NaP_i, pH 6.8, plus (○) nothing, (●) 20 μM L-glutamic acid, (△) 20 μM D-glutamic acid, (▽) 20 μM L-aspartic acid, and (□) 20 μM GABA. (B) Influx: 37 μg of membrane protein was diluted into 200 μL of 0.1 M NaCl plus 0.5 μCi of L-[3,4-$^3H_2$]glutamic acid and the same additions [same symbols as in (A)].

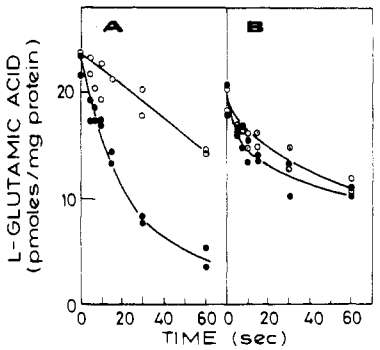

FIGURE 5: Effect of internal ion composition of L-glutamic acid induced L-glutamic acid efflux from passively loaded vesicles. Membrane vesicles were loaded passively, with L-[3,4-$^3H_2$]glutamic acid and the internal media indicated below, by freezing and thawing. Subsequently, dilution-induced efflux into either 90 mM NaCl plus 10 mM NaP_i, pH 6.8 (○), or the same plus 50 μM L-glutamic acid (●) was measured. (A) The internal medium contained 90 mM NaCl plus 10 mM NaP_i, pH 6.8; (B) the internal medium contained 90 mM LiCl plus 10 mM LiP_i, pH 6.8.

acid induced efflux of L-glutamic acid does not require external sodium (data not shown).

The experiment depicted in Table I also shows that the K_m for L-glutamic acid influx is lowered by external sodium and internal potassium, while V_{max} is increased by these conditions. These results further support the direct effects of sodium and potassium on the L-glutamic acid transporter.

(2) *Effect of Internal Sodium Ions.* Since release of internal L-glutamic acid by external L-glutamic acid does not require external sodium, it is of interest to consider whether this process requires internal sodium. As shown previously, net efflux of L-glutamic acid requires internal sodium. Notwithstanding the fact that during active loading the vesicles were not preloaded with sodium, apparently enough of it enters during the influx stage (1 min of incubation into 0.1 M NaCl) to support the subsequent dilution-induced efflux. However, it is possible to circumvent the stage of preexposure to sodium by loading the vesicles with L-[3,4-$^3H_2$]glutamic acid passively by the freeze–thaw technique (Kanner & Kifer, 1981; Kanner & Marva, 1982). In the experiment illustrated in Figure 5, vesicles were loaded with L-[3,4-$^3H_2$]glutamic acid and either a lithium or a sodium medium. Subsequently the vesicles are diluted into sodium phosphate with or without L-glutamic acid.

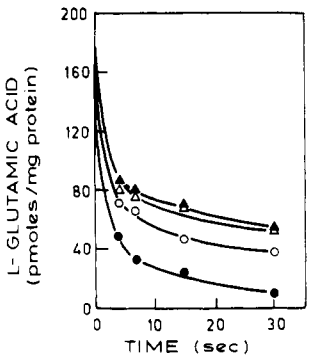

FIGURE 6: Effect of nigericin on L-glutamic acid induced L-glutamic acid efflux. Active loading and efflux were performed as in Figure 1 by using 24 μg of membrane protein. The composition of the efflux medium (pH 6.8) was (○) 0.1 M NaP_i plus 50 μM L-glutamic acid, (●) the same plus 5 μM nigericin, (△) 0.1 M LiP_i plus 50 μM L-glutamic acid, and (▲) the same plus 5 μM nigericin.

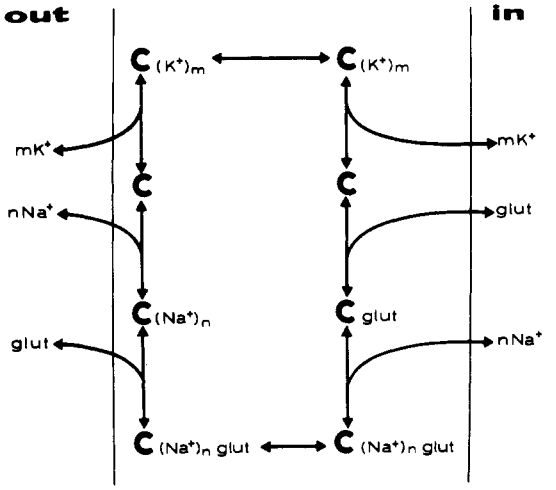

FIGURE 7: One of the possible models for the mechanism of L-glutamic acid translocation. As discussed in the text the binding/debinding order of sodium and L-glutamic acid as indicated here for the inside and the outside may explain the data, but other possibilities exist. The main point is that the translocation step for potassium is distinct from that for sodium and L-glutamic acid.

With the lithium-loaded vesicles, some efflux of L-glutamic acid occurs, probably by a leakage pathway. The efflux is only slightly enhanced by external L-glutamic acid (Figure 5B). On the other hand, external L-glutamic acid clearly stimulates efflux of L-glutamic acid from the sodium-loaded vesicles (Figure 5A). Thus, it appears that the process requires internal sodium. Further support for this contention is obtained in the experiment illustrated in Figure 6. Here the vesicles are actively loaded with L-glutamic acid. Dilution into a medium containing sodium and L-glutamic acid causes a very fast release. When the ionophore nigericin is included in the dilution medium, a condition which should increase internal sodium, this release is even faster (Figure 6). The effect is not due to an artifact of nigericin since this ionophore has no effect when external sodium is substituted by lithium (Figure 6).

Discussion

All the results on influx, efflux, and exchange of L-glutamic acid can be explained by the model depicted in Figure 7. According to this model an influx cycle is intiated by the binding of sodium ions to the external face of the transporter, followed by the binding of L-glutamic acid. This results in formation of the translocation complex. Subsequently the transporter reorients resulting in translocation of the sodium

ions and the L-glutamic acid to the inside. Sodium ions are released first followed by the L-glutamic acid. In order for a new translocation cycle to start, the transporter has to reorient back in order for the sodium and L-glutamic acid binding sites to be exposed to the outside. This is achieved by the binding of internal potassium, translocation of the potassium loaded transporter, and the release of the potassium to the outside. Efflux is to occur via reversal of this mechanism (going clockwise).

This model explains the observation that efflux of L-glutamic acid can occur under two sets of conditions: (a) external potassium and internal sodium ions (Kanner & Marva, 1982; Figure 1) or (b) external L-glutamic acid. In the absence of external potassium hardly any efflux of L-glutamic acid occurs (Figure 1), but exchange is very fast (Figure 2). This implies that a step distinct from the L-glutamic acid translocation step—such as the return of the translocator without sodium and L-glutamic acid—is rate determining for net efflux. The rate of efflux is stimulated by external potassium so that it becomes comparable to that of exchange. This implies that potassium acts at the rate-limiting step for net flux. Potassium appears to be directly involved in the translocation cycle; it is moving in the opposite direction as sodium and L-glutamic acid (Kanner & Marva, 1982). Therefore, we postulate that potassium binds to the unloaded transporter and enables it to return so that its binding sites for sodium and L-glutamic acid become available after the potassium has been translocated and released. Thus, with external potassium, efflux of L-glutamic acid occurs involving all the steps outlined in the model, going clockwise. When external L-glutamic acid provokes efflux, this occurs via exchange mechanism—radioactive L-glutamic acid binds from the inside, is translocated to the outside, and is released there; subsequently unlabeled glutamic acid binds on the outside and translocates back, and upon its release to the inside, another molecule of radioactive L-glutamic acid can exit.

One of the most interesting observations is that, although the effect is exerted via the transporter (Figures 3 and 4), no external sodium is required (Figure 2). This implies very strongly that sodium is released on the outside more slowly than L-glutamic acid. In other words, radioactive L-glutamic acid, upon translocation from the inside, is released on the outside and unlabeled L-glutamic acid rebinds before sodium is released from the transporter. The result excludes an ordered mechanism where sodium is released on the outside prior to the L-glutamic acid. On the other hand, it cannot be excluded that the order is random. If the probability that on the outside L-glutamic is debinding first is equal to that for a sodium ion, one would expect that the initial rate of loss of radioactive L-glutamic acid into a medium containing lithium and L-glutamic acid will be half of that into a medium containing sodium and L-glutamic acid, provided $n = 1$. Since those initial rates are too fast to be measured (Figure 2) we have no information on this. Moreover, the number of sodium ions participating in a translocation cycle is probably greater than one. In view of the electrogenicity of the process—positive charge moving inward (Kanner & Sharon, 1978)—the simplest stoichiometry will be three sodium ions and one glutamate anion moving inward and one potassium moving outward.

With regard to the order of events on the inside, these are most easily explained by assuming that at least one of the sodium ions is released on the inside prior to L-glutamic acid. Thus, both with actively (Figure 6) and with passively loaded vesicles (Figure 5) the release of internal glutamic acid exhibits a dependence on the internal sodium concentration. However, one other possibility which is also consistent with this result cannot be excluded; L-glutamic acid may be released on the inside prior to sodium but rebinding of L-glutamic acid on the inside would be much slower than debinding of the sodium.

From a very recent study (Kanner & Marva, 1982) comparing the ionic requirements for influx and efflux, it appeared as if the L-glutamic acid transporter is functionally symmetrical. However, the exchange studies described here indicate an asymmetry with regard to sodium. The role of potassium has been clarified as well. The results of the previous paper (Kanner & Marva, 1982) are consistent with the possibility that potassium is transported simultaneously with L-glutamic acid but also with the possibility that the transporter translocates potassium at a step distinct from the L-glutamic acid translocation step. From the present study it can be concluded that the last possibility is the correct one.

Finally, it should be noted that Wheeler (1979), performing kinetic experiments with intact synaptosomes, arrived at a binding order on the outside of Na, Na, and L-glutamic acid. Although the obligatory role of potassium in the translocation cycle was not taken into consideration, it is of interest that this order agrees very well with the conclusions reached by an entirely different approach on synaptic plasma membrane vesicles.

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