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Endogenous L-glutamate transport in oocytes of *Xenopus laevis*

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The existence of an endogenous Na^+-glutamate cotransporter in the oocytes of *Xenopus laevis* is demonstrated. The transporter does not accept D-glutamate as substrate. The dependence on substrate displays two saturating components with low ($K_{1/2}$ = 9 mM) and high ($K_{1/2}$ = 0.35 μM) affinities for L-glutamate. The dependence on external Na^+ exhibits a saturating component with a $K_{1/2}$ value of about 5 mM and a component that has not saturated up to 110 mM Na^+. In voltage-clamped oocytes, it is possible to demonstrate that Na^+-dependent L-glutamate transport is directly coupled to countertransport of Rb^+. The analysis of the voltage dependence of the Na^+,K^+-dependent L-glutamate uptake suggests that positive charges are moved inwardly during the transport cycle.

Introduction

Na^+-driven cotransport of L-glutamate has been demonstrated in epithelia of kidney and intestine [1], in synaptic membranes [2], in glia cells [3], and cells of liver [4] and placenta [5,6]. In addition to its dependence on external Na^+, L-glutamate uptake depends on the presence of internal K^+ [7–11]. Since outwardly directed K^+ gradients stimulate L-glutamate uptake [7] and Rb^+ efflux is stimulated when L-glutamate is added to the external medium [9], countertransport of K^+ has been suggested [10]. However, final proof is still missing since one cannot exclude that the observed stimulation of Rb^+ efflux in fact results from L-glutamate induced depolarization of the membrane potential.

Since the membrane potential depolarizes during stimulation of the transport of anionic amino acids across epithelial membranes [12,13], one can conclude that the overall Na^+- and K^+-dependent uptake of L-glutamate is electrogenic. The transport of charges across the cell membrane implies that transport activity has to depend on membrane potential; this has been shown for glutamate uptake in renal and intestine epithelia (see Ref. 14) and for current mediated by the glutamate transporter in glia cells [15].

To characterize carrier-mediated transport systems, the oocytes of *Xenopus laevis* have been demonstrated to represent an excellent model system. The large size of these cells allows one to investigate potential dependence of tracer fluxes and electrogenicity of amino-acid cotransporters under voltage-clamp conditions [16,17]. Since the introduction of the oocytes as an expression system for foreign mRNA [18], membrane receptors, ion channels, and transporters have been expressed in these cells. Na^+-driven cotransport also could be characterized in the oocytes after injection of foreign mRNA [19–23]. The investigation of foreign cotransport systems in the oocytes is complicated by the fact that these cells exhibit a variety of endogenous Na^+-driven cotransport systems [16,24–28].

Successful expression of membrane transport after injection of specific mRNA does not mean a priori that this mRNA codes for the whole transport protein. For the Na^+/K^+ pump, e.g., it could be demonstrated that injection of foreign mRNA for only the β-subunit already leads to expression of endogenous catalytic α-subunits and additional functional pumps [29,30]. Therefore, to judge whether a new foreign transport system is expressed after injection of mRNA, detailed knowledge of the endogenous transport systems is necessary.

In this paper, we show that the oocytes of *Xenopus laevis* exhibit endogenous Na^+-dependent L-glutamate uptake. This transport can occur at rates of similar magnitude as reported for oocytes that had been injected with foreign mRNA [31], and shows characteristics similar to those of somatic cells. In the oocytes,

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we can demonstrate that Na^+-dependent L-glutamate uptake is coupled to Rb^+ countertransport under voltage-clamp conditions. Part of these results have been presented previously [32].

Materials and Methods

Oocytes

Females of the clawed toad *Xenopus laevis* were anesthetized by incubating the animal in water containing 2 g/l *m*-aminobenzoic acid ethylester methanesulfonate (MS222, Sandoz Ltd., Basel, Switzerland) and parts of the ovary were removed. Fullgrown oocytes arrested in the prophase of the first meiotic division (stage V and VI, after Dumont [33]) were selected after removal of enveloping tissue by treatment of the ovarial pieces with collagenase (0.6–0.8 U/ml in oocyte Ringer's solution (ORi, see below) and subsequent washing in Ca^{2+}-free ORi. All experiments were performed at room temperature (about 21°C).

Measurements of uptake

To measure uptake of substrate, oocytes were incubated at room temperature in ORi containing L- or D-glutamate, or L-aspartate labeled with ^3H (about 185 kBq in 500 μl) After suitable lengths of time, 10 oocytes were removed from the medium. The cells were washed, placed individually into counting vials, and dissolved in 0.1 ml (5%) sodium dodecyl sulfonate solution. The radioactivity taken up by the oocytes was then determined by liquid scintillation counting. All data presented in the results refer to average values obtained from at least 10 single oocytes. To determine $K_{1/2}$ values for the stimulation of L-glutamate uptake, rates of uptake (k) were described by the equation:

$$k = k_{max} \cdot [X]^n / (K_{1/2}^n + [X]^n) \quad (1)$$

where [X] represents the Na^+ or L-glutamate concentration. The parameter values were calculated using a curve-fitting procedure based on least-squares methods.

Measurements of $^{86}Rb^+$ efflux

For measurements of L-glutamate-dependent K^+ efflux, $^{86}Rb^+$ was used as a tracer. For this purpose oocytes were preloaded with $^{86}Rb^+$ by incubating the cells for 6 h in Na^+- and K^+-free solution containing 7.1 mM $^{86}Rb^+$ (about 700 kBq per 100 μl). Single oocytes were then incubated successively in small volumes (250–500 μl) of K^+-free efflux media. For each oocyte $^{86}Rb^+$ release into K^+-free ORi was measured first, then into K^+-free ORi plus 10 mM L-glutamate, and finally into Na^+- and K^+-free ORi plus 10 mM L-glutamate. Occasionally measurements of $^{86}Rb^+$ release at the end of the experiment into control K^+-free ORi gave about the same rate of efflux as at the beginning of the experiment. For each type of efflux medium, the rate of Rb^+ efflux was evaluated from five consecutive determinations of the amounts of radioactivity released to the medium within 5 min. The data given in the Results represent averages ± S.E. of the five determinations performed on three different oocytes, respectively.

Electrophysiological measurements

Measurements of membrane potential and voltage-clamp experiments were performed by conventional two-microelectrode techniques [34]. For determination of current-voltage dependencies, rectangular voltage pulses of variable amplitude and 500 ms duration were applied every 4 s, and steady-state current was averaged during the last 100 ms. Between the pulses, the holding potential was set to −60 mV. For uptake measurements under voltage clamp, oocytes were exposed to ORi containing L-[^3H]glutamate; within less than one minute voltage-clamp was established and the cell was then kept under constant voltage-clamp conditions for 10 min.

Solutions

The composition of the ORi solution was (in mM): 110 NaCl, 3 KCl, 2 $CaCl_2$, 5 morpholinopropanesulfonic acid (Mops, adjusted to pH = 7.6). If lower Na^+ concentrations were used, NaCl was replaced by equimolar concentrations of tetramethylammonium chloride (TMA-Cl).

Results

The full-grown prophase-I arrested oocytes show variable L-glutamate uptake. Depending on the batch of oocytes, the rate of uptake by a single cell at 10 mM L-glutamate in the external medium can vary by more than one order of magnitude from 15.5 ± 0.7 for one batch to 282.6 ± 26.6 pmol/min for another batch. To give average values for results obtained from experiments on oocytes of different animals, all data were normalized to control conditions (usually 110 mM Na^+ and 10 mM L-glutamate), and later on rescaled to the averaged value at the control conditions. The uptake of L-glutamate is composed of two components that are similar in size. One component (40%) is independent of the presence of extracellular Na^+, for the other component (60%) extracellular Na^+ is necessary. The Na^+-dependent component of uptake follows a nearly linear time course in the presence of 140 nM L-[^3H]glutamate for about half an hour (Fig. 1). After 10 min, the amount of L-glutamate taken up by a single oocyte is sufficiently large to be analyzed accurately. Therefore, rates of uptake were determined after incubation of oocytes for 10 min in solution containing the

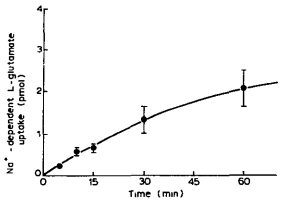

Fig. 1. Time course of Na^+-dependent L-glutamate uptake was determined as difference of uptake in presence and absence of 110 mM Na^+. The L-glutamate concentration in the external medium was 140 nM. All data points represent averages (±S.E.) from 20 oocytes of three different animals.

radioactively labeled substrate. In the following, we will characterize only the Na^+-dependent transport of L-glutamate as determined by the difference between uptake in presence and absence of 110 mM Na^+.

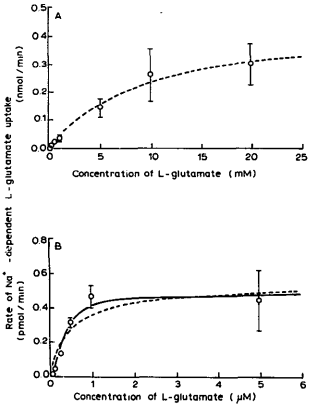

Fig. 2. Dependence of Na^+-dependent L-glutamate uptake on L-glutamate concentration determined as the difference of uptake in presence and absence of 110 mM Na^+. Rate of uptake is shown for a range of (A) millimolar concentrations and of (B) micromolar concentrations. Data points represent averages from 20 to 30 oocytes (±S.E.). Broken lines represent non-linear least-squares fits of Eqn. 1 to the data with $n = 1$, the solid line in (B) with $n = 2$. The fitted $K_{1/2}$ values were for low affinity 9.1±2.7 mM, for high affinity 0.44±0.22 μM ($n = 1$) or 0.35±0.05 μM ($n = 2$).

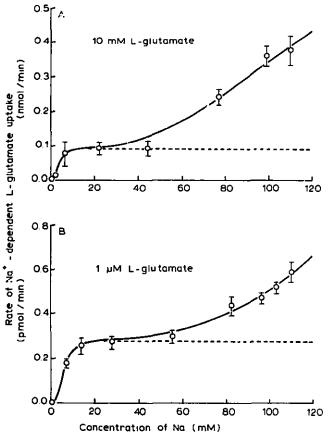

Fig. 3. Na^+ dependence of Na^+-dependent L-glutamate uptake with (A) 10 mM and (B) 1 μM L-glutamate in the external medium. Data points represent averages from 25 oocytes (±S.E.) of four animals. The broken lines represent non-linear least-squares fits of Eqn. 1 to the data below 60 mM Na^+ with $n = 3$. The corresponding $K_{1/2}$ values in presence of 10 mM and 1 μM L-glutamate were 4.2±0.2 mM and 5.9±0.4 mM, respectively. The solid lines represent the sum of this component plus a second component which has not saturated up to 110 mM.

Dependence of glutamate uptake on glutamate concentration

Fig. 2 illustrates the dependence of the rate of Na^+-dependent L-glutamate uptake on L-glutamate concentration for two different concentration ranges. In the millimolar range (Fig. 2A) saturation is obtained at about 20 mM, and the data can be described by the Michaelis-Menten equation (Eqn. 1 with a Hill coefficient of $n = 1$). A least-squares fit yields a $K_{1/2}$ value of about 9 mM. Analysis of the Na^+-dependent uptake at micromolar substrate concentrations demonstrates a second saturating component (Fig. 2B). The dependence on L-glutamate can again be described by the Michaelis-Menten equation (see broken line) with a $K_{1/2}$ value of about 0.4 μM though a better fit is obtained with $n = 2$ (solid line) yielding a slightly lower $K_{1/2}$ value (see legend to Fig. 2). The data demonstrate endogenous Na^+-dependent L-glutamate transport in *Xenopus* oocytes with low and high substrate affinities. The k_{max} value of the high-affinity component is three orders of magnitude lower than the k_{max} of the low-affinity component.

Dependence of glutamate uptake on Na^+ concentration

If the extracellular Na^+ concentration (normally 110 mM) is reduced, the rate of L-glutamate uptake decreases. This is shown for two glutamate concentrations (10 mM and 1 μM) in Fig. 3. These concentrations are of the same order of magnitude as the $K_{1/2}$ values for the low- and high-affinity components, respectively (compare Fig. 2). A reasonable description of L-glutamate uptake on Na^+ concentration is obtained by superimposition of two components. At Na^+ concentrations below 60 mM, transport may be described for both L-glutamate concentrations by a saturating component (Eqn. 1) with a $K_{1/2}$ value of about 5 mM and a Hill coefficient of $n = 3$ (broken line). At higher Na^+ concentrations, L-glutamate uptake continues to increase with no clear sign of saturation up to 110 mM Na^+. Whether or not this second component is also mediated by a saturating transport mechanism cannot be decided since application of significantly higher concentrations of Na^+ will increase the osmolarity of the solution too much and damage the oocyte.

Specificity of the glutamate transporter

To determine whether or not the Na^+-dependent glutamate transporter is specific for L-glutamate, we examined the effects of adding 0.1 mM of unlabeled L-glutamate, L-aspartate, or D-glutamate to solution containing 250 nM L-[^{3}H]glutamate. As expected, strong inhibition by more than 80% is obtained with 0.1 mM L-glutamate; inhibition (by 50%) is also achieved with 0.1 mM L-aspartate. On the other hand, 0.1 mM D-glutamate does not inhibit the Na^+-dependent L-glutamate uptake. The results suggest that the L-glutamate transporter may accept L-aspartate as well, but does not carry D-glutamate.

Dependence of ^{86}Rb efflux on glutamate

For the Na^+-dependent glutamate transporter in somatic cells, dependence of L-glutamate uptake on intracellular K^+ concentration has been demonstrated and an antiport of K^+ has been suggested [3,5,7,9–11,35]. To investigate whether in the oocytes Na^+-dependent L-glutamate uptake is accompanied by an antiport of K^+, oocytes were preloaded with $^{86}Rb^+$ as a tracer for K^+ efflux. The rate of Rb^+ release was determined under different external substrate conditions. In K^+-free ORi without L-glutamate, a low rate of Rb^+ release of 2.4 ± 0.2 pmol/min is detectable. If 10 mM L-glutamate is added to the incubation solution, a dramatic increase in Rb^+ release to 12.9 ± 1.6 pmol/min is obtained. This increase in Rb^+ release is not obtained if Na^+ is replaced by TMA^+ (2.8 ± 0.5 pmol/min) suggesting that the Na^+-dependent L-glutamate uptake is coupled to the Rb^+ (or K^+) efflux.

As has been demonstrated for other cells [12,13,15], Na^+-glutamate cotransport is electrogenic also in the *Xenopus* oocytes (see below). Since stimulation of L-glutamate uptake results in membrane depolarization, the increased Rb^+ efflux in the presence of L-glutamate could be due to an increased driving force rather then representing Rb^+ antiport by the L-glutamate transporter. To exclude this possibility, efflux measurements were performed on oocytes that were kept at -70 mV by two-microelectrode voltage clamp during the efflux measurement. Also under voltage clamp $^{86}Rb^+$ efflux is facilitated during stimulation of the Na^+-dependent L-glutamate uptake. In Na^+- or L-glutamate-free solution the rate of $^{86}Rb^+$ efflux is 3.9 ± 1.7 pmol/min but increases to 55.0 ± 6.7 pmol/min in the presence of 110 mM Na^+ and 10 mM L-glutamate. The more pronounced stimulation of Rb^+ efflux by L-glutamate under voltage clamp may partially reflect variability of oocytes from different females, partially to be due to the fact that the Na^+,K^+-dependent L-glutamate transport is potential-dependent (see Fig. 5C below) and that the membrane potential in the unclamped oocytes is only about -20 mV under the experimental conditions used here.

Electrogenicity of glutamate uptake

In order to explore whether or not the Na^+,K^+-dependent L-glutamate transport is electrogenic, we investigated the effect of transport stimulation on membrane potential. If 10 mM L-glutamate is added to ORi, the membrane potential depolarizes; depending on the batch of oocytes, the depolarization can vary

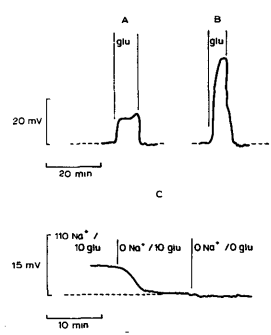

Fig. 4. (A) and (B) Effect of 10 mM L-glutamate on membrane potential in two oocytes obtained from different animals. The membrane potential in ORi was about -60 mV (broken line). (C) Effect of 10 mM L-glutamate in presence and absence of 110 mM Na^+. For this type of experiment K^+-selective channels were blocked by 5 mM $BaCl_2$ and 20 mM tetraethylammonium chloride, the membrane potential in Na^+-containing solution without L-glutamate was about -30 mV (broken line).

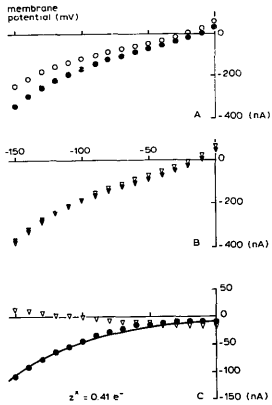

Fig. 5. Voltage dependence of membrane current. All bath solutions contained 10 μM ouabain to inhibit the Na^+/K^+ pump and 5 mM $BaCl_2$ plus 20 mM tetraethylammonium chloride to block K^+-selective channels. (A) Current-voltage dependencies with 10 mM L-glutamate in the external solution in presence (filled symbols) and absence of 110 mM Na^+ (open symbols). (B) Current-voltage dependencies without L-glutamate in the external solution in presence (filled symbols) and absence of 110 mM Na^+ (open symbols). (C) Voltage dependence of current mediated by the Na^+-dependent glutamate transporter (filled circles) determined in the presence of the L-glutamate as the difference of current in presence and absence of Na^+. Data were corrected for the small effect of Na^+ removal on membrane current (open triangles). The solid line represents a non-linear least-squares fit of $I = I(0\ mV) \exp(z^*EF/RT)$ (E is the membrane potential, F/RT have their usual meaning).

between 10 and 30 mV (see Figs. 4A and B). Since L-glutamate-induced depolarizations are occasionally observed also in the absence of extracellular Na^+, it cannot be excluded that part of the membrane polarization is due to activation by glutamate of anion-selective channels in the oocytes [36]. But also in cells that are insensitive to L-glutamate in the absence of extracellular Na^+, glutamate-induced depolarizations can be observed in the presence of Na^+ (Fig. 4C). This suggests that the Na^+- and L-glutamate-dependent transport is electrogenic and involves net inward movement of positive charges.

Voltage dependence of glutamate uptake

Since L-glutamate uptake is electrogenic, the rate of transport should depend on membrane potential. This was supported by preliminary experiments which showed that uptake of L-glutamate in the presence of Na^+ was reduced if the membrane potential was depolarized. For a more detailed investigation of the potential dependence of Na^+,K^+-dependent L-glutamate transport, the potential dependence of current mediated by the carrier was measured in voltage-clamp experiments. Figs. 5A and B show an example of current-voltage curves of total membrane current in external solutions with and without 10 mM L-glutamate in presence of 110 mM Na^+ (A), and with and without 110 mM Na^+ in absence of L-glutamate (B). In addition, all bath solutions contained 10 μM ouabain to block active Na^+/K^+ translocations; under these conditions, removal of extracellular Na^+ has only a small effect on membrane currents in the absence of L-glutamate (see Fig. 5B and open triangles in 5C). Since *Xenopus* oocytes occasionally exhibit anion channels that can be activated by glutamate [36] (compare also open symbols in Fig. 5A with those in Fig. 5B), current generated by the transporter was estimated as the difference of membrane current in presence and absence of extracellular Na^+ while 10 mM L-glutamate was present throughout (see Fig. 5A and filled circles in Fig. 5C). The voltage dependence of current mediated by the L-glutamate transporter demonstrates that membrane depolarization leads to an inhibition of transport. The voltage dependence can be described by a single exponential assuming that an effective charge of about 0.4 of an elementary charge moves in the electrical field during a transport cycle.

Discussion

In this paper endogenous Na^+,K^+-dependent L-glutamate transport was detected in the oocytes of *Xenopus laevis*. This transport was characterized since detailed knowledge about the endogenous transport is a prerequisite if the oocytes are to be used as an expression system for foreign L-glutamate transporters. In addition, oocytes represent an ideal model system to investigate properties of Na^+,K^+-coupled L-glutamate transport since tracer flux measurements can be performed on single cells under voltage-clamp conditions. The low permeability of the oocyte membrane for cations directly allows to demonstrate Na^+, L-glutamate coupled efflux of the K^+ using $^{86}Rb^+$ as tracer.

Our data show that the endogenous Na^+,K^+-dependent L-glutamate transport in the oocytes is similar but not identical to previously characterized Na^+-dependent L-glutamate transport in somatic cells. The similarities include substrate specificity [6,8,9]; it appears that in addition to L-glutamate L-aspartate is accepted as substrate, D-glutamate is not carried. For stimulation of L-glutamate uptake by external L-glutamate in somatic cells $K_{1/2}$ values mostly in the micromolar range (1–20 μM) were reported [2,3,5,9,15]. In addition to this high affinity component, a second, low affinity transport system for anionic amino acids has

been postulated with $K_{1/2}$ values in the millimolar range [37,38] but has not been demonstrated unequivocally [6]. For the endogenous Na^+-dependent L-glutamate transporter in the *Xenopus* oocytes, a high-capacity component with an affinity in the millimolar range ($K_{1/2} = 9$ mM) and a component with a nearly three orders of magnitude lower capacity but high affinity ($K_{1/2} = 0.4$ μM) was detected.

Whereas in glia and kidney cells $K_{1/2}$ values for Na^+ stimulation of at least 40 mM were demonstrated [3,8], we found in the oocytes superimposition of two components with respect to transport stimulation by external Na^+: a saturating component with a $K_{1/2}$ value of about 5 mM and a component which had not saturated at 110 mM Na^+. The affinity for Na^+ of the saturating component is independent of whether the high- or low-affinity transport for L-glutamate is investigated. This supports the hypothesis that high- and low-affinity transport represent two transport modes of the same transport system rather than two separate transporters. In both cases best fits are obtained with a Hill coefficient of about 2–3. A similar Hill coefficient was also obtained by electrical measurements in glia cells [3]. The observed Hill coefficients are consistent with the hypothesis that together with the uptake of one negatively charged L-glutamate three Na^+ ions are cotransported into the cell and one K^+ ion is countertransported out of the cell [3,9].

Since it has been observed previously that internal K^+ stimulates the L-glutamate uptake into membrane vesicles [2,5,7,9,11,35] and the L-glutamate induced current in glia cells [3], antiport of K^+ has been discussed (see, for example, Ref. 10). K^+ efflux could not directly be studied in the relatively leaky membrane vesicles but after reconstitution of the Na^+-dependent L-glutamate transporter in proteoliposomes [9]. In these experiments, an increased $^{86}Rb^+$ efflux could be demonstrated after L-glutamate was added to the external medium suggesting a direct coupling of L-glutamate uptake with K^+ efflux. Uncertainty, however, remained since it cannot be excluded that the observed increase in Rb^+ efflux is due to membrane depolarization that accompanies the application of glutamate. In the present investigation, L-glutamate induced Rb^+ efflux could be measured under voltage clamp conditions, and stimulation of the efflux at a fixed membrane potential gives final proof that Rb^+ antiport is coupled to L-glutamate uptake in the oocytes.

Whether or not transport is electrogenic has been deduced in other preparations from voltage dependence of L-glutamate uptake (see, for example, Ref. 14); this, however, is not an unequivocal characteristic since also electrically silent transport can depend on membrane potential [39]. As has been demonstrated for the Na^+-dependent L-glutamate transporter in kidney and retina [12,13,15], we found that also the endogenous glutamate transporter in the *Xenopus* oocytes is electrogenic since stimulation of the transporter leads to depolarization of the membrane potential. This depolarization is due to Na^+-and glutamate-dependent inwardly directed current that could be measured under voltage-clamp conditions. For the determination of the current-voltage (*I-V*) relationship, the transporter was stimulated in presence of L-glutamate by addition of Na^+ rather than addition of L-glutamate in the presence of Na^+ [15]. This avoids the additional activation of anion-selective channels that are sensitive to glutamate [36]. The steepness in the *I-V* relationship is compatible with the interpretation that an effective charge of about 0.41 of a positive elementary charge is move inward in the electrical field during one transport cycle; the same effective charge can be estimated from the data published by Brew and Attwell [15].

In conclusion, the oocytes of *Xenopus laevis* have an endogenous Na^+-glutamate cotransporter which apparently shares the main features of Na^+-glutamate cotransporters thus far described; these features include countertransport of K^+ and electrogenicity. Quantitative differences exist with respect to substrate dependencies. In expression studies, these differences may help to separate endogenous from expressed Na^+,K^+-dependent L-glutamate transport.

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