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Electrogenic L-Glutamate Uptake in *Xenopus laevis* Oocytes Expressing a Cloned Rat Brain L-Glutamate/L-Aspartate Transporter (GLAST-1)*

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The transport of L-glutamate into *Xenopus laevis* oocytes expressing the cloned L-glutamate/L-aspartate transporter (GLAST-1) from rat brain was studied using the voltage clamp technique. At a holding potential of −90 mV, a bath application of 100 μM L-glutamate induced an inward current (I_{GLAST}) with an amplitude ranging from −5 to −30 nA. I_{GLAST} did not require extracellular Ca^{2+}, Mg^{2+}, or Cl^-, was larger at negative potentials, and did not reverse up to +80 mV. The current was dependent on external L-glutamate and Na^+ with half-maximal amplitudes at 11 μM L-glutamate and 41 mM Na^+. I_{GLAST} saturated at 100 μM L-glutamate and 80 mM Na^+. The Hill coefficient for Na^+ and L-glutamate was 3.3 and 1.3, respectively, suggesting that 3 Na^+ accompany the transport of 1 L-glutamate molecule. At low $[Na^+]_o$, I_{GLAST} was enhanced by reducing $[K^+]_o$, an indication for the countertransport of K^+. Reducing external pH from 7.4 to 6.0 did not change the amplitude of I_{GLAST}. This argues against a glutamate/proton cotransport. The results provide evidence for GLAST-1 carrying out a high affinity, sodium-dependent L-glutamate transport with a proposed stoichiometry of 3 Na^+, 1 L-glutamate$^-$/1 K^+.

L-Glutamate mediates neurotransmission at the vast majority of excitatory synapses in mammalian central nervous system (for review see Collingridge and Lester (1988)). The excitatory action of this amino acid is involved in complex physiological processes like learning and the establishment of memory (Monaghan *et al.*, 1989). On the other hand, L-glutamate has potent excitatoxic properties leading to neurodegeneration and brain damage (for review see Choi (1988)).

To terminate the excitatory signal, L-glutamate is rapidly removed from the synaptic clefts. Sodium-dependent L-glutamate transporters residing in the plasma membranes of the presynaptic nerve ending and surrounding glia cells are thought to be responsible for this transport process (Flott and Seifert, 1991). The structural specificity and ion dependence of this transport system have been investigated by [^{14}C]glutamate uptake studies using brain slices (Balcar and Johnston, 1972), synaptosomes (Bennett *et al.*, 1973), isolated cells (Gordon and Balázs, 1983; Flott and Seifert, 1991), and cell lines (Waniewski and Martin, 1984) for more than two decades. Further information about the mechanism of transport is obtained from electrophysiological measurements. It has been shown in Müller cells from salamander retina that L-glutamate evokes an inward current, which is most probably associated with L-glutamate uptake into the cytosol (Brew and Attwell, 1987; Barbour *et al.*, 1988, 1991; Schwartz and Tachibana, 1990). This electrogenic uptake of L-glutamate was dependent on external Na^+ and internal K^+ (Barbour *et al.* (1988, 1991) but see Schwartz and Tachibana (1990)).

The recent cloning of cDNAs coding for three rodent L-glutamate transporters offers for the first time the opportunity to investigate independently single members of this family upon expression in heterologous systems (Storck *et al.*, 1992; Pines *et al.*, 1992; Kanai and Hediger, 1992). Using electrophysiological techniques we describe important functional properties of the cloned L-glutamate/L-aspartate transporter (GLAST-1)[1] from rat brain expressed in *Xenopus laevis* oocytes. We demonstrate that the cloned transporter is electrogenic and has a high affinity for L-glutamate. The transport of L-glutamate depends on the transmembrane gradients of sodium and potassium ions. There is no evidence for the cotransport of protons.

MATERIALS AND METHODS

20–72 h after injection of GLAST-1 cRNA (Storck *et al.*, 1992) the oocytes were superfused by gravity flow with a solution composed of (mM): NaCl (120), $CaCl_2$ (1.8), KCl (2), HEPES-Tris (10), pH 7.4. Oocytes were voltage-clamped using a two-electrode voltage clamp amplifier (Warner Instruments Corp., Hamden, CT). Microelectrodes filled with 3 M KCl had resistances ranging from 1 to 2 megaohms. The software and hardware package ISO1 (MFK, Frankfurt, Germany) was used to generate the voltage commands to acquire and evaluate the data. Data were filtered at 10 Hz (−3 db) and digitized at 100 Hz. Assuming that L-glutamate has no other electrical effects apart from activating the transporter, I_{GLAST} can be obtained as the difference of the membrane current before and during application of L-glutamate.

In experiments in which the sodium or chloride concentrations of the bath solution were varied, these were equimolarly substituted by choline or methanesulfonate, respectively. All measurements were done at room temperature (20–22 °C). Data are expressed if not otherwise noted as mean ± S.D. All chemicals were purchased from Sigma.

RESULTS

The Glutamate-induced Inward Current Is Generated by the Activity of GLAST-1—Bath application of 100 μM L-glutamate induced an inward current into *Xenopus* oocytes (stage V–VI) that peaked within 2 s to a plateau of −7 nA and remained stable up to 2 h as long as L-glutamate was present in the bath with less than a 20% decline in amplitude (Fig. 1*A*). Upon washout of L-glutamate the inward current disappeared. No inward currents were observed upon L-glutamate application in oocytes injected with water (n = 8, per batch) or in non-injected oocytes (n = 5, per batch).

The L-glutamate-induced current did not depend on extracellular Ca^{2+}, Mg^{2+}, or Cl^-, suggesting that inward current did not flow through L-glutamate-gated channels. Under control conditions 100 μM L-glutamate induced a current with a mean am-

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[1] The abbreviations used are: GLAST-1, a L-glutamate/L-aspartate transporter; MES, 2-(*N*-morpholino)ethanesulfonic acid.

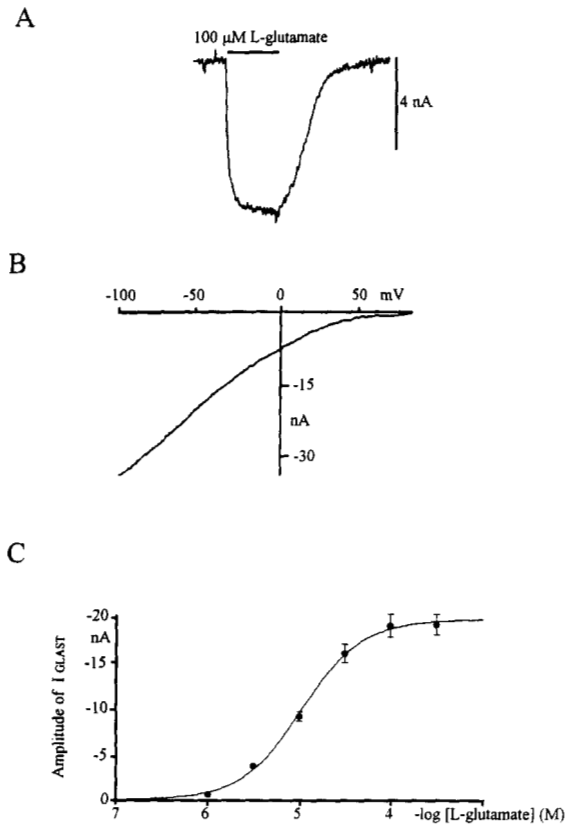

FIG. 1. **The inward current is generated by the activity of GLAST-1.** *A*, current induced by 100 μM L-glutamate under control conditions (holding potential = −90 mV). *B*, current voltage relationship obtained by depolarizing the membrane with a voltage ramp continuously from −100 to +80 mV. The difference of the currents under control conditions and during application of 100 μM L-glutamate is plotted as a function of voltage. *C*, concentration response curve for L-glutamate. The *dots* represent the mean values of 39 oocytes. The *solid line* is fit to the data by minimizing squared errors according to the equation shown in the text with a apparent K_m value of 11 μM and an I_{max} of −20 nA (holding potential = −90 mV).

plitude of −7.2 ± 1.7 nA (n = 6). Reducing $[Ca^{2+}]_o$ (substituted by 1.8 mM Mg^{2+} + 1 mM EGTA) resulted in an inward current of similar amplitude (−7.6 ± 2.5 nA), and likewise changing $[Cl^-]_o$ from 119.6 to 4.6 mM left the inward current with an amplitude of −7.2 ± 1.8 nA unimpaired.

Using ramp depolarizations (within 10 s from −100 to +80 mV) the L-glutamate-induced current is larger at negative potentials and did not reverse up to +80 mV (Fig. 1*B*). For unselective cation channels opened by L-glutamate the reversal potential should be around 0 mV (for review see Monaghan (1989)). Further evidence for the inward current reflecting the L-glutamate uptake into the oocyte (I_{GLAST}) came from the application of glutamate agonists (*trans*-1-amino-1,3-cyclopentane dicarboxylate, *N*-methyl-D-aspartate, and kainate). They completely failed to induce currents in oocytes injected with GLAST-1 cRNA (n = 7).

The amplitude of I_{GLAST} depended on the extracellular L-glutamate concentration. Fig. 1*C* shows the concentration response curve obtained from 12 different oocytes at a holding potential of −90 mV. In order to compare the different amplitudes of I_{GLAST} for each oocyte the amplitudes were normalized to the maximal current induced with 316 μM L-glutamate. When plotted as a function of the L-glutamate concentration the data fit with a curve obeying the equation,

$$I = I_{max}[\text{L-glutamate}]^n/([\text{L-glutamate}]^n + K_m{}^n) \quad \text{(Eq. 1)}$$

where I_{max} is the normalized maximum current amplitude, [L-glutamate] the L-glutamate concentration in the bath, K_m the apparent affinity constant, and n the Hill coefficient. The best fit was obtained with a K_m of 11 μM and n = 1.3. This result is compatible with the hypothesis that L-glutamate interacts with one high affinity binding site per transporter.

Dependence of I_{GLAST} on External Ions—Exchanging extracellular Na^+ with choline$^+$ completely blocked I_{GLAST} induced by bath application of 15 μM L-glutamate. At $[Na^+]_o$ larger than 10 mM the current rose steeply and saturated at $[Na^+]_o$ greater than 90 mM (Fig. 2*A*). The relation between the amplitude of I_{GLAST} and $[Na^+]_o$ could be best described by the following equation.

$$I = I_{max}[Na]^n/([Na]^n + K_m{}^n) \quad \text{(Eq. 2)}$$

The best fit yielded a K_m of 41 mM and a Hill coefficient of 3.3, suggesting that L-glutamate is cotransported with 3 sodium ions.

At 120 mM $[Na^+]_o$, raising $[K^+]_o$ from 2 to 32 mM had no effect on I_{GLAST}. At 2 mM $[K^+]_o$, 100 μM L-glutamate induced a current with a mean amplitude of −8.8 ± 1.2 nA (n = 5), elevating $[K^+]_o$ to 32 mM resulted in an I_{GLAST} of −8.6 ± 1.1 nA, and reducing its concentration back to 2 mM $[K^+]_o$ left I_{GLAST} with a mean amplitude of −9.2 ± 1.2 nA. However, at reduced $[Na^+]_o$, I_{GLAST} did depend on the K^+ gradient. In order to further reduce the sodium gradient the Na^+/K^+ pump was inhibited by storing the oocytes overnight at 4 °C. At 20 mM $[Na^+]_o$ and 2 mM $[K^+]_o$ application of 100 μM L-glutamate evoked a current with an

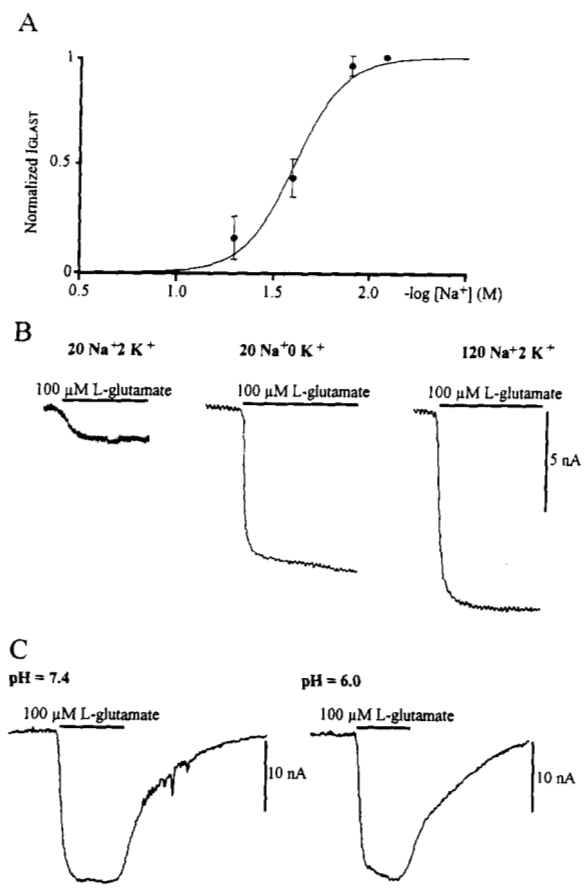

FIG. 2. **Dependence of I_{GLAST} on external ions.** *A*, concentration response curve for Na^+. The *dots* represent the means the *bars* the standard deviation from 5 oocytes. The current of each oocyte was normalized to the current amplitude at 120 mM $[Na^+]_o$. *B*, I_{GLAST} recorded at the indicated $[Na^+]_o$ and $[K^+]_o$ in the same oocyte. *C*, original traces recorded at a pH of 7.4 (*left*) and 6.0 (*right*) upon bath application of 100 μM L-glutamate.

amplitude of −1.7 nA (Fig. 2*B*, *left*). Under these conditions, however, the omission of K^+ from the bath solution produced dramatic changes in I_{GLAST}. Applying L-glutamate induced a current with an amplitude of −7.2 nA. In five similar experiments at reduced $[K^+]_o$ I_{GLAST} was enhanced by a factor of 3.5 ± 0.8. Changing back to control conditions (120 mM Na^+, 2 mM K^+) resulted in an inward current with an amplitude of −8.5 nA (Fig. 2*B*, *right*).

Reducing extracellular pH has no effect on the amplitude of I_{GLAST}. At pH 7.4 100 μM L-glutamate induced a current with an amplitude of −21.2 nA (Fig. 2*C*, *left*). Changing pH to 6.0 (10 mM MES buffer) has no appreciable effect on the amplitude of I_{GLAST} (−22.3 nA, Fig. 2*C*, *right*). This finding suggests that GLAST-1 does not transport protons together with L-glutamate into the cell.

DISCUSSION

Our results demonstrate for the first time that the GLAST-1-induced transport of L-glutamate in *X. laevis* oocytes is electrogenic. Application of 100 μM L-glutamate induced an inward that is independent of extracellular Ca^{2+}, Mg^{2+}, or Cl^-. This suggests that the current did not result from the opening of L-glutamate-gated channels but reflects the L-glutamate uptake into the oocytes. The current flowing through L-glutamate-gated unselective cation channels has a reversal potential around 0 mV (for review see Monaghan *et al.* (1989)). In contrast, the current studied here did not reverse up to +80 mV. A similar voltage dependence has been shown for the glutamate uptake into glial cells of the salamander retina (Barbour *et al.*, 1988, 1991; Schwartz and Tachibana, 1990).

At a holding potential of −90 mV the half-maximal current was obtained at a L-glutamate concentration of 11 μM. This value is lower than the value estimated from tracer flux measurements (77 ± 27 μM (Storck *et al.*, 1992)) but similar to the 6.9 μM reported for a cloned intestinal L-glutamate transporter (Kanai and Hediger, 1992) or the 5–20 μM reported for salamander glial cells (Barbour *et al.*, 1991). The low K_m of 11 μM indicated that the GLAST-1 from rat brain has a high affinity for L-glutamate.

There is evidence that the transport of L-glutamate depends on the presence of extracellular Na^+ (for review see Nicholls and Attwell (1990)). Also the electrogenic uptake of L-glutamate into glial cells depends on the presence of extracellular Na^+ with half-maximal inhibition at 43–50 mM (Barbour *et al.*, 1991; Schwartz and Tachibana, 1990), with Hill coefficients between 2 and 3. Our results fit best with a curve showing half-maximal inhibition at 41 mM $[Na^+]_o$ with a Hill coefficient of 3.3, suggesting that the transport of L-glutamate might go along with the binding of 3 Na^+. These results cannot exclude the possibility that only 2 Na^+ are transported in countertransport of pH changing anions as proposed by Bouvier *et al.* (1992).

From tracer flux studies it is evident that the uptake of L-glutamate depends on $[K^+]_o$ (Kanner and Sharon, 1978; Nicholls and Attwell, 1990). However, voltage clamp experiments gave conflicting results. Barbour *et al.* (1991) reported that K^+ is necessary for the L-glutamate-induced current, while in the same preparation Schwartz and Tachibana (1990) did not find any influence of K^+. Our finding that I_{GLAST} is unimpaired by high $[K^+]_o$ at saturating "physiological" $[Na^+]_o$ does not exclude K^+ as a countertransported ion. With 120 mM Na^+ and 100 μM L-glutamate the transporter is maximally stimulated so that increasing $[K^+]_o$ is not sufficient to decrease the high transport rate. The situation is different when the transport rate is not maximally stimulated, *e.g.* at reduced $[Na^+]_o$. Now a reduction of $[K^+]_o$ dramatically increases I_{GLAST} suggesting that K^+ is countertransported. Since the inward current associated with the uptake of L-glutamate requires the translocation of 1 netto positive charge, at least 3 Na^+ per molecule of L-glutamate have to be transported into the oocyte 1 in countertransport of 1 K^+.

Erecinska *et al.* (1983) have reported that protons were transported together with aspartate whereas according to Gazzola *et al.* (1981) L-glutamate is transported as an anion without a proton. Using a pK_a for the γ-carboxyl group of the L-glutamate group of 4.31, one can calculate that at a pH of 7.4 the ratio of protonated to deprotonated L-glutamate is approximately 1:1230, *i.e.* when adding 100 μM L-glutamate to the bath 0.08 μM is protonated. At pH 6.0 this relation is 1:49. Assuming that L-glutamate is transported as a zwitterion the amplitude I_{GLAST} should dramatically increase at acid pH because the amount of the protonated form has increased by a factor of 25. If L-glutamate is transported as an anion, there should be no appreciable influence on the amplitude of the current, because the amount of the protonated L-glutamate decreases only from 100 to 98 μM. Since acid pH has no detectable influence on the amplitude of I_{GLAST} it is likely that L-glutamate is transported as a negatively charged molecule. This hypothesis is in accordance with the result reported by Schwartz and Tachibana (1990) in salamander glial cells.

In this study we have characterized basic functional properties of the GLAST-1 cloned from rat brain and expressed in *X. laevis* oocytes. Our results provide evidence that GLAST-1 is a high affinity sodium-dependent L-glutamate transporter with a proposed stoichiometry of 3 Na^+, 1 L-glutamate$^-$/1 K^+.

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