Water transport by the human Na^+-coupled glutamate cotransporter expressed in *Xenopus* oocytes

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1. The water transport properties of the human Na^+-coupled glutamate cotransporter (EAAT1) were investigated. The protein was expressed in *Xenopus laevis* oocytes and electrogenic glutamate transport was recorded by two-electrode voltage clamp, while the concurrent water transport was monitored as oocyte volume changes.
2. Water transport by EAAT1 was bimodal. Water was cotransported along with glutamate and Na^+ by a mechanism within the protein. The transporter also sustained passive water transport in response to osmotic challenges. The two modes could be separated and could proceed in parallel.
3. The cotransport modality was characterized in solutions of low Cl^- concentration. Addition of glutamate promptly initiated an influx of 436 ± 55 water molecules per unit charge, irrespective of the clamp potential.
4. The cotransport of water occurred in the presence of adverse osmotic gradients. In accordance with the Gibbs equation, energy was transferred within the protein primarily from the downhill fluxes of Na^+ to the uphill fluxes of water.
5. Experiments using the cation-selective ionophore gramicidin showed no unstirred layer effects. Na^+ currents in the ionophore did not lead to any significant initial water movements.
6. In the absence of glutamate, EAAT1 contributed a passive water permeability (L_p) of $(11.3 \pm 2.0) \times 10^{-6}$ cm s^{-1} (osmol l^{-1})$^{-1}$. In the presence of glutamate, L_p was about 50% higher for both high and low Cl^- concentrations.
7. The physiological role of EAAT1 as a molecular water pump is discussed in relation to cellular volume homeostasis in the nervous system.

Cotransporters are membrane proteins in which the transmembrane fluxes of various substrates are coupled: the downhill flux of one substrate energizes the uphill flux of another. Evidence is accruing, however, that cotransporters, in addition to their recognized functions, also play a direct role in water transport. All cotransporters of the symport type tested so far exhibit cotransport of water, in which a fixed number of water molecules are translocated together with non-aqueous substrates by a process that is independent of external parameters. In addition, the cotransporters can function as passive water channels. The K^+–Cl^- and H^+–lactate cotransporters have been found to cotransport 500 water molecules per turnover (Zeuthen, 1991, 1994; Zeuthen *et al.* 1996). The human Na^+–glucose cotransporter, the rabbit Na^+–glucose cotransporter and the Na^+–dicarboxylate cotransporter cotransport 210, 390 and 175 water molecules per turnover, respectively (Loo *et al.* 1996; Zeuthen *et al.* 1997; Meinild *et al.* 1998, 2000). Osmotic water transport has been described in detail for the Na^+–glucose and the Na^+–GABA transporters (Loike *et al.* 1996; Loo *et al.* 1996; Loo *et al.* 1999*b*).

Here we study the water transport properties of a Na^+–amino acid cotransporter, the human glutamate transporter EAAT1. This protein is primarily found in glial cells (Rothstein *et al.* 1994; Lehre *et al.* 1995) but its location in peripheral tissue has also been reported (Arriza *et al.* 1994). Glutamate serves a variety of physiological functions. Besides its role as a constituent of proteins, glutamate is also one of the most abundant neurotransmitters in the mammalian central nervous system. Five different isoforms of the human glutamate transporter (EAAT1–5) have been cloned (Arriza *et al.* 1994; Fairman *et al.* 1995; Arriza *et al.* 1997). It is well established that the cotransporter is electrogenic (Brew & Attwell, 1987), yet the exact stoichiometry is disputed. The cotransport of two Na^+ (Stallcup *et al.* 1979; Erecinska *et al.* 1986) or three Na^+ (Barbour *et al.* 1988; Zerangue & Kavanaugh, 1996) has been suggested. This is associated

with cotransport of H^+ (Nelson *et al.* 1983; Zerangue & Kavanaugh, 1996) and accompanied by counter-transport of K^+ (Kanner & Bendahan, 1982; Barbour *et al.* 1988). In addition to the cotransport function, the glutamate transporters possess an anion channel, which is activated in the presence of the substrate (Fairman *et al.* 1995; Wadiche *et al.* 1995*a*; Wadiche & Kavanaugh, 1998).

EAAT1 was expressed and studied in *Xenopus laevis* oocytes. The data on water transport agree with the conclusions drawn from experiments on the other cotransporters cited above, but show some additional novel features. In addition to the demonstration of cotransport and osmotic transport of water, we show that the passive water permeability is different in different states of the protein, and that conductive Cl^- fluxes in the protein do not lead to water transport. From the point of view of protein dynamics, the findings emphasise the importance of water–protein interactions for the conformational changes underlying glutamate transport.

METHODS

The human glutamate transporter (EAAT1) was subcloned into a vector optimized for oocyte expression (pNB1), *in vitro* transcribed, and expressed in *Xenopus laevis* oocytes (50 ng RNA per oocyte) as previously described (Hediger *et al.* 1989). *Xenopus* oocytes were collected under anaesthesia from frogs that were humanely killed after the final collection. Oocytes were incubated in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM Hepes; pH 7.4) at 19°C for 3–7 days before experiments were performed. The experimental chamber was perfused by control solution (90 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes; pH 7.4, 193 mosmol l^{-1}), a total of 96 mM Cl^-. This solution will be referred to as the high Cl^- solution. Isotonic test solutions were obtained by adding 100–200 μM L-glutamate (RBI, Natick, MA, USA). Experiments with low Cl^- concentrations were performed with 90 mM sodium gluconate substituting for 90 mM NaCl. This low Cl^- solution contained 6 mM Cl^-. Hyper- and hyposmotic test solutions were produced by addition or removal of mannitol.

To test the effects of hyposmolarity on the substrate-induced current (I_s), oocytes were adapted to transport in solutions where 90 mM NaCl was replaced by 50 mM NaCl plus 100 mM mannitol. Osmolarities of the test solutions were determined with an accuracy of 1 mosmol l^{-1}. When the influence of unstirred layers and electrode artifacts were tested, the cation-selective ionophore gramicidin D (300 nM, Sigma no. G-5002, St Louis, MO, USA) was added to the bathing solutions. After about 10 min, gramicidin had decreased the input resistance of the oocyte by a factor of 15–60 and was removed from the bathing solution. Ouabain (200 μM, Sigma no. O-3125) was used in order to test contributions from the Na^+–K^+-ATPase.

The experimental chamber, the voltage clamp, and the optical system for the volume measurements have been described in detail elsewhere (Parent *et al.* 1992; Zeuthen *et al.* 1997; Mackenzie *et al.* 1998). In short, the oocytes were impaled by two microelectrodes, one providing the clamp current and the other measuring the membrane potential. The microelectrodes were filled with 0.5 or 1 M KCl. Consequently the current electrodes do not contribute to the net solute flux since the clamp current will be carried by equal and opposite fluxes of K^+ and Cl^-. The experimental chamber had a volume of 30 μl and solution changes were 90% complete in 5 s, given a flow rate of 12 μl s^{-1}. The solutions were fed into the chamber via a mechanical valve with a dead space of 5 μl.

The oocyte was observed from below via a low magnification objective and a charge coupled device camera. To achieve a high stability of the oocyte image, the upper surface of the bathing solution was determined by the flat end of a Perspex rod, which also provided an illuminated background. Images were captured directly from the camera to the random access memory of a computer. The oocyte was focused at the circumference and assumed to be spherical. The volume was recorded and calculated on-line at a rate of one point per second with an accuracy of 3 in 10 000. Only oocytes in a good condition and with well-defined and clean surfaces were used. The diameter of the oocytes was 1.34 ± 0.02 mm ($n = 13$). The osmotic water permeabilities (L_p) are given per true membrane surface area (Loo *et al.* 1996). This is about nine times the apparent area due to membrane foldings (Zampighi *et al.* 1995). The data were corrected for the L_p of the native oocytes that were measured for each batch. L_p values are given in units of cm s^{-1} (osmol l^{-1})$^{-1}$. To obtain L_p in units of cm s^{-1}, the values should be divided by the partial molar volume of water, V_w, of 18 cm^3 mol^{-1}. The coupling ratio of the EAAT1 is taken as the number of water molecules cotransported per unit charge by the protein. Accordingly, the coupling ratio equals $FJ_{H_2O}(V_w I_s)^{-1}$, where J_{H_2O} is the water flux, I_s is the clamp current induced by application of glutamate, and F is Faraday's constant.

All numbers are given as means ± S.E.M. with n equal to the number of oocytes tested unless otherwise stated.

RESULTS

Cotransport of water under isotonic conditions

In voltage-clamped oocytes expressing EAAT1, glutamate elicited an abrupt inward current, I_s (Fig. 1*A*), and an inward flux of water detected as a linear increase in oocyte volume (jagged line in Fig. 1*B*). The volume increase was a direct function of the inward current: the integrated current (Q_s) described the volume change. This suggests that a fixed number of water molecules were coupled to the influx of each unit charge (smooth line in Fig. 1*B*). No current or volume changes were detected in uninjected oocytes (data not shown).

Evaluation of the coupling ratio (water molecules per unit charge) is complicated by the fact that glutamate not only stimulates cotransport, but also opens a Cl^- channel in the EAAT1 protein (Wadiche *et al.* 1995*a*; Wadiche & Kavanaugh, 1998). Consequently, the total current through the protein is the sum of a conductive and a cotransport component. To study the effect of each of these components, we performed experiments at both high (96 mM) and low (6 mM) external Cl^- concentrations; oocytes were adapted to the low Cl^- solution for 16–24 h prior to experiments. The intracellular Cl^- activity was estimated by adding the Ca^{2+}-selective ionophore A23187 (2 μM) to the bathing solution (Wadiche *et al.* 1995*a*). The rise in intracellular Ca^{2+} concentration led to activation of endogenous Cl^- channels, which in turn caused the membrane potential to approach the reversal potential for Cl^- (E_{Cl}). In high Cl^- solutions, E_{Cl} was in the range −30 to −10 mV, as previously reported (Wadiche *et al.*

1995*a*). In oocytes adapted to the low Cl^- concentrations the intracellular Cl^- concentration was probably too low to contribute significantly to the clamp current. With the endogenous Cl^- channels activated, these oocytes exhibited clamp currents (at −100 mV) that were ten times lower than those obtained in high Cl^- concentrations (3 oocytes).

In oocytes adapted to low Cl^- concentrations, the glutamate-induced increase in clamp current (I_s) was mediated mainly by cotransport. These oocytes exhibited a linear relationship between I_s and the water flow, J_{H_2O}, with a constant coupling ratio of 436 ± 55 water molecules transported per unit charge ($n = 8$) (Fig. 2). The effect was tested for I_s in the range 40–800 nA obtained by clamp voltages between 0 and −110 mV; the relation between I_s and clamp voltage is shown in Fig. 3*A*.

In oocytes adapted to high Cl^- concentration, glutamate-induced currents (I_s) are the sum of a conductive Cl^- current and a cotransport current. The two components were estimated by comparing the relationship between I_s and clamp voltage obtained at low and high Cl^- concentrations (Fig. 3*A*). At large negative clamp voltages the conductive current was about 60 % of the total current, in agreement with earlier reports (Wadiche *et al.* 1995*a*).

At clamp potentials equal to E_{Cl} (about −20 mV), the current generated by Cl^- in the conductive pathway is negligible. Consequently, the coupling ratio obtained at −20 mV, 423 ± 25 water molecules per charge ($n = 10$), was similar to the one obtained at low Cl^- concentrations. For clamp potentials more negative than the reversal potential, there was a conductive current in the same direction as the cotransport current, maintained by a Cl^- efflux (Fig. 3*A*). Accordingly, the ratio between the flux of water and the total current was lower at high negative voltages than that estimated at E_{Cl}. For clamp voltages more positive than E_{Cl}, the conductive current was opposite to the cotransport current and the coupling ratio appears higher than that estimated at E_{Cl}. The relationship between J_{H_2O} and I_s at high Cl^- concentrations could be predicted from: (1) the relation between J_{H_2O} and I_s determined in low Cl^- concentrations, and (2) the assumption that the conductive Cl^- current in the EAAT1 does not give rise to

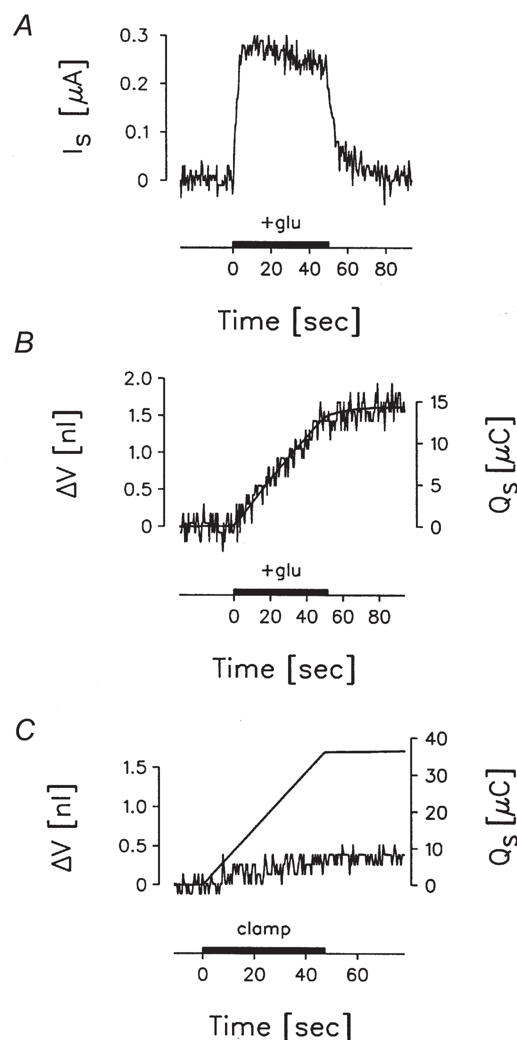

Figure 1. Glutamate-induced current and water flow under isotonic conditions

A, an EAAT1-expressing oocyte was clamped continuously at −30 mV at an external Cl^- concentration of 6 mM. Glutamate (200 μM) was introduced abruptly into the bath (horizontal bar). This caused an inward current, I_s, that increased rapidly towards a maximum of 280 nA. *B*, I_s was associated with a linear increase in oocyte volume (Δ*V*, jagged line), indicative of a constant rate of water influx of about 30 pl s^{-1}. The volume increase and I_s were abolished when L-glutamate was removed from the bath. The integrated current (Q_s, smooth line in *B*) describes the volume changes if it is assumed that a fixed number of water molecules enter per unit charge. *C*, there was no initial change in oocyte volume when the inward Na^+ current was mediated by a gramicidin channel. An inward current of about 750 nA was obtained (not shown) by changing the intracellular electrical potential from the resting potential (about −45 mV) to −90 mV by means of the voltage clamp (horizontal bar). The current did not give rise to any immediate change in cell volume (jagged line). The magnitude of the integrated current shows that only a small number of water molecules are transferred per unit charge. The oocyte treated with gramicidin expressed EAAT1 to the same degree as the one used in *A* and *B*, but the cotransporter was kept inactive as no substrate was present.

water transport (see legend to Fig. 3*B*). A representative example (out of 10 oocytes) of the relation between J_{H_2O} and I_s obtained in high Cl^- concentrations is shown in Fig. 3*B*. The oocyte had an expression similar to that used for the studies in low Cl^- concentrations (Fig. 2).

The glutamate-induced clamp current, I_s, exhibited run-down with time. Given a 40 s glutamate stimulation every 3 min, a 30 % reduction over the first 30 min period was not unusual. After that, the run-down decreased markedly. The phenomenon has also been reported for the rabbit Na^+–glucose transporter (Mackenzie *et al.* 1998); its mechanism is not understood. To mitigate the problem, the measurements of curves such as those in Fig. 3*A* and *B* were performed at a randomized sequence of clamp potentials and after the initial period of run-down.

Test for specific effects of current on water transport

The observed water flow did not result from current-induced increases or decreases in substrate concentrations adjacent to the oocyte membrane. Such unstirred layer effects were estimated by inserting the cation-selective ionophore gramicidin (Finkelstein & Andersen, 1981) into the EAAT1-expressing oocyte, while the cotransporter was held inactivated by the absence of glutamate. The level of gramicidin incorporation was adjusted in order to decrease the input resistance of the oocyte by a factor of between 15 and 60. By applying a negative clamp voltage, large inward Na^+-mediated currents were generated via the ionophore; replacement of external Na^+ by choline ions completely abolished the gramicidin-mediated current ($n = 7$).

The Na^+ fluxes by the gramicidin gave rise to no, or only small, initial water fluxes; an example is shown in Fig. 1*C*. The water fluxes obtained in six oocytes for clamp currents in the range 320–1330 nA are compiled in Fig. 2 where they are compared with the initial values of J_{H_2O} obtained from activation of the EAAT1 by glutamate. Gramicidin-mediated currents below 600 nA gave rise to no initial water fluxes, while currents in the range 600–1330 nA did evoke initial water fluxes, up to an average of 8 pl s^{-1} at the largest currents. Regression analysis shows that the gramicidin-generated currents were, on average, thirteen times less efficient in generating water fluxes than cotransport currents (see Fig. 2).

In order to estimate the significance of the current generated by the Na^+–K^+-ATPase, ouabain (200 μM) was added during the gramicidin-induced clamp current. Ouabain increased the clamp current by 13.6 ± 5.5 nA ($n = 5$). Pump currents of this magnitude account for less than 3 % of the currents used in this study.

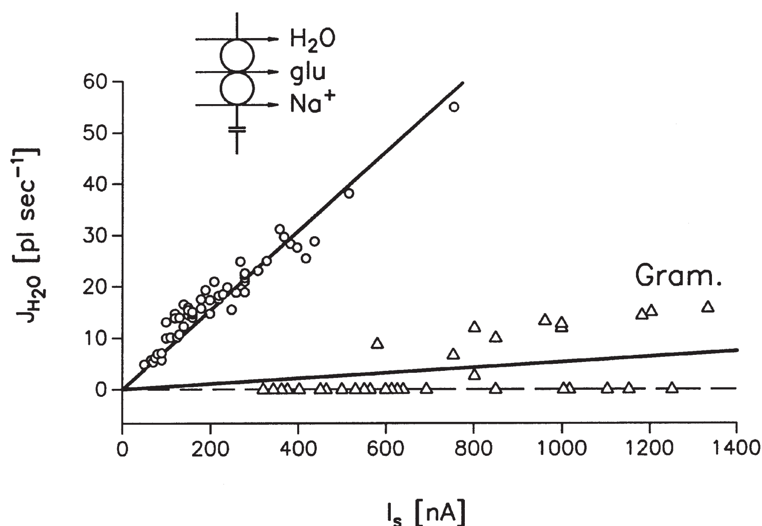

Figure 2. Cotransport of water as a function of glutamate-induced clamp currents (I_s), low external Cl^-

The oocytes were bathed in low external Cl^- concentrations (6 mM) where the current is determined by the cotransport component (see inset and text). ○, the rate of water influx, J_{H_2O}, plotted as a function of I_s; experiments as in Fig. 1. Clamp voltages were in the range 0 to −110 mV (the relation between I_s and clamp voltage is given in Fig. 3*A*). J_{H_2O} (10^{-3} pl s^{-1}) was a linear function of the clamp current = 77.4 ± 1.6 I_s (nA) ($r = 0.97$, 51 measurements in 8 oocytes). The coupling ratio was 436 ± 55 ($n = 8$). △, initial rates of water transport in gramicidin-treated oocytes (Gram.) as a function of clamp currents induced by sudden applications of clamp voltage; experiments as in Fig. 1*C*. EAAT1 was expressed but kept inactivated by the absence of glutamate. The regression line for J_{H_2O} is given by (5.8 ± 1.1) × 10^{-3} pl s^{-1} nA^{-1}) ($r = 0.55$, 32 measurements in 6 oocytes).

Osmotic transport

In addition to its capacity to act as a molecular water pump, EAAT1 also sustains a passive water transport when exposed to transmembrane osmotic gradients. The passive water permeability (L_p) of EAAT1-expressing oocytes was measured under different conditions: with and without glutamate present (200 μM), and at high and low Cl^- concentrations. In general the external clamp was not applied in order to minimize the cotransport component, yet the effect of external clamping was tested in specific cases. Initially, the oocyte was adapted to the experimental condition to be tested. When the oocyte had achieved a constant volume ($\Delta V/\Delta t$ less than 1 pl s^{-1}), mannitol was added to (or removed from) the bathing solution. The induced volume changes ($\Delta V/\Delta t = J_{H_2O}$) were linear, and osmotic gradients ($\Delta\pi$) as large as 20 mosmol lasting as long as 1 min were employed. The L_p values were evaluated as $\Delta J_{H_2O}/\Delta\pi$ (Fig. 4). The values given in the following have been corrected for the L_p of the native oocyte (top trace in Fig. 4*A*). This was $(3.3 \pm 0.5) \times 10^{-6}$ cm s^{-1} (osmol $l^{-1})^{-1}$ ($n = 5$), in agreement with previous studies (Zhang & Verkman, 1991; Loo *et al.* 1996; Meinild *et al.* 1998). This L_p was about 20% of the permeability of the EAAT1-expressing oocytes, and was independent of Cl^- and glutamate concentrations.

High Cl^- concentrations (96 mM) and glutamate absent. L_p averaged $(11.3 \pm 2.0) \times 10^{-6}$ cm s^{-1} (osmol $l^{-1})^{-1}$. These data were from eight oocytes, the expression level of which were characterized by glutamate-induced currents (I_s) in the range 350–800 nA measured at clamp potentials around E_{Cl}. In general, a larger expression level was associated with a larger L_p. Normalized to an oocyte with an I_s of 500 nA, L_p was calculated as $(9.0 \pm 1.5) \times 10^{-6}$ cm s^{-1} (osmol $l^{-1})^{-1}$. The oocytes tested had membrane potentials between −25 and −40 mV. The membrane potential did not change during the osmotic challenge. Conversely, we found no differences in L_p

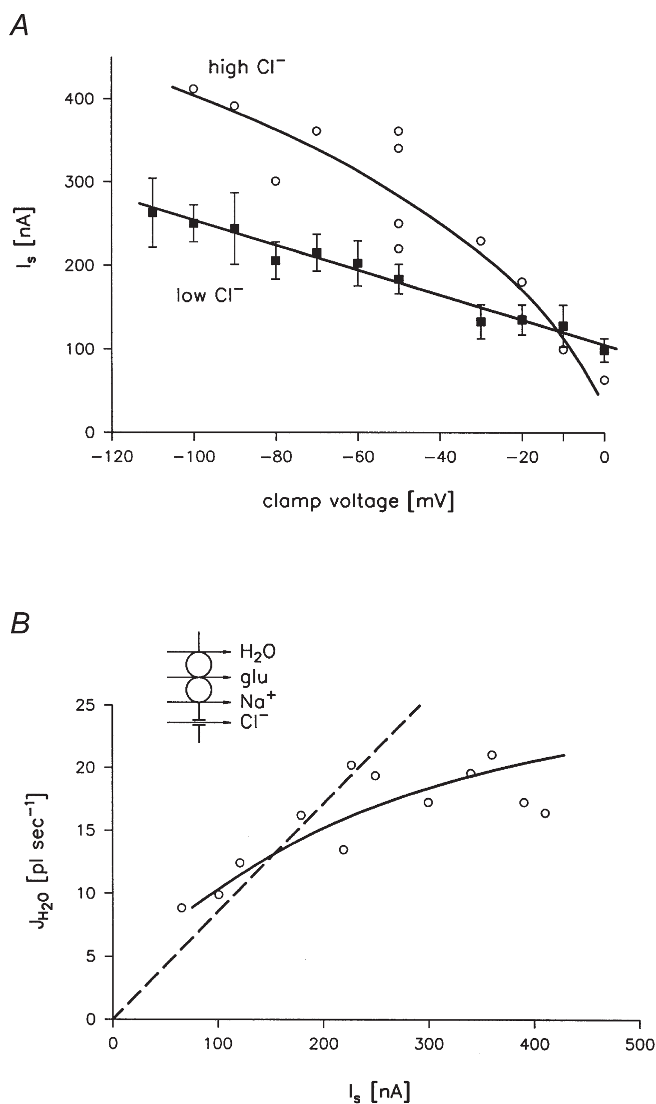

Figure 3. Cotransport of water as a function of glutamate-induced clamp currents (I_s), high external Cl^-

A, the relation between I_s and clamp voltage obtained in high Cl^- and low Cl^- concentrations. The data obtained at low Cl^- concentrations (6 mM) are the averaged data from the oocytes presented in Fig. 2. The data obtained at high Cl^- concentrations (96 mM) are from an oocyte from the same batch as those used for the low Cl^- experiments. Curves are drawn by eye. *B*, water flux (J_{H_2O}) *versus* glutamate-induced current (I_s) for an oocyte bathed in high external Cl^- concentrations (96 mM). Data are from the oocyte above, which is typical for ten oocytes. The curved line through the points is the J_{H_2O} *versus* I_s relation constructed from two assumptions: (1) the cotransport of water is linked to the Na^+–glutamate transport and given by the relation measured in low Cl^- (Fig. 2) and (2) the conductive Cl^- current in the EAAT1 does not give rise to water transport. Compare, for example, the data obtained at a clamp voltage of −100 mV and at E_{Cl}, which is around −20 mV. The cotransport component of the current at −100 mV is about 100 nA larger than at E_{Cl} (Fig. 3*A*, ■). Accordingly, J_{H_2O} would be predicted to be 7 pl s^{-1} larger at −100 mV, about 20 pl s^{-1}, see Fig. 2. The total current, however, at −100 mV is 400 nA. The dashed line through zero is the J_{H_2O} *versus* I_s relation in low Cl^- solutions (from Fig. 2).

values of oocytes whether unclamped or clamped to a membrane potential in range −25 to −40 mV ($n = 4$).

High Cl^- concentrations (96 mM) and glutamate present. The presence of glutamate (200 μM) increased L_p by a factor of 1.4 ± 0.1 ($n = 6$) compared with the glutamate-free case presented above (Fig. 4*A*). The comparison is performed in paired experiments so the difference is highly significant ($P < 0.01$). Glutamate concentrations of 100 and 300 μM were also tested; $K_{0.5}$ for the effect on L_p was below 100 μM. Volume changes obtained by osmotic gradients of various size are shown in Fig. 4*B* and plotted in Fig. 6*A* (○).

The application of glutamate to the EAAT1-expressing oocyte under conditions of no clamp depolarized oocytes to $+24 \pm 5$ mV ($n = 16$). Even at this positive potential there is probably a slow rate of inwardly directed cotransport. The turnover rate can be estimated to be up to 20% of the maximal rate (Wadiche *et al.* 1995*a*); Mitrovic *et al.* 1998). After about 3–5 min, however, the oocyte volume increase tended to stabilize at a value of around 4 pl s^{-1}. L_p was recorded during this phase. It should be emphasized that activity of the cotransporter does not prevent the measurement of L_p. L_p was derived from the *change* in water flow induced by a *change* in bath osmolarity; gradients of up to ±20 mosmol l^{-1} were employed. Gradients of this magnitude do not affect the rate of cotransport. This was estimated from the sensitivity of I_s to osmotic challenges. Under clamped conditions it took gradients of 100 mosmol l^{-1} mannitol to reduce I_s by 6.9 ± 0.8% ($n = 6$). I_s increased by a similar percentage if the external osmolarity was decreased by 100 mosmol l^{-1}.

Low Cl^- concentrations (6 mM) and glutamate absent. The L_p recorded at low external Cl^- appeared to be lower than that recorded in the presence of Cl^-. Normalized to an oocyte with an I_s of 500 nA, L_p was calculated as $(7.5 \pm 0.7) \times 10^{-6}$ cm s^{-1} (osmol l^{-1})$^{-1}$ (data from Table 1).

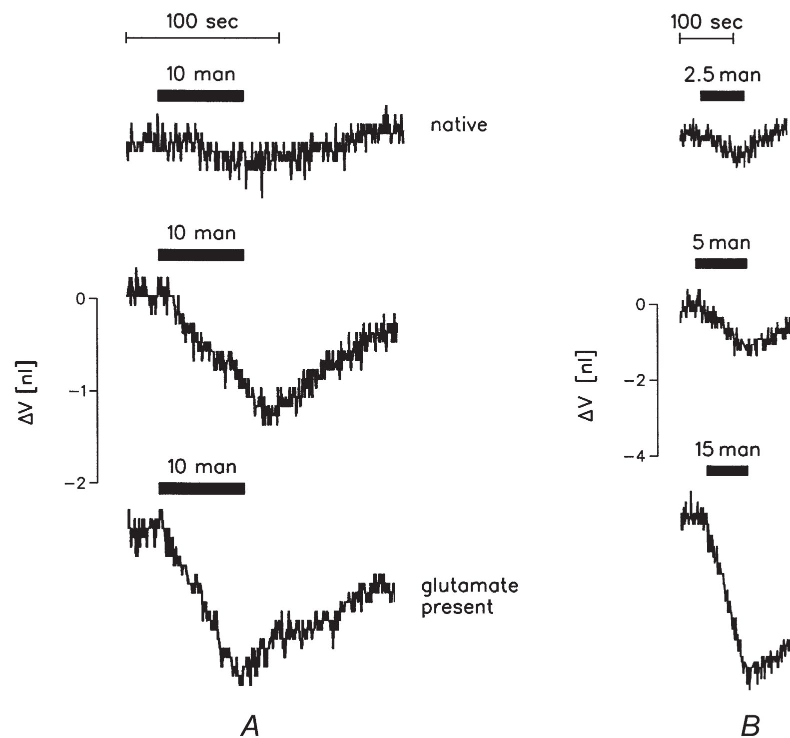

Figure 4. Passive water permeability of EAAT1-expressing oocytes

A, effects of the presence of glutamate. L_p was determined from the linear volume change elicited by the addition of mannitol to the bathing solution. Upper trace, native oocyte. Middle trace, EAAT1-expressing oocyte. Lower trace, the same oocyte as above, but with glutamate (200 μM) present throughout, i.e. in both the bathing and test solutions. The oocyte was unclamped in order to minimize the cotransport component. Accordingly, a well-defined baseline was achieved, indicative of little or no change in oocyte volume (see text). It is seen that the presence of glutamate increases L_p. The native and EAAT1-expressing oocytes were from the same batch. *B*, effects of magnitude of osmotic gradient. Volume changes are in response to hyperosmolar challenges of 2.5, 5 or 15 mosmol l^{-1}. The recordings are from the same oocyte and are plotted in Fig.6*A* (○). It appears that the same L_p was determined irrespective of the magnitude of the osmotic challenge. Glutamate was present in bathing and test solutions throughout, no external clamp applied (see text).

Table 1. Cotransport of water (J_{H_2O}) and osmotic water permeability (L_p) of EAAT1-expressing oocytes

Oocyte no.	J_{H_2O} initial (pl s^{-1})	L_p (−clamp, −glu) (10^{-6} cm s^{-1} (osmol $l^{-1})^{-1}$)	L_p (−clamp, +glu) (10^{-6} cm s^{-1} (osmol $l^{-1})^{-1}$)	L_p (+clamp, +glu) (10^{-6} cm s^{-1} (osmol $l^{-1})^{-1}$)	Clamp voltage (mV)	I_s (nA)
High Cl^-						
1	64.7 ± 1.8 (7)	19.5 ± 0.2 (6)	28.9 ± 0.6 (7)	24.9 ± 1.0 (7)	−30	795
2	26.0 ± 0.9 (13)	n.d.	9.9 ± 0.4 (5)	12.1 ± 0.3 (13)	−30	575
3	30.8 ± 0.6 (9)	7.3 ± 0.1 (9)	11.5 ± 0.1 (8)	11.9 ± 0.2 (9)	−30	875
4	26.7 ± 1.0 (7)	7.3 ± 0.1 (9)	11.5 ± 0.1 (8)	10.1 ± 0.3 (7)	−30	340
5	22.0 ± 1.5 (7)	9.8 ± 0.2 (10)	13.4 ± 0.3 (8)	14.7 ± 0.3 (7)	−20	535
Low Cl^-						
6	22.1 ± 2.1 (8)	3.8 ± 0.1 (11)	5.7 ± 0.1 (11)	n.d.	−70	330
7	18.5 ± 1.9 (7)	2.5 ± 0.3 (5)	5.0 ± 0.4 (11)	4.4 ± 0.4 (7)	−50	440
8	19.5 ± 2.8 (3)	5.7 ± 0.04 (23)	6.9 ± 0.02 (17)	n.d.	−50	470
9	30.0 ± 1.1 (8)	6.7 ± 0.2 (8)	12.4 ± 0.3 (5)	14.9 ± 0.4 (8)	−50	750

The L_p was determined under three conditions: L_p (−clamp, −glu) gives the value obtained from mannitol challenges under conditions of no external clamp and no glutamate present (Fig. 4*A*, middle trace); L_p (−clamp, +glu) gives the value obtained from mannitol challenges, glutamate present throughout, but no external clamp applied (Fig. 4*A*, lower trace); L_p (+clamp, +glu) gives the value obtained from simultaneous applications of glutamate and mannitol under voltage clamp conditions (Fig. 5). Oocytes 3 and 4 represent recordings from the same oocyte at different times: early in the experiment the clamp currents (I_s) were high; after about half an hour of glutamate stimulation under clamped conditions I_s had stabilized at a lower level.

It was not possible, however, to apply the high and low Cl^- conditions on the same oocyte in a way that would allow the oocyte to be its own control; oocytes adapted to low Cl^- were rather fragile. A firm conclusion on the effects of Cl^- requires further experiments.

Low Cl^- concentrations (6 mM) and glutamate present. The presence of glutamate increased the L_p by a factor of 1.6 ± 0.2 ($n = 7$) compared with the values above (Table 1). The comparison was performed in paired experiments so the differences are highly significant ($P < 0.01$).

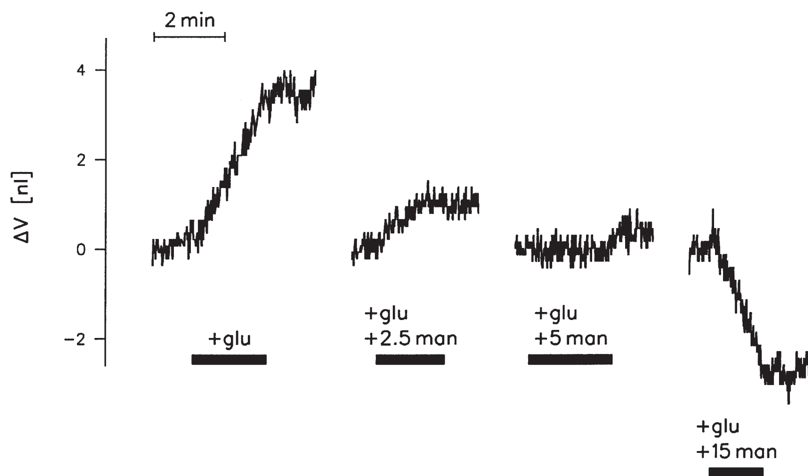

Figure 5. Uphill transport of water by EAAT1

The EAAT1-expressing oocyte was initially bathed in glutamate-free control solutions under clamped conditions (−30 mV). At the time indicated by the horizontal bar, glutamate (200 μM) was added to the bathing solution (+glu). This induced an immediate swelling of the oocyte; this part of the experiment is analogous to that illustrated in Fig. 1*A* and *B*. In the following three recordings the stimulation by glutamate was combined with a hyperosmotic challenge of 2.5, 5 and 15 mosmol l^{-1} of mannitol, respectively (+man). The glutamate-induced current I_s (not shown) was about 300 nA in all four experiments (see text). It appears that the inwardly directed cotransport of water can proceed despite adverse osmotic gradients. The rates of water transport are plotted in Fig. 6*A* as a function of the osmotic challenge.

Separation of cotransport and osmotic water transport

In order to test whether the cotransport and osmotic components could be treated as independent modalities, we performed experiments in which cotransport and osmotic transport were activated simultaneously. In the control situation, oocytes were bathed in a glutamate-free solution and clamped to a negative potential. The cotransport component was determined by adding glutamate (200 μM) under isotonic conditions (Fig. 5, first trace), in analogy to the experiments shown in Fig. 1*A* and *B*. In the subsequent experiments the osmolarity of the bathing solution was changed simultaneously with the application of glutamate. Results from nine oocytes serving as their own controls are listed in Table 1.

The oocyte would swell in the face of small hyperosmotic gradients and shrink at the larger gradients; values in the range ± 20 mosmol l^{-1} were tested. At an osmotic gradient of +5 mosmol l^{-1} the osmotic efflux and the cotransported influx of water would be about equal, and the oocyte volume would remain stable (Fig. 5). The rate of swelling plotted as a function of the osmotic gradient defines a line (Fig. 6*A*, ●), the slope of which determines an L_p (Table 1, L_p +clamp, +glu). This L_p was not significantly different from the L_p determined in the presence of glutamate without the external clamp turned on (Table 1, L_p −clamp, +glu). This shows that the osmotic pathway of EAAT1 in the presence of glutamate can be described by a single L_p value that is independent of the osmotic gradient and of the rate of cotransport.

The cotransport component of the water transport was also independent of the magnitude and direction of the osmotic challenge. The data in Fig. 5 are explained most simply by a model in which a constant component of water cotransport is superimposed upon the osmotic component of the water transport (Fig. 6). As described above, the cotransport current was independent of osmotic gradients in the range employed; it took 100 mosmol l^{-1} to produce significant changes in I_s.

Temperature dependence

The glutamate-induced cotransport current and associated water flow were studied as a function of temperature (range 13–24°C). The Arrhenius activation energy, E_a, for I_s was 25 ± 4 kcal mol^{-1} (105 ± 17 kJ mol^{-1}; $n = 6$). In two of the oocytes, E_a for J_{H_2O} was found to be the same as that for I_s. In four of the oocytes the low temperature reduced J_{H_2O} below detection level, about 2 pl s^{-1}, which means that E_a for J_{H_2O} was larger or equal to that of I_s. The high values of E_a suggest that the charge translocation and the associated water transport take place with substantial conformational changes of the

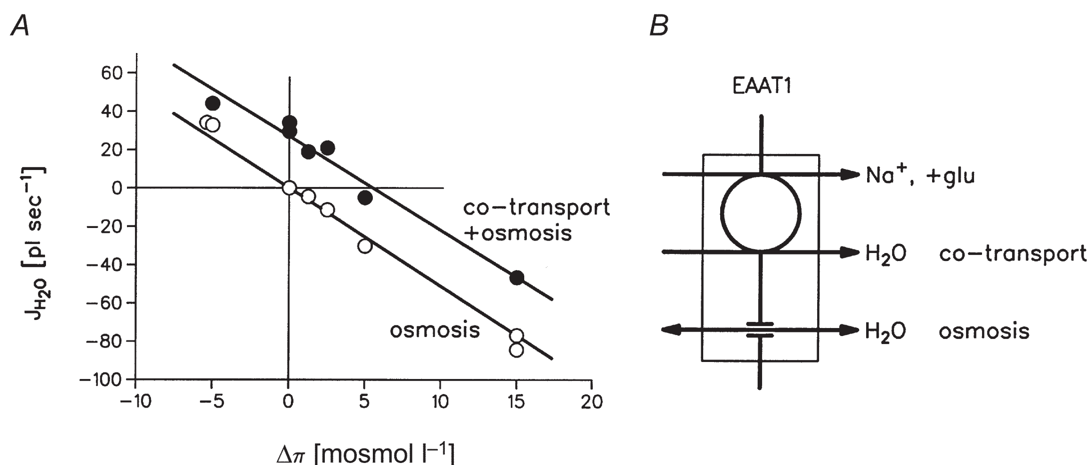

Figure 6. Independence of the cotransport component and the osmotic component of water transport in EAAT1

A, summary of the data obtained in Figs 4*B* and 5, where the cotransport and the passive components of water transport were studied as a function of the transmembrane osmotic difference. The (minor) fraction of the water flux going through the native oocyte membrane has been subtracted. The upper curve (●) represents the water fluxes ($J_{H_2O} = \Delta V/\Delta t$) obtained by the simultaneous activation of the cotransport and the osmotic water transports by sudden application of glutamate and mannitol (data from Fig. 5). J_{H_2O} as a function of $\Delta\pi$ is given by the regression line $J_{H_2O} = 26.7$ pl s^{-1} − 4.8 pl s^{-1}(mosmol l^{-1})$^{-1}$ ($r = -0.99$). The lower curve (○) represents J_{H_2O} values obtained from osmotic challenges only; data from Fig. 4*B*. J_{H_2O} is given by the line −5.5 pl s^{-1} (mosmol l^{-1})$^{-1}$ ($r = -0.997$). The two sets of data are from the same oocyte. The slopes of the two lines were similar and represent the L_p of EAAT1 in the presence of glutamate. The constant vertical displacement represents the cotransport component of water transport. Similar sets of data were obtained in 7 other oocytes (Table 1). *B*, a model of the protein in which the cotransport and the osmotic water transport work in parallel and independently.

protein, and reflect the comparatively low turnover number (about 15 s^{-1}) for these transporters (Wadiche *et al.* 1995*b*). E_a for the passive osmotic water permeability of the EAAT1-expressing oocytes was not significantly different from that of the uninjected oocytes, which is about 10 kcal mol^{-1} (42 kJ mol^{-1}; Zhang & Verkman, 1991). This makes a quantitative evaluation difficult, but suggests that E_a for the osmotic water transport is low, indicative of minor conformational changes associated with this type of transport. This is in analogy with findings for other symporters (Meinild *et al.* 1998; Loo *et al.* 1999*a*,*b*).

DISCUSSION

We show that water can be transported across cell membranes by the human glutamate transporter EAAT1. The transport proceeds by two mechanisms, cotransport and osmosis, which operate in parallel and independently (Fig. 6*B*).

EAAT1 as a molecular water pump

Cotransport of one unit charge led to the cotransport of about 436 water molecules. This was demonstrated directly at low Cl^- concentrations where the clamp current could be identified with the cotransport current (Fig. 2). The coupling ratio was independent of the presence of Cl^-: a similar coupling ratio was observed in solutions with high Cl^- concentrations at clamp potentials equal to E_{Cl}, where the conductive Cl^- fluxes through the protein were minimized (Fig. 3*B*). In addition, the cotransport of water was independent of clamp potentials, currents via the Cl^- channel of the protein, and external osmolarity. Most importantly, cotransport of water could proceed uphill, against the water chemical potential difference (Figs 5 and 6*A*).

The cotransport of water appears to be energized by coupling to the electrochemical driving force by a mechanism within the protein, which requires major conformational changes. If we assume a stoichiometry with cotransport of 2 Na^+, 1 H^+, 1 glutamate, 436 water molecules, and countertransport of 1 K^+ (for references, see Introduction), the Gibbs equation gives:

$$2RT\ln(Na^+_o/Na^+_i) + RT\ln(H^+_o/H^+_i) + RT\ln(glu^-_o/glu^-_i)$$
$$-RT\ln(K^+_o/K^+_i) + nRT\ln(W_o/W_i) = F(\Psi_i - \Psi_o), \qquad (1)$$

where 'o' defines the outside and 'i' the inside compartment, W is the water concentration (proportional to exp(−osmolarity/n_w)), n_w is the molarity of water (= 55 M), n is the coupling ratio (= 436), and Ψ is the electrical potential (Zeuthen, 1996; Meinild *et al.* 1998; Rudnick, 1998). With a membrane potential of −50 mV, tenfold concentration differences for Na^+, glutamate (inwardly directed) and K^+ (outwardly directed) and no concentration difference for H^+, it can be calculated that the inward water flux would proceed in spite of adverse osmotic gradients of up to 1500 mosmol l^{-1}. The Na^+ and the electrical gradients alone would account for 900 mosmol l^{-1}. With the assumption of cotransport of 3 Na^+, the numbers would be 2100 and 1800 mosmol l^{-1}. These estimates comply with the finding that the cotransport was insensitive to osmotic challenges of the order of 20 mosmol l^{-1} (Fig. 5*A*). The dependence of the cotransport on osmotic gradients only became apparent at gradients as large as ±100 mosmol l^{-1}.

The properties of EAAT1 as a molecular water pump were compatible with those observed for other electrogenic Na^+-coupled cotransporters listed in the introduction. In the EAAT1, however, the L_p was large relative to its capacity for water cotransport compared with the Na^+–glucose cotransporter SGLT1. In EAAT1 it took an external hyperosmolarity of 5 mosmol l^{-1} to produce an osmotic efflux that matched the cotransported influx (Fig. 6*A*). In SGLT1 it took an external hyperosmolarity of 15 mosmol l^{-1} to match osmosis with cotransport (Meinild *et al.* 1998). In other words, the capacity for uphill water transport in EAAT1 is three times smaller than in SGLT1.

Estimation of unstirred layer effects and electrode artifacts

Large currents maintained by gramicidin did not give rise to any significant initial water flows (Fig. 2). This suggests that the current flow as such in the EAAT1 does not give rise to initial water flows.

In order to compare the effects of gramicidin-generated currents and cotransport currents, the stoichiometry of the EAAT1 has to be considered. With 3 Na^+ and 1 glutamate (negatively charged) two osmotically active particles are transferred for each unit charge; with 2 Na^+ and 1 glutamate, three osmotically active particles are transferred for each unit charge; the transport of K^+ and H^+ may cancel out (see below). In the worst case, then, the volume effects observed in the gramicidin experiments should be multiplied by a factor of three in order to mimic the EAAT1 experiments. As seen from Fig. 2, the J_{H_2O} values induced by the EAAT1 are more than one order of magnitude larger than the effects observed with gramicidin. We suggest that currents as such cannot explain the initial values of J_{H_2O}.

Three minor effects may be relevant for the above comparison. (i) The estimated number of osmotic particles transported by the EAAT1 is probably an overestimate. EAAT1 exports 1 K^+ and imports 1 H^+, but due to binding, the H^+ may not exert any osmotic effects in the oocyte. (ii) The Na^+ ions transported by the gramicidin will become surrounded by Cl^- or other osmotically active counter ions at the inside of the membrane. The Na^+ ions carried by the EAAT1 already have glutamate as counter ions, consequently the excess density of other counter ions will be smaller. From (i) and (ii) it appears that in the worst case, the volume effects observed in the gramicidin experiments should be multiplied by a factor of only two

in order to mimic the EAAT1 experiments. (iii) If glutamate diffuses twice as slowly as Na^+, the volume effects observed in the gramicidin experiments should be multiplied by a factor of four in order to mimic the EAAT1 experiments. A recent estimate, however, indicates that glucose (which is larger than glutamate) makes unstirred layers only 10–20 % larger than those of Na^+ (Zeuthen *et al.* 2001).

Unstirred layer effects have been suggested to arise at least in part from the folded nature of the oocyte membrane (Diamond, 1996). The plasma membrane of the oocyte contains microvilli, which are about 10 μm long and 1 μm wide (Soreq & Seidman, 1992). Such dimensions, however, cannot amplify unstirred layer effects significantly (for a review, see House, 1974), in agreement with our findings of no unstirred layer effects.

The relation between water transport and current observed in solutions with high Cl^- concentrations could be explained quantitatively by a combination of the cotransport component and the current via the Cl^- channel part of the protein (see above and legend to Fig. 3). This suggests that the electrogenic Cl^- current through the EAAT1 does not give rise to water transport.

In order to minimize net solute flow from the microelectrodes, KCl was used as filling solution. This ensures that currents are carried by equal and opposite fluxes of K^+ and Cl^-. The lack of initial changes in oocyte volume with large gramicidin currents supports this notion.

The osmotic water permeability of EAAT1

Glutamate increased the L_p of the EAAT1 irrespective of the rate of cotransport. We determined the L_p in the presence of glutamate under two transport conditions. In one condition the oocyte was unclamped and allowed to reach a positive steady state potential of about +25 mV (L_p −clamp, +glu; Table 1). Under these conditions the rate of cotransport is slow, about 20 % of the maximal rate (Wadiche *et al.* 1995; Mitrovic *et al.* 1998). In the other situation L_p was determined under conditions of maximal rate of cotransport obtained at negative clamp potentials (L_p +clamp, +glu). The two L_p values were the same but about 50 % larger than the L_p determined in the absence of glutamate.

The osmotic water permeability is the weighed average of the L_p values of the conformational states occupied under the given circumstances. Our finding suggests that the presence of glutamate shifts the conformational equilibrium towards more water-permeable states. Full activation of the cotransporter does not change the L_p, but it adds a constant component of water transport that is independent of the osmotic gradient (Fig. 6*A*). Further experiments would be required to obtain a precise correlation between the L_p and the distribution of conformational states of the protein, and to obtain the L_p values of individual conformational states.

For equal values of substrate-induced currents, EAAT1-expressing oocytes had a 6-fold higher L_p than oocytes expressing the human Na^+–glucose cotransporter (cf. Meinild *et al.* 1998). This could reflect a higher expression level for the EAAT1 transporter. The EAAT1 has a turnover rate, 15 s^{-1} (Wadiche *et al.* 1995*b*), lower than that of the Na^+–glucose transporter, which is about 60 s^{-1} (Parent *et al.* 1992). Consequently, it would take a higher expression level of EAAT1 to achieve the same current. Substrate-induced changes in L_p values were not observed with the Na^+–glucose transporters (Loo *et al.* 1999*b*). Possibly, the expression levels in these studies are too low to detect analogous changes in the L_p.

Molecular mechanisms

The molecular mechanisms underlying water transport in the EAAT1 and other symporters are as yet unknown. Two models based on mobile barriers or loops have been suggested (Zeuthen, 1996). The models are based on the fact that a well-defined number of water molecules are bound or released by enzymes during conformational changes. This applies both to aqueous enzymes (Colombo *et al.* 1992; Rand *et al.* 1993; Qian *et al.* 1995) as well as to membrane-bound enzymes (Zimmerberg & Parsegian, 1986; Kornblatt & Hoa, 1990); values in the range of 10–1200 water molecules have been reported. The models suggest that binding of glutamate at the outside is associated with the opening and filling of an aqueous pathway in the protein. This agrees with our finding of a glutamate-induced increase in L_p and with studies of the accessibility of specific amino acid residues in neurotransmitter transporters, which indicate the existence of a water-filled pore in the proteins (Golovanevsky & Kanner, 1999; Slotboom *et al.* 1999). When glutamate is subsequently released to the other side of the membrane, the process is reversed: the cavity closes and the water is released. To accommodate 400 water molecules as bulk water, the cavity would have to be about 10^4 Å^3 large. If, in analogy to haemoglobin (Colombo *et al.* 1992), the water was held as loosely bound surface water, it would need a surface of about 4000 Å^2, roughly equivalent to the van der Waal surface of three α-helices, 80 Å long.

Physiological relevance

Due to the wide distribution of Na^+–glutamate transporters, the ability to transport water may have several physiological consequences in relation to both epithelial absorption and cellular water homeostasis. The EAAT1 is primarily located in glial cells (Rothstein *et al.* 1994; Lehre *et al.* 1995) where the water channel AQP4 has also been found (Jung *et al.* 1994; Frigeri *et al.* 1995; Nielsen *et al.* 1997). Glial cells have been proposed to be osmometers of the central nervous system (Jung *et al.* 1994; King & Agre, 1996). Osmotic imbalances of these cells could occur from uncompensated uptake of solutes, such as glutamate, which would lead to disturbances of the water homeostasis of the brain. This perturbation would be alleviated, at least in part, by cotransport of

water in the EAAT1. A precise evaluation requires knowledge of the osmolarity of the transported solution, and therefore of the stoichiometry of the EAAT1 (see introduction). If 2 Na^+ and 1 H^+ were cotransported per glutamate, while 1 K^+ was countertransported, 141 water molecules would enter per particle, 212 if 3 Na^+ ions were translocated. These values should be compared with human plasma (320 mosmol l^{-1}), which contains about 170 water molecules per osmotically active particle. In conclusion, water transport by the EAAT1 renders the glutamate re-uptake nearly isotonic and hence non-disturbing to the osmosensation of the glial cells.

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