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Serine-O-sulphate transport by the human glutamate transporter, EAAT2

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1 Expression of the recombinant human excitatory amino aid transporters, EAAT1 and EAAT2, in *Xenopus laevis* oocytes allows electrogenic transport to be studied under voltage clamp conditions.

2 We have investigated the transport of the pharmacological substrate, L-serine-O-sulphate transport by EAAT1 and EAAT2. The EC_{50} values for L-serine-O-sulphate transport by EAAT2 showed a steep voltage-dependence, increasing from 152 ± 11 μM at -100 mV to 1930 ± 160 μM at 0 mV. In contrast to EAAT2, EC_{50} values for L-serine-O-sulphate transport by EAAT1 were relatively constant over the membrane potential range of -100 mV to 0 mV. The EC_{50} values for L-glutamate and D-aspartate transport, by EAAT2, were also relatively constant over this membrane potential range.

3 Chloride ions modulated the voltage-dependent changes in EC_{50} values for transport by EAAT2. This effect was most apparent for L-serine-O-sulphate transport, and to a lesser extent for L-glutamate and not at all for D-aspartate transport by EAAT2.

4 Extracellular sodium and proton concentrations also modulated the voltage-dependence of L-serine-O-sulphate EC_{50} values for EAAT2.

5 We speculate that these different properties of L-serine-O-sulphate transport by EAAT2 compared to other substrates may be due to the much stronger acidity of the sulphate group of L-serine-O-sulphate compared to carboxyl groups of L-glutamate or D-aspartate.

6 These results highlight some of the differences in the way different glutamate transporter subtypes transport substrates. This may be used to understand further the transport process and develop subtype selective inhibitors of glutamate transport.

Keywords: Glutamate transport; L-serine-O-sulphate; EAAT1; EAAT2

Introduction

Glutamate transporters play an important role in the central nervous system by mediating uptake of glutamate released at excitatory synapses. Glutamate may be taken up by transporters in the pre- or postsynaptic membranes or alternatively glutamate that diffuses out of the synapse is taken up by transporters located in surrounding glial cells (Rothstein *et al*., 1994). A family of glutamate transporter cDNAs have recently been cloned and their amino acid sequences are 40–60% identical (Pines *et al*., 1992; Storck *et al*., 1992; Kanai & Hediger, 1992; Fairman *et al*., 1995; Arriza *et al*., 1997). The functional properties of five of the cloned human glutamate transporters (termed EAAT1–5) have been directly compared by expressing cRNAs encoding the transporters in *Xenopus* oocytes (Arriza *et al*., 1994; 1997; Fairman *et al*., 1995).

The energy required for the transport of glutamate into the cell is derived from the coupling of glutamate flux to the sodium, potassium and pH gradients across the cell membrane. From radiolabelled flux measurements and electrophysiological studies the probable stoichiometry for the transport process is 1 glutamate molecule co-transported with 2 or 3 sodium ions and 1 proton with counter-transport of 1 potassium ion to complete the cycle (Kanner & Sharon, 1978; Stallcup *et al*., 1979; Barbour *et al*., 1988; Zerangue & Kavanaugh, 1996). In addition, the different transporter subtypes allow a varying number of chloride ions and possibly hydroxyl ions through the transporter (Bouvier *et al*., 1992; Fairman *et al*., 1995; Wadiche *et al*., 1995a; Eliasof & Jahr, 1996; Billups *et al*., 1996; Arriza *et al*., 1997). Under voltage clamp conditions, the direction of chloride ion movement through the transporter does not appear to influence the velocity of [^{3}H]-glutamate uptake, suggesting that chloride ion fluxes through glutamate transporters are not thermodynamically coupled to the flux of glutamate. Different transporter substrates also appear to be differentially coupled to the activation of chloride fluxes (Fairman *et al*., 1995; Wadiche *et al*., 1995a; Billups *et al*., 1996).

The different transporter subtypes show quite distinct pharmacological profiles. The most striking pharmacological difference between the transporter subtypes is the selective sensitivity of the EAAT2 subtype to competitive blockade by dihydrokainate, kainate and *threo*-3-methylglutamate (Arriza *et al*., 1994; Vandenberg *et al*., 1997). These compounds block glutamate transport in a voltage-independent manner, suggesting that they block transport by competing with glutamate at an extracellular recognitition site. In contrast to kainate and *threo*-3-methylglutamate, the binding of sodium ions to EAAT2 is voltage-dependent (Wadiche *et al*., 1995b), which may be interpreted as being due to sodium ions binding to a site on the transporter that is within the electrical field of the membrane. This sort of information indicates that there are a number of different binding sites for the various ions and substrates as they pass through the pore of the transporter.

In terms of substrate movement through the transporters, a more subtle difference between EAAT2 and the other transporter subtypes has been observed for the transport of L-serine-O-sulphate (SOS) (Arriza *et al*., 1994; Vandenberg *et al*., 1995). At -60 mV, the EAAT1 transporter showed a 6 fold lower EC_{50} for SOS transport than observed for EAAT2.

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In addition, SOS generated a similar maximal transport current to that of glutamate when transported by EAAT1, but only 60% of the maximal glutamate transport current when transported by EAAT2 (Vandenberg *et al*., 1995). In this study, we have investigated further the transport of SOS by EAAT2 and showed that both the rate of transport and the EC_{50} for transport of SOS are steeply voltage-dependent. We have characterized the ionic basis for the voltage-dependence and have used this to develop further an understanding of the differences in the way in which different glutamate transporters transport substrates.

Methods

Expression of transporters and electrophysiological recording

Complementary DNAs encoding the human glutamate transporters, EAAT1 and EAAT2, were subcloned into pOTV for expression in *Xenopus laevis* oocytes as previously described (Arriza *et al*., 1994). The plasmids were linearized with BamH1 and cRNA was transcribed from each of the cDNA constructs with T7 RNA polymerase and capped with 5′7-methyl guanosine by use of the mMESSAGE mMACHINE (Ambion Inc., Austin TX, U.S.A.). Fifty nanograms of cRNA encoding either EAAT1 or EAAT2 were injected into defolliculated Stage V *Xenopus* oocytes. Two to seven days later, current recordings were made at 22°C with a Geneclamp 500 interfaced with a IBM compatible computer by use of a Digidata 1200 A/D controlled by pCLAMP software (Version 6.0.2) (Axon Instruments). The standard Frog Ringer recording solution contained (in mM) NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5; pH 7.55. Under these recording conditions only net inward flux of substrates is expected at membrane potentials up to +60 mV (Zerangue & Kavanaugh, 1996). In general the transport currents for EAAT2 showed little (<10%) run down or desensitization during the course of an experiment. In some instances EAAT1 transport current did 'desensitize' (up to ~10%) during the 30 s application of glutamate. This effect was most apparent at high expression levels and if observed we used the peak current as the measure of transport current. In experiments where the role of pH was investigated the pH of the recording solution was adjusted by the addition of HCl. In experiments in which sodium was altered, equimolar concentrations of sodium ions were replaced with Tris or in the case where the sodium concentration was increased to 140 mM, 42 mM sodium gluconate was added to the Frog Ringer solution, with the extracellular chloride kept at 104 mM. In chloride substitution experiments, chloride was replaced with equimolar gluconate and junction potentials were minimized by the use of a 3 M KCl-agar bridge from the recording chamber to a reservoir containing 3 M KCl and Ag/AgCl electrodes connected to a bath clamp headstage. The oocytes were voltage clamped at −30 mV and the current-voltage relations were determined by subtraction of steady state current measurements in the absence of substrate, obtained during 200 ms voltage pulses to potentials between −100 mV and +60 mV, from corresponding current measurements in the presence of substrate as previously described (Vandenberg *et al*., 1995).

Analysis of kinetic data

Current (I) as a function of substrate concentration ([S]) was fitted by least squares to $I = I_{max}[S]/EC_{50} + [S])$, where I_{max} is the maximal current and EC_{50} is the concentration of substrate generating half the maximal current. I_{max} and EC_{50} values are expressed as mean ± s.e.mean and were determined by fitting data from individual oocytes. For serine-O-sulphate (SOS) dose-responses, SOS concentrations up to 3 mM were used, except in the experiments in reduced extracellular sodium where concentrations up to 6 mM SOS were used. EC_{50} values were calculated for membrane potentials in the range −100 mV to 0 mV. At positive membrane potentials the current amplitudes were small with proportionally large errors, which produced accurate estimates of EC_{50} values.

Chemicals

L-Glutamate-Na salt, D-aspartic acid, L-serine-O-sulphate-K salt, HEPES-hemi Na salt, tris and gluconate salts were obtained from Sigma/Aldrich, Sydney, Australia. All other buffer components were of analytical or h.p.l.c. grade.

Results

The transport of substrates into oocytes expressing glutamate transporters generates dose-dependent currents, which may be used to estimate the EC_{50} for transport of a given substrate. It is also possible to measure the voltage-dependence of transport by measuring transport currents at various membrane potentials. Whilst currents generated by a maximal dose of substrate were voltage-dependent for all substrates studied thus far, the EC_{50} values for transport of most substrates by either EAAT1 or EAAT2 showed little variation (<3 fold) over the membrane potential range of −100 mV to 0 mV. However, the EC_{50} for SOS transport by EAAT2 was steeply voltage-dependent, varying from 152 ± 11 μM ($n = 6$) at −100 mV to 1960 ± 160 μM ($n = 6$) at 0 mV (Figure 1). We investigated this effect more closely in order to gain a better understanding of the differences with which different transporter subtypes transport substrates. A simple explanation of this result could be that the voltage-dependence of EC_{50} values arises through a surface charge effect by SOS chelating divalent cations. This possibility was investigated by increasing

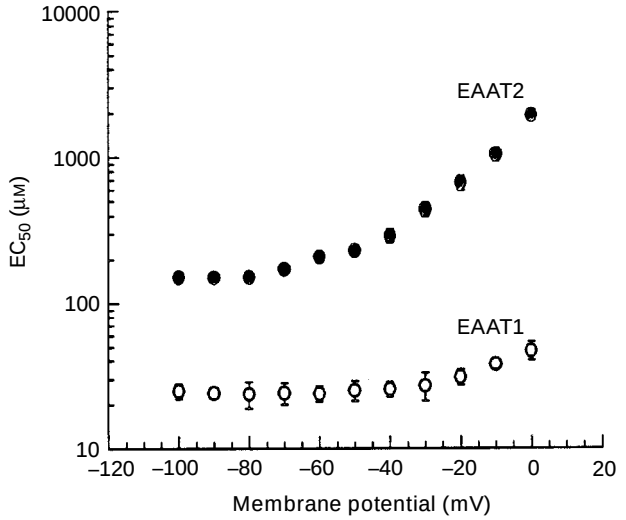

Figure 1 Voltage-dependence of EC_{50} values for L-serine-O-sulphate transport by EAAT2 and EAAT1. EC_{50} values were calculated from current measurements as described in Methods. Vertical lines show s.e.mean, with $n = 3$ for EAAT1 and $n = 6$ for EAAT2. Note the y axis is logarithmic.

the calcium ion concentration in the extracellular solution from 2 mM to 5 mM. Similar voltage-dependence of SOS EC_{50} values was seen irrespective of the extracellular calcium ion concentration (results not shown), making this possibility unlikely. Additional evidence that the voltage-dependent changes in SOS EC_{50} values were not due to SOS chelating divalent cations comes from the observation that SOS EC_{50} values for transport by EAAT1 were relatively voltage-independent (Figure 1).

Chloride-dependence of SOS transport by EAAT2

The voltage-dependence of SOS EC_{50} values showed an apparent biphasic relationship. At membrane potentials in the range -100 mV to -70 mV the slope of the EC_{50}-voltage curve was a modest 1 μM mV^{-1}, whereas in the membrane potential range -30 mV to 0 mV the slope of the curve was 50 μM mV^{-1}. This suggests that more than one factor is involved in determining the voltage-dependent changes in EC_{50} values.

Chloride ions have been demonstrated to have differing contributions to the net transport current depending on the membrane potential at which transport is measured. At membrane potentials greater than the reversal potential for chloride ions ($> \sim -25$ mV) for *Xenopus* oocytes (Barish, 1983, Fairman *et al*., 1995; Wadiche *et al*., 1995a), chloride will move in the same direction through the transporter as the substrate. At membrane potentials more negative than the chloride reversal potential ($< \sim -25$ mV), chloride ions will move in the opposite direction. Thus, we investigated the possibility that the voltage-dependent changes in SOS transport are due to an altered chloride flux through the transporter compared to the chloride flux activated by other substrates. Oocytes expressing EAAT2 were incubated overnight in chloride free solution (gluconate substituted for chloride ions) to reduce the intracellular chloride concentration. It has been estimated that this procedure reduces the intracellular chloride concentration from $\sim$40 mM to $\sim$4 mM (Wadiche *et al*., 1995a). SOS transport currents were then measured in the same chloride free solution. Under these conditions SOS transport showed some distinct differences from transport measured in the standard Frog Ringer solution. The EC_{50} for SOS transport was measured in both the standard Frog Ringer solution and under chloride-free conditions at -20 mV; -20 mV is close to the reversal potential of chloride ions for oocytes in the standard Frog

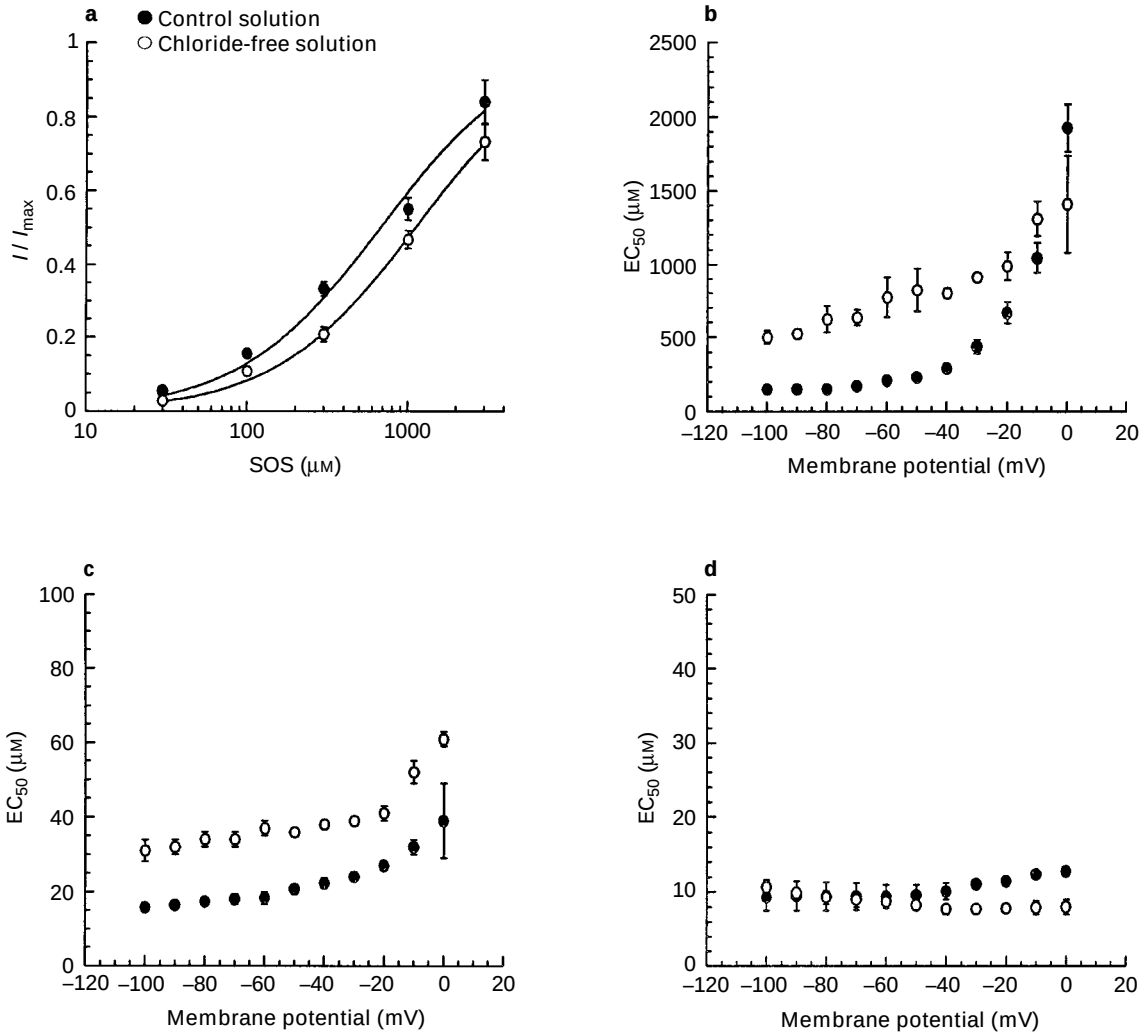

Figure 2 Effect of chloride ions on L-serine-O-sulphate (SOS) transport by EAAT2. (a) Dose-dependent SOS transport currents were measured at -20 mV, in standard Frog Ringer solution and chloride-free Frog Ringer solution after incubation of the oocytes in the same chloride-free solution overnight (see Methods for details of the buffer solutions). The effects of chloride ions on voltage-dependent changes in EC_{50} values were measured for SOS (b), L-glutamate (c) and D-aspartate (d) transport by EAAT2. EC_{50} values were calculated from current measurements in: the standard Frog Ringer solution; chloride-free Frog Ringer, after oocytes were stored overnight in the same chloride free solution. Vertical lines show s.e.mean, with $n = 3$.

Ringer solution, therefore at this membrane potential chloride ions should not contribute to the transport current. The EC_{50} value for SOS transport in the presence of chloride was lower than the corresponding EC_{50} value measured at the same membrane potential, but in the absence of chloride (Figure 2a). This suggests that under conditions where there is no net flux of chloride ions through the transporter, chloride ions still modulate the transport of SOS. When SOS transport was measured with the voltage step protocol in the chloride-free Frog Ringer solution two distinct differences were observed compared to SOS transport measured in the standard Frog Ringer solution. The EC_{50} for transport was increased at all membrane potentials in the range -100 mV to 0 mV, with greatest changes occurring at more negative potentials. In addition, the steepness of the voltage-dependence was also reduced from a 12 fold change to a 2.8 fold change over the membrane potential range of -100 mV to 0 mV. L-Glutamate transport was also modulated by chloride ions, but to a much lesser extent than observed for L-serine-O-sulphate. At -100 mV there was a 2 fold difference in the EC_{50} in the presence and absence of chloride, but the slope of the voltage-dependence of EC_{50} values was relatively constant over the membrane potential range of -100 mV to 0 mV. In contrast, the transport of D-aspartate (Figure 2d), also Wadiche *et al*., 1995a) in similar chloride free conditions showed minimal changes in EC_{50} for transport. These results demonstrate that there is a complex set of interactions between substrate, chloride ions and the transporter and that the nature of these interactions may be different for the various transporter substrates.

Sodium and pH dependence of SOS transport by EAAT2

The above results suggests that SOS interacts with EAAT2 in a different manner to that of other substrates such as D-aspartate or L-glutamate. Therefore, it seemed important to investigate whether such differences may also cause differences in the way in which SOS transport is coupled to sodium and pH gradients across the membrane. Glutamate transport is coupled to the transport of sodium ions with a probable stoichiometry of 3 sodium : 1 glutamate (Zerangue & Kavanaugh, 1996). Sodium binding to EAAT2 has been demonstrated to be voltage-dependent (Wadiche *et al*., 1995b) and under reduced extracellular sodium concentrations it has been found that the EC_{50} for L-glutamate transport by the rabbit glutamate transporter, EAAC1 is voltage-dependent (Kanai *et al*., 1995). Therefore, if SOS transport does not couple to all three sodium ions as efficiently or alters the co-operativity of sodium interactions with the transporter, as compared to glutamate or aspartate, SOS transport may be particularly sensitive to extracellular sodium concentrations. We investigated this possibility by measuring EC_{50} values for SOS transport currents with extracellular sodium concentrations of 50 mM, 98 mM and 140 mM. Increased extracellular sodium decreased the EC_{50} values for SOS transport at all membrane potentials in the range -100 mV to 0 mV and also reduced the voltage-dependent changes in EC_{50} values. At 50 mM sodium and 98 mM sodium SOS transport EC_{50} values were steeply voltage-dependent, but at 140 mM sodium the EC_{50} values showed significantly reduced voltage-dependence (Figure 3). In contrast, L-glutamate and D-aspartate EC_{50} values were relatively constant over the same membrane potential range with either 98 mM sodium or 140 mM sodium in the extracellular solution. However, with 50 mM sodium, the EC_{50} values for both D-aspartate and L-glutamate were voltage-dependent. L-Glutamate EC_{50} values increased from 13.7 ± 2.9 μM at -100 mV to 50.2 ± 9.6 μM at 0 mV ($n = 3$) and D-aspartate EC_{50} values increased from 7.0 ± 1.6 μM to 15.0 ± 5.1 μM ($n = 3$) over the same membrane potential range,

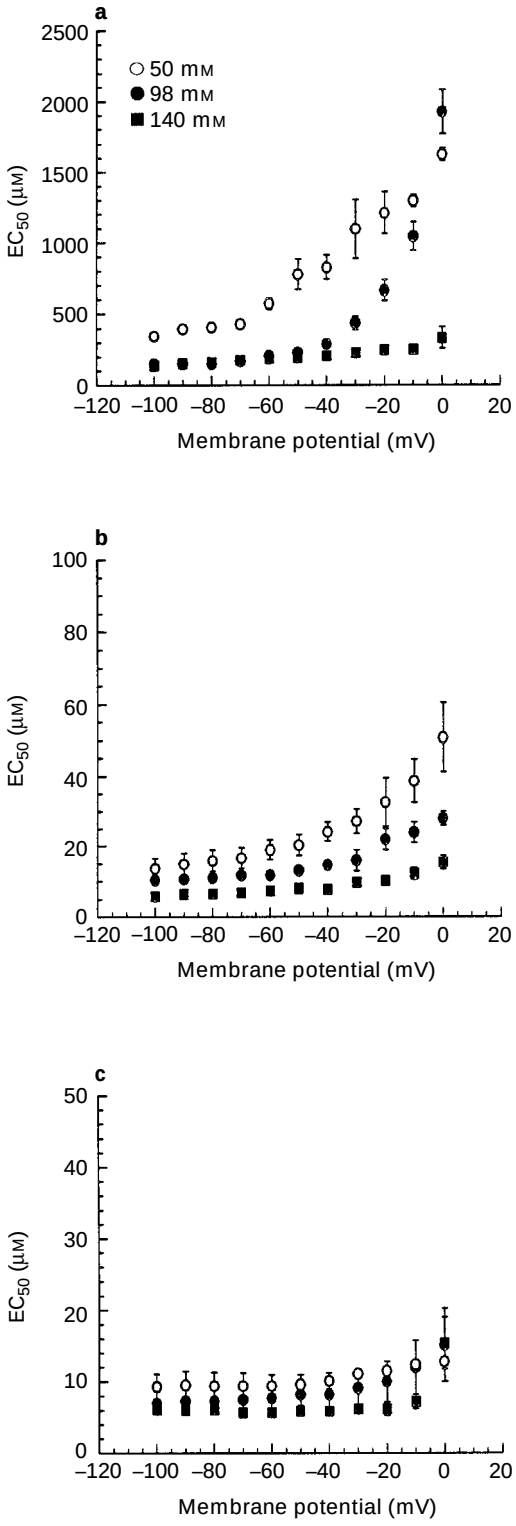

Figure 3 Sodium modulation of L-serine-O-sulphate (a), L-glutamate (b) and D-aspartate (c) transport by EAAT2. EC_{50} values were calculated at membrane potentials between -100 mV and 0 mV from dose-dependent current measurement with extracellular solutions containing 50 mM sodium, 98 mM sodium and 140 mM sodium (see Methods for buffer details, the key is the same for (a), (b) and (c)). Vertical lines show s.e.mean ($n = 3$ for 50 mM sodium and 140 mM sodium, $n = 6$ for 98 mM sodium).

with the reduced extracellular sodium concentration. Thus, voltage-dependent SOS transport shows an enhanced sensitivity to extracellular sodium, compared to voltage-dependent transport of either D-aspartate or L-glutamate.

Glutamate transport is also coupled to the co-transport of 1 H^+, possibly as a carboxylate-ion pair and under appropriate conditions may provide part of the driving force for transport (Zerangue & Kavanaugh, 1996). Therefore, we investigated whether altering the pH of the extracellular solution changed the SOS transport parameters. Decreasing the pH of the external solution from pH 7.55 to pH 6.2 caused a decrease in EC_{50} values for SOS transport at all membrane potentials between −100 mV and 0 mV and also reduced the voltage-dependent changes in EC_{50} values (Figure 4). Although the EC_{50} values for L-glutamate or D-aspartate transport currents measured at pH 6.2 were reduced compared to values measured at pH 7.55, there was very little voltage-dependent change in EC_{50} values at either pH 7.55 or pH 6.2 (Figure 4).

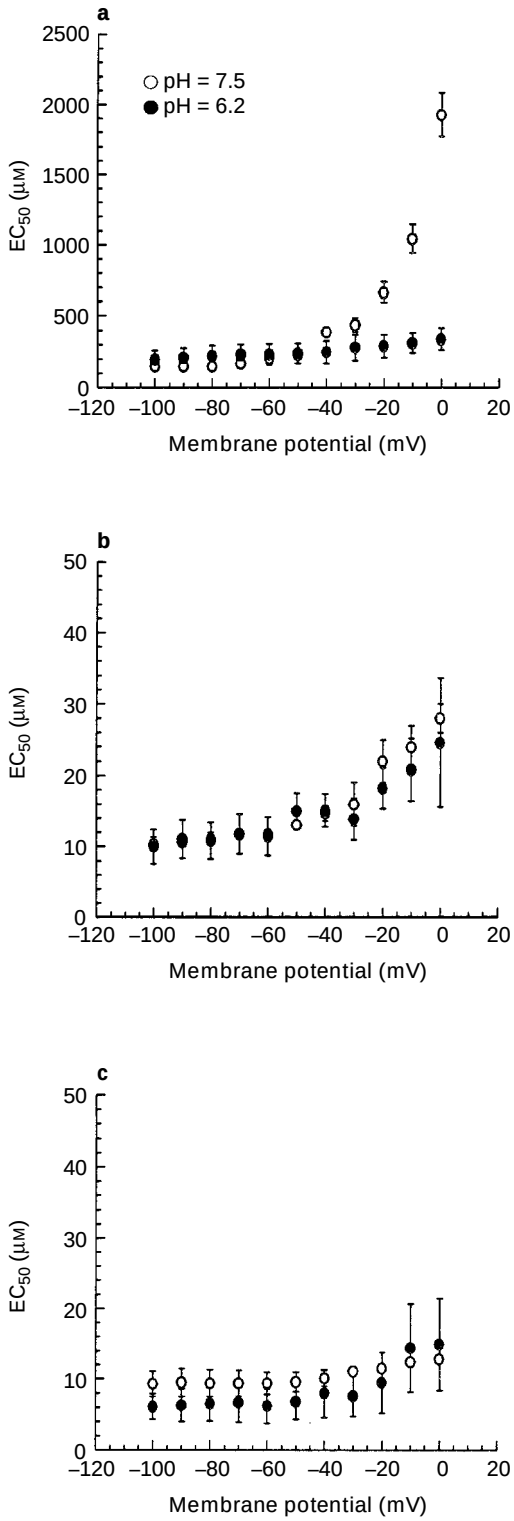

Figure 4 pH modulation of L-serine-O-sulphate (SOS) (a), L-glutamate (b) and D-aspartate (c) transport by EAAT2. EC_{50} values were calculated at membrane potentials between −100 mV and 0 mV from dose-dependent current measurements in the standard Frog Ringer solution at pH 7.55 and pH 6.2. Vertical lines show s.e.mean ($n=3$, except for SOS transport at pH 7.55, where $n=6$).

Discussion

The results presented demonstrate that SOS is transported by the human glutamate transporter EAAT2 in a subtly different manner to that of other substrates and is also different to SOS transport by other glutamate transporters investigated to date. The steep voltage-dependence of SOS transport highlights an important difference in the way different substrates may interact with the pore of the different glutamate transporter subtypes and, as such, may also provide a very useful probe in further studies in identifying the structural basis for the interactions of substrates with the pore of the different transporter subtypes.

In order to appreciate some of the differences in the ways in which SOS, L-glutamate and D-aspartate interact with excitatory amino acid transporters it may be instructive to consider the structures of these substrates (Figure 5). SOS is a structural analogue of L-glutamate with the $-O\text{-}SO_3^-$ moiety in SOS substituting for the $-CH_2\text{-}CO_2^-$ moiety in L-glutamate. Both SOS and L-glutamate have one atom more than D-aspartate in their backbone chain of atoms. SOS and L-glutamate differ from D-aspartate in having the L-configuration at the amino acid carbon. The D-isomer of SOS is inactive against L-glutamate uptake into rat brain slices (Balcar & Johnston, 1972). As the sulphur-oxygen double bonds in the $-SO_3^-$ of SOS are longer than the carbon-oxygen double bonds in the $-CO_2^-$ of L-glutamate (ca 1.450 Å vs ca 1.328 Å, respectively), the sulphate grouping will be somewhat larger overall than the carboxylate group. In addition, the oxygen atoms of the sulphate group are bonded to the sulphur atom in a tetrahedal configuration compared to the planar configuration of the oxygen atoms bound to the carbon atom in the carboxylate group. Furthermore, the oxygen atom in the CH_2-O-S part of the SOS backbone chain of atoms could offer different interactions, e.g. through the lone pair of electrons on the oxygen, with a transporter to the equivalent carbon atom in L-glutamate. However, the major difference between SOS, L-glutamate and D-aspartate, is that the $-O\text{-}SO_3^-$ group in SOS is a much stronger acid than the equivalent CO_2^- groups of L-glutamate and D-aspartate. The pK_a of the CO_2^- group of L-glutamate is ca 4.28, whereas the pK_a of $-O\text{-}SO_3^-$ group in SOS is likely to be less than 0 based on pK_a values reported for propranolol-0-sulphate and related sulphur esters (Manners *et al*., 1989). Although neither the sulphate nor carboxylate groups are likely to be protonated to any significant extent at physiological pH, under an appropriately acidic local environment, which may possibly occur in the pore of a transporter (Zerangue & Kavanaugh, 1996 – see discussion below), the sulphate group will have a much reduced capacity to combine with a proton compared to the equivalent carboxylate group in either L-glutamate or D-aspartate. The much stronger acidity of SOS compared to L-glutamate and D-

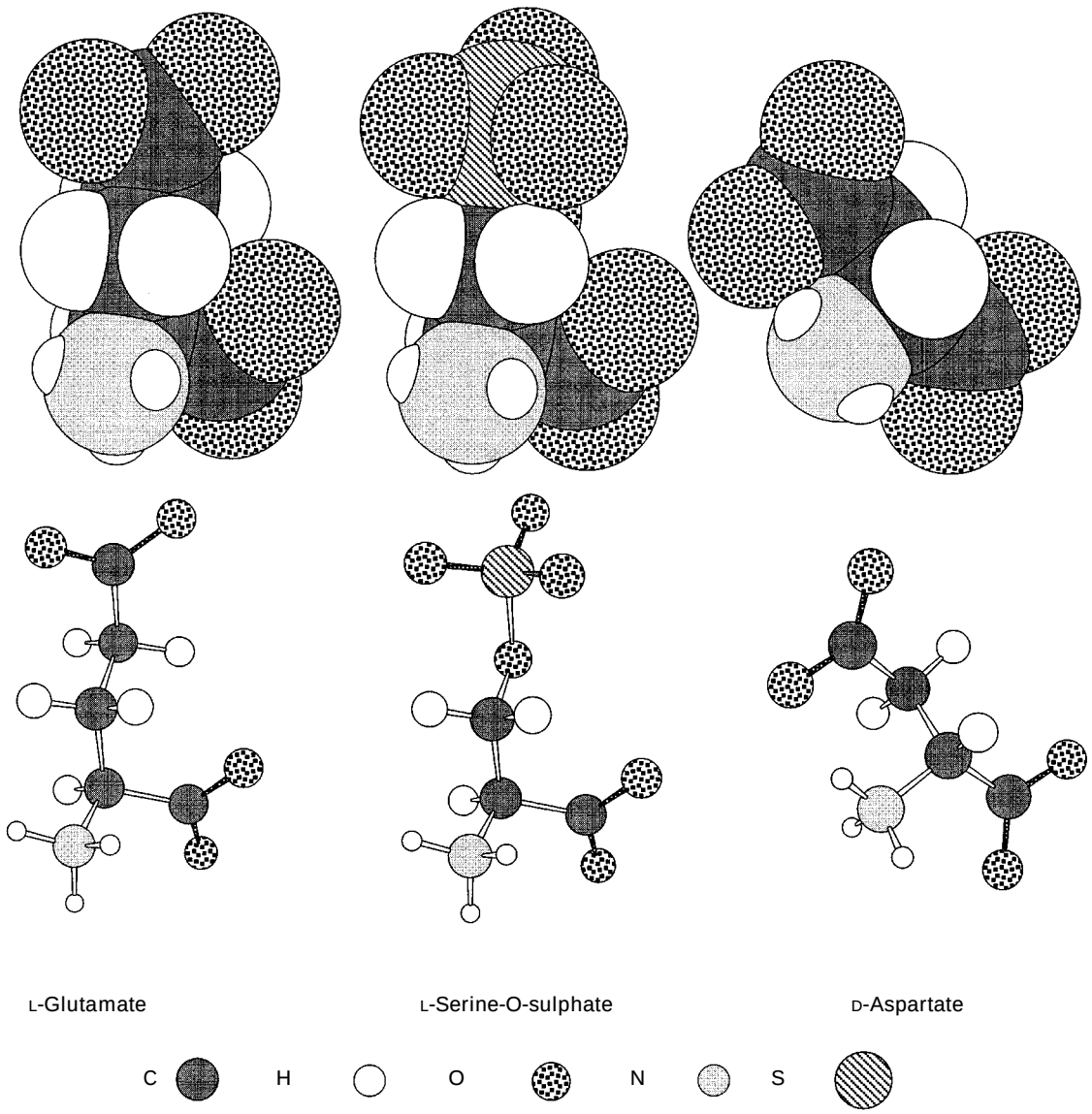

Figure 5 Structures of L-glutamate, L-serine-O-sulphate and D-aspartate. Figures generated in Chem3D Plus (Cambridge Scientific Computing, Inc, Cambridge, Mass. U.S.A., version 3.1.2, 1994). Upper structures are space filling models showing the Van der Walls radii of the atoms, while lower structures are ball and stick models with cylindrical bonds showing stereochemistry. The conformations shown are the mimimum energy conformations of the fully ionized molecules.

aspartate could have a significant influence on transport, e.g. in the ways in which SOS is transported in different ionic environments.

Following the initial observation of a steep voltage-dependence in the EC_{50} for SOS transport by EAAT2 over the membrane potential range of -100 mV to 0 mV we have characterized the ionic basis for this effect by altering the concentrations of the various ions involved in the transport of substrates through the transporter. When measuring SOS transport currents we were actually measuring the total sum of charges moving across the membrane, which may not necessarily be directly proportional to the flux of SOS at each given membrane potential. The various ions involved may have differing contributions to the current sum at different membrane potentials (Fairman *et al*., 1995; Wadiche *et al*., 1995a). The voltage-dependence of SOS EC_{50} values showed an apparent biphasic relationship. As chloride ions can move in different directions through the transport, independent of the direction of substrate movement, it is not unreasonable to postulate that chloride ions may have differing effects on the way in which substrates pass through the transporter depending on the direction with which chloride ions are moving. Our results show that chloride ions decrease the EC_{50} for SOS transport, which presumably (but not necessarily) reflects an increase in affinity of SOS for the transporter. These decreases in EC_{50} values in the presence of chloride were most apparent at membrane potentials at which chloride ions are expected to move out of the cell (i.e. in the opposite direction to that of SOS). In addition chloride ions appeared to increase the steepness of the voltage-dependence of SOS transport. This effect was most apparent at membrane potentials at which chloride ions are expected to move into the cell (i.e. in the same direction of that of SOS). These results suggest that there are multiple chloride recognition/modulatory sites on the transporters. Clearly, there must be a chloride recognition site within the pore of the transporter to allow passage of these ions through the membrane, but other additional modulatory sites are also likely to exist. If there are multiple chloride

recognition sites within the pore or at a site that may modulate the structure of the pore, the extra bulk and electron density of the sulphate group, compared to the carboxyl group of aspartate or glutamate, may differentially alter the accessibility or affinity of chloride for such a recognition site(s). Additional evidence for chloride modulatory sites on glutamate transporters comes from the observation that a chimeric glutamate transporter derived from EAAT1 and EAAT2 allows an uncoupled flux of monovalent cations that appears to be modulated by both intracellular and extracellular chloride ions (Vandenberg *et al*., 1995). In another study, Billups *et al*. (1996) found that the outward chloride current activated by glutamate transport in Muller cells of the Salamander retina shows a less than Nernstian dependence on the extracellular chloride concentration. They speculated that this may be due to the existence of a site on the transporter that allows chloride ions to modulate transporter function. Thus, there is increasing evidence for the existence of chloride modulatory sites on glutamate transporters. However, at this stage the role of such modulation in transporter function has not been fully resolved.

Sodium ions also appear to play an important role in determining the voltage-dependence of SOS transport. Increased extracellular sodium concentrations reduced both the EC_{50} values and the voltage-dependence of the EC_{50} values for SOS transport. This result could be explained if SOS and chloride interact with the transporter in such a way that disrupts the coupling of the 3 sodium ions to the movement of SOS. As sodium binding to EAAT2 is voltage-dependent (Wadiche *et al*., 1995b), a reduction in affinity or alteration in the coupling efficiency of one of the three sodium ions for the transporter could effectively alter the voltage-dependent rate of SOS flux through the transporter and cause an apparent reduction in affinity of SOS for EAAT2. At elevated sodium concentrations, the altered sodium site would become saturated and provide a more efficient coupling of sodium movement to that of SOS, thus reducing the voltage-dependence in the EC_{50} values. In the cases of L-glutamate and D-aspartate transport, only when the extracellular sodium concentration was reduced to 50 mM were the EC_{50} values weakly voltage-dependent. Thus, in contrast to SOS, 98 mM sodium may be sufficient for efficient coupling for L-glutamate and D-aspartate transport and as such the EC_{50} values are less voltage-dependent.

A decrease in extracellular pH caused both a reduction in EC_{50} values for SOS and a reduction in the voltage-dependent changes in EC_{50} values. The decreases in EC_{50} values at low pH may be partly explained by an increased driving force for the transport process. Such an increase in the driving force may also explain the small decreases in EC_{50} values which were observed for L-glutamate and also D-aspartate transport by EAAT2. However, there are a number of possible explanations for the reduction in the voltage-dependent changes in EC_{50} values for SOS transport. One simple explanation is that a decrease in extracellular pH causes a change in the protonation state of an amino acid residue that is accessible to the extracellular solution. It has been suggested that histidine 326 of GLT1 (GLT1 is the rat homologue of EAAT2), which may be titrated in the range of pH 6.2 to pH 7.55, plays an important role in glutamate transport (Zhang *et al*., 1994). The altered protonation state, of an amino acid residue such as histidine 326 at pH 6.2, may induce a conformation of the transporter acid that allows the larger sulphate group of SOS to fit into its recognition site. Once bound, SOS may then couple efficiently to sodium ions to maintain a similar affinity of SOS for the transporter over the membrane potential range used in this study. An alternative explanation comes from the suggestion that glutamate is transported in a protonated form (Zerangue & Kavanaugh, 1996). If such a model were correct, then there must be local environments within the pore of the transporter that are sufficiently acidic to maintain the protonated form of the carboxyl group as it traverses the membrane. Glutamate 404 of GLT1 (GLT1 is the rat homologue of EAAT2) has been demonstrated to play an important role in substrate discrimination (Pines *et al*., 1995) and may also form part of an acidic environment of the pore. However, the acid dissociation constant of the sulphate group of SOS is at least 4 orders of magnitude lower than the acid dissociation constant of the carboxyl group of L-glutamate or D-aspartate. Protonation of the sulphate, even in the acidic environment of the pore is unlikely. Therefore, although protonation of the substrate may not be essential for transport it may alter the properties of the transport process. In the case of SOS transport by EAAT2, the unprotonated sulphate may bind weakly to the transporter within the electric field of the membrane creating a voltage dependent step in the transport process and voltage-dependent changes in EC_{50} values. At lower pH the voltage-dependent changes in EC_{50} values for SOS were less apparent. This may be explained by the higher concentration of protons providing a greater driving force for the transport of substrates (Zerangue & Kavanaugh, 1996), which may overcome weak voltage-dependent interactions between the sulphate moiety of SOS and the transporter.

Other possible explanations for the roles of chloride ions, sodium ions and protons in determining the voltage-dependence of SOS transport are also possible. However, one of the major impediments to providing a full mechanistic description of the molecular basis for voltage-dependent SOS transport currents is the lack of radiolabelled SOS. The use of labelled SOS would allow a direct measure of the SOS K_m at various membrane potentials irrespective of the other ions involved. Without such a compound, any models to explain the molecular basis for the voltage-dependent transport of SOS must remain speculative.

The results presented in this study raise the interesting question as to why voltage-dependent transport of SOS is most apparent with EAAT2 compared to other transporter subtypes. The answer to this question must lie in the different properties of either the initial substrate recognition sites at the external surface of the transporter or within the pore of the transporters. A number of pharmacological and physiological differences between the transporter subtypes have been described, which may provide clues about the differing structures of the different transporter subtypes. Of the human cloned glutamate transporters, EAAT2 is the least permeant to chloride ions (Fairman *et al*., 1995; Wadiche *et al*., 1995a; Arriza *et al*., 1997). In addition, the amplitude of transport currents differ significantly between transporter subtypes. For example, the maximal currents generated by glutamate transport by EAAT1 may be up to 3 μA at hyperpolarized potentials, whereas similar currents measurements for EAAT2 are up to an order of magnitude smaller (eg. see Wadiche *et al*., 1995). Assuming similar expression levels, such differences may reflect different rates of turnover of the transporters. Finally, distinct pharmacological differences between transporter subtypes have also been observed. In particular, 4-methylglutamate is a potent blocker of transport by EAAT2, but is a high affinity substrate for EAAT1. This pharmacological difference may reflect a more stringent structural requirement by EAAT2 in determining whether or not a molecule can pass through the transporter or not. Thus, EAAT2 may have more stringent requirements for substrate recognition, but at the

expense of speed of turnover and chloride permeability. In contrast, EAAT1 may have more relaxed structural requirements for substrate recognitition so as to maintain a high turnover rate and high chloride permeability. As far as SOS fits into such a scheme, it is envisaged that SOS meets the structural requirements for a substrate for EAAT2, but not quite as effectively as glutamate, causing an alteration in the coupling of sodium ions, protons and also chloride ions to the movement of SOS through the transporter, with a resultant voltage-dependence in EC_{50} values. Less stringent requirements for substrate recognition by EAAT1 may accommodate the bulkier and more electronegative sulphate group of SOS, allowing normal coupling of the various ions to the transport process. Further developments in characterization of the molecular structures of the pores of glutamate transporters may provide a clearer understanding of these events.

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