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Molecular Basis for Differential Inhibition of Glutamate Transporter Subtypes by Zinc Ions

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ABSTRACT

Zinc ions (Zn^{2+}) are stored in synaptic vesicles with glutamate in a number of regions of the brain. When released into the synapse, Zn^{2+} modulates the activity of various receptors and ion channels. Excitatory amino acid transporters (EAATs) maintain extracellular glutamate concentrations below toxic levels and regulate the kinetics of glutamate receptor activation. We have investigated the actions of Zn^{2+} on two of the most abundant human excitatory amino acid transporters, EAAT1 and EAAT2. Zn^{2+} is a noncompetitive, partial inhibitor of glutamate transport by EAAT1 with an IC_{50} value of 9.9 ± 2.3 μM and has no effect on glutamate transport by EAAT2 at concentrations up to 300 μM. Glutamate and aspartate transport by EAAT1 are associated with an uncoupled chloride conductance, but Zn^{2+} selectively inhibits transport and increases the relative chloride flux through the transporter. We have investigated the molecular basis for differential inhibition of EAAT1 and EAAT2 by Zn^{2+} using site-directed mutagenesis and demonstrate that histidine residues of EAAT1 at positions 146 and 156 form part of the Zn^{2+} binding site. EAAT2 contains a histidine residue at the position corresponding to histidine 146 of EAAT1, but at the position corresponding to histidine 156 of EAAT1, EAAT2 has a glycine residue. Mutation of this glycine residue in EAAT2 to histidine generates a Zn^{2+} sensitive transporter, further confirming the role of this residue in conferring differential Zn^{2+} sensitivity.

Zn^{2+} is found in many parts of the brain and has been reported to modulate glutamate receptors (Peters *et al.*, 1987; Westbrook and Mayer, 1987), γ-aminobutyric acid and glycine receptors (Smart and Constanini, 1983; Laube *et al.*, 1995), glutamate transporters (Balcar and Johnston, 1972; Gabrielsson *et al.*, 1986; Spiridon *et al.*, 1998), and the Na^+K^+ATPase (Hexum, 1974); thus, it may modulate neurotransmission in a number of different ways (reviewed by Frederickson, 1986). In particular regions of the brain, most notably in the mossy fibers of the hippocampus, Zn^{2+} is co-localized with glutamate in synaptic vesicles and is released into the synapse in a calcium-dependent manner; it may reach concentrations of up to 118 μM (Howell *et al.*, 1984; Spiridon *et al.*, 1998). The different glutamate receptor subtypes show differential sensitivity to Zn^{2+}. The NMDA receptor subtype is potently inhibited by Zn^{2+} in a rapid and reversible manner, yet the kainate and quisqualate receptor subtypes are relatively insensitive to Zn^{2+} (Westbrook and Maher, 1987). Furthermore, Zn^{2+} has differential effects on different subtypes of NMDA receptors (Chen *et al.*, 1997).

Balcar and Johnston (1972) and Gabrielsson *et al.* (1986) reported inhibition of [^{3}H]glutamate transport in brain slices and synaptosomes, respectively, by moderate concentrations of Zn^{2+} (30–100 μM). However, in these experimental systems, Zn^{2+} may have a number of effects, including inhibition of Na^+K^+ATPase activity, which may indirectly reduce glutamate transport activity by disrupting the Na^+ and K^+ gradients required for glutamate uptake. More recently, Zn^{2+} has been demonstrated to inhibit glutamate transport in Müller cells and cone cells of the salamander retina (Spiridon *et al.*, 1998). The effects of Zn^{2+} in this study were rapid in onset and fully reversible, which suggests a direct role in transport inhibition. Zn^{2+} does not seem to compete with glutamate, sodium, potassium or protons binding to the transporter, which implies that Zn^{2+} allosterically modulates transporter function. In addition to inhibiting glutamate transport, Zn^{2+} seems to have opposing effects on the chloride conductance associated with the glutamate transporter in the two cell types investigated in the salamander retina. In Müller cells, the chloride conductance is increased by Zn^{2+}; in the cone cells, however, the chloride conductance is inhibited by Zn^{2+}. The transporter in Müller cells is likely to be similar in properties to EAAT1/GLAST1, whereas the cone-cell glutamate transporter is most similar to EAAT5 (Eliasof *et al.*, 1997).

We have investigated the effects of Zn^{2+} on two of the most abundant human glutamate transporter subtypes, EAAT1

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ABBREVIATIONS: EAAT1; excitatory amino acid transporter 1, EAAT2; excitatory amino acid transporter 2, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; GLAST, glutamate aspartate transporter.

and EAAT2 (Arriza *et al.*, 1994) expressed in *Xenopus laevis* oocytes. Zn^{2+} is a noncompetitive inhibitor of glutamate transport by EAAT1, but has no effect on transport by EAAT2. Zn^{2+} seems to cause an increase in the relative chloride component of the EAAT1 transport current while inhibiting the transport component. In addition, analysis of the Zn^{2+} sensitivity of point mutated transporters has been used to characterize the molecular basis for differential Zn^{2+} inhibition of glutamate transport by EAAT1 compared with EAAT2.

Materials and Methods

Chemicals. L-glutamate-Na salt, D-aspartic acid, HEPES-hemi Na salt, gluconate salts and methanesulfonic acid were obtained from Sigma/Aldrich, Sydney, Australia. Zinc sulfate (analytical grade) was obtained from Standard Laboratories, Melbourne, Australia. Stock solutions of zinc sulfate in frog Ringer buffer were made up fresh on the day of use. All other buffer components were of analytical grade or acceptable for use in high performance liquid chromatography.

Expression of transporters and electrophysiological recording. cDNAs encoding the human glutamate transporters, EAAT1 and EAAT2, were subcloned into pOTV for synthesis of RNA and expression in *X. laevis* oocytes as described previously (Arriza *et al.*, 1994). Site-directed mutagenesis was carried out using the Altered Sites kit (Stratagene, La Jolla, CA) as described in the manufacturer's instructions. Two to seven days after RNA injection, current recordings were made at 22° with a Geneclamp 500 interfaced with an IBM-compatible computer using a Digidata 1200 A/D controlled by pCLAMP software (version 6.0.2; Axon Instruments). The standard frog Ringer recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.55. In experiments in which chloride was removed, the oocytes were dialyzed overnight in a chloride-free frog Ringer solution in which all the chloride was replaced with gluconate (96 mM Na-gluconate, 2 mM

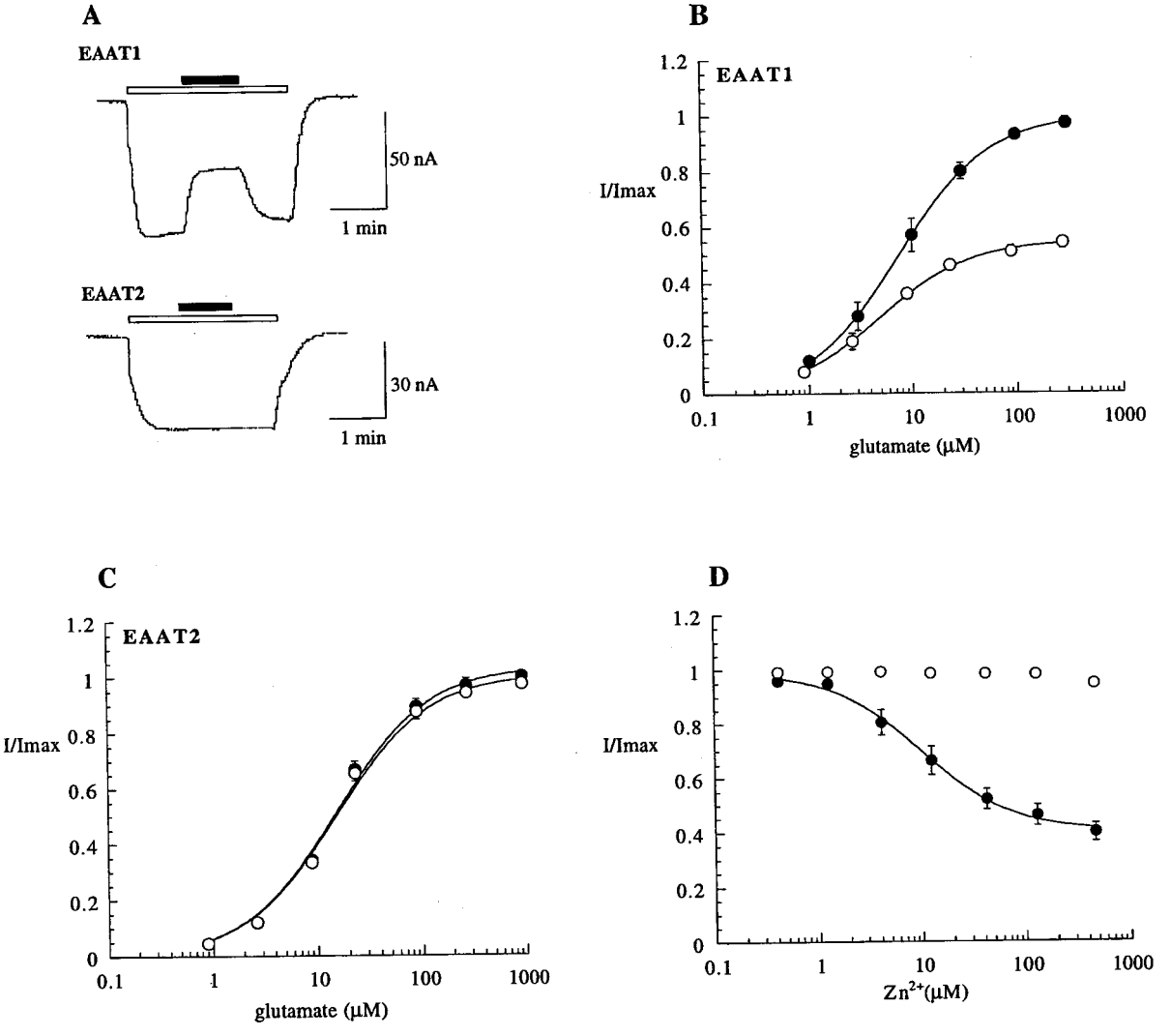

Fig. 1. Zn^{2+} inhibits glutamate transport by EAAT1 and has no effect on EAAT2. A, Representative current traces at −60 mV for the effect of 100 μM Zn^{2+} (*filled bar*) on 30 μM glutamate (*open bar*) transport by oocytes expressing EAAT1 or EAAT2. For the purposes of this figure, we selected traces from cells in which Zn^{2+} alone had no effect on the base-line current. Zn^{2+} has no effect on the EC_{50} values for glutamate transport by either EAAT1 (B) or EAAT2 (C) but reduces the maximal glutamate transport current for EAAT1. Dose-dependent glutamate transport currents at −60 mV were measured in the presence (○) and absence (●) of 100 μM Zn^{2+}. Data from individual oocytes were normalized to a maximal dose of glutamate in the absence of Zn^{2+} and fit to the equation $I = I_{max} \cdot [S]/(EC_{50} + [S])$ (see Methods). The data plotted represent the mean ± standard error (n = 4). D, Glutamate (1 mM) transport currents at −60 mV for EAAT1 (●) and EAAT2 (○) were measured in the presence of increasing doses of Zn^{2+}. Current measurements were normalized to the current in the absence of Zn^{2+} and the mean ± standard error from four cells plotted. The errors for EAAT2 are smaller than the size of the symbol.

K-gluconate, 1 mM Mg (gluconate)$_2$, 1.8 mM Ca (gluconate)$_2$, and 5 mM HEPES, pH 7.55). It has been estimated that this procedure reduces the intracellular chloride concentration from 40 mM to 4 mM (Wadiche *et al.*, 1995). The next day, recordings were made using a chloride-free Ringer solution in which the chloride was replaced with either gluconate, as described above, or methanesulfonate (96 mM methanesulfonic acid, 2 mM KOH, 1 mM $MgSO_4$, 1.8 mM Ca-acetate, 5 mM HEPES and the pH adjusted with NaOH to pH 7.55). When recording using the chloride-free solutions, 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-msec voltage pulses to potentials between −100 mV and +50 mV, from corresponding current measurements in the presence of L-glutamate or D-aspartate. In experiments concerned with the pH dependence of Zn^{2+} inhibition of glutamate transport, the pH of the bath solution was adjusted with HCl. In some oocytes, Zn^{2+} blocked a current that was present in uninjected oocytes, water-injected oocytes, and also oocytes injected with either EAAT1 or EAAT2. Therefore, all base-line current measurements were made in the presence of the appropriate Zn^{2+} concentration so that only glutamate transport currents were measured.

Analysis of kinetic data. Zn^{2+} chelates glutamate; therefore, to estimate the free Zn^{2+} and free glutamate concentrations, we have used the correction methods described by Dawson *et al.* (1986) and also employed by Spiridon *et al.* (1998). When calculating values for IC_{50}, EC_{50}, and %Inhibition, we have used the free Zn^{2+} and free glutamate concentrations. Current (I) as a function of L-glutamate concentration ([S]) was fitted by least squares to $I = I_{max} \cdot [S]/(EC_{50} + [S])$, where I_{max} is the maximal current and EC_{50} is the concentration of L-glutamate generating half the maximal current. I_{max} and EC_{50} values are expressed as mean ± standard error and were determined by fitting data from individual oocytes. For Zn^{2+} dose responses, current (I) as a function of Zn^{2+} concentration ($[Zn^{2+}]$) was fitted by least squares to $I = I_{max(Z)} - [(I_{max(Z)} \cdot [Zn^{2+}])/(IC_{50} + [Zn^{2+}])] + I_r$, where $I_{max(Z)}$ is the maximal current generated by L-glutamate that is inhibited by Zn^{2+}, IC_{50} is the concentration of Zn^{2+} that reduced $I_{max(Z)}$ by 50% and I_r is the residual glutamate transport current in the presence of a maximal dose of Zn^{2+}. %Inhibition = $I_{max(Z)}/(I_{max(Z)}+I_r)$. IC_{50} and %Inhibition values are expressed as mean ± standard error.

Results

Application of L-glutamate to *X. laevis* oocytes expressing the human glutamate transporters, EAAT1 or EAAT2, generates dose-dependent inward currents when voltage clamped at −60 mV (Arriza *et al.*, 1994). Co-application of 100 μM Zn^{2+} with 30 μM glutamate reduced the EAAT1 transport current but had no effect on EAAT2 (Fig. 1A). The inhibition of transport currents in EAAT1 are rapid in onset and fully reversible, which indicates a direct action of Zn^{2+} on the transporter. EC_{50} values for glutamate transport measured in the presence and absence of 100 μM Zn^{2+} were similar (Table 1) but the maximal transport current was decreased by approximately 50% in the presence of 100 μM Zn^{2+} compared with its absence (Fig. 1B). This suggests that Zn^{2+} is acting as a noncompetitive inhibitor of glutamate transport. Inhibition of EAAT1 transport currents by Zn^{2+} is dose dependent with an IC_{50} value of 9.9 ± 2.3 μM and maximal inhibition of 59 ± 4% (Fig. 1D). In contrast, Zn^{2+}, at concentrations up to 300 μM, had little or no effect on glutamate transport currents for oocytes expressing EAAT2 (Fig. 1, *C* and *D*). Thus, the actions of Zn^{2+} on EAAT1 seem to be similar (although less potent for EAAT1) to that observed for glutamate transporters of salamander retina Müller cells.

Glutamate transport is coupled to 2–3 Na^+, 1 H^+ (or possibly counter-transport of 1 OH^-) and the counter-transport of 1 K^+ (Kanner and Sharon, 1978; Barbour *et al.*, 1988; Zerangue and Kavanaugh, 1996). The different transporter subtypes also allow a varying degree of chloride ion flux, which is thermodynamically uncoupled to the rate of glutamate transport (Fairman *et al.*, 1995; Wadiche *et al.*, 1995; Billups *et al.*, 1996; Eliasof and Jahr, 1996). In salamander-retina Müller cells, Zn^{2+} seems to increase the chloride conductance activated by glutamate transport (Spiridon *et al.*, 1998). We investigated the effects of Zn^{2+} on the chloride conductance activated by transport by EAAT1. D-aspartate transport by EAAT1 activates a significantly greater chloride conductance than L-glutamate transport (Wadiche *et al.*, 1995), which allows more accurate measures of the chloride conductance. The D-aspartate transport current reverses direction at 3 ± 2 mV (n = 4), with the outward current caused by the uncoupled chloride flux (Wadiche *et al.*, 1995). The membrane potential at which the current reverses direction may be used as an indicator of the chloride conductance relative to the transport component of the current. In the presence of 100 μM Zn^{2+} the D-aspartate transport current reverses direction at −14 ± 4 mV (n = 4). This shift in transport current reversal potential toward the chloride reversal potential for *X. laevis* oocytes (~ −20 mV; Barish, 1983; Fairman *et al.*, 1995; Wadiche *et al.*, 1995) suggests that in the presence of Zn^{2+}, chloride ions contribute a larger proportion of the transport current compared with conditions in which Zn^{2+} is absent. At membrane potentials greater than the reversal potentials for D-aspartate, the net transport currents in the presence and absence of Zn^{2+} converge (Fig. 2). This suggests that although the chloride conductance *relative* to substrate flux has increased, the *absolute* chloride conductance has not increased significantly.

The voltage dependence of glutamate transport block by Zn^{2+} was also measured. For these experiments, we used oocytes that had been dialyzed in chloride-free Ringer solution (gluconate substituted for chloride; see Materials and Methods for buffer details) to avoid chloride effects on the transport currents, which would otherwise create an appar-

TABLE 1

Kinetic parameters for glutamate transport and inhibition of glutamate transport by Zn^{2+} for EAAT1, EAAT2 and point-mutated transporters.

EC_{50} values were estimated using the first equation listed in Materials and Methods. Measurements for EC_{50}* values were made in the presence of 100 μM Zn^{2+}. IC_{50} and %Inhibition values were estimated using the second equation in Materials and Methods. The number of cells used for each measurement was between four and eight, except where stated.

Transporter	Glutamate		Zn^{2+}	
	EC_{50}	EC_{50}*	IC_{50}	%Inhib
	μM		μM	
EAAT1	15 ± 3	9.6 ± 0.4	9.9 ± 2.9	59 ± 4
EAAT1-H156A	24 ± 2	23 ± 1	237 ± 27	52 ± 3
EAAT1-H146A	31 ± 4	16 ± 3	>300	
EAAT1-H146D	33 ± 5	21 ± 4	199 ± 60	37 ± 4
EAAT1-E86Q	4.8 ± 0.4	3.7 ± 0.2	44 (n = 2)	50 (n = 2)
EAAT1-E86D	18 ± 4	10 ± 3	21.9 ± 0.8	51 ± 4
EAAT1-E303A	18 ± 3	6.7 ± 0.6	21 ± 2	71 ± 2
EAAT1-E184Q, C186A	28 ± 2	19 ± 3	18 ± 4	52 ± 3
EAAT2	23 ± 1	24 ± 2	>300	
EAAT2-G154H	17 ± 1	16 ± 1	44 ± 7	59 ± 3

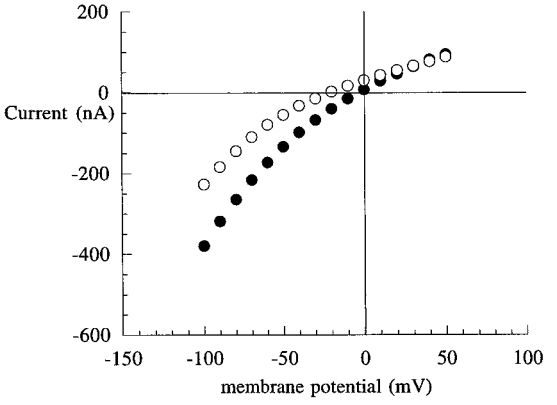

Fig. 2. Zn^{2+} modulates the transport activated chloride conductance of EAAT1. Zn^{2+} inhibits D-aspartate transport by EAAT1 and shifts the current reversal potential toward the chloride reversal potential. The transport currents elicited by 1 mM D-aspartate in the presence (○) and absence (●) of 100 μM Zn^{2+} were measured for membrane potentials between −100 mV and +50 mV in 10 mV increments, using the voltage pulse protocol described in the methods section with the standard frog Ringer solution. Data from a representative cell are shown.

ent voltage dependence, separate from any Zn^{2+} effects. Zn^{2+} inhibition of glutamate transport by EAAT1 was then measured using a chloride-free Ringer solution in which the chloride was replaced with either gluconate or methanesulfonate. After taking into account the chelation of Zn^{2+} by gluconate, as described by Dawson *et al.* (1986), and Spiridon *et al.* (1998), the IC_{50} for Zn^{2+} inhibition of 1 mM glutamate transport currents, measured using gluconate-substituted buffer solutions, was at least an order of magnitude greater than similar measurements using the chloride-containing frog Ringer solution. In contrast, the IC_{50} value measured using a buffer with methanesulfonate substituted for chloride was similar to IC_{50} measurements made in the presences of chloride. Therefore, we used the methanesulfonate-substituted frog Ringer solution to measure the voltage dependence of Zn^{2+} inhibition of glutamate transport. The IC_{50} for Zn^{2+} inhibition of glutamate transport was relatively constant over the membrane potential range of −100 mV to +50 mV, varying from 10.8 ± 2.6 μM at −100 mV to 15.7 ± 6.0 μM at +50 mV (Fig. 3). The %Inhibition was also relatively constant over this membrane potential range at around 60%. This suggests that the Zn^{2+} binding site on the transporter is not influenced by the electric field of the membrane and is likely to be near the external surface of the transporter. Again, similar voltage dependence of Zn^{2+} inhibition of glutamate transport by Müller cells was observed, consistent with the identification of the transporter in this cell type as similar to EAAT1.

It has been demonstrated that a reduction in pH reduces Zn^{2+} inhibition of transport in Müller cells and that this is unlikely to reflect competition between Zn^{2+} and protons for a site on the transporter (Spiridon *et al.*, 1998). Zn^{2+} inhibition of glutamate transport currents for EAAT1 was similarly abolished at pH 6.0 (Fig. 4). This suggests that the protonation state of the Zn^{2+} binding site on EAAT1 is altered at pH 6.0 compared with pH 7.5, which indicates the role of histidine and also possibly cysteine residues in forming part of the Zn^{2+} binding site (e.g., see Wang *et al.*, 1995). To identify amino acid residues that may form part of a Zn^{2+} binding site on glutamate transporters and also explain the differential Zn^{2+} sensitivity of EAAT1 compared with EAAT2, we targeted histidine, cysteine, and negatively charged amino acid residues for mutagenesis using the following criterion: residues located in regions likely to be present within extracellular domains and regions that are distinct from the putative pore region of the transporters (carboxyl-terminal domain; Pines *et al.*, 1995; Vandenberg *et al.*, 1995; Kavanaugh *et al.*, 1996; Mitrovic *et al.*, 1998).

Histidine 146, of EAAT1, is conserved among all the cloned glutamate transporters, except EAAT3, and is located at the extracellular edge of the putative transmembrane domain 3 (Seal *et al.*, 1996; Arriza *et al.*, 1997; Wahle and Stoffel, 1997). Application of glutamate to oocytes expressing the EAAT1-H146A mutant generates dose dependent transport currents of similar magnitude and with an EC_{50} value simi-

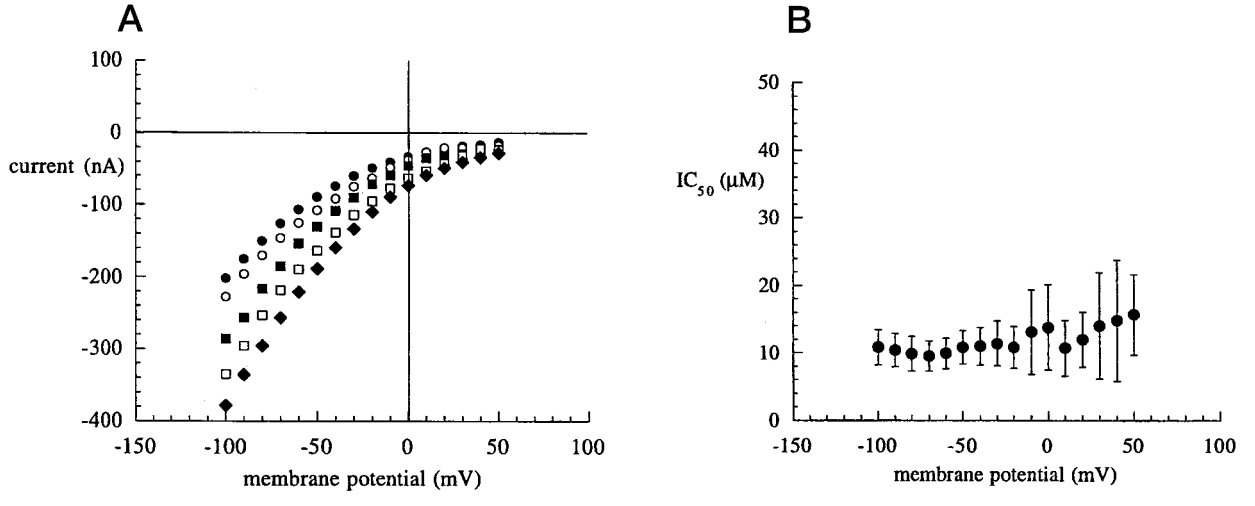

Fig. 3. Zn^{2+} inhibition of glutamate transport is voltage independent. A, Dose dependent Zn^{2+} inhibition of 1 mM glutamate transport by a representative oocyte expressing EAAT1 was measured in a chloride-free frog Ringer solution using oocytes that had been placed in chloride-free Ringer solution overnight to reduce the intracellular chloride. For the purposes of this figure, concentrations of Zn^{2+} shown are 300 μM (●), 100 μM (○), 30 μM (■), 10 μM (□) and 3 μM (◆). B, Current measurements at the indicated membrane potentials were fit to the equation $I = I_{max(Z)} - [(I_{max(Z)} \cdot [Zn^{2+}])/(IC_{50} + [Zn^{2+}])] + I_r$ (see Methods) to obtain estimates for IC_{50} values for each cell. Zn^{2+} concentrations from 1 μM to 1 mM were used in calculations of IC_{50} values. *Error bars*, mean ± standard error from four cells.

lar to that of oocytes expressing wild type EAAT1. However, the EAAT1-H146A mutant shows marked reduction in sensitivity to Zn^{2+}, with concentrations up to 1 mM required to cause any significant inhibition of the glutamate transport currents (Figs. 5 and 6; Table 1). Thus, the H146A mutation selectively alters the Zn^{2+} sensitivity without affecting glutamate transport. Therefore, it is likely that in the wild type EAAT1 transporter, Zn^{2+} interacts with this histidine residue. Further evidence in favor of this conclusion comes from analysis of Zn^{2+} inhibition of an additional mutant, in which the histidine residue has been changed to an aspartic acid residue. Aspartic acid residues have also been identified at the Zn^{2+} binding sites of various zinc finger proteins; therefore, it was expected that this mutant might yield more detailed information about the Zn^{2+} binding site. The H146D mutant is significantly less sensitive to Zn^{2+} (IC_{50} = 199 ± 60 μM) than the wild type (IC_{50} = 9.9 ± 2.3 μM). These results demonstrate that subtle changes in the side chain of the amino acid residue at position 146 are sufficient to significantly alter the Zn^{2+} sensitivity of EAAT1.

The amino acid residue in EAAT2 at the position corresponding to histidine 146 of EAAT1 is also a histidine; therefore, the presence of this histidine residue alone cannot explain the differential Zn^{2+} sensitivity of EAAT1 compared with EAAT2. An additional EAAT1 mutant, H156A, showed reduced Zn^{2+} sensitivity with an IC_{50} value of 237 ± 37 μM (Figs. 5 and 6; Table 1), which is approximately 24-fold higher than for the wild type EAAT1. The maximal glutamate transport currents and the EC_{50} for glutamate of this mutant were not significantly different from that of wild type EAAT1 (Fig. 6; Table 1), which suggests that the mutation has specifically changed the Zn^{2+} binding site without altering the overall structure or expression of the transporter. Alignment of the amino acid sequences of the glutamate transporters shows some distinct differences between subtypes corresponding to this histidine residue (Arriza *et al.*, 1997). The amino acid residue in EAAT2 that corresponds to histidine 156 of EAAT1 is glycine; as such, the presence or absence of a histidine residue at this position may explain the differential Zn^{2+} sensitivity of glutamate transporter subtypes. We tested this hypothesis by introducing a histidine into EAAT2 at position 154 in place of the glycine residue, expecting to cause an increase in Zn^{2+} sensitivity compared with wild type EAAT2. As predicted, Zn^{2+} inhibits glutamate transport of the EAAT2 mutant G154H (Fig. 5), with an IC_{50} value of 44 ± 7 μM and a maximal inhibition of 59 ± 3% (Fig. 6; Table 1). Further, Zn^{2+} (at 100 μM) decreases the I_{max} for glutamate transport currents without affecting the EC_{50} value for glutamate (Table 1), as observed for EAAT1. Thus, it may be concluded that the Zn^{2+} binding site on EAAT1 includes two histidine residues and the differential inhibition of glutamate transporter subtypes may be explained by the presence or absence of a histidine residue corresponding to position 156 of EAAT1. Additional mutations in EAAT1 that did not affect Zn^{2+} sensitivity include E86Q (extracellular loop 1); E184Q, C186A (extracellular loop 2); E303A (extracellular loop 3) (Table 1).

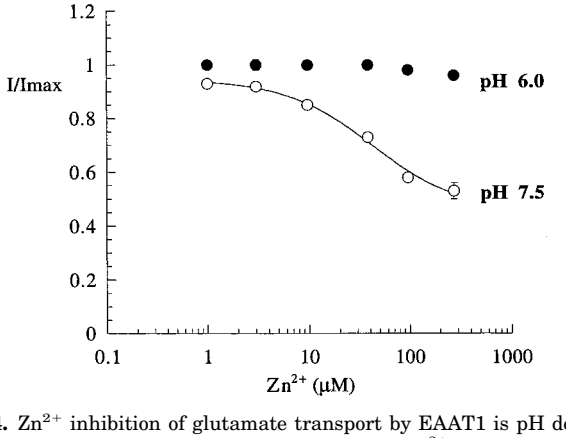

Fig. 4. Zn^{2+} inhibition of glutamate transport by EAAT1 is pH dependent. The inhibition of glutamate transport by Zn^{2+} at −60 mV was measured at pH 7.5 (○) and at pH 6.0 (●); see Methods for details. *Error bars*, mean ± standard error from four cells.

Discussion

This study has demonstrated that Zn^{2+} is a noncompetitive, partial inhibitor of glutamate transport by EAAT1 and has no effect on glutamate transport by EAAT2. In addition, although Zn^{2+} inhibits transport by EAAT1, it seems to increase the relative chloride conductance of EAAT1. The results observed for EAAT1 are similar in many respects to the effects of Zn^{2+} on the salamander-retina Müller cell glutamate transporter (Spiridon *et al.*, 1998). The major difference is that Zn^{2+} is less potent on EAAT1 than the Müller cell transporter. The cone cell glutamate transporter does show some differences with respect to the effects on the chloride conductance. The chloride conductance activated by glutamate transport in cone cells is inhibited whereas for EAAT1 and the Müller cell transporter the chloride conductance is increased. This discrimination by Zn^{2+} in modulation of the different transporter functions is particularly interesting. An expla-

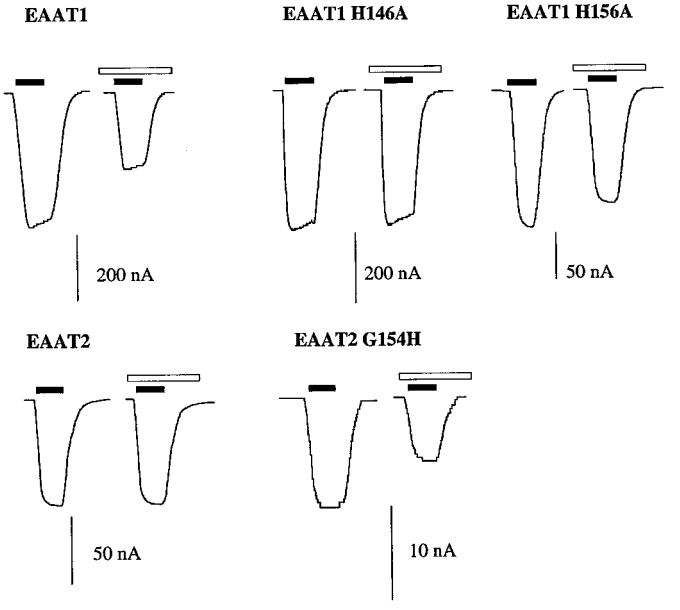

Fig. 5. Point mutations of histidine residues at positions 146 and 156 of EAAT1 and glycine 154 of EAAT2 alter Zn^{2+} inhibition of glutamate transport. Representative traces from cells expressing EAAT1, EAAT1 H146A, EAAT1 H156A, EAAT2, and EAAT2 G154H. Glutamate (100 μM; *filled bar*) transport currents at −60 mV are presented in the presence and absence of 100 μM Zn^{2+} (*open bar*). Note that the current amplitudes for EAAT1 and the EAAT1 mutants are significantly larger than the currents amplitudes for EAAT2 and the EAAT2 mutant.

nation suggested by Spiridon *et al.* (1998) for the different effects on transport versus chloride conductance activation, is that Zn^{2+} may stabilize a conformation of the transporter that slows the passage of glutamate through the transporter. With glutamate being bound to the transporter for a longer period a more efficient activation of the chloride conductance may be achieved.

Molecular basis for Zn^{2+} modulation of EAAT1. To better understand the molecular basis for the mechanism of action of Zn^{2+} on glutamate transporters, we have begun to characterize the Zn^{2+} binding site on EAAT1. In this study, we demonstrated that two histidine residues of EAAT1 at positions 146 and 156 are likely to form part of the Zn^{2+} binding site. Although the histidine residue at position 146 is conserved between glutamate transporter subtypes (except EAAT3), a histidine residue corresponding to position 156 of EAAT1 is found in only EAAT1 and EAAT4. We postulate that the nature of the amino acid residue at this position is an important determinant of Zn^{2+} sensitivity. Consistent with this suggestion is that a glycine-to-histidine substitution at this position in EAAT2 generates a Zn^{2+} sensitive EAAT2 mutant. EAAT5 has a glutamate residue at the position corresponding to histidine 156 of EAAT1; because glutamate residues have also been identified as forming Zn^{2+} binding sites on other proteins (e.g., Vazeux *et al.*, 1996) it may be expected that Zn^{2+} would also interact with EAAT5. It would be of interest to confirm this prediction, because the glutamate transporter found in cone cells of the salamander retina is inhibited by Zn^{2+} (Spiridon *et al.*, 1998) and is likely to be closely related to EAAT5 (Eliasof *et al.*, 1997). Although this study has identified two amino acid residues required for the formation of the Zn^{2+} binding site on EAAT1 and also the molecular basis for differential sensitivity between transporter subtypes, it is anticipated, based on the structures of other zinc binding sites on other proteins (see Branden and Tooze, 1991) that at least one other amino acid residue is required to bind Zn^{2+}.

Although the roles of individual amino acid residues involved in forming the pore region of the transporters are not well understood, the highly conserved carboxyl-terminal domain of glutamate transporters [residues 354–499 of EAAT1 (Arriza *et al.*, 1997)] has been suggested to form a pore structure, containing binding sites for glutamate, sodium ions, potassium ions, and protons (Pines *et al.*, 1995; Kavanaugh *et al.*, 1996; Mitrovic *et al.*, 1998). From the one-dimensional structure derived from the amino acid sequence,

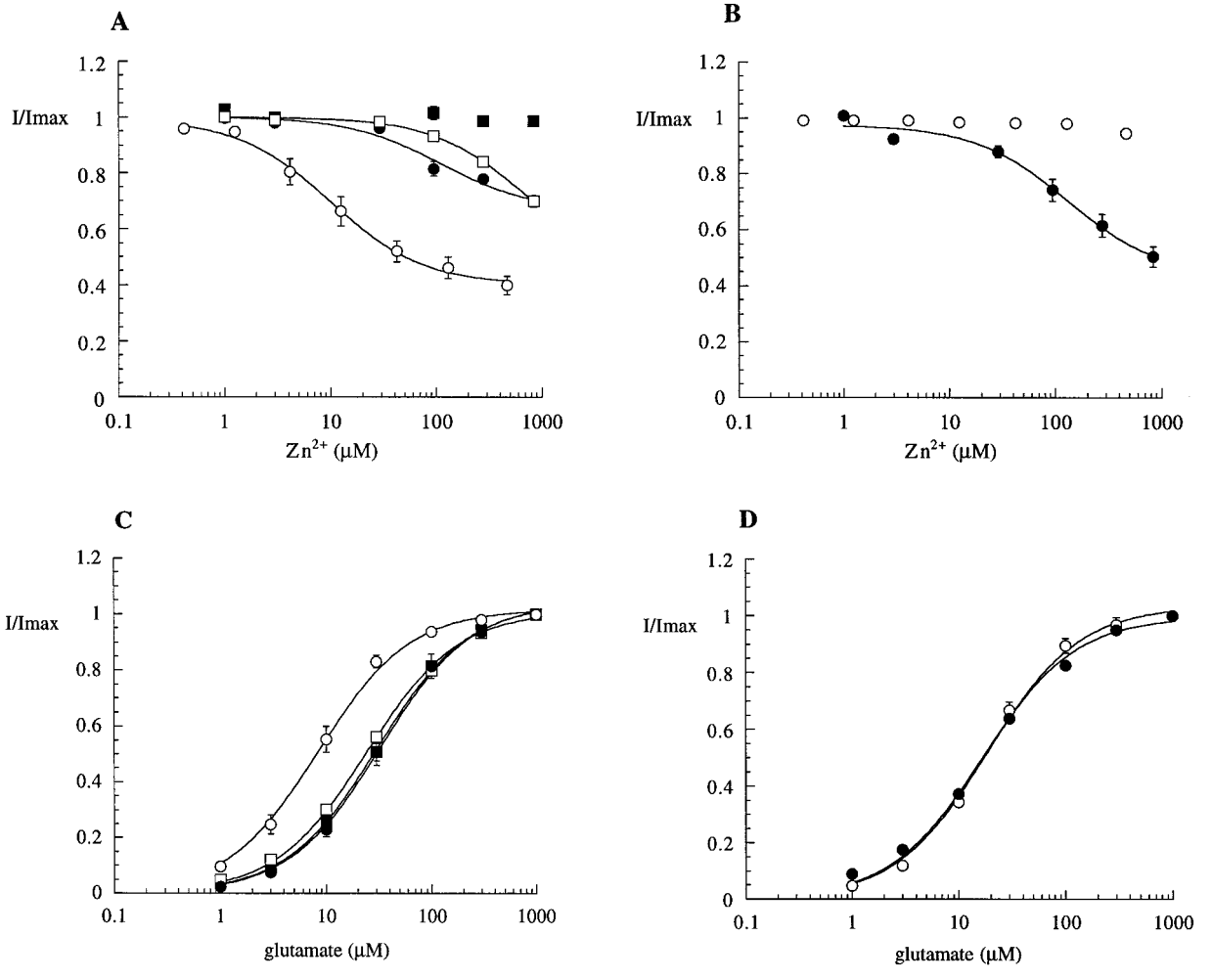

Fig. 6. Glutamate and Zn^{2+} dose response curves for the mutated glutamate transporters. Glutamate dose response curves (A) for EAAT1 (○) EAAT1 H146A (■), EAAT1 H156A (□) and (B) EAAT2 (○) and EAAT2 G154H (●). Current amplitudes were normalized to the maximal transport current for each cell. Data represent mean values ± standard error (n = 4–8). Zn^{2+} dose dependent inhibition of glutamate transport currents (C) for EAAT1 (○), EAAT1 H146A (■), EAAT1 H156A (□) and EAAT1 H146D (●) and (D) EAAT2 (○) and EAAT2 G154H (●). Errors are mean ± standard error from four cells. A summary of EC_{50} values for glutamate transport and IC_{50} values for Zn^{2+} inhibition of glutamate transport is presented in Table 1.

the Zn^{2+} binding site is distinct from the putative pore region of the transporter. In some respects this would be expected, given that Zn^{2+} is a noncompetitive modulator of transporter function. It will be of considerable interest to understand how the various regions of the transporters fold up in the three-dimensional structure such that occupation of an apparently distal site by Zn^{2+} is able to modulate the properties of the pore. One possibility is that although the third transmembrane domain may not be directly involved in forming the pore of the transporter, it might be closely associated with other domains that do form the glutamate, sodium ion, potassium ion, or proton binding sites. The binding of Zn^{2+} to the extracellular edge of the third transmembrane domain may alter the conformation of this transmembrane region, which may in turn alter the conformation of the pore to regulate substrate and ion passage through the transporter.

Physiological implications of the differential modulation of glutamate transporter subtypes by Zn^{2+}. The modulation of neurotransmission by zinc ions has been most extensively studied in the CA3 region of the rat hippocampus (e.g., Assaf and Chung, 1984; Frederickson, 1986). Although both GLAST1 (the rat equivalent of EAAT1) and glutamate transporter1 (GLT-1; rat equivalent of EAAT2) are expressed in the CA3 region of hippocampus, GLT-1 is the more abundant transporter (Rothstein *et al.*, 1994; Chaudhry *et al.*, 1995). Thus, the differential modulation of glutamate transport by Zn^{2+} ions in the CA3 region is unlikely to play a major role in shaping normal glutamatergic neurotransmission.

In the retina, however, GLAST1 (or EAAT1) is the most abundant transporter and is expressed in Müller cells, whereas GLT1 (or EAAT2) is present at much lower levels and seems to be expressed in bipolar cells (Rauen *et al.*, 1996). Although zinc is found presynaptically in photoreceptors of the retina, the concentrations of extracellular zinc achieved are not well established (Wu *et al.*, 1993; Spiridon *et al.*, 1998). If extracellular zinc concentrations achieved in the retina are similar to those achieved for the CA3 region of the hippocampus, the differential sensitivity of glutamate transporters to inhibition by zinc ions may provide a very important role in modulating retinal neurotransmission and may also provide a novel mechanism for controlling or regulating the various intracellular pools of glutamate. Glutamate transport by Müller cells provides one of the major mechanisms for clearance of extracellular glutamate in the retina and inhibition of this process would alter the time course of glutamate within the synapse, which would presumably alter the kinetics of glutamate receptor activation. Glutamate taken up into Müller cells is converted to glutamine, which is then transported back to the neurons and converted back to glutamate. This pathway seems to be a major source of glutamate for bipolar and ganglion cells (Pow and Robinson, 1994). Therefore, the inhibition of EAAT1 (expressed by Müller cells) by Zn^{2+} will reduce uptake of glutamate into Müller cells and reduce the amount of glutamine that may be transported back into the neurons for subsequent conversion back to glutamate. This process would result in more glutamate uptake by other cells containing Zn^{2+}-insensitive transporters. Thus, Zn^{2+} may provide an important regulator of the spatial regulation of both synaptic glutamate concentrations and intracellular pools of glutamate.

The inhibition of glutamate transport by Zn^{2+} under pathological conditions may have a number of effects on glutamatergic neurotransmission. High concentrations of Zn^{2+}, which would be generated under conditions such as ischemia, would decrease the rate of clearance of glutamate, which may be expected to potentiate glutamate excitotoxicity (Choi *et al.*, 1987). However, these effects would have to be counterbalanced with the inhibition of NMDA receptors by Zn^{2+} (Westbrook and Mayer, 1987) and also the inhibition of reverse glutamate transport by Zn^{2+} (Spiridon *et al.*, 1998). Thus, a possible scenario is that, under mild ischemic conditions, although Zn^{2+} may prolong the clearance rate of glutamate from the synapse, the inhibition of NMDA receptors and the block of reverse glutamate transporter operation may limit the excitotoxic damage that may otherwise occur.

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