0026-895X/97/050809-07$3.00/0

MOLECULAR PHARMACOLOGY, **51**:809–815 (1997).

Contrasting Modes of Action of Methylglutamate Derivatives on the Excitatory Amino Acid Transporters, EAAT1 and EAAT2

ROBERT J. VANDENBERG, ANN D. MITROVIC, MARY CHEBIB, VLADIMIR J. BALCAR, and GRAHAM A. R. JOHNSTON

Departments of Pharmacology (R.J.V., A.D.M., M.C., G.A.R.J.) and Anatomy and Histology (V.J.B.), University of Sydney, Sydney, New South Wales, 2006, Australia

Received November 25, 1996; Accepted January 22, 1997

SUMMARY

We have investigated the mechanism of action of a series of glutamate derivatives on the cloned excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2), expressed in *Xenopus laevis* oocytes. The compounds were tested as substrates and competitive blockers of the glutamate transporters. A number of compounds showed contrasting mechanisms of action on EAAT1 compared with EAAT2. In particular, (2*S*,4*R*)-4-methylglutamate and 4-methylene-glutamate were transported by EAAT1, with K_m values of 54 μM and 391 μM, respectively, but potently blocked glutamate transport by EAAT2, with K_b values of 3.4 μM and 39 μM, respectively. Indeed, (2*S*,4*R*)-4-methylglutamate is the most potent blocker of EAAT2 yet described. (±)-Threo-3-methylglutamate also potently blocked glutamate transport by EAAT2 (K_b = 18 μM), but was inactive on EAAT1 as either a substrate or a blocker at concentrations up to 300 μM. In contrast to (2*S*,4*R*)-4-methylglutamate, L-threo-4-hydroxyglutamate was a substrate for both EAAT1 and EAAT2, with K_m values of 61 μM and 48 μM, respectively. It seems that the chemical nature and also the orientation of the group at the 4-position of the carbon backbone of glutamate is crucial in determining the pharmacological activity. The conformations of these molecules have been modeled to understand the structural differences between, firstly, compounds that are blockers versus substrates of EAAT2 and, secondly, the pharmacological differences between EAAT1 and EAAT2.

L-glutamate is the predominant excitatory neurotransmitter in the brain and the control of extracellular glutamate concentrations is crucial for maintaining normal neurotransmission. Prolonged exposure of neurons to elevated glutamate concentrations can lead to excitoxicity (1–3), which has been implicated in the pathogenesis of a number of neurological disorders, such as ischemia after a stroke (see ref. 4 for review). Glutamate can be removed from the synaptic cleft by sodium-dependent transporters located at the pre- and postsynaptic terminals; alternatively, glutamate that has diffused out of the synapse may be taken up by transporters located in the glial cell membrane (5, 6).

A number of glutamate transporter cDNAs have been cloned and their amino acid sequences are 40–60% identical (7–11). The functional properties of four of the human glutamate transporters (termed EAAT1–4), which have been directly compared by expressing cRNAs in *Xenopus laevis* oocytes, show a number of pharmacological and electrophysiological differences (10–13). Most of the previously described pharmacological inhibitors of glutamate transport (14–17) were demonstrated to inhibit transport by being transported themselves and thus may be classified as competitive substrates. However, two conformationally restricted analogues of glutamate, kainate and dihydrokainate, inhibit transport of glutamate by blocking the transporter but are not themselves transported (10, 11, 18). Furthermore, kainate and dihydrokainate are active on EAAT2 in the low micromolar range, whereas ~10 mM is required to block glutamate transport by EAAT1, EAAT3 and EAAT4 (10, 11). Analysis of the voltage- dependence of the interaction between kainate and EAAT2 suggests that the apparent fraction of the electric field of the membrane sensed by kainate was only 3.4%. It has been concluded, therefore, that kainate binds at the initial recognition site for glutamate at the extracellular surface of the transporter (11).

A set of methyl derivatives of glutamate have recently been characterized in terms of binding to ionotropic glutamate receptors. One of these compounds, 2S4R4MG, is a potent and selective ligand for the kainate receptor subtype of ionotropic receptors with an IC_{50} for the displacement of [^{3}H]kainate of 35 nM (19). 2S4R4MG has also been reported to be a weak inhibitor of the low affinity vesicular glutamate transporter (20). As such, we have investigated the mecha-

This study was supported by the Australian National Health and Medical Research Council and the Ramaciotti Foundation.

ABBREVIATIONS: EAAT1, excitatory amino acid transporter 1; EAAT2, excitatory amino acid transporter 2; 2S4R4MG, (2*S*,4*R*)-4-methylglutamate; tPDC, L-*trans*-pyrrolidine 2,4-dicarboxylic acid; T3MG, (±)*threo*-3-methylglutamate; E3MG (±)*erythro*-3-methylglutamate; LT4HG, L-*threo*-4-hydroxyglutamate; E4HG, (±)*erythro*-4-hydroxyglutamate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

nism of action of a series of related methyl and hydroxyl derivatives of glutamate on EAAT1 and EAAT2. A number of compounds have been identified that are able to block glutamate transport by EAAT2 and are either not recognized by or are competitive substrates of EAAT1. In contrast to kainate and dihydrokainate, the compounds used in this study are simple derivatives of glutamate with methyl or hydroxyl substitutions at the 3- and 4-positions of the carbon backbone. We have modeled the conformations of the methylglutamate derivatives in comparison to kainate and also another rigid analogue of glutamate that is able to be transported by EAAT1 and EAAT2, tPDC (10, 21). This has allowed definition of the minimal structural differences between substrates and blockers of glutamate transport and may also lead to the development of new class of compounds designed to interact with glutamate transporters.

Materials and Methods

Chemicals and reagents. Glutamate was obtained from Sigma Chemical (St. Louis, MO) 2S4R4MG, (2*S*,4*S*)-4-methylglutamate, T3MG, and 2*S*-α-methylglutamate and tPDC were obtained from Tocris Cookson (Bristol, UK). L-kainate was obtained from Sigma. (±)-4-Methylene glutamate, LT4HG, and E4HG were kindly donated by Professor David Curtis, Australian National University, Canberra, Australia.

Expression and transport measurements of EAAT1 and EAAT2 in oocytes. Complementary DNAs encoding the human glutamate transporters, EAAT1 and EAAT2, were subcloned into pOTV for expression in *X. laevis* oocytes as previously described (10, 22). The plasmids were linearized with *Bam*HI and cRNA was transcribed from each of the cDNA constructs with T7 RNA polymerase and capped with 5′,7-methyl guanosine using the mMESSAGE mMACHINE (Ambion, Austin, TX). cRNA (50 ng) encoding either EAAT1 or EAAT2 was injected into defolliculated Stage V *X. laevis* oocytes. Two to seven days later, transport was measured by two-electrode voltage-clamp recording using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) and a MacLab 2e recorder (ADI Instruments, Sydney, NSW, Australia) and controlled using pCLAMP 6.01 interfaced to a Digidata 1200 (Axon Instruments). In most experiments, oocytes were voltage-clamped at −60 mV and continuously superfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5). For transport measurements, this buffer was changed to one containing the indicated concentration of substrate and/or blocker. The voltage dependence of block of glutamate transport was measured by clamping the membrane potential at −30 mV and then applying a series of 100-msec voltage pulses from −100 mV to 0 mV and measuring the steady state current at each membrane potential. This protocol was applied both before and during the application of the compound in question and then the base-line current at each membrane potential was subtracted from the current in the presence of the compounds to get a measure of the transport specific current at the various membrane potentials.

Analysis of kinetic data. Current (I) as a function of substrate concentration ([S]) was fitted by least squares to $I = I_{max}.[S]/(K_m + [S])$, where I_{max} is the maximal current and K_m is the Michaelis transport constant. The I_{max} values for the various substrates are expressed relative to the current generated by a maximal dose of L-glutamate in the same cell. I_{max} and K_m values are expressed as mean ± standard error and were determined by fitting data from individual oocytes. The potent competitive transport blockers were characterized by Schild analysis (23) and the K_b estimated from the Schild regression plot. The less potent blockers were assumed to be competitive and K_i values calculated from IC_{50} values using the equation $K_i = IC_{50}/(1+ [glutamate]/K_m)$, (24), where K_i is the inhibition constant, IC_{50} is the concentration giving half maximal inhibition, K_m is the transport constant for glutamate and [glutamate] was 30 μM. The fraction of the membrane electric field sensed by transport blockers when bound to the transporters was estimated using the Woodhull equation, $K_i = K_i^0 \exp(-z\partial FE/RT)$ (25), where K_i is the inhibition constant, K_i^0 is the inhibition constant at 0 mV, z is the charge on the blocker, ∂ is the fraction of the membrane field, F is Faraday's constant, E is the membrane potential, R is the gas constant and T is temperature in °K. In applying this equation to our data on transport blockers, we have adopted the assumptions made in the original derivation of the equation (for details, see ref. 25).

Molecular modeling. A computer-assisted study was carried out on tPDC, kainate, (2*S*,3*R*)-3-methylglutamate and 2S4R4MG using the program Chem-X (Chemical Design, Oxford UK) to determine the conformations that a compound may adopt when a substrate or a blocker on EAAT1 and EAAT2. The three-dimensional matrices of the compounds were optimized using the molecular mechanics optimization routines in Chem-X. The conformers of each compound were then subjected to conformational search routines about the bonds at which torsional rotations were possible. Both tPDC and kainate are conformationally constrained analogues of glutamate and are ideal for molecular modeling studies. It is proposed that these are the conformations of the compounds that are acting as substrates on EAAT1 and EAAT2 and as blockers on EAAT2. A search was then undertaken to determine the low energy conformations of each analogue of glutamate, that fitted both tPDC and kainate.

We have proposed that for a compound to be a substrate, the compound needs to attain an extended conformation as in tPDC. To block transport, the compound needs to attain a partially folded conformation, as in kainate. The pyrrolidine ring of both tPDC and kainate can attain several conformations. We have used the conformation in which the nitrogen is out of the plane. This configuration allows the other substituents to be away from each other in a low energy conformation (21). The fitted-root-mean square distance between tPDC and (2S,3R)-3-methylglutamate and between tPDC and 2S4R4MG were 0.0748 and 0.0752, respectively. The fitted root-mean-square distance between kainate and (2S,3R)-3-methylglutamate and between kainate and 2S4R4MG were 0.0641 and 0.0876, respectively. Figs. 5 and 6 were generated from the data obtained by Chem-X analysis and used in Alchemy (Version 3.02) and Chem 3-D (Cambridge Scientific Computing, Cambridge, MA).

Results

Application of L-glutamate to *X. laevis* oocytes voltage clamped at −60 mV and expressing either EAAT1 or EAAT2 generates a dose-dependent inward current (10, 22). We screened a series of glutamate derivatives at a concentration of 100 μM for the generation of similar inward currents (Fig. 1). Compounds that did not generate a current were further tested as blockers of glutamate dependent transport currents. After identification of active compounds, the mechanism of actions on EAAT1 and EAAT2 were further investigated.

3-Methylglutamate derivatives. Application of 100 μM T3MG to oocytes expressing either EAAT1 or EAAT2 did not generate currents. However, T3MG did cause a dose-dependent block of the current induced by 30 μM glutamate when applied to oocytes expressing EAAT2 (Fig. 2; Table 1). Schild analysis of the effect of increasing doses of T3MG on glutamate dose responses showed that T3MG is a competitive blocker of transport with a K_b of 18.3 ± 1.0 μM at −60mV (n = 6) (Fig. 3; Table 1). The voltage dependence of the block was also measured and K_b changed from 22.4 μM to 17.1 μM over the membrane potential range of −100 mV to −30mV

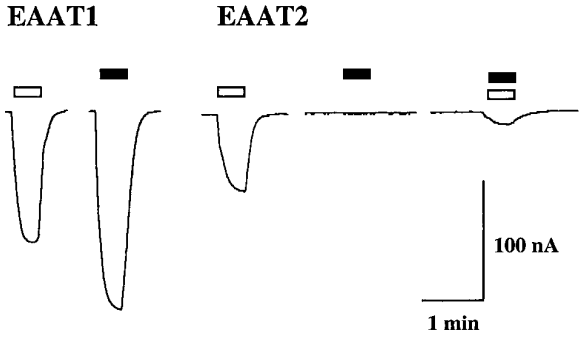

Fig. 1. 2S4R4MG is substrate for EAAT1 and a blocker of EAAT2. Application of 30 μM glutamate (□) to oocytes expressing EAAT1 or EAAT2 generated an inward current when clamped at −60 mV. Application of 100 μM 2S4R4MG (■) to oocytes expressing EAAT1 generated a current similar to that of glutamate. When 2S4R4MG is applied to oocytes expressing EAAT2, no current was generated, but coapplication of 100 μM of 2S4R4MG and 30 μM glutamate caused a reduction in the amplitude of the current compared with glutamate alone.

(Fig. 4). Assuming a simple electrostatic interaction between the blocker and the transporter, this data may be fitted to the Woodhull equation (see Materials and Methods) (25) to estimate $z\partial$, where z is the valance of the charge on the blocker and ∂ is the fraction of the membrane electric field sensed by that charge. This value may be used to estimate the fraction of the membrane electric field sensed by T3MG when bound to the transporter (25). The $z\partial$ for T3MG was 0.10 ± 0.01 (n = 3). This value is only slightly larger than the $z\partial$ value of 0.03 obtained for kainate binding to EAAT2 (11). The $z\partial$ value obtained for T3MG should be used only as a qualitative measure of the fraction of the electric field sensed by the blocker when bound to EAAT2. In this analysis, we have used the term "$z\partial$" because the charge (z) associated with the blocker when bound to the transporter has not been established. Nevertheless, the relatively small values of $z\partial$ obtained for T3MG binding to EAAT2 are consistent with the blocker binding at a site that is located at the edge of the membrane electric field (i.e., near the extracellular surface of the protein).

T3MG had no effect on glutamate transport by EAAT1 (data not shown); as such, T3MG displays pharmacological properties similar to those of kainate and dihydrokainate. Application of 100 μM E3MG did not generate transport currents; the transport currents of glutamate were not inhibited by 100 μM E3MG for both EAAT1 and EAAT2 (data not shown). Thus, the orientation of the methyl group at position 3 of the carbon backbone of glutamate appears to be critical for binding to EAAT2.

4-Methylglutamate derivatives. 2S4R4MG had effects on EAAT2 that were similar to those of T3MG. 2S4R4MG competitively blocked glutamate transport without being transported. The K_b for 2S4R4MG binding to EAAT2 was 3.4 ± 0.2 μM at −60 mV (n = 6) (Fig. 3; Table 1); from the voltage dependence of the interaction, $z\partial$ = 0.11 ± 0.02 (n = 3) (Fig. 4). The similarities of the $z\partial$ values for T3MG, 2S4R4MG, and also kainate suggest that all three blockers are likely to bind to the same or closely related sites on EAAT2 near the extracellular surface of the protein. Application of 2S4R4MG to oocytes expressing EAAT1 showed a dose dependent inward transport current with a K_m of 54 ± 17 μM (n = 4) and a maximal current of 80% of the maximal current generated by glutamate (Fig. 2; Table 1). Thus, 2S4R4MG is a substrate for EAAT1 but is a competitive blocker of EAAT2. The (2*S*,4*S*)-4-methylglutamate enantiomer was inactive at 100 μM either as a blocker or as a substrate for both EAAT1 and EAAT2 (data not shown). The activity of 4-methylene-glutamate was also characterized; although similar modes of action on EAAT1 and EAAT2 were observed compared with 2S4R4MG, the potency at both transporters was reduced (Fig. 2). The K_m for 4-methyleneglutamate transport by EAAT1 was 391 ± 51 μM (n = 3) (Table 1) and the K_b for binding to EAAT2 was 39 ± 7 μM (n = 3) (Table 1).

4-hydroxyglutamate derivatives. L-*threo*-3-hydroxyaspartate is a potent substrate of glutamate transporters (10, 14–17, 26–28). In light of the above results with methyl and methylene substitutions at the 4 position of the carbon backbone of glutamate, we investigated the actions of LT4HG and E4HG on EAAT1 and EAAT2. LT4HG is a potent substrate for both EAAT1 and EAAT2 with K_m values of 61 ± 14 μM (n = 3) and 48 ± 5 μM (n = 3) (Fig. 2; Table 1) and maximal currents of 78% and 90% of the maximal glutamate transport current, respectively. Although E4HG was somewhat less potent (K_m = ~1000 μM for EAAT1 and EAAT2; Table 1), it

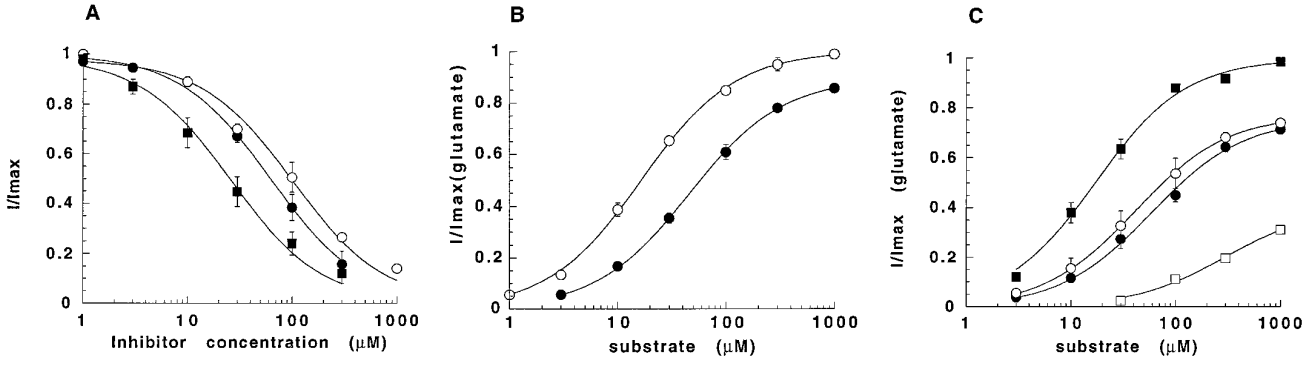

Fig. 2. Dose-dependent inhibition of glutamate transport and dose-dependent transport currents induced by various glutamate derivatives applied to oocytes expressing EAAT1 or EAAT2. A, Inhibition of glutamate transport currents of EAAT2 by 2S4R4MG (■), T3MG (●), and 4-methyleneglutamate (○). B, EAAT2 dose-dependent transport currents generated by the application of glutamate (○) and LT4HG (●). C, EAAT1 dose-dependent transport currents generated by the application of glutamate (■), 2S4R4MG (○), LT4HG (●), and 4-methyleneglutamate (□). The responses were measured as described in Materials and Methods and fitted to the equation $I = I_{max} \cdot [substrate]/(K_m + [substrate])$ for substrates or $I = I_{max} \cdot \{I_{max} \cdot [glutamate]/(IC_{50} + [glutamate])\}$, where [glutamate] = 30 μM for transport blockers. The IC_{50} values were converted to K_i using the Cheng-Prusoff equation, as described previously. The K_m and K_i values for all compounds investigated are presented in Table 1.

TABLE 1

Transport parameters for the various glutamate derivatives used in this study

K_m and I_{max} values for the various competitive substrates were determined as described in Materials and Methods. I_{max} values are relative to the maximal current generated by glutamate. T3MG, 2S4R4MG, and kainate blocked glutamate transport by EAAT2; Schild analysis (22) was used to establish competitive antagonism and also to determine the K_b for binding of the compound to the transporter (see Fig. 3). The K_i for (±)-4-methyleneglutamate block of glutamate transport by EAAT2 was derived from the IC_{50} using the Cheng-Prusoff equation, assuming a competitive antagonism mechanism (23). The number of cells tested for each compound on both EAAT1 and EAAT2 was between three and eight, except for E4HG, in which only one cell expressing EAAT1 and one cell expressing EAAT2 were tested.

	EAAT1		EAAT2	
	K_m	I_{max}	K_m/K_b	I_{max}
	μM		μM	
L-Glutamate	20 ± 3	1	18 ± 3	1
L-Kainate	>3000		17.4 ± 3.4	0
tPDC	26.3 ± 0.8	0.74 ± 0.03	2.3 ± 0.5	0.23 ± 0.02
T3MG	No activity (300)		18.3 ± 1.0	0
E3MG	No activity (100)		No activity (100)	
2S4R4MG	54 ± 17	0.80 ± 0.05	3.4 ± 0.2	0
(2*S*,4*S*)-4-Methylglutamate	No activity (100)		No activity (100)	
(±)-4-Methylene-glutamate	391 ± 51	0.43 ± 0.02	39 ± 7	0
LT4HG	61 ± 14	0.78 ± 0.06	48 ± 5	0.90 ± 0.04
E4HG	> 1000	~1.0	> 1000	~1.0
(*S*)-α-Methyl glutamate	No activity (100)		No activity (100)	

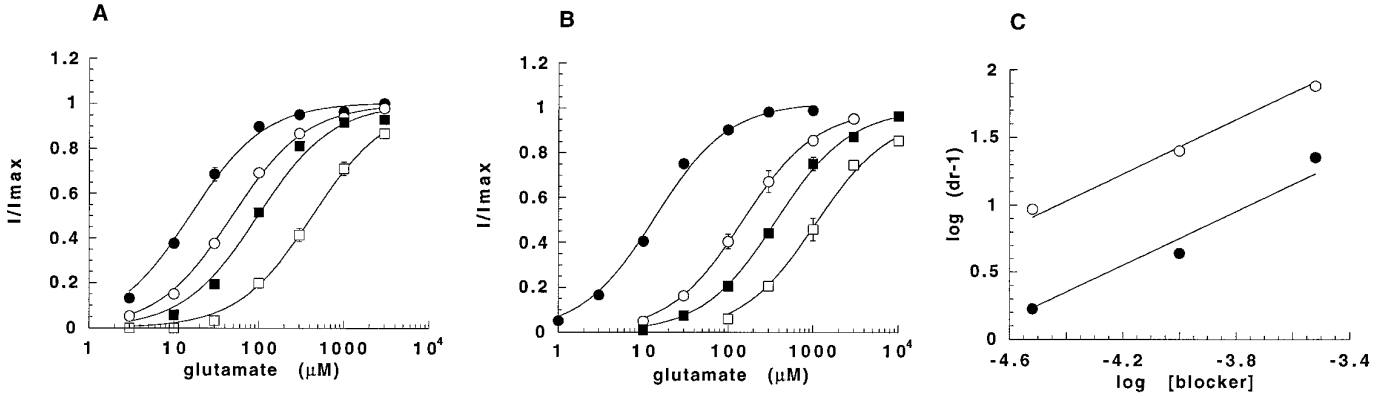

Fig. 3. T3MG and 2S4R4MG are competitive blockers of glutamate transport. Glutamate dose responses of oocytes expressing EAAT2 and voltage clamped at −60 mV were measured as described in Materials and Methods in the presence of increasing doses of T3MG (A) and 2S4R4MG (B). Concentrations of blockers were 0 μM (●), 30 μM (○), 100 μM (■) and 300 μM (□). Data presented are mean ± standard error (three separate experiments for each concentration of blocker). The K_m values for glutamate at the various concentrations of blocker were used to calculate log(dr − 1), where dr is the dose ratio for glutamate at the various blocker concentrations. C, The log(dr −1) versus log (blocker) plot for T3MG (●) and 2S4R4MG (○) have slopes of 0.91 and 1.11 respectively, which indicates that the compounds are competitive blockers.

did generate current amplitudes similar to those observed for glutamate.

Discussion

This study has identified a set of glutamate derivatives that have contrasting mechanisms of action on the human glutamate transporters EAAT1 and EAAT2. Methyl substitution at the 4-position or the 3-position of the glutamate backbone, or methylene substitution at the 4-position yields compounds that are potent blockers of glutamate transport by EAAT2. In fact, 2S4R4MG is the most potent glutamate transport blocker that has been described. Its K_b of 3.4 μM is approximately 3-fold lower than the K_b for dihydrokainate binding to EAAT2 (10). T3MG is actually an equal mix of the two enantiomers (2*S*,3*R*)-3-methylglutamate and (2*R*,3*S*)-3-methylglutamate; considering that D-glutamate is a very weak substrate of EAAT2 (10), it is most likely that (2*R*,3*S*)-3-methylglutamate will be similarly inactive. Therefore the K_b for (2*S*,3*R*)-3-methylglutamate is likely to be less than the K_b of 18 μM for the mixture. In contrast to the effects on EAAT2, the 4-methyl and 4-methylene substituted glutamate derivatives are substrates of EAAT1 and T3MG is not recognized at all by EAAT1.

It seems that the chemical nature of the group at the 4-position is a critical determinant of the activity of the compound. This is highlighted by a comparison of the modes of action of LT4HG and 2S4R4MG on EAAT1 and EAAT2. 2S4R4MG is a blocker on EAAT2 and substrate on EAAT1, whereas LT4HG is a substrate of both EAAT1 and EAAT2. Thus, it seems that in the case of EAAT2, a hydroxyl group at position-4 is tolerated for transport; however, although a methyl group at this position does not prevent binding to the transporter, passage through the pore of the transporter is prevented. For EAAT1, there are no such limitations on the passage through the pore.

We have modeled the conformations of the various glutamate derivatives used in this study in an attempt to understand the structural basis for the differences between substrates and blockers of glutamate transport and also the structural basis for the pharmacological differences between EAAT1 and EAAT2. The glutamate derivatives used in this study are flexible compounds and can exist in multiple con-

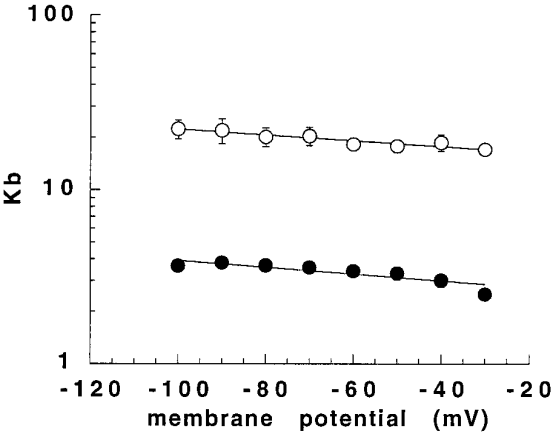

Fig. 4. Voltage dependence of the block of glutamate transport through EAAT2 by 2S4R4MG (●) and T3MG (○). The measurement of voltage dependent transport currents was as described in Materials and Methods. Schild regression of glutamate dose responses (from three cells) at membrane potentials from −100 mV to −30 mV in the presence of increasing doses of 2S4R4MG or T3MG was used to calculate K_b values for the compound in question. Data presented are mean ± standard error from three cells. The K_b values were fitted to the Woodhull equation, as described in the text, to get an estimate of the fraction of the membrane electric field sensed by the blockers when bound to EAAT2. ∂z values were 0.11 ± 0.02 and 0.10 ± 0.01 for 2S4R4MG and T3MG, respectively.

formations. The conformations of the glutamate derivatives were fitted to kainate and tPDC as these conformationally restricted analogues of glutamate may define the blocker and substrate conformations for the glutamate transporters, EAAT1 and EAAT2 (Fig. 5). The glutamate backbone of tPDC is in an extended conformation, whereas in kainate, the glutamate backbone is in a partially folded conformation. We propose that the terminal carboxyl group of kainate cannot attain the same conformation as found in tPDC because of the steric constraints imposed by the isopropene moiety attached to the pyrrolidine ring of kainate. The carbon backbones of the various glutamate derivatives used in this study can all be fitted to the conformations of both kainate and tPDC (Fig. 5). For the compounds that block glutamate transport by EAAT2, we have assumed that the partially folded conformation is adopted and for compounds that are transported by either EAAT1 or EAAT2 we have assumed that the extended conformation is the active conformation. Although the glutamate backbone of (2*S*,4*S*)-4-methylglutamate and E3MG are able to fit to both the extended and partially folded conformations, the methyl groups do not align with kainate or tPDC pyrrolidine rings; we must conclude that the lack of affinity of these compounds as either substrates or blockers with either EAAT1 or EAAT2 is caused by unfavorable orientation of these methyl groups.

If T3MG is fitted to kainate, the methyl group aligns with the ring structure of kainate at the corresponding position. In this sense, kainate is a conformationally restricted analogue of T3MG. This may explain the similar mechanisms of action of T3MG and kainate on EAAT2. If 2S4R4MG is fitted to kainate the methyl group lies in the same region as the isopropene and pyrrolidine ring of kainate. The interaction of the methyl groups of T3MG and 2S4R4MG with EAAT2, when in the partially folded conformation, may prevent the terminal carboxyl group from interacting with a site required for transport to occur but still permit binding to occur. The lack of interaction between EAAT1 and T3MG (or kainate) as a transport blocker may be caused by the inability of the carboxyl group, when in the partially folded conformation, to interact with the transporter. When T3MG is in the extended

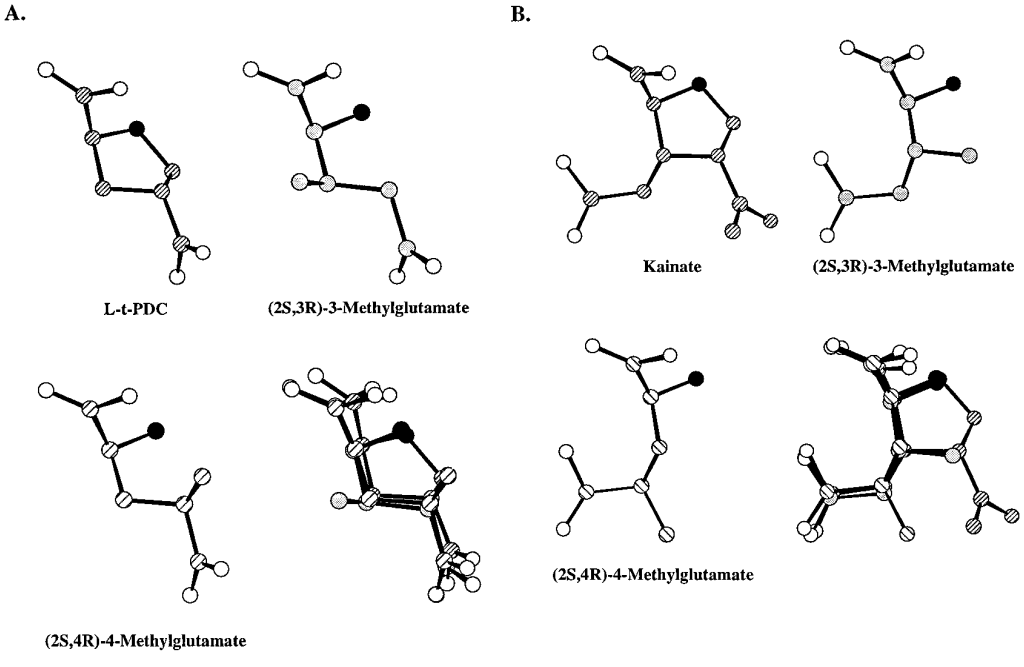

Fig. 5. Molecular models of 2S4R4MG, (2*S*,3*R*)-3-methylglutamate, kainate, and tPDC. We have only modeled the (2*S*,3*R*)-3-methylglutamate enantiomer of T3MG and assumed that the (2*R*,3*S*)-3-methylglutamate will be inactive because D-glutamate shows no activity. A, The structures of (2*S*,3*R*)-3-methylglutamate and 2S4R4MG are fitted to the tPDC conformation, with the overlayed structures showing the structural alignment. B, The structures of (2*S*,3*R*)-3-methylglutamate and 2S4R4MG are fitted to the kainate conformation, with overlayed structures. The modeling was carried out as described in Materials and Methods. The conformations are presented so as to highlight the possible structural basis for the differences in pharmacological activity of the methylglutamate derivatives on the glutamate transporters EAAT1 and EAAT2.

conformation, the methyl group may be forced into a sterically unfavorable site on EAAT1. In contrast, as 2S4R4MG is able to be transported by EAAT1, the methyl at position 4 in the glutamate backbone is tolerated. There are no high affinity blockers of glutamate transport by EAAT1 described as yet and a set of compounds that may yield such a blocker may need longer alkyl groups than a methyl group at the 4-position of the glutamate backbone in the 2*S*,4*R* configuration (Fig. 5A and Fig. 6). This type of compound may allow binding of the glutamate backbone in an extended folded conformation, but also has additional interactions with EAAT1 through the longer alkyl group. These additional interactions may prevent the passage of the compound through the transporter pore.

A comparison of the pharmacological properties of LT4HG and 2S4R4MG may provide additional information about the differences between EAAT1 and EAAT2 in how they bind and transport glutamate. If we assume that 2S4R4MG adopts alternate conformations when interacting with EAAT1 and EAAT2 and that LT4HG only adopts the extended conformation, then a number of structural predictions about EAAT1 and EAAT2 may be made. The glutamate binding/transport site on EAAT1 is able to tolerate either a hydroxyl group or a methyl group at carbon 4 of the glutamate backbone; as such, the size and orientation of the group is important, but its chemical nature is not. The situation with EAAT2 is more complex. The chemical nature of the group at carbon 4 is important for determining whether the compound is a blocker or a substrate. One explanation is that the methyl group stabilizes the "kainate conformation" by binding to some hydrophobic region in the binding site and orientating the carboxyl group to the partially folded conformation. The hydroxyl group may not be able to stabilize this partially folded conformation and, as such, prefers the extended "tPDC conformation"; the hydroxyl group in this conformation is in a different position that may be tolerated by the transporter. An alternative explanation is that both compounds interact with the transporter in the partially folded "kainate conformation." In the case of LT4HG, the hydroxyl group may substitute for part of the carboxyl group and still allow transport to occur. The methyl group of 2S4R4MG cannot substitute for the carboxyl group because of its very different chemical nature. A compound that could test this idea would have a hydroxyl group at the 4 carbon and an additional hydroxyl group replacing the carboxyl group (Fig. 6).

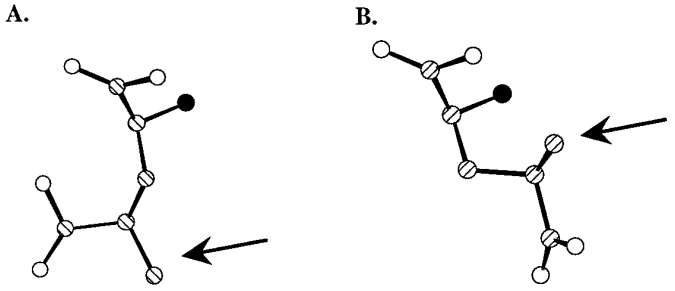

Fig. 6. The putative active conformations of 2S4R4MG in the extended and partially folded forms. A, The 4-methyl group (*arrow*), when in the extended "tPDC conformation," may be investigated in the development of possible EAAT1 blockers. Longer or branched chain alkyl groups at this position may yield an EAAT1 blocker. B, The 4-methyl group (*arrow*), when in the partially folded "kainate conformation," could be developed in designing compounds that may explain the structural basis for the differences between substrates and blockers of EAAT2. In this case, the size of the chemical group would be kept to a minimum, but the chemical nature of the group could be altered.

This study and others (e.g., ref. 10) highlight some of the pharmacological differences between EAAT1 and EAAT2, which raises the question as to which part of the transporter proteins are responsible for these differences. If we assume that the highly conserved domains are important for the functional properties of the transporters, then it may be predicted that amino acid changes within these conserved domains may play an important role in determining some of the pharmacological differences. Furthermore, given that 2S4R4MG, T3MG, and kainate binding to EAAT2 is relatively voltage- independent, they are all likely to bind near the external surface of the transporter. Therefore, it may be expected that the amino acid residues responsible for determining the pharmacological differences between EAAT1 and EAAT2 would be within a relatively conserved domain on the transporter that is exposed to the extracellular surface. However, the transmembrane topology of the glutamate transporters has only been partially determined (29). The topology of the most highly conserved domain of the transporters (residues 354 to 499 of EAAT1), which is thought to form at least part of the pore of the transporter (22, 30), has not been resolved; therefore, it is difficult to make rational predictions about which particular amino acid residues within this domain are likely to be responsible for conferring the pharmacological differences. One way to reduce the number of possible amino acid residues that may be important is to construct a series of chimeric transporters using EAAT1 and EAAT2.

This study has characterized the mechanisms of action of a number of glutamate derivatives on the human glutamate transporters, EAAT1 and EAAT2. It seems that EAAT1 and EAAT2 recognize methyl- and hydroxyl-derivatives of glutamate in subtly different ways, which is demonstrated by the contrasting modes of action of 2S4R4MG on EAAT1 compared with EAAT2. Some of these subtle differences may be used to develop more selective blockers and substrates of glutamate transporters.

Acknowledgments

We wish to thank Kong Li and Drs. Mac Christie and Nick Sangster for help in maintaining the *X. laevis* colony.

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Send reprint requests to: Dr. Robert Vandenberg, Department of Pharmacology, D06, University of Sydney, Sydney, New South Wales, 2006, Australia. E-mail: robv@pharmacol.su.oz.au