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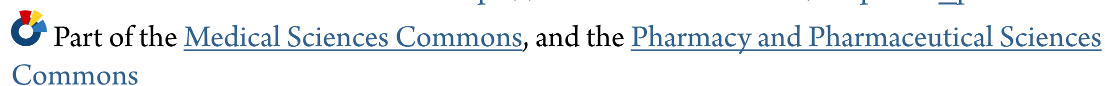

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Constitutive Ion Fluxes and Substrate Binding Domains of Human Glutamate Transporters*

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Application of L-glutamate activates ionic currents in voltage-clamped *Xenopus* oocytes expressing cloned human excitatory amino acid transporters (EAATs). However, even in the absence of L-glutamate, the membrane conductance of oocytes expressing EAAT1 was significantly increased relative to oocytes expressing EAAT2 or control oocytes. Whereas transport mediated by EAAT2 is blocked by the non-transported competitive glutamate analog kainate (K_i = 14 μM), EAAT1 is relatively insensitive (K_i > 3 mM). Substitution of a block of 76 residues from EAAT2 into EAAT1, in which 18 residues varied from EAAT1, conferred high affinity kainate binding to EAAT1, and application of kainate to oocytes expressing the chimeric transporter blocked a pre-existing monovalent cation conductance that displayed a permeability sequence $K^+ > Na^+ > Li^+ \gg$ choline$^+$. The results identify a structural domain of glutamate transporters that influences kainate binding and demonstrate the presence of a constitutive ion-selective pore in the transporter.

Transport of L-glutamate in the central nervous system and periphery is mediated by a family of membrane proteins postulated to contain from 6 to 10 transmembrane domains (for review, see Ref. 1). An important goal in understanding the molecular basis of transporter function is the identification of the structural domains involved in substrate recognition and in the formation of the pore or conduction pathway through which amino acids and other ions pass. Glutamate uptake is accompanied by an influx of sodium ions and an efflux of potassium and hydroxyl ions (2–5). Voltage clamp recording from cells with sufficiently high transporter density can be used to measure membrane currents associated with glutamate uptake. A stoichiometry proposed for uptake involves co-transport of $2Na^+:1Glu^-$ with countertransport of 1 K^+ and 1 OH^-, resulting in translocation of one net positive charge (5). However, recent studies of a cloned human glutamate transporter have demonstrated that the quantity of charge translocated with glutamate is voltage-dependent (6). This variable charge stoichiometry occurs as a consequence of an additional current arising from a thermodynamically uncoupled chloride flux activated by transporter substrates (7, 8). Different transporter subtypes exhibit intrinsic differences in the magnitude of the chloride flux relative to flux of glutamate (7).

In addition to differences in ion conduction properties, pharmacological differences exist between the human excitatory amino acid transporter subtypes. Many compounds that inhibit radiolabeled glutamate transport (*e.g.* serine *O*-sulfate, L-*trans*-pyrrolidine-2,4-dicarboxylic acid, L-cysteic acid, and D-aspartate) also induce inward currents, suggesting that they act as competitive substrates for transport (9). In contrast, kainate and dihydrokainate, two conformationally restricted glutamate analogues (10), selectively and competitively block transport of glutamate by the EAAT2[1] subtype but are not themselves transported. EAAT1 and EAAT3 are insensitive to these compounds (K_i > 3 mM) (9). Schild analysis of the inhibition of EAAT2 by kainate has demonstrated that the K_d is relatively voltage-independent, which suggests that kainate binds to an external site on the transporter outside the membrane electric field (6). The competitive action of kainate with respect to glutamate (9) and similar voltage independence of the K_m for glutamate activation[2] suggest that kainate may interact with EAAT2 at the same site in the outer pore of the transporter where glutamate first binds.

Studies on various cloned sodium-dependent transporters have demonstrated the presence of substrate-independent currents (11–14), suggesting that these carriers possess intrinsic pores through which ions pass (for review see Ref. 15). Identification of differences in pore conduction properties between the transporter subtypes will facilitate identification of residues involved in forming the pore structure. In this study we demonstrate that EAAT1, but not EAAT2, allows substrate-independent ion fluxes. We have constructed a chimeric transporter that mediates a glutamate-independent conductance blocked by kainate, thereby identifying a domain involved in substrate binding and allowing analysis of the voltage dependence and ionic selectivity of this substrate-independent current.

EXPERIMENTAL PROCEDURES

Construction of a Chimeric Transporter—An *in vivo* recombination procedure (17) was used to construct chimeric glutamate transporters. The EAAT1 and EAAT2 cDNAs were cloned in tandem into the pCMV plasmid (9) with a unique *Xba*I site between the cDNAs. The tandem construct was linearized with *Xba*I, and 100 ng of DNA was used to transform competent DH5α cells. Colonies were screened for plasmids that contained a single cDNA and sequenced to determine the junction sites (Sequenase, U. S. Biochemical Corp.). A number of plasmids were isolated, and all the chimeras generated using this method had junction sites within a highly conserved domain from serine 366 to glutamine 415 (EAAT1 numbering). One of these chimeras contained an EAAT1 sequence at the amino terminus and junctioned with EAAT2 after serine 366. An *Eco*RI site was incorporated into the EAAT2 segment in this chimera at a position corresponding to an endogenous *Eco*RI site in EAAT1 (residues 442–443), and this construct was used to generate a three-part chimera using a polymerase chain reaction-based strategy. Briefly, an antisense oligonucleotide incorporating silent base changes

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[1] The abbreviations used are: EAAT1, EAAT2, and EAAT3, human excitatory amino acid transporters 1–3; μS, microsiemens.

[2] M. Kavanaugh, unpublished observations.

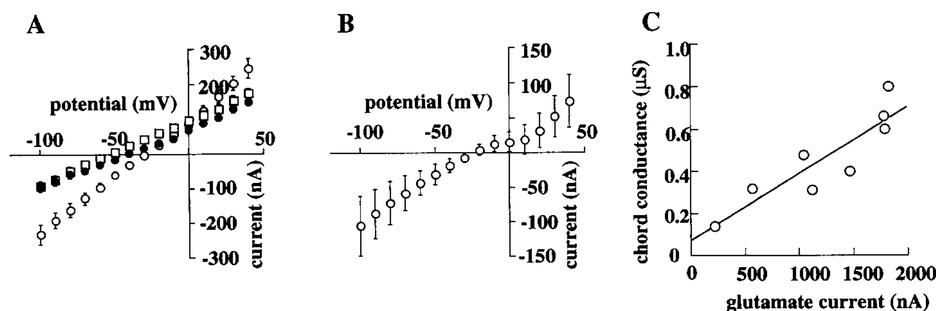

FIG. 1. **EAAT1 mediates an uncoupled ion flux.** *A*, current-voltage plots of oocytes 4 days following injection of equal amounts of cRNA encoding EAAT1 (*open circles*), EAAT2 (*open squares*), or uninjected controls (*filled circles*). Data points represent mean ± S.E. (n = 6). *B*, voltage dependence of glutamate-independent current in oocytes expressing EAAT1 obtained by subtraction of the mean current-voltage curve of uninjected oocytes from that of oocytes expressing EAAT1. *C*, the amplitude of the uncoupled current in individual oocytes between 1 and 5 days following injection of EAAT1 cRNA (measured as the difference in chord conductance compared with matched control oocytes) is proportional to the level of expression of the transporter (measured as the amplitude of the current elicited by 300 μM L-glutamate at −100 mV) in the same oocytes.

to introduce the *Eco*RI site was paired with a sense oligonucleotide corresponding to the vector polylinker and used in a polymerase chain reaction with the chimera as template. The resulting product was subcloned into EAAT1, and the entire coding region was sequenced to confirm the structure of the construct. The three-part chimera, termed E1–2-1, was subcloned into pOTV for oocyte expression (9).

Electrophysiological Recording—50 ng of capped cRNA transcribed with T7 polymerase from each of the cDNA constructs was injected into defolliculated stage V *Xenopus* oocytes. Two to seven days later transport was measured by two-electrode voltage clamp recording, and kinetic analyses were performed as described previously (16). In experiments involving ion substitution, sodium was partially replaced with equimolar choline, lithium, or potassium. The permeabilities of test ions relative to Na^+ were determined from the shift in reversal potential, ΔV, of the kainate-sensitive leak current induced by substitution of one-half the external sodium with ion *X* from a modified form of the Goldman-Hodgkin-Katz equation, $P_X/P_{Na} = 2(\exp(\Delta VF/RT)) - 1$. Mean shifts for each test ion were calculated by measurement of reversal potentials in 4–6 oocytes following measurement in control (ND96) solution. In experiments in which the chloride concentration was altered, chloride was substituted with either gluconate or methane sulfonate. To avoid offset potentials, a 3 M KCl/agar bridge was used to connect the bath to ground via a 3 M KCl reservoir containing a silver/silver chloride electrode.

RESULTS

Substrate-independent Ion Flux—In order to test for the presence of substrate-independent ionic currents mediated by the EAAT1 and EAAT2 subtypes, the membrane conductance of oocytes expressing these transporters was compared with the conductance of uninjected oocytes in the absence of amino acid (Fig. 1*A*). The chord conductance (measured between −40 and +40 mV) in a group of uninjected control oocytes was 1.8 ± 0.4 μS (mean ± S.E., n = 6). In oocytes from the same group that expressed EAAT2, the chord conductance was unchanged from control (1.9 ± 0.4 μS; n = 6), while in oocytes expressing comparable levels of EAAT1, this value was increased significantly (3.4 ± 0.6 μS; $n = 6$; $p < 0.05$). Similar results were seen in different batches of oocytes from three frogs. Subtraction of the mean current-voltage relationship of uninjected oocytes from that of oocytes expressing EAAT1 revealed the voltage dependence of the uncoupled ion flux (Fig. 1*B*). This current was approximately linear over the voltage range −100 to +40 mV and reversed at −16.9 ± 4.9 mV (n = 8). In order to test whether the glutamate-independent current in oocytes expressing EAAT1 was mediated by the transporter, the difference in conductance from the controls was compared with the amplitude of the current elicited by 300 μM L-glutamate at −100 mV in oocytes expressing different levels of transporter. There was a linear correlation in individual oocytes (Fig. 1*C*), demonstrating that the amplitude of the glutamate-independent current was related to the transporter expression level (r = 0.89). These results suggest that EAAT1, but not EAAT2, mediates a substrate-independent ion flux.

Expression and Kinetic Parameters of a Chimeric Transporter—Kainate competitively antagonizes the EAAT2-mediated current induced by L-glutamate with a K_d of 14 μM but has no effect on the steady state current in the absence of glutamate at membrane potentials between −160 mV and +80 mV (6). In contrast to EAAT2, glutamate transport by EAAT1 is relatively insensitive to kainate (9). Application of up to 3 mM kainate alone to oocytes expressing EAAT1 does not induce any steady-state current over this voltage range (results not shown). In order to further investigate the properties of the glutamate-independent current mediated by EAAT1 and to identify domains involved in the interaction of the transporter with kainate, chimeric transporters were constructed and assessed for their kainate sensitivity. One such chimera, termed E1–2-1, is comprised of the EAAT1 sequence from the amino terminus through Ser-366 followed by the EAAT2 sequence through Ala-441, followed by the EAAT1 sequence through the carboxyl terminus (Fig. 2). E1–2-1 contains 18 residues from EAAT2 between Ser-366 and Ala-441 that differ from the corresponding EAAT1 residues. Injection of RNA transcribed from the E1–2-1 chimeric cDNA into oocytes resulted in a >10-fold increase in ^{3}H-labeled L-glutamate uptake (data not shown). Application of glutamate to voltage-clamped oocytes expressing E1–2-1 resulted in inward currents similar to those observed in oocytes expressing the parent transporters (Fig. 3). The K_m for L-glutamate exhibited by the chimeric transporter was 9 ± 3 μM (n = 6), slightly lower than that for EAAT1 (19 ± 3 μM) and EAAT2 (17 ± 2 μM). Because the substrate serine *O*-sulfate displays marked differences in kinetic parameters between EAAT1 and EAAT2 (9), chimera-mediated currents induced by this compound were examined. EAAT1 displayed a K_m of 35 ± 2 μM for serine *O*-sulfate and an I_{max} (relative to glutamate) of 1.02 ± 0.05, whereas the corresponding values for EAAT2 were 237 ± 14 μM and 0.54 ± 0.02. The K_m and I_{max} values for serine *O*-sulfate transport by E1–2-1 were 17 ± 3 μM and 1.0 ± 0.1 (n = 5), suggesting that the structural domain of the chimera involved in determining the kinetic parameters for substrate translocation may be comprised of EAAT1-derived sequences.

Kainate Actions on the Chimeric Transporter—In contrast to EAAT1 and EAAT2, application of kainate to oocytes expressing the E1–2-1 chimeric transporter generated a small outward current at −60 mV (Fig. 3*C*). This outward current was increased in a dose-dependent and saturable fashion by kainate (EC_{50} = 120 ± 16 μM at −60 mV; n = 4). In addition, co-application of 10 μM glutamate with 3 mM kainate to the E1–2-1 chimera similarly resulted in an outward current rather

FIG. 2. *A*, schematic diagram of the chimeric EAAT1/EAAT2 transporter E1–2-1. *Filled areas* correspond to the EAAT1 cDNA sequence, and the *open area* corresponds to the EAAT2-derived domain inserted into EAAT1 (see "Experimental Procedures"). *Lines above* represent major hydrophobic domains identified using the Goldman-Engelman-Steitz algorithm (MacVector). *B*, predicted amino acid sequences of EAAT1 and EAAT2 corresponding to the substituted domain and junction region (*numbers* correspond to residues in EAAT1). *Dashes* reflect identical residues between EAAT1 and EAAT2.

FIG. 3. **Substitution of a block of residues from EAAT2 into EAAT1 confers kainate sensitivity to the chimeric transporter E1-2-1.** Application of kainate alone does not induce currents in either EAAT1 (*top*) or EAAT2 (*middle*), while co-application of kainate with glutamate blocks the transport current mediated by EAAT2 but not EAAT1. Application of kainate alone or co-applied with glutamate resulted in an outward current in cells expressing E1–2-1 as a result of blockade of a constitutive uncoupled current in addition to block of the glutamate-induced current (see text). Oocytes were voltage clamped at −60 mV and superfused with compounds for duration indicated by the *bars*.

than a simple block of the inward transport current as seen with EAAT2. The outward current induced by kainate at −60 mV resulted from a conductance decrease due to the block of a constitutive inward current, as revealed by subtraction of the current-voltage plot in the presence of kainate from that in its absence (Fig. 4*A*). The kainate-sensitive current reversed direction at −17.9 ± 1.0 mV (n = 16) in ND96 bathing solution, similar to the reversal potential of the EAAT1-associated uncoupled flux (−16.9 ± 4.9 mV, Fig. 1*B*). Thus, the small segment of EAAT2 sequence in the E1–2-1 chimera confers sensitivity of the uncoupled current to block by kainate.

The nature of the uncoupled current through the chimeric transporter was examined by ion substitution experiments. Partial replacement of Na^+_{out} by choline (reducing $[Na^+]_{out}$ from 100.5 to 22.5 mM) caused a 38.2 ± 4.2 mV (n = 6) shift in the reversal potential, indicating that sodium ions contribute to the substrate-independent leak current. A plot of the reversal potential of the current blocked by kainate *versus* the logarithm of the external sodium concentration revealed that the reversal shifted 58.2 mV/decade change in $[Na^+]_o$, in accord

FIG. 4. *A*, voltage dependence of L-glutamate-independent (uncoupled) and L-glutamate-dependent currents mediated by the chimeric E1–2-1 transporter. The glutamate-induced current in a representative oocyte was obtained by subtraction of the current-voltage curve in the absence of L-glutamate from that in the presence of 300 μM L-glutamate; the uncoupled current (in the same cell) was obtained by subtraction of the current-voltage curve in the presence of 5 mM kainate from that in the absence of kainate. *B*, the kainate-sensitive leak conductance is carried by Na^+ ions. The reversal potential of the kainate-sensitive uncoupled currents was measured as in *A* using external bath solution (ND96) with $[K^+]$ = 0 and in which $[Na^+]$ was varied by equimolar substitution with $choline^+$. The *line* shows least squares fit to data points (mean ± S.E.; n = 5) with a slope of 58.2 mV.

with the prediction of the Nernst equation for a Na^+-selective electrode (Fig. 4*B*). The monovalent cation selectivity of the uncoupled conductance was further studied by equimolar replacement of one-half the external Na^+ with K^+, Li^+, or choline. From the reversal potential shifts, the permeability sequence was determined to be K^+ (1.64) > Na^+ (1) > Li^+ (0.83) ≫ choline (<0.04).

Substitution of Cl^-_{out} with gluconate or methanesulfonate had no effect on the reversal potential of the uncoupled current (n = 5). However, after depletion of intracellular chloride by 16-h incubation in gluconate-substituted medium, removal of extracellular chloride reversibly abolished the kainate-sensitive uncoupled current (n = 5). These results indicate that, although chloride ions are not permeant, they are required for expression of the uncoupled monovalent cation current. When measured in chloride-free conditions to abolish the leak current, kainate blocked the inward current induced by 30 μM L-glutamate with a K_i of 97 ± 18 μM (n = 3), similar to the potency observed for the inhibition of the substrate-independent leak current in the normal ND96 bath solution (126 ± 6 μM). This result is consistent with the hypothesis that block of the leak conductance and glutamate transport are both mediated by kainate binding at a single site on the transporter.

DISCUSSION

Comparison of the membrane conductance of control oocytes with oocytes expressing EAAT1 and EAAT2 suggests that EAAT1, but not EAAT2, mediates an ionic flux in the absence of glutamate. This uncoupled flux is likely to be mediated directly by the transporter since its amplitude is proportional

to the level of transporter expression as measured by the glutamate-induced current. Using cDNAs encoding the human EAAT1 and EAAT2 subtypes, a chimeric glutamate transporter was constructed that is sensitive to the EAAT2-selective blocker kainate. The chimeric transporter is composed predominantly of EAAT1, with the EAAT2 sequence substituted between the seventh and ninth hydrophobic domains (residues 366–441; Fig. 2). This transposed domain encompasses a highly conserved region among members of the glutamate transporter family. For example, the motif FIAQ (residues 415–418) is conserved among the rat homologs of EAAT1 (GLAST (18)) and EAAT2 (GLT-1 (19)), the rabbit homolog of EAAT3 (EAAC1 (20)), and the *Escherichia coli* glutamate transporter gltP (21). Within the chimeric sequence derived from EAAT2, there are 18 residues that differ from the corresponding residues in EAAT1. Substitution of this discrete domain conferred a >50-fold increase in affinity for kainate. However, because the affinity of kainate is still 7–8-fold lower than that observed for EAAT2, it is likely that additional amino acid residues influence kainate sensitivity. The study of further chimeras in conjunction with site-directed mutagenesis will be required to determine the precise role of these and other residues in the interaction of kainate with the transporter.

The kainate sensitivity of the chimeric transporter has allowed identification of the glutamate-independent current as a flux of monovalent cations through the transporter. Substrate-independent currents have been observed to be mediated by cloned sodium-dependent transporters for glucose (11), serotonin (12), γ-aminobutyric acid (13), dopamine (14), and phosphate.[3] Similar to results in the present study, a sodium-dependent leakage current attributed to the glutamate transporter(s) endogenous to salamander retinal glial cells has also been reported (22). The presence of uncoupled currents in various sodium co-transporters may provide clues about the structural requirements for the permeation path involved in translocation of large substrate molecules in addition to small inorganic ions. The similarity of the inhibition constants for kainate block of the glutamate-dependent and -independent currents suggests that the ions that carry the uncoupled current are likely to permeate the same pore region of the transporter as glutamate. While transporter-mediated substrate translocation is commonly modeled by an alternating access scheme (23), the permeation and possible gating mechanisms underlying substrate-independent ion fluxes are not well understood. The steady-state uncoupled cation conductance of the glutamate transporter is relatively insensitive to membrane potential. Nevertheless, the requirement of the cation flux for chloride suggests a possibility of gating, perhaps via an allosteric effect of chloride binding. Alternatively, interactions between cations and chloride may occur in the transporter pore as have been observed in a neuronal chloride channel (24). Although both EAAT1 and EAAT2 mediate a chloride flux, this flux requires amino acid for activation (7) and is thus distinguished from the uncoupled cation current that occurs in the absence of amino acid. Further work to elucidate the molecular mechanisms underlying these fluxes, including localization of the precise residues that comprise the permeation pathway, will allow the construction of increasingly more detailed models of transport.

[3] E. Klamo and M. Kavanaugh, unpublished observations.

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