Functional diversity of excitatory amino acid transporters: ion channel and transport modes

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Fairman, W. A., and S. G. Amara. Functional diversity of excitatory amino acid transporters: ion channel and transport modes. Am. J. Physiol. 277 (Renal Physiol. 46): F481–F486, 1999.—Recent studies of glutamate transporters in the central nervous system indicate that in addition to their fundamental role in mediating neurotransmitter uptake, these proteins may contribute to the modulation of a variety of cellular processes. Activation of the excitatory amino acid (EAA) carriers generates an electrogenic current attributable to ion-coupled cotransport. In addition to this transport-associated current, a substrate-gated thermodynamically uncoupled anion flux has been identified that has been proposed to dampen neuronal excitability. Arachidonic acid has been reported to modulate a variety of membrane proteins involved in cellular signaling. Here we discuss recent findings that indicate arachidonic acid stimulates a previously uncharacterized proton-selective conductance in the Purkinje cell-specific subtype, EAAT4. The unique channel-like properties of the EAATs, their unexpected localization, and physiological evidence propose a modulatory role for the EAATs in neuronal signaling and suggest a broader role for glutamate transporters than simply the clearance of synaptically released glutamate. Thus, the identification of this arachidonate-stimulated proton conductance extends the complexity of mechanisms through which glutamate transporters modulate neuronal excitability.

proton conductance; neuronal excitability; glutamate transporter; ligand-gated chloride conductance; arachidonic acid

Excitatory amino acid transporters in the central nervous system (CNS) maintain extracellular glutamate concentrations below excitotoxic levels and contribute to the clearance of glutamate released during neurotransmission. At least five structurally distinct subtypes of human glutamate transporters, EAAT1–EAAT5 (1, 2, 11), have been identified and characterized by molecular cloning. Transport of substrates by these carriers is proposed to be thermodynamically coupled to the cotransport of two to three sodium ions (4, 13, 23), one proton (23), and the countertransport of a potassium ion (4); thus this process is electrogenic. However, the amount of charge moved when substrates are applied to these carriers is greater than would be predicted from the flux of coupled ions and substrate: this additional current elicited during substrate application arises from a thermodynamically uncoupled anion flux (for review, see Ref. 17). The relative proportion of the current generated by ion-coupled substrate transport or the ligand-gated chloride conductance varies for each cloned EAAT subtype. For EAAT1, EAAT2, and EAAT3 the chloride flux is a relatively small component of the currents, whereas for two of the neuronal transporters, EAAT4 and EAAT5, the currents elicited by substrates are almost entirely comprised of a gated anion flux. The association between these two neuronal EAATs and a ligand-gated chloride conductance suggests a broader role for transporters in regulating neuronal excitability and signaling mechanisms. Current data support the idea that the chloride conductance is intrinsic to the transporters, but there has been no formal structural or biochemical evidence to suggest whether single or multiple permeation pathways exist for anions, substrates and cotransported ions and/or whether accessory proteins are necessary for the generation of the multiple conductances associated with these carriers (for review, see Ref. 17). Alternative models for the transporter-associated conductances are represented in Fig. 1.
In addition to the ion channel-like properties attributed to members of this transporter family, the cellular and anatomical localization of the different EAAT subtypes is also consistent with novel, unexpected functions for these carriers. Earlier work using synaptic preparations, pathway lesioning experiments, and autoradiographic studies of uptake sites had suggested that excitatory amino acid transporters were predominantly localized on presynaptic neurons and in glia surrounding synapses. This notion that neuronal glutamate transporters are distributed on the terminals of glutamatergic neurons has been challenged by recent studies in human and rat brain, which demonstrate that neuronal carriers identified in the brain, EAAT3 and EAAT4, are not found on axons or presynaptic terminals, but instead are located on the soma and dendrites of neurons (16, 19). Furthermore, EAAT4 is found predominantly on a GABA-ergic cell type, the cerebellar Purkinje cell, and appears concentrated in dendritic areas that receive major glutamatergic inputs. An additional observation linking glutamate carriers to other components of postsynaptic signaling complexes is the observation that EAAT5, a retinal transporter, contains a PDZ-binding motif at its COOH terminus (2). The same motif has been implicated in receptor and ion channel clustering at the synapse and has been shown to mediate the binding of the cytoplasmic COOH terminus of N-methyl-D-aspartate (NMDA) receptor subunits and of Shaker-type potassium channels to a group of abundant synaptic proteins that include PSD95 and PSD93. The apparent postsynaptic localization of the neuronal EAATs and their expression in nonglutamatergic cells suggests that they do not simply serve as sites of neurotransmitter reuptake and may function in other ways.

Arachidonic acid released during neuronal activity alters the electrical and biochemical properties of a variety of membrane proteins involved in cellular signaling including several neurotransmitter transporters. For the glutamate transporters EAAT1, EAAT2, and EAAT3, arachidonic acid was reported to modulate the kinetics of substrate transport as assessed by uptake of radiolabeled glutamate and/or by measurement of substrate-elicited currents (22). Arachidonic acid appears to have very different actions on the function of EAAT4. In EAAT4-expressing oocytes, the application of physiologically relevant concentrations of arachidonic acid produces an approximately twofold stimulation in the substrate-gated currents at \(-60\) mV which cannot be attributed to the modulation of substrate transport or of the ligand-gated anion current associated with this carrier (10). In addition, preliminary studies in our laboratory suggest that a similar stimulation of the substrate-gated current is observed when arachidonic acid is applied to the retinal carrier, EAAT5. In the case of EAAT4, arachidonic acid stimulates a novel ligand-activated conductance selective for protons. This effect does not require the metabolism of arachidonic acid, and is not blocked by inhibitors of endogenous oocyte ion exchangers (10). This observation that arachidonic acid stimulates a proton conductance associated with EAAT4 expands the repertoire of ion channel-like properties associated with the EAAT family, and suggests yet another mechanism, perhaps involving local pH changes, through which synaptic excitability can be modulated by substrate binding and transport.

Actions of arachidonic acid on EAAT4. The effect of arachidonic acid on EAAT4-expressing oocytes was assessed by measuring substrate-gated currents and radiolabeled uptake. At \(-60\) mV, arachidonic acid stimulated \(\text{L-Asp-}\)gated currents \(200 ± 64\%\) \((n = 56)\) (Fig. 2A), with an \(EC_{50}\) of \(1.7 ± 0.3\) µM. Several lines of evidence suggest that this stimulation is a direct consequence of the application of arachidonic acid and not the result of arachidonic acid metabolism. Arachidonic acid was able to potently stimulate the \(\text{L-Asp-}\)
gated current in the presence of inhibitors of lipoxygenase, cyclooxygenase, and monoxygenase inhibitors. Other polyunsaturated fatty acids were able to stimulate the L-Asp-gated current with a rank order of potency that paralleled their degree of unsaturation (docosahexaenoic acid > arachidonic acid > linoleic acid > linolenic acid > oleic acid). In addition, the inactive analog of arachidonic acid, arachidonic acid ethyl ester, was inactive in cells that showed a subsequent stimulation by arachidonic acid.

Previous work demonstrated that with EAAT1, arachidonic acid decreased the maximal uptake velocity, and with EAAT2, it increased the affinity for L-glutamate with no change in the maximal transport rate (22). Surprisingly, although arachidonic acid potentiated substrate-activated currents in EAAT4-expressing oocytes, it had little effect on transport activity. No significant differences in either the affinity or the maximal transport rate for L-[3H]Asp was observed in the presence or absence of a maximal concentration of arachidonic acid (100 µM). Similarly, arachidonic acid had no effects on the kinetics or sodium dependence of the currents induced by L-glutamate (10).

Additional experiments ruled out the possibility that the stimulation of the L-Asp-induced current by arachidonic acid might be caused by a stimulation of the ligand-gated anion conductance. As shown in the current-voltage relation in Fig. 2B, the coapplication of arachidonic acid produced robust increases in the current amplitude at the most negative potentials, with no apparent stimulation of outward currents at potentials more positive than the reversal potential ($E_{\text{rev}}$). In cells in which the chloride equilibrium potential, $E_{\text{Cl}}$, was directly measured to be $-14 \pm 0.4$ mV, the coapplication of arachidonic acid shifted $E_{\text{rev}}$ of the L-Asp-induced current to $-4.2 \pm 1$ mV ($n = 5$), away from $E_{\text{Cl}}$. Furthermore, equimolar substitution of extracellular chloride with the impermeant ion gluconate completely

![Fig. 2. A: representative current trace of the stimulation of the L-Asp-induced current by arachidonic acid in an EAAT4-expressing oocyte held at $-60$ mV. B: representative current-voltage (I-V) curve for 10 µM L-Asp in presence (●) and absence (○) of 100 µM arachidonic acid. C: representative curves illustrating effect of extracellular Cl⁻ replacement by the impermeant ion gluconate on the I-V relation for L-Asp in presence (●) and absence (○) of 100 µM arachidonic acid. D–F: representative I-V curves demonstrating pH dependence of L-Asp-induced and arachidonic acid-stimulated current in chloride-depleted conditions. I-V relations for L-Asp-induced current (●, $I_{\text{Asp}}$) and arachidonic acid (AA)-stimulated current (○, $I_{\text{AA}}$) are shown at pH 6.5 (D), 7.5 (E), and 8.5 (F). G: representative I-V relations in a single cell illustrating the effect of application of $^2$H$_2$O substituted Ringer on L-Asp-induced currents in presence (●) and absence (○) of arachidonic acid and the subsequent application of L-Asp in presence (●) and absence (○) of arachidonic acid in proton-rich Ringer.](http://ajpendo.physiology.org/DownloadedFrom/10.1152/ajpendo.00022.16)
abolished the outward current induced by L-Asp, whereas coapplication of arachidonic acid and L-Asp still elicited an outward current (Fig. 2C). Varying extracellular chloride concentration (extracellular [Cl\(^{-}\)) from 104 to 14 mM produced a shift in \(E_{\text{rev}}\) of \(-57 \pm 2\) mV per 10-fold change in \([\text{Cl}^{-}]\) \((n = 7)\), whereas varying extracellular [Cl\(^{-}\)] in the presence of arachidonic acid produced only a modest shift in \(E_{\text{rev}}\). Finally, arachidonic acid was still able to shift \(E_{\text{rev}}\) to more positive potentials and stimulate the amplitude of the L-Asp-induced current at potentials more negative than \(E_{\text{rev}}\) when extracellular chloride was replaced with nitrate (10), a more permeant anion (20). These results provide compelling evidence that the arachidonic acid-stimulated conductance is not mediated by chloride ions.

Arachidonic acid stimulates a novel proton-selective conductance. Ion substitution experiments indicate that arachidonic acid stimulates a conductance that is selective for protons. Varying extracellular sodium, calcium, and potassium ions had no significant effects on the \(E_{\text{rev}}\) of the current stimulated by the coapplication of arachidonic acid and L-Asp. Varying extracellular proton concentration from pH 6.5 to 8.5 had no effect on the \(E_{\text{rev}}\) of the L-Asp-induced current, but significantly altered the amplitude of the current. In contrast, the coapplication of arachidonic acid produced a marked shift in \(E_{\text{rev}}\) with alterations in extracellular pH, indicating the protons (or OH\(^{-}\)) is likely to be the charge-carrying species of the arachidonic acid-induced current. To more clearly establish the proton selectivity of the arachidonic acid-stimulated current, substrate-elicited currents in EAAT4-expressing oocytes were studied under chloride-depleted conditions. The coapplication of arachidonic acid and L-Asp in gluconate-substituted Ringer produced a shift of \(-38 \pm 0.3\) mV per pH unit when extracellular pH was varied from 6.5 to 8.5 (Fig. 2, D–F) (10). Unexpectedly, under these conditions, similar pH-dependent shifts for the L-Asp-induced currents roughly paralleled those observed for the coapplication of arachidonic acid with substrate, suggesting that L-Asp may gate a proton flux that is stimulated by exogenous arachidonic acid.

Deuterium substitution has been used to investigate proton flux through a variety of channels (6, 7). Because deuterons exhibit reduced permeability relative to protons, whereas the mobility of O\(_2\)H\(^{-}\) and OH\(^{-}\) is comparable, we examined the effect of \(^2\)H\(_2\)O substitution on both the L-Asp- and arachidonic acid-stimulated cur-

Fig. 3. Proposed model by which sustained depolarization or arachidonic acid release may regulate a proton conductance mediated by the excitatory amino acid transporter, EAAT4. PLA\(_2\), phospholipaseA\(_2\).
rent. No significant effects on [3H]L-Asp flux were observed in the presence of 2H2O-substituted Ringer solution. In contrast, although 80% 2H2O substitution produced no significant alterations in the L-Asp-induced current, the arachidonic acid-stimulated current was completely abolished (Fig 2G) in cells showing a significant stimulation of the L-Asp-induced current with the subsequent application of arachidonic acid in proton-rich Ringer (10).

To rule out the possibility that endogenous oocyte ion exchangers participate in the arachidonic acid-stimulated proton conductance, a variety of inhibitors of endogenous oocyte ion exchangers were tested for their ability to inhibit the stimulation of the L-Asp-induced current by arachidonic acid. No significant diminution in the ability to inhibit the stimulation of the L-Asp-induced current was completely abolished (Fig 2G) in cells showing a significant stimulation of the L-Asp-induced current with the subsequent application of arachidonic acid in proton-rich Ringer (10).

Physiological significance of EAAT-associated ion conductances. Emerging data indicate that transporters and their associated conductances may contribute to intercellular signaling in the nervous system beyond the canonical role of the carriers in terminating synaptic chemical signals through transmitter reuptake and recycling. In retinal bipolar cells, a transporter-gated chloride conductance has been proposed to mediate the cone component of the ON bipolar cell light response (12). Cone photoreceptor cells from the tiger salamander respond to the glutamate they release with hyperpolarizing responses through the activation of a glutamate-gated Cl− conductance; this current appears to act as feedback mechanism to limit further depolarization and consequent glutamate release (15). Evidence for a retinal glutamate transporter with an associated glutamate-gated Cl− conductance has come from molecular cloning studies in retina, which have identified and characterized at least seven distinct glutamate transporter subtypes from salamander retina (9). A human homolog of one of the retinal-specific carriers, human EAAT5, also appears to have a substantial substrate-activated chloride current and is a promising candidate for the carrier that serves as a glutamate sensor in photoreceptors. In other brain regions, such as the Purkinje cells that express EAAT4, the activation of a Cl− conductance has been proposed to dampen neuronal excitability. As discussed above, arachidonic acid further modulates the function of EAAT4 to stimulate an additional substrate-gated conductance carried predominantly by protons (10).

EAAT4 is concentrated postsynaptically in the extrajunctional spaces of climbing fiber- and parallel fiber-Purkinje cell synapses (19). In a recent patch-clamp study using rat cerebellar Purkinje cells, Kataoka et al. (14) found that either sustained depolarization or exogenous application of arachidonic acid induced an approximately twofold increase in the maximum current mediated by the EAATs. This increase was blocked by inhibitors of phospholipase A2 or phospholipase C but not protein kinase C or Ca2+/calmodulin-dependent kinase. Sustained depolarization was proposed to activate arachidonic acid release and stimulate the EAAT4-associated anion conductance by increasing the affinity for L-Asp. Although L-Asp clearly activated an anion conductance in these cells, the identity of the charge-carrying species underlying the increased current observed with arachidonic acid was not formally demonstrated. Since our studies imply that arachidonic acid stimulates a proton conductance associated with the Purkinje cell transporter EAAT4, the transporter-mediated currents observed in Purkinje cell cultures with sustained depolarization or the exogenous application of arachidonic acid are likely to be mediated by protons.

The multiple functional properties associated with the EAAT4 carrier are particularly interesting when considering the selective postsynaptic localization of EAAT4 in cerebellar Purkinje neurons. Inhibition of EAAT4 transporters at climbing fiber and parallel fiber-Purkinje cell synapses has been reported to slow the decay time and decrease the amplitude of the EPSC (18). At these synapses the coactivation of AMPA and metabotropic glutamate receptors and the concurrent increase in cytosolic calcium concentration has been proposed to be an important mechanism for arachidonic acid release (8). In addition, increased levels of arachidonic acid have been implicated in the induction of long-term depression (LTD) in this region and have been reported to increase in pathological conditions (3). Alterations in pH have been proposed to be an important means of regulating many cellular functions. Although it is difficult to predict whether the activation of a proton conductance through EAAT4 might acidify the intracellular milieu of a Purkinje cell dendrite sufficiently to modulate the activity of pH-sensitive cellular functions, the complex geometry of the dendrite makes it conceivable that local alterations in intracellular pH might modulate other proteins involved in synaptic excitability, such as glutamate and GABA receptors (5). Thus the complex properties associated with EAAT4 provide several mechanisms by which activation of this protein might modulate neuronal excitability: the termination of synaptic transmission by glutamate transport, the dampening of cellular excitability with its ligand-gated anion conductance, and the stimulation of a proton conductance by arachidonic acid could all serve as mechanisms by which to regulate neuronal excitability.

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