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Arachidonic Acid Activates a Proton Current in the Rat Glutamate Transporter EAAT4*

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The excitatory amino acid transporter EAAT4 is expressed predominantly in Purkinje neurons in the rat cerebellum (1–3), and it participates in postsynaptic reuptake of glutamate released at the climbing fiber synapse (4). Transporter-mediated currents in Purkinje neurons are increased more than 3-fold by arachidonic acid, a second messenger that is liberated following depolarization-induced Ca2+ activation of phospholipase A2 (5). In this study we demonstrate that application of arachidonic acid to oocytes expressing rat EAAT4 increased glutamate-induced currents to a similar extent. However, arachidonic acid did not cause an increase in the rate of glutamate transport or in the chloride current associated with glutamate transport but rather activated a proton-selective conductance. These data reveal a novel action of arachidonate on a glutamate transporter and suggest a mechanism by which synaptic activity may decrease intracellular pH in neurons where this transporter is localized.

Glutamate transporters play critical roles in synaptic transmission and in maintaining glutamate homeostasis in the brain (6). They are encoded by genes belonging to a family of acidic and neutral amino acid transporters (7), and they exhibit specific localization patterns. Glutamate transporters found on glia include EAAT1 (excitatory amino acid transporter 1)/GLAST and EAAT2/Glt-1 (8, 9), and transporters found on neurons include the widely expressed EAAT3/EAAC1 (8), the cerebellar-specific EAAT4 (1–3), and the retinal EAAT5 (10).

Arachidonate is released following activation of postsynaptic glutamate receptors (11). In synaptosomal preparations, arachidonate inhibits glutamate uptake (12–14). However, it exerts differential effects on cloned glutamate transporter subtypes, enhancing EAAT2 and inhibiting EAAT1 transport (15). Arachidonate inhibits uptake in salamander retinal glial cells (16). These cells predominantly express an EAAT1 homolog (sEAAT1) that is similarly inhibited by arachidonate when it is exogenously expressed in oocytes (10, 15). Because arachidonate is released during synaptic activity and can modulate synaptic transmission (17, 18), understanding its effects on various glutamate transporter subtypes is important. Recently, Kataoka et al. reported an activity-dependent enhancement of glutamate transporter currents in rat cerebellar Purkinje neurons that was mediated by arachidonate (5). The present study was designed to examine the mechanism of the effects of arachidonate on the cloned rat EAAT4 transporter, which is expressed at high levels in Purkinje neurons.

EXPERIMENTAL PROCEDURES

The rat EAAT4 cDNA1 was subcloned into pOG (19) that contains a multiple cloning site between flanking Xenopus β-globin 5′ and 3′ untranslated sequences. Capped mRNA was transcribed using T7 polymerase and injected into stage V or VI oocytes (approximately 50 ng/oocyte). Recordings and radiolabel uptake assays were made 4–7 days later as described (15). Extracellular Ringer’s solution contained (in mM) 100 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2. Buffers were present at 5 mM and consisted of MES/HEPES (pH 6.5), Na/HEPES (pH 7.5), or HEPES/Tris (pH 8.5). Solutions containing indicated ion substitutions were changed by bath exchange. Recordings were made using a two-microelectrode voltage clamp circuit (20), and records were analyzed using CLAMP 6.0 software (Axon Instruments).

RESULTS

Three to four days following injection of RNA transcribed from the rat excitatory amino acid transporter EAAT4 cDNA Xenopus oocytes displayed >30-fold increased uptake of 1 μM [3H]l-glutamate. In oocytes voltage-clamped at −60 mV, EAAT4 currents induced by application of 30 μM l-glutamate were increased upon co-application of 100 μM arachidonate (Fig. 1A). This effect was reversible, although its onset and offset were slower than the solution exchange times as monitored by the glutamate response (Fig. 1A). Application of 100 μM arachidonate alone in oocytes expressing rEAAT4 resulted in a small but significant inward current (Fig. 1B; −2.2 nA ± 0.6, n = 4). This inward current was not observed in uninjected oocytes (1.1 nA ± 0.9, n = 9). At −60 mV, arachidonate increased the magnitude of the steady-state current induced by 30 μM glutamate to 324 ± 41% of its control value (n = 11). Currents elicited by glutamate in the presence and absence of arachidonate at a series of membrane potentials showed that arachidonate enhanced the current amplitude to a greater extent at more negative potentials (Fig. 1, C and D; also see Fig. 4). These results are consistent with a study on Purkinje neuron transporter currents (5) and a recent report on the human EAAT4 transporter (21).

The arachidonate concentration dependence in the presence of a saturating concentration of glutamate (30 μM) revealed that the arachidonate effect on the current was saturable, with an EC50 of 135 ± 21 μM (n = 3; Fig. 2A). In the presence of

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1 C.-L. Lin, A. V. Tzingounis, J. D. Rothstein, and M. P. Kavanaugh, submitted for publication.

2 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
Arachidonate Effects on EAAT4

Arachidonate increased the Imax without changing the glutamate and arachidonate and fitted to the Michaelis-Menten equation. Arachidonate, respectively (5nM L-glutamate) and (mean 6S.E., n = 3). To examine whether arachidonate affected the transport of L-glutamate in oocytes expressing rEAAT4, uptake of 1 mM [3H]L-glutamate was assayed in the presence or absence of 300 μM arachidonate (Fig. 2, C and D). In marked contrast to its effects on the currents, arachidonate had no significant effect on the uptake of L-glutamate into oocytes. Uptake of 1 mM [3H]L-glutamate was 524 ± 152 fmol/min in control conditions and 468 ± 86 fmol/min in the presence of 300 μM arachidonate (n = 6, p = 0.74). Uptake of 30 μM L-glutamate uptake was also not significantly changed by 300 μM arachidonate (1753 ± 506 fmol/min and 1477 ± 271 fmol/min in control and arachidonate, respectively; n = 5; p = 0.64). Arachidonate also had no effect on L-glutamate uptake in uninjected oocytes (Fig. 2, C and D).

These results demonstrate that arachidonate increased a glutamate-dependent rEAAT4 current without affecting glutamate uptake. This is in contrast to the effects of arachidonate on the EAAT1 and EAAT2 subtypes, in which L-glutamate uptake. This is in contrast to its effects on the currents, arachidonate had no...
glutamate transport current, because the outward current was abolished when extracellular chloride was substituted by glu-
conate (n = 4; data not shown). To examine whether arachidonic acid selectively increased the uncoupled Cl⁻ conductance, the voltage dependence of the current induced by glutamate was examined in the presence and absence of arachidonate. The arachidonate-dependent current was inwardly rectifying and did not reverse at the Cl⁻ equilibrium potential (−20 mV), indicating that the conductance increased by arachidonate was not Cl⁻-selective (Fig. 3A). To further rule out an action of arachidonate on the transporter-mediated anion conductance, extracellular Cl⁻ was substituted by the more permeant ion NO₃⁻ (4, 20). Similar to results with the human EAAT4 transporter, NO₃⁻ was more permeant than Cl⁻. Replacement of extracellular Cl⁻ by NO₃⁻ increased the glutamate-induced outward current and shifted the reversal potential to more negative potentials, from −14.8 ± 1.1 mV to −7.2 ± 2.4 mV (n = 3), and this shift was not influenced by changing the Na⁺ gradient by substitution of 48 mM Na⁺ with choline (n = 3; data not shown). Hence, the arachidonate-mediated increase of the L-glutamate current was selective for ions other than sodium or chloride.

Glutamate transporters mediate a coupled flux of protons with glutamate (23). To examine whether a proton-selective conductance was involved in the arachidonate potentiation of the L-glutamate current, currents were measured with varying extracellular pH between 6.5 and 8.5. Altering the extracellular proton concentration markedly influenced voltage dependence of the arachidonate-dependent current. As the extracellular proton concentration increased, the potential at which the glutamate current recorded in the presence of arachidonate crossed the control glutamate current shifted to more positive potentials (Fig. 4). With extracellular pH at 7.5, close to the value of the intracellular pH (24), the arachidonate-dependent current crossed the control current at 2.3 ± 3.9 mV (n = 11), and this reversal potential changed 53 mV/pH unit (Fig. 4D). These results indicate that the major component of the conductance amplified by arachidonate was proton-selective.

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**DISCUSSION**

The present results show that arachidonic acid activates a proton-selective conductance during EAAT4-mediated transport of L-glutamate, extending the recognized types of currents associated with glutamate transporters. In addition to the current associated with the Na⁺/H⁺ exchange (15), thermodynamically uncoupled Cl⁻ currents (20–22, 25, 26) as well as cation leak currents (27, 28) have been associated with glutamate transport. Different subtypes exhibit variability in both their anion (20) and cation conductances (28). Several other neurotransmitter transporters mediate uncoupled proton currents (29, 30), but EAAT4 is the first glutamate transporter reported to exhibit this property.

EAAT4 is predominantly localized to the cerebellum, where it is expressed in Purkinje cell bodies and dendrites (1, 2, 3). Transporter-mediated reuptake of glutamate released at climbing and parallel fiber synapses onto Purkinje cells plays a role in speeding the decay of postsynaptic responses (31, 32). The transporters participating in this process are located both in glial cells surrounding the synapse (33, 34) and in the postsynaptic dendrites and cell body (4, 35). The pharmacological and electrophysiological properties of the synthetically activated transport current in Purkinje cells suggest that it is mediated in large part by EAAT4 (4).

Arachidonic acid is liberated by activation of phospholipase A₂ during neuronal activity (5, 36), and it can modulate activity-dependent changes in synaptic strength in Purkinje cells (18). Whether exogenously applied or generated by depolarization, arachidonic acid increased the amplitude of glutamate transporter-mediated currents in rat Purkinje cells (5). The amplitude of the increase seen in Purkinje cells was similar to that reported here with the exogenously expressed EAAT4. Together these results suggest that synaptic activity may lead to activation of a glutamate transporter-mediated proton influx in neurons that express EAAT4. Because pH strongly influences the activity of many types of ion channels (for review see Ref. 37), this property of the transporter could provide an additional mechanism to modulate postsynaptic responses. Furthermore this phenomenon could contribute to intracellular acidification during ischemia as a consequence of the pathological elevation of glutamate and arachidonate.

To further study the effect of arachidonate on the transporter-mediated anion conductance, extracellular Cl⁻ was substituted by the more permeant ion NO₃⁻ (4, 20). Similar to results with the human EAAT4 transporter, NO₃⁻ was more permeant than Cl⁻. Replacement of extracellular Cl⁻ by NO₃⁻ increased the glutamate-induced outward current and shifted the reversal potential to more negative potentials, from −14.8 ± 1.1 mV to −7.2 ± 2.4 mV (n = 3), and this shift was not influenced by changing the Na⁺ gradient by substitution of 48 mM Na⁺ with choline (n = 3; data not shown). Hence, the arachidonate-mediated increase of the L-glutamate current was selective for ions other than sodium or chloride.
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