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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 Ca^{2+} activation of phospholipase A_2 (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 cDNA[1] was subcloned into pOG, a vector derived from pBSTA (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 $CaCl_2$, 1 $MgCl_2$. Buffers were present at 5 mM and consisted of MES[2]/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 pCLAMP 6.0 software (Axon Instruments). [³H]L-glutamate (1 Ci/mmol; Amersham Pharmacia Biotech) uptake assays were performed at 25 °C. Following a 5-min incubation in the indicated concentration of [³H]L-glutamate (10 μCi/ml), oocytes were rapidly washed three times in cold Ringer and lysed in 1% SDS, and scintillation spectroscopy was performed. Arachidonic acid (Calbiochem) was stored at −20 °C in 100 mM stock solutions in Me_2SO and dissolved in recording solution by sonication immediately prior to use. All other compounds were from Sigma.

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. 1*A*). This effect was reversible, although its onset and offset were slower than the solution exchange times as monitored by the glutamate response (Fig. 1*A*). Application of 100 μM arachidonate alone in oocytes expressing rEAAT4 resulted in a small but significant inward current (Fig. 1*B*; −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 EC_{50} of 135 ± 21 μM (n = 3; Fig. 2*A*). 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.
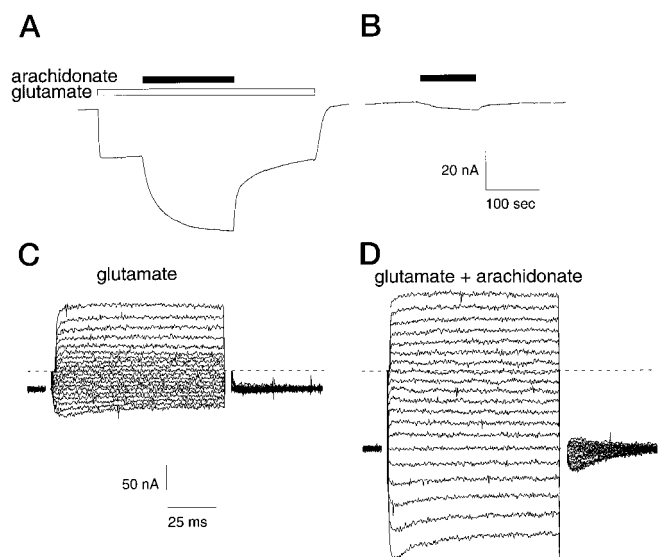
FIG. 1. **Arachidonic acid enhances the magnitude of gluta-mate-induced currents recorded in voltage-clamped oocytes ex-pressing rat EAAT4.** *A*, representative cell clamped at −60 mV; compounds were superfused for the times indicated by the *open* (30 μM L-glutamate) and *closed bars* (100 μM arachidonic acid). *B*, application of arachidonate alone induced a current much smaller than observed with co-application of L-Glu. *C*, subtracted (30 μM glutamate-control) currents recorded during 90-ms voltage jumps between −120 mV and +70 mV. *D*, currents recorded in the same cell as *C* with 100 μM arachidonic acid present. The *dashed line* indicates zero current; capacitive artifacts have been removed for clarity. Holding potential, −70 mV.
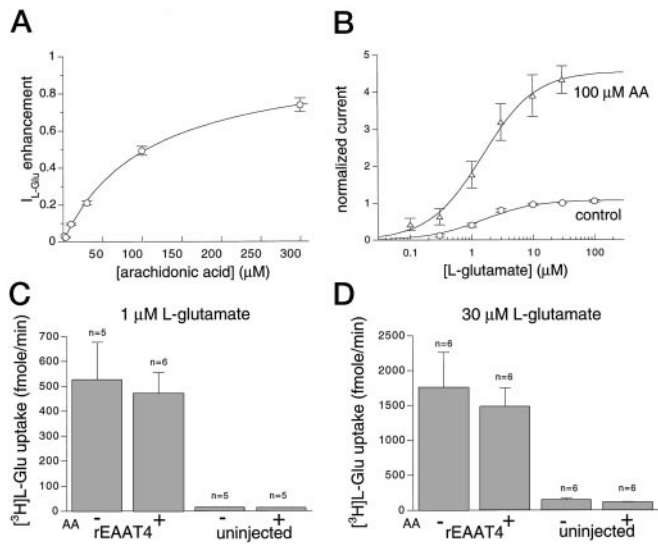
FIG. 2. **Arachidonate dose-dependently increases the transport current without increasing glutamate flux.** *A*, arachidonate concentration dependence of transporter currents activated by 30 μM glutamate (normalized to the maximal current at $V_m = -70$ mV). Points (mean ± S.E., $n = 3$) are fitted to the Michaelis-Menten equation with a $K_{0.5}$ of 135 μM. *B*, L-glutamate concentration dependence of currents in the presence and absence of 100 μM arachidonate. Currents (mean ± S.E., $n = 6$) were normalized to the maximum current in the absence of arachidonate and fitted to the Michaelis-Menten equation. Arachidonate increased the I_{max} without changing the glutamate $K_{0.5}$ value (1.5 μM).

arachidonate, the apparent affinity of the transporter for glutamate was unaffected; the EC_{50} of the current was 1.5 ± 0.2 and 1.5 ± 0.3 μM in control and with 100 μM arachidonate, respectively ($n = 6$). The effects of arachidonate on the transport current seemed to be direct rather than through a metabolite, because coapplication of the cyclooxygenase inhibitor in-
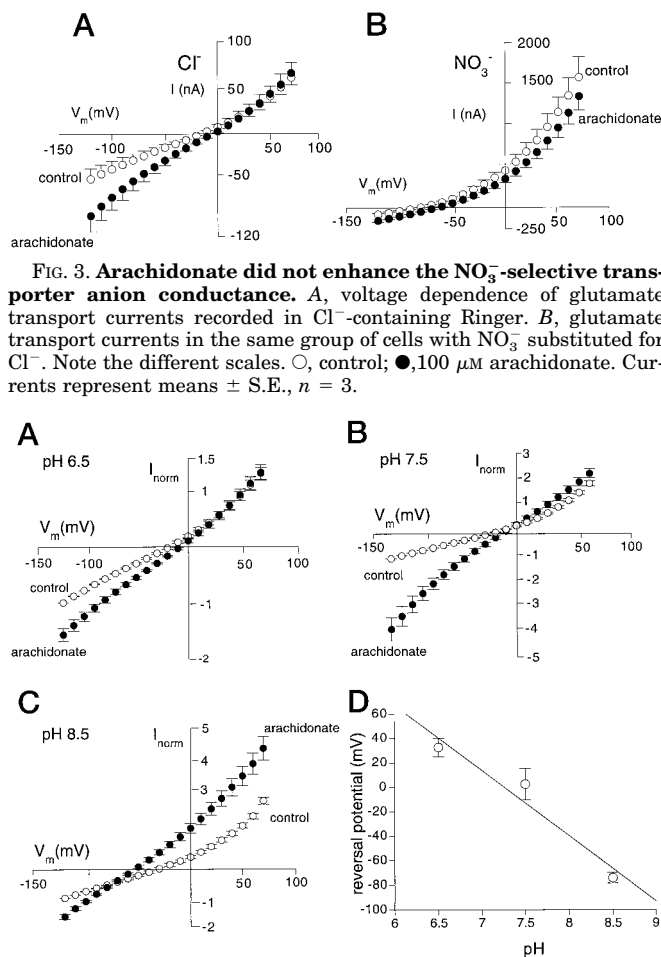
FIG. 3. **Arachidonate did not enhance the NO_3^--selective transporter anion conductance.** *A*, voltage dependence of glutamate transport currents recorded in Cl^--containing Ringer. *B*, glutamate transport currents in the same group of cells with NO_3^- substituted for Cl^-. Note the different scales. ○, control; ●, 100 μM arachidonate. Currents represent means ± S.E., $n = 3$.

FIG. 4. **Arachidonate enhances a proton-selective current.** Voltage dependence of currents induced by 30 μM glutamate in the presence and absence of 100 μM arachidonate at pH 6.5 (*A*), 7.5 (*B*), and 8.5 (*C*). *D*, extracellular pH shifted the potential at which currents in the presence of arachidonate crossed over the control transport currents. The *l* shows least squares fit with slope of −53.4 mV/pH unit.

domethacin (100 μM) together with the lipoxygenase inhibitor nordihydroguaretic acid (50 μM) had no effect on the potentiation induced by arachidonic acid (96 ± 2% of control enhancement, $n = 3$). To examine whether arachidonate affected the transport of L-glutamate in oocytes expressing rEAAT4, uptake of 1 μM or 30 μM [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 μM [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 and currents are decreased or increased in parallel (15). Hence, the ionic nature of the rEAAT4 conductance increased by arachidonate was investigated further. Similar to the human EAAT1-EAAT4 subtypes (20, 22), rat EAAT4 mediates an uncoupled Cl^- conductance in addition to the sodium-coupled

glutamate transport current, because the outward current was abolished when extracellular chloride was substituted by gluconate ($n = 4$; data not shown). To examine whether arachidonate 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. 3*A*). To further rule out an action of arachidonate on the transporter-mediated anion conductance, extracellular Cl^- was substituted by the more permeant ion NO_3^- (4, 20). Similar to results with the human EAAT4 transporter (4), NO_3^- was more permeant than Cl^-. Replacement of extracellular Cl^- by NO_3^- increased the glutamate-induced outward current and shifted the reversal potential to more negative potentials, from -14.8 ± 1.1 to -81.8 ± 1.1 mV ($n = 3$; Fig. 3*B*). Coapplication of 100 μM arachidonate with glutamate slightly inhibited the outward NO_3^- current, further supporting that conclusion that the conductance increased by arachidonate was not anion-selective (Fig. 3*B*). The reversal potential of the glutamate-induced current was shifted approximately $+10$ mV by arachidonate (from -14.8 ± 1.1 mV to -4.7 ± 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 current 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. 4*D*). These results indicate that the major component of the conductance amplified by arachidonate was proton-selective.

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 $Na^+/H^+/K^+$-coupled translocation of glutamate (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 found on 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 synaptically 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 A_2 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.

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