

## Differential Modulation of Human Glutamate Transporter Subtypes by Arachidonic Acid\*

(Received for publication, December 2, 1994)

Noa Zerangue‡, Jeffrey L. Arriza‡, Susan G. Amara§, and Michael P. Kavanaugh‡¶

From the ‡Vollum Institute and §Howard Hughes Medical Institute, Oregon Health Sciences University, Portland, Oregon 97201

**Arachidonic acid has been proposed to be a messenger molecule released following synaptic activation of glutamate receptors and during ischemia. Here we demonstrate that micromolar levels of arachidonic acid inhibit glutamate uptake mediated by EAAT1, a human excitatory amino acid transporter widely expressed in brain and cerebellum, by reducing the maximal transport rate approximately 30%. In contrast, arachidonic acid increased transport mediated by EAAT2, a subtype abundantly expressed in forebrain and midbrain, by causing the apparent affinity for glutamate to increase more than 2-fold. The results demonstrate that the response of different glutamate transporter subtypes to arachidonic acid could influence synaptic transmission and modulate excitotoxicity via positive or negative feedback according to the transporter(s) present in a particular region.**

Reuptake of L-glutamate in brain is mediated by a recently isolated family of membrane proteins (1–3). Because of its role as an excitatory neurotransmitter, regulation of the L-glutamate concentration in the synaptic space is critical for normal neurotransmission (for review see Ref. 4). Another important aspect of glutamate transport relates to its role in limiting neurotoxicity, which results from elevated levels of this transmitter during ischemia (5). Recently, members of an excitatory amino acid transporter (EAAT)<sup>1</sup> gene family expressed in human brain have been molecularly cloned and expressed (6). These human transporters as well as corresponding rat homologs are differentially expressed in various brain regions (6, 7).

Arachidonic acid is released by synaptic activation of ionotropic and metabotropic glutamate receptors (8, 9) as well as during ischemia (10, 11). Inhibition of glutamate uptake by arachidonic acid has been proposed to play a role in modulation of synaptic transmission (12) and neurotoxicity (10, 11). However, previous studies of arachidonic acid actions on glutamate transport have relied upon experimental systems, which are likely to reflect the activity of multiple subtypes of transporters

such as brain slice and synaptosomal preparations (13–15). The present study characterizes the actions of arachidonic acid on three cloned human brain glutamate transporter subtypes (6) expressed in *Xenopus* oocytes and reveals a heretofore unknown heterogeneity in the transporter response to arachidonic acid.

### EXPERIMENTAL PROCEDURES

**Expression of EAAT Subtypes in Oocytes and HEK293 Cells**—Capped RNA was transcribed from linearized pOTV plasmids containing the coding region of EAAT1–3 (6) using T7 polymerase (Boehringer Mannheim). 50 ng of RNA was injected into stage V oocytes, and experiments were performed 2–6 days later. Stably transformed HEK-293 cells expressing various EAATs were obtained by transfection with pCEP4ΔE constructs that confer hygromycin resistance and utilize the cytomegalovirus promoter to drive transporter expression. pCEP4ΔE was derived from pCEP4 (Invitrogen) by restriction digest with *EcoRV* and *StuI* and religation to delete the EBNA-1 gene and OriP. EAAT coding sequences were isolated as *KpnI/BamHI* fragments and subcloned into pCEP4ΔE. HEK-293 cells stably transformed with the pCEP4ΔE-EAAT plasmids were selected using hygromycin (Sigma) at a concentration of 250 μg/ml.

**L-[<sup>3</sup>H]Glutamate Uptake Measurements in Oocytes and HEK293 Cells**—Uptake was measured in control (uninjected) oocytes and in oocytes expressing EAAT1–3 during a 10-min incubation in ND-96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES pH 7.4) containing 10 μM L-[<sup>3</sup>H]glutamate. Radiolabeled L-glutamate (Amersham) was diluted with an appropriate concentration of unlabeled L-glutamate to give a final specific activity of 100 μCi/μmol. Uptake was terminated by three rapid washes in ice-cold buffer followed by lysis in 0.1% SDS and scintillation counting.

Uptake assays were performed in transfected HEK-293 cells grown to a density of approximately 10<sup>5</sup> cells/well by incubation with 10 μM L-[<sup>3</sup>H]glutamate in serum-free Dulbecco's modified Eagle's medium for 10 min followed by three washes in ice-cold buffer.

**Electrophysiology**—Two-electrode voltage clamp recordings were made with an Axon Instruments Geneclamp interfaced to a Macintosh computer with a MacLab 2E analog/digital converter (ADInstruments, Milford, MA). Oocytes were voltage-clamped at –60 mV and continuously superfused with ND-96 recording solution or solution containing test compounds at various concentrations. The normalized mean concentration response of currents induced by L-glutamate was fitted by least squares to the equation,  $I = I_{max}([Glu]/([Glu] + K_m))$ .  $K_m$  values are expressed as mean ± S.E. from fits to individual oocytes. The effects of arachidonic acid on glutamate-induced currents were determined by co-application as indicated by bars above current traces. The percentage decrease (EAAT1) or increase (EAAT2) in the transport current was calculated by comparing the current amplitudes induced by 30 μM L-glutamate co-applied with varying concentrations of arachidonic acid to the control transport current amplitudes in the same oocytes. Data were fitted to the expression, % change = % change<sub>max</sub> / ([arachidonic acid]<sup>n</sup> / ([arachidonic acid]<sup>n</sup> +  $K_{0.5}^n$ )).  $K_{0.5}$  values are expressed as mean ± S.E. from fits to individual oocytes. Experiments were repeated in batches of oocytes from five different frogs. Arachidonic acid (Calbiochem) was stored in 100 mM stock solutions in dimethyl sulfoxide and dissolved in recording solution by sonication immediately prior to use. All other compounds were from Sigma.

### RESULTS

*Xenopus* oocytes were injected with cRNAs encoding the human excitatory transporters EAAT1, EAAT2, and EAAT3 (6). The inward transport current resulting from superfusion of 30 μM L-glutamate in voltage-clamped oocytes expressing EAAT1 was decreased 20–30% by co-application of 100 μM arachidonate (Fig. 1). In contrast, the transport current mediated by EAAT2 was increased approximately 2-fold by arachidonate, while the EAAT3 current was increased only slightly (Fig. 1). Both the inhibition and the increase in transport currents had

\* This work was supported by National Institutes of Health Grant GM48709. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Vollum Inst., Oregon Health Sciences University, 3181 SW Sam Jackson Pk. Rd., Portland, OR 97201. Tel.: 503-494-4601; Fax: 503-494-2285; E-mail: kavanaugh@ohsu.edu.

<sup>1</sup> The abbreviation used is: EAAT1, EAAT2, EAAT3, human excitatory amino acid transporters 1–3.

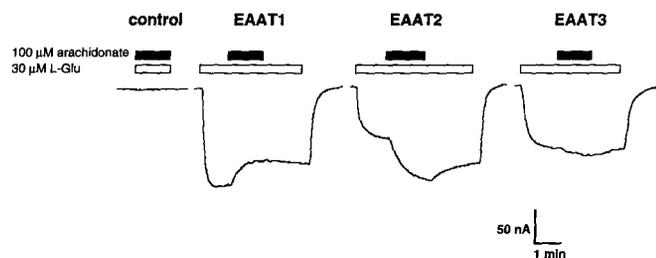


FIG. 1. Differential effects of arachidonic acid on glutamate-induced currents recorded in voltage-clamped uninjected oocytes (control) and oocytes injected with RNA transcribed from human EAAT cDNAs. Cells were clamped at  $-60$  mV, and compounds were superfused for the times indicated by open ( $30 \mu\text{M}$  L-glutamate) and closed ( $100 \mu\text{M}$  arachidonic acid) bars. Identical results were seen in batches of oocytes from five frogs.

a rapid onset and reversed slowly upon washout of arachidonate (Fig. 1). These effects were due to specific interaction with the transporters, as application of arachidonate alone or with glutamate did not induce a current in uninjected oocytes (Fig. 1). In addition, arachidonate alone did not induce any current in oocytes expressing the transporters nor were the glutamate transport currents altered by co-application of vehicle ( $0.1\%$  dimethyl sulfoxide) with glutamate (not shown). In order to determine whether the arachidonate-induced changes in transport currents mediated by EAAT1 and EAAT2 resulted from changes in glutamate uptake, measurements were made of radiolabeled L-glutamate transport mediated by these subtypes. Uptake of  $10 \mu\text{M}$  L- $^3\text{H}$ glutamate into oocytes expressing the transporters was linear for at least 20 min and was increased 10–100-fold over control (uninjected or water-injected) oocytes. In agreement with voltage clamp measurements, addition of arachidonic acid ( $100 \mu\text{M}$ ) resulted in differential effects on the transporters (Fig. 2). Specific uptake in oocytes expressing EAAT1 was decreased from  $275 \pm 9$  to  $210 \pm 10$  fmol/oocyte/s (mean  $\pm$  S.E.,  $n = 6$ ). In contrast, uptake in oocytes expressing EAAT2 was increased from  $75 \pm 6$  to  $143 \pm 11$  fmol/oocyte/s ( $n = 6$ ; Fig. 2). Similar effects were seen in measurements of  $10 \mu\text{M}$  L- $^3\text{H}$ glutamate into the human embryonic kidney cell line HEK-293 stably transfected with EAAT1 or EAAT2. Co-application of  $100 \mu\text{M}$  arachidonate reduced EAAT1-mediated uptake from  $158 \pm 8$  to  $123 \pm 9$  pmol/ $10^5$  cells ( $n = 6$ ), while uptake of L- $^3\text{H}$ glutamate mediated by EAAT2 was increased from  $120 \pm 6$  to  $175 \pm 6$  pmol/ $10^5$  cells ( $n = 6$ ). The background in control (non-transfected) cells was not significantly changed by addition of arachidonate ( $10.9 \pm 1.0$  control versus  $9.9 \pm 1.0$  pmol/ $10^5$  cells;  $n = 6$ ).

The effects of arachidonate on transport in oocytes expressing EAAT1 and EAAT2 were dose-dependent and saturable. The  $K_{0.5}$  for the maximal inhibition of the EAAT1 current was  $16 \pm 6 \mu\text{M}$ , while the  $K_{0.5}$  for maximal increase of the EAAT2 current was  $59 \pm 6 \mu\text{M}$  ( $n = 5$ ; Fig. 3, A and B). The kinetic mechanism underlying the modulation of transport was investigated by analyzing the effect of arachidonic acid on the glutamate concentration response. Co-application of  $100 \mu\text{M}$  arachidonic acid with varying doses of glutamate to oocytes expressing EAAT1 resulted in a  $29 \pm 1\%$  decrease in the maximal current relative to control, while the apparent affinity for glutamate was not significantly altered ( $K_m = 42 \pm 3$  versus  $37 \pm 1 \mu\text{M}$ ,  $n = 5$ ; Fig. 4A). In contrast, the EAAT2 transport currents were enhanced via a decrease in the transport affinity constant for glutamate from  $36 \pm 7$  to  $14 \pm 1 \mu\text{M}$  ( $n = 5$ ; Fig. 4B).

The pharmacological mechanism of action of arachidonic acid was investigated by testing structural analogues and inhibitors of its metabolism. The increase in EAAT2-mediated transport of  $10 \mu\text{M}$  L-glutamate caused by  $100 \mu\text{M}$  arachidonic acid was

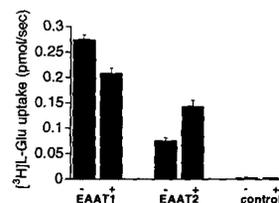


FIG. 2. Arachidonic acid inhibits uptake of L- $^3\text{H}$ glutamate into oocytes expressing EAAT1 and stimulates uptake mediated by EAAT2. Uptake in control (uninjected) oocytes was unaffected by arachidonic acid.

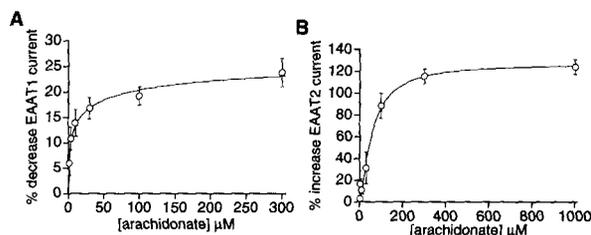


FIG. 3. Concentration response for arachidonic acid effects on glutamate transport currents mediated by EAAT1 (A) and EAAT2 (B). The percentage decrease or increase was calculated by comparing the current amplitudes induced by  $30 \mu\text{M}$  L-glutamate co-applied with varying concentrations of arachidonic acid to the control transport current amplitudes in response to  $30 \mu\text{M}$  L-glutamate in the same oocytes. Data points represent mean  $\pm$  S.E. ( $n = 3-4$ ).

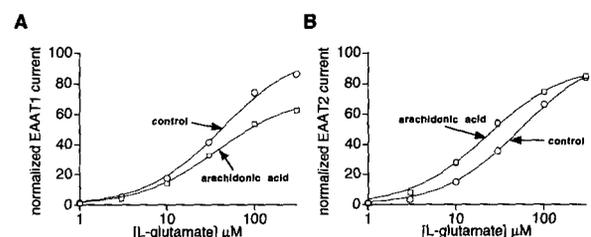


FIG. 4. Differential effects of arachidonic acid on transport kinetic parameters for EAAT1 (A) and EAAT2 (B). Data points representing normalized mean  $\pm$  S.E. for 5 oocytes were fit to  $I = I_{max}([Glu]/([Glu] + K_m))$ . A, co-application of  $100 \mu\text{M}$  arachidonic acid reduced the maximal EAAT1 current  $29 \pm 1\%$  without significantly changing the apparent affinity for glutamate. B, glutamate concentration dependence of EAAT2 transport currents was shifted by  $100 \mu\text{M}$  arachidonic acid from  $36 \pm 7$  to  $14 \pm 1 \mu\text{M}$  without significantly affecting the  $I_{max}$ .

compared with equal concentrations of linolenic, linoleic, or arachidonic acid. The order of efficacy relative to arachidonic acid was linolenic acid ( $91 \pm 21\%$ )  $>$  linoleic acid ( $49 \pm 9\%$ )  $\gg$  arachidonic acid ( $1 \pm 5\%$ ;  $n = 3$ ). The same rank order of efficacy relative to arachidonic acid was observed for inhibition of EAAT1: linolenic ( $109 \pm 19\%$ )  $>$  linoleic ( $43 \pm 14\%$ )  $\gg$  arachidonic ( $5 \pm 5\%$ ;  $n = 3$ ). Neither the cyclooxygenase inhibitor indomethacin ( $100 \mu\text{M}$ ) nor the lipoxygenase inhibitor nordihydroguaritic acid ( $50 \mu\text{M}$ ) affected arachidonate inhibition of EAAT1 ( $n = 3$ ) or stimulation of EAAT2 ( $n = 6$ ). These results, together with the rapid onset of modulation (Fig. 1), suggest that both effects on glutamate transport are mediated directly by arachidonic acid itself, rather than via a metabolite.

#### DISCUSSION

Arachidonic acid has been proposed to be a messenger molecule that influences synaptic transmission released by synaptic activation of ionotropic and metabotropic glutamate receptors (8, 9). Its release also occurs following ischemia (10, 11). Arachidonic acid-mediated inhibition, but not stimulation, of glutamate transport has been reported previously (13–16). Similar to the results in the present study, these inhibitory

actions appear to be mediated by arachidonic acid itself, as are its potentiating actions on synaptic transmission in hippocampus (12). Although net L-[<sup>3</sup>H]glutamate uptake into rat brain synaptosomes and slices is reduced by arachidonic acid (13–15), regional heterogeneity in transporter expression would not be resolved in such an assay (6, 7). EAAT1 is relatively abundant in many human brain regions, particularly in cerebellum, while EAAT2 is highly abundant in forebrain and midbrain regions including cortex and basal ganglia (6). EAAT3 is uniformly expressed in brain and periphery at lower levels (6). These distributions are consistent with the immunohistochemical localization in rat brain of the analogous glutamate transporters with which they share >90% sequence identities (7).

The glutamate receptor-mediated stimulation of arachidonate synthesis in glial cells (17) and neurons (8, 9, 18) suggests that arachidonic acid could modulate uptake in both cell types according to which transporter subtype is expressed. In one well defined system, the salamander retinal Mueller cell, arachidonic acid directly inhibits glutamate transport currents (16). In accord with this result, a transcript encoding a glutamate transporter homologous to EAAT1 is highly abundant in these cells, and expression of this salamander transporter in *Xenopus* oocytes confirms that it is negatively modulated by arachidonate.<sup>2</sup> While some studies on cultured mammalian glial cells have demonstrated arachidonic acid inhibition of glutamate uptake (15, 19), a recent study utilizing cultured astroglial cells demonstrated that inhibition of arachidonic acid synthesis during ischemia potentiates toxicity, suggesting a possible neuroprotective action of arachidonic acid (20).

The present results suggest the possibility that arachidonic acid could differentially influence the rate of clearance of synaptically released glutamate, which can in turn influence the kinetics of glutamatergic transmission at some synapses (21, 22). Moreover, modulation of interstitial glutamate levels could lead to changes in synaptic efficacy by tonic activation of receptors (23) or receptor desensitization (24). The actions of

arachidonic acid on glutamate transport kinetics would allow for complex regulation of synaptic transmission as well as excitotoxicity via either positive or negative feedback according to which transporter subtypes are present in a particular pathway.

*Acknowledgments*—We thank Alan North, Gary Westbrook, Scott Eliasoff, Jacques Wadiche, and Nancy Zahniser for discussion.

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<sup>2</sup> N. Zerangue, J. Arriza, S. Amara, and M. Kavanaugh, unpublished results.