A Postsynaptic Excitatory Amino Acid Transporter with Chloride Conductance Functionally Regulated by Neuronal Activity in Cerebellar Purkinje Cells

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Excitatory amino acid (EAA) neurotransmitters induce postsynaptic depolarization by activating receptor-mediated cation conductances, a process known to underlie changes in synaptic efficacy. Using a patch-clamp method, we demonstrate here an EAA-dependent postsynaptic anion conductance mediated by EAA transporters present on cerebellar Purkinje cell bodies and dendrites in culture. This transporter-mediated current was modulated by neuronal activity: it exhibited facilitation for >20 min after transient depolarization accompanied by Ca^{2+} influx. Evidence is presented suggesting that the transporter facilitation is mediated by arachidonate release after Ca^{2+}-dependent activation of phospholipase A_2, which exists in Purkinje cells. This postsynaptic reuptake system may represent a novel modulatory mechanism of synaptic transmission as well as prevent neuronal excitotoxicity.

Key words: Purkinje cell; excitatory amino acid; postsynaptic transporter; chloride conductance; arachidonate; phospholipase A_2; patch clamp

Excitatory amino acid (EAA) transporters on the plasma membrane of presynaptic termini and glial cells have been reported to relieve the postsynaptic excitation by removing EAAs from the extracellular space promptly after their release from presynaptic termini. The Na^+-dependent EAA transporters have been cloned from rabbits and rats, i.e., GLAST (Storck et al., 1992), GLT1 (Pines et al., 1992), and EAAC1 (Kanai and Hediger, 1992), and from humans, i.e., EAAT1–4 (Arriza et al., 1994; Fairman et al., 1995). Recently, the EAA transporters have been reported to have Cl^- conductance in cone photoreceptors (Sarantis et al., 1988; Picaud et al., 1995) and glial cells (Eliasof and Jahr, 1996) of the salamander retina, and in *Xenopus* oocytes expressing human brain EAA transporters (Fairman et al., 1995; Wadiche et al., 1995). The Cl^- conductance may reduce the membrane depolarization induced by the Na^+-dependent electrogenic transport and may maintain efficient transport activity, because the transport activity has been reported to be reduced by membrane depolarization (Nicholls and Attwell, 1990; Schwartz and Tachibana, 1990; Szatkowski et al., 1990); however, the functional linkage between the neuronal EAA transport with Cl^- conductance and the neuronal or synaptic activity remains unclear.

Using the whole-cell patch-clamp method on cultured rat cerebellar Purkinje cells, we isolated the EAA-induced ionic current carried mainly by Cl^-. This current was associated with the postsynaptic EAA transport. Furthermore, we proposed a novel regulation of the transport system by the postsynaptic activity.

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MATERIALS AND METHODS

Culture of rat cerebellar Purkinje cells. Cerebellar Purkinje cells were cultured on several coverslips coated with poly-D-lysine (P-6407; Sigma, St. Louis, MO) as reported previously (Weber and Schachner, 1984; Hirano et al., 1986; Kataoka and Ohmori, 1996). We used Purkinje cells after 3–5 weeks in culture for the experiments reported here.

Whole-cell patch-clamp electrode recording. Cultured Purkinje cells were voltage-clamped by the whole-cell patch-clamp method. Composition of the internal pipette solution was as follows: 160 mM CsCl or choline chloride, 5 mM EGTA, 10 mM HEPES (buffered to pH 7.4 with CsOH). Composition of the external solution was as follows: 153 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 17 mM glucose, 1 μM tetrodotoxin, 20 μM bicuculline, 200 μM 2-amino-5-phosphonovalerate (APV), 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 10 mM HEPES (buffered to pH 7.4 with KOH). The Ca^{2+}-free external solution had the following composition: 155 mM NaCl, 6 mM $MgCl_2$, 17 mM glucose, 1 mM EGTA, 1 μM tetrodotoxin, 20 μM bicuculline, 200 μM APV, 20 μM CNQX, and 10 mM HEPES (buffered to pH 7.4 with KOH). The concentration of K^+ in both the Ca^{2+}-containing and Ca^{2+}-free external solution was 5 mM. The patch-pipette resistance in the external solution was 3–4 MΩ, and the series resistance in whole-cell mode was 8–15 MΩ. Liquid junction potentials between internal and external solutions were corrected by use of the method reported previously (Hagiwara and Ohmori, 1982). Data in the electrophysiological study were obtained at 22–24°C.

Application of L-aspartate (L-Asp). L-Asp was applied to cultured Purkinje cells by the pressure-puff method. L-Asp was dissolved in the external solution and applied to the cell soma by pressure applied internally to the delivery pipette. The tip diameter of the delivery pipette was 3–5 μm, and the pipette was placed 50 μm away from the cell soma. Sufficient pressure was applied to the pipette to apply L-Asp of a defined concentration to the target cell without the L-Asp being diluted by the surrounding medium. L-Asp applied by this method induced a current that was rapidly generated and attained a constant level during the application, indicating that L-Asp was likely applied to the cell at the defined concentration in the pipette. Application of 1–100 μM L-Asp does not activate the ionotropic glutamate receptors in the presence of antagonists (APV and CNQX) or metabotropic glutamate receptors (Sugiyama et al., 1989; Yuzaki and Mikoshiba, 1992) in Purkinje cells.

Immunohistochemistry. A double immunohistochemical staining for cytosolic phospholipase A_2 ($cPLA_2$) and calbindin-D was performed by the combination of the immunogold–silver staining and the avidin–

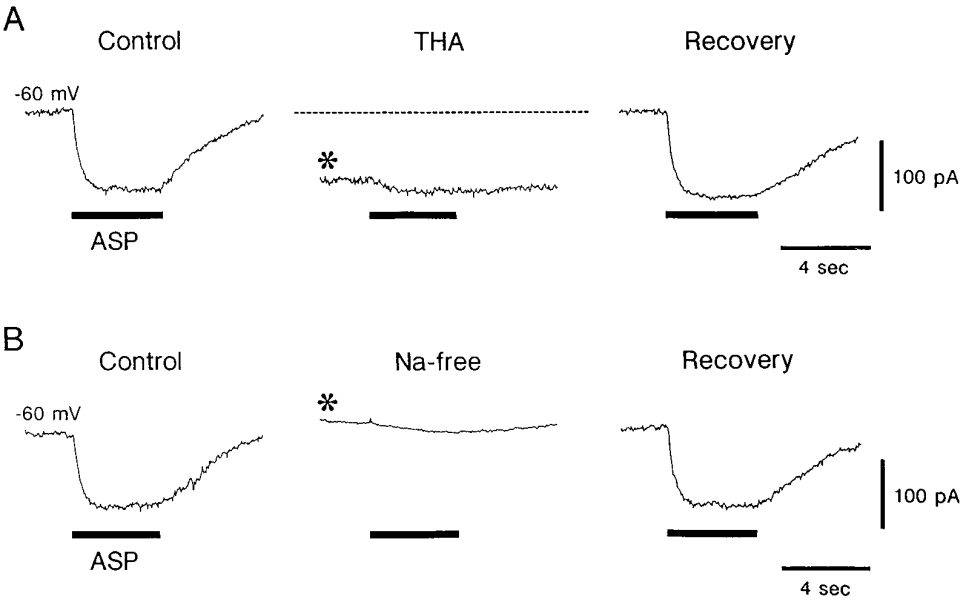

Figure 1. Na^+-dependent EAA transporter-mediated current in Purkinje cells. L-Asp (10 μM) was applied by a pressure-puff method to Purkinje cells voltage-clamped at −60 mV during the times indicated by the *bars*. *A*, An application of 100 μM D(+)-threo-β-hydroxyaspartate (*THA*) to the external solution induced an inward current of 110 pA (*asterisk*) and suppressed further current generation by subsequent application of L-Asp. *B*, The replacement of the extracellular Na^+ with equimolar $choline^+$ (*Na-free*) induced an outward current of 10 pA (*asterisk*) and suppressed the L-Asp-induced current.

biotin–peroxidase complex methods as reported by Sako et al. (1986), with slight modifications. Cultured cerebellar neurons including Purkinje cells were fixed with 4% formaldehyde in Ca^{2+}, Mg^{2+}-free PBS [PBS(−)] for 3 hr at room temperature. After washes with PBS(−), the cells were incubated with PBS(−) containing 4% normal goat serum for 1 hr at room temperature and then were incubated with 1 μg/ml anti-$cPLA_2$ IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS(−) containing 2% normal goat serum for 48 hr at 4°C in a humidified chamber. After incubation for 2 hr at room temperature with biotinylated goat anti-rabbit IgG, the cells were incubated for 2 hr at room temperature with 1 nm gold particle-conjugated streptoavidin (British BioCell International, Cardiff, UK) diluted 1:50 in PBS(−). The gold particles were visualized after treatment with a silver enhancing kit (British BioCell). Thereafter, the cells were incubated overnight at 4°C with anti-calbindin-D IgG (Sigma; 1:200) in PBS(−) containing 2% normal goat serum. The immunohistochemical detection was performed by the avidin–biotin–peroxidase complex method using 3,3′-diaminobenzidine.

Western blot analysis. Rats were decapitated under deep anesthesia with ethyl ether, and the cerebellum was quickly removed and homogenized in 50 mM Tris-Cl, pH 7.5, 0.32 M sucrose, 5 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml E-64 using a Teflon homogenizer. The proteins in the homogenate were precipitated by incubation with 10% (w/v) trichloroacetate (final concentration) on ice for 15 min. The precipitates were collected by centrifugation and solubilized by brief sonication in 9 M urea solution containing 2% (w/v) Triton X-100 and 5% (v/v) 2-mercaptoethanol. After the addition of a solution containing 10% (w/v) lithium dodecylsulfate and 0.1% (w/v) bromophenol blue (one-fifth volume of the solubilization solution), each sample solution was neutralized with 2 M Tris, followed by sonication to disrupt genomic DNA. After separation by 10–20% gradient SDS-PAGE, the proteins were transferred to a Fluoro Trans W polyvinylidene difluoride membrane (Pall Co., Port Washington, NY) by electroblotting using a Bio-Rad transblot SD system (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked for 1 hr at room temperature with a blocking buffer containing 0.5% (w/v) casein and 0.3% (w/v) Tween 20 in PBS(−) and then incubated for 2 hr at room temperature with anti-$cPLA_2$ antibody (1:500 dilution). After it was washed with a blocking buffer, the membrane was incubated with alkaline phosphatase-conjugated second antibody for 1 hr at room temperature. Immunoreactive proteins were detected with a Phototope-Star Western blot detection kit (New England Biolabs, Beverly, MA).

Chemicals. APV, CNQX, and D(+)-threo-β-hydroxyaspartate (THA) were purchased from Tocris Cookson (Bristol, UK). Arachidonate, mepacrine, and indomethacin were from Sigma. U73122, nordihydroguaiaretic acid, and arachidonyl trifluoroketone were purchased from Biomol Research Laboratories (Plymouth Meeting, PA), and TPA, H-7, and KN-62 were from Research Biochemicals (Natick, MA).

RESULTS

EAA transporter-mediated current in cerebellar Purkinje cells

L-glutamate (L-Glu), L-Asp, and L-homocysteate were previously shown to induce a current likely mediated by the EAA transporter in cultured rat cerebellar Purkinje cells by the whole-cell patch-clamp method (Kataoka et al., 1995; Kataoka and Ohmori, 1996). This current was suppressed by neither an antagonist for NMDA (APV) nor one for non-NMDA-type glutamate receptors (CNQX). Recently, Takahashi et al. (1996) also reported the EAA transporter-mediated current containing anion flux in Purkinje cells in rat cerebellar slices. In the present study, the nature of the current induced by the pressure-puff application of 10 μM L-Asp in cultured Purkinje cells was characterized in the solution containing APV and CNQX. L-Asp at that concentration does not activate the metabotropic glutamate receptor in Purkinje cells (Sugiyama et al., 1989; Yuzaki and Mikoshiba, 1992).

L-Asp could not induce the current in Purkinje cells bathed in a solution containing THA (n = 4 cells) (Fig. 1*A*) or D-aspartate (D-Asp; data not shown); both are potent substrates for the high-affinity glutamate transporters (Kanai et al., 1993), and they also induce inward currents in the cells (Fig. 1*A*, *asterisk*). The replacement of extracellular Na^+ with $choline^+$ suppressed this current completely and reversibly (n = 3 cells) (Fig. 1*B*). The local application of L-Asp by pressure-puff or ionophoresis induced the current all over the subcellular regions, including the proximal and distal dendrites (Kataoka et al., 1995). These data were obtained at 22–24°C. When the experiments were performed at 28–30°C, L-Asp could induce the same Na^+-dependent current, which was not suppressed by APV and CNQX and was generated also by THA and D-Asp. These electrophysiological observations indicate that Purkinje cells, which are inhibitory neurons releasing GABA as a neurotransmitter, have a Na^+-dependent, high-affinity EAA transporter on their plasma membrane and take up postsynaptically the EAAs released from innervating excitatory inputs.

Three Na^+-dependent, high-affinity glutamate transporters (GLAST, GLT1, and EAAC1) have been reported to be depen-

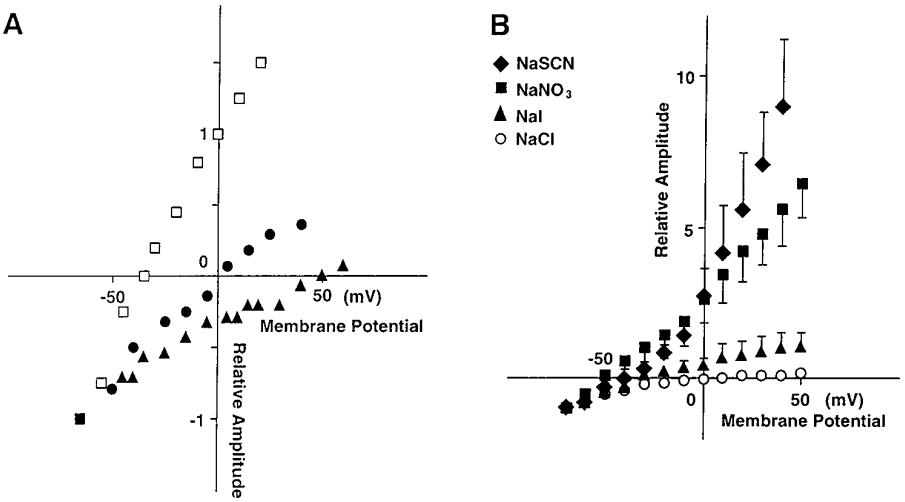

Figure 2. EAA transporter coupled with anion conductance. *A*, Current–voltage relationships for 10 μM L-Asp with internal and external solutions containing different Cl^- concentrations; ●, internal 160 mM Cl^- and external 160 mM Cl^- (E_{Cl} = 0 mV); ▲, internal 160 mM Cl^- and external 20 mM Cl^- (E_{Cl} = 53 mV) by the replacement of external 142 mM Cl^- with equimolar gluconate$^-$; □, internal 14 mM Cl^- and external 160 mM Cl^- (E_{Cl} = −62 mV) by the replacement of internal 146 mM Cl^- with equimolar glucronate$^-$. Relative current amplitude was plotted after the normalization of the amplitude at −65 mV as −1 (■). Data indicated by ● and ▲ were obtained from the same Purkinje cell. *B*, Current–voltage relationships for 10 μM L-Asp with internal 160 mM choline Cl and external 155 mM NaSCN (◆), $NaNO_3$ (■), NaI (▲), or equimolar NaCl (the normal external solution; ○). Relative current amplitude was plotted with the SD after the normalization of the amplitude at −70 mV as −1. All data in *A* and *B* were obtained after the membrane depolarization to −20 mV for 5 sec (see Fig. 3).

dent on the intracellular K^+ concentration (Barbour et al., 1988; Kanai and Hediger, 1992; Klöckner et al., 1993). In sister Purkinje cells in the same culture dish, the transporter-mediated current was induced to almost the same extent by three intracellular cations in the pipette solution more than 10 min after the start of whole-cell recording at −65 mV: K^+, 37 ± 17 pA (n = 6 cells); Cs^+, 48 ± 13 pA (n = 5 cells); choline$^+$, 39 ± 14 pA (n = 3 cells).

Transporter-mediated current carried by anions including Cl^-

The transporter-mediated current induced by L-Asp was reversed at 5–10 mV, and the current–voltage relationship showed a moderate inward rectifying property with equimolar Cl^- concentrations on both sides of the membrane (Fig. 2). D-Asp and THA (data not shown) showed a similar current–voltage relationship as found for L-Asp. When internal or external Cl^- was exchanged for glucronate or gluconate, the reversal potential of the current was shifted; however, it did not completely follow the Nernst equation for Cl^- (Fig. 2*A*). The reversal potential of L-Asp-induced current was −42 ± 5 mV (n = 7 cells) with 14 mM internal Cl^- and 160 mM external Cl^- concentrations (14 Cl_i/160 Cl_o; the calculated E_{Cl} was −62 mV), 7 ± 3 mV (n = 9 cells) with 160 Cl_i/160 Cl_o (E_{Cl} = 0 mV), and 48 ± 11 mV (n = 3 cells) with 160 Cl_i/20 Cl_o (E_{Cl} = 53 mV). The deviation of reversal potentials in the positive direction from E_{Cl} in the two former cases indicates that the transporter-mediated current consists of at least two current components: the dominant one is the current carried by Cl^-, and the other is likely carried by the co-transport of EAA and Na^+. To observe the current mainly carried by the EAA and Na^+ co-transport, L-Asp was applied to the cells near the E_{Cl} (−62 mV) with 14 Cl_i/160 Cl_o. The current amplitude was 15 ± 6 pA (n = 7 cells) and was equivalent to 10% of the current (140 ± 68 pA; n = 9 cells) generated at −60 mV with 160 Cl_i/160 Cl_o (E_{Cl} = 0 mV). This indicates that most (90%) of the transporter-mediated current induced by L-Asp was carried by Cl^- at −60 mV with 160 Cl_i/160 Cl_o. If this uptake system works by the electrochemical energy of co-transported Na^+, the driving force should be diminished as the membrane potential changes in a positive direction (Brew and Attwell, 1987; Barbour et al., 1991), and this would explain the moderate inward-going rectification of the current–voltage relationship with equimolar Cl^- concentrations on both sides of the membrane (Fig. 2*A,B*).

Several kinds of anionic substitutions (SCN^-, NO_3^-, and I^-) for Cl^- in the external solution brought about considerable enhancement of the outward currents (Fig. 2*B*). The sequence ($SCN^- > NO_3^- > I^- > Cl^-$) of relative current amplitude at positive potentials was similar to that for the cloned glutamate transporter from human cortex (EAAT1) (Wadiche et al., 1995) and for the retinal glial glutamate transporter of tiger salamander (Eliasof and Jahr, 1996). On the other hand, exchanging Cl^- for NO_3^- in the pipette solution enhanced the inward current 7–8 times at −65 mV (data not shown).

Postsynaptic depolarization enhances the transporter-mediated current

Membrane depolarization to −30 or to −20 mV for >8 msec enhanced the amplitude of the transporter-mediated current induced by 10 μM L-Asp to 300% of the control when evaluated at 0.5–1.0 min after the depolarization (Fig. 3*A,B*). Membrane depolarization for <8 msec failed to enhance the current. We failed to observe a precise correlation between the duration of depolarization and the amplitude of current enhancement [3.19 ± 0.20 times the control by 10 msec depolarization (n = 5 cells) and 3.14 ± 0.47 times by 5 sec depolarization (n = 8 cells)], which seemed to be because of the incomplete regulation of the actual duration of depolarization, because the large membrane conductance was induced by the depolarization for >8 msec in Purkinje cells. In the following experiments, cells were depolarized for 5 sec to accomplish the complete enhancement in every tested cell. THA (100 μM) occluded completely and reversibly the current induced by 10 μM L-Asp also after the membrane depolarization (data not shown), indicating that the current enhanced by depolarization was also mediated by the transporter.

Although the amplitude of current enhancement was maximum at 1 min after the depolarization and then diminished gradually with time for 5 or 10 min, the enhancement was maintained for >20 min after the membrane depolarization (Fig. 3*B*). The current enhancement by depolarization was prevented completely in a Ca^{2+}-free external solution or by the presence of 300 μM Cd^{2+} in the normal external solution containing Ca^{2+} (Fig. 3*A,C*). BAPTA (30 mM), a potent Ca^{2+} chelator, in the internal

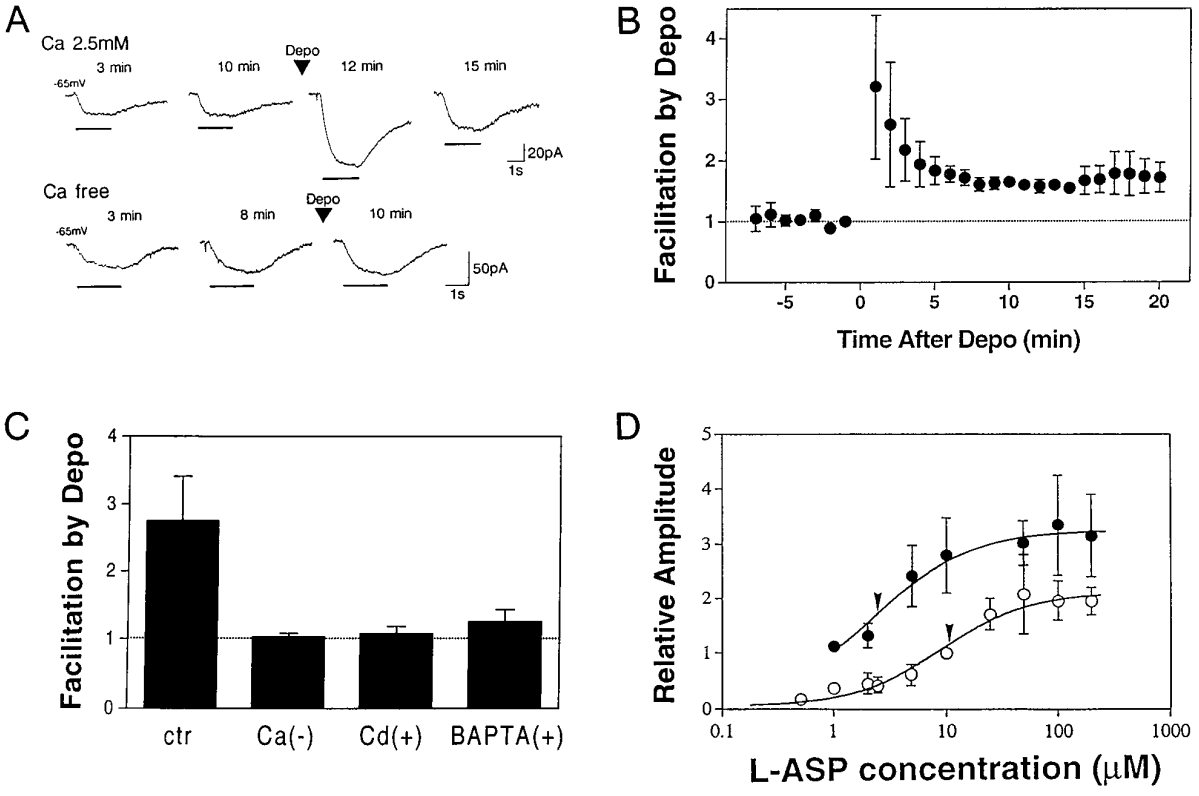

Figure 3. Transporter-mediated current is enhanced by membrane depolarization. *A*, L-Asp (10 μM) was applied to Purkinje cells voltage-clamped at −65 mV during the times indicated by the *bars*. Time from the start of whole-cell recording is indicated above each current trace. Purkinje cells were depolarized to −20 mV for 5 sec, as indicated by *Depo*, at 11 min and at 9 min after the start of whole-cell recording in the normal external solution containing 2.5 mM Ca^{2+} (*Ca 2.5 mM*) and in Ca^{2+}-free external solution (*Ca free*), respectively. *B*, Relative current amplitude was plotted before and after the membrane depolarization (0 min) by the normalization of the amplitude at 1 min before the depolarization as 1. Error bars indicate SDs (n = 4 cells). *C*, Amplitude of the current enhancement at 1 min after the depolarization was plotted with the SD, by the normalization of that before the depolarization as 1. Cells were depolarized in the Ca^{2+}-containing (normal) external solution (*ctr*; n = 4 cells), in the Ca^{2+}-free external solution [*Ca(−)*; n = 3 cells], in the normal external solution containing 300 μM Cd^{2+} [*Cd(+)*; n = 4 cells], and with the internal solution containing 30 mM BAPTA [*BAPTA(+)*; n = 4 cells]. *D*, Concentration–response relationships of the L-Asp-induced current, before (○) and 1 min after (●) the depolarization. L-Asp was pressure-puff-applied to Purkinje cells at 10 μM as a control and at a test concentration by use of two delivery pipettes. Current amplitude was normalized with respect to that induced at 10 μM before the depolarization. Mean value of the relative current amplitude was semilogarithmically plotted against the concentration of L-Asp. Data at each concentration were obtained from three to five cells. SDs are shown as error bars except for the one at 10 μM before the depolarization. If the bars of SDs were shorter than the size of plot symbols, they were not used. Curves of concentration–response relationships were fitted by the Hill equation by use of the Quasi–Newton method: before the depolarization (○), the maximum relative amplitude (I_{max}) was 2.1, and the K_m value was 10.6 μM (*arrowhead*) with a Hill coefficient of 1.1; 1 min after the depolarization (●), the respective values were 3.3 and 2.4 μM (*arrowhead*), with a Hill coefficient of 1.0.

solution inhibited the current enhancement as well (Fig. 3*C*). These studies indicate that the enhancement of EAA transporter-mediated current by membrane depolarization resulted from an increase in the intracellular Ca^{2+} concentration mediated by Ca^{2+} influx through voltage-gated Ca^{2+} channels.

Concentration–response relationships for L-Asp-induced transporter currents before and 1 min after the membrane depolarization (Fig. 3*D*) revealed that the Ca^{2+} influx by membrane depolarization reduced the K_m value from 10.6 to 2.4 μM and increased the maximum current amplitude (I_{max}) by 1.6 times, without any change in the Hill coefficient (=1.0–1.1). The reduced K_m indicates that the membrane depolarization facilitates both the EAA and Na^+ co-transport and the anion conductance by the increase in affinity for EAA. To observe easily the contribution of the anion conductance to an increase in the I_{max}, we enhanced the anion conductance-mediated inward current by 7–8 times using NO_3^- for Cl^- in the patch-pipette solution (data not shown). In this case, almost all the transporter-mediated current was induced through the anion conductance. The anion conductance-mediated current induced by L-Asp (100 μM) for maximal effect (Fig. 3*D*) was increased by 1.6 times (1.58 ± 0.21) after the membrane depolarization, indicating that the increase in I_{max} resulted at least from the increase in anion conductance in the transport system.

Arachidonate enhances the transporter-mediated current

Arachidonate in the extracellular medium enhanced the transporter-mediated current in a concentration-dependent manner, in both the Ca^{2+}-containing (Fig. 4*A,B*) and the Ca^{2+}-free external solution (data not shown). In this event, arachidonate, not its metabolites, seems to be an enhancer, because 25 μM arachidonate with both inhibitors for cyclo-oxygenase (10 μM indomethacin) and lipoxygenase (10 μM nordihydroguaiaretic acid) also enhanced the current (2.31 ± 0.16 times; n = 5 cells) to the same extent as that without these inhibitors (2.50 ± 0.19 times; n = 4 cells).

The preincubation of Purkinje cells with arachidonate reduced

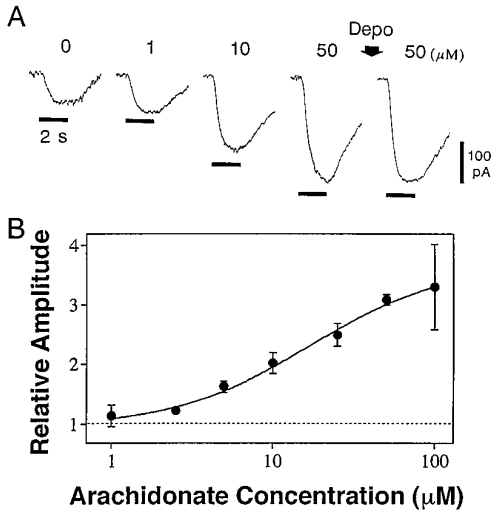

Figure 4. Arachidonate mimics the facilitating effect of membrane depolarization on the transporter-mediated current. *A*, L-Asp-induced current was enhanced by arachidonate application to the normal external solution at several concentrations, which are indicated on each current trace, in a Purkinje cell voltage-clamped at −65 mV. L-Asp was pressure-puff-applied during the times indicated by the *bars*. Arachidonate did not induce any current without the L-Asp application in Purkinje cells. The membrane depolarization (*Depo*) to −20 mV for 5 sec did not enhance the current any more after the enhancement by 50 μM arachidonate. *B*, The extent of enhancement, with the SD, of 10 μM L-Asp-induced current by arachidonate is semilogarithmically plotted against the extracellular arachidonate concentrations. Data at each concentration were obtained from three to five cells. The curve was fitted by the Hill equation by use of the Quasi–Newton method; the maximum relative amplitude was 3.7, and the $K_{0.5}$ value was 19 μM with a Hill coefficient of 1.0.

the extent of depolarization-induced current enhancement in a concentration-dependent fashion (Fig. 5*A*). On the other hand, the depolarization-induced current enhancement before the application of arachidonate occluded the additional current enhancement (0.97 ± 0.12; $n = 4$ cells) by 50 μM arachidonate as well. These findings imply that arachidonate may act as the main mediator of the depolarization-induced enhancement of transporter-mediated current. An inhibitor for PLA_2 and PLC (7–10 μM U73122 in the external solution) suppressed the depolarization-induced enhancement of the transporter-mediated current in the Ca^{2+}-containing external solution (Fig. 5*B*). Two other inhibitors (100 μM mepacrine and 35 μM arachidonyl trifluoroketone in the external solution), which are more selective for PLA_2, also suppressed the enhancement (Fig. 5*B*); however, protein kinase C activator (TPA), protein kinase C inhibitor (H-7), and calcium/calmodulin-dependent protein kinase II inhibitor (KN-62) did not affect the extent of current enhancement by the membrane depolarization: the extent of enhancement was 2.74 ± 1.13 ($n = 4$ cells) times the control with 1 μM TPA-containing external solution, 3.09 ± 1.49 ($n = 9$ cells) with 30 μM H-7-containing internal or external solution, and 2.75 ± 0.63 ($n = 5$ cells) with 50 μM KN-62-containing internal solution.

The rich expression of $cPLA_2$ was immunohistochemically demonstrated in the soma and dendrites of the Purkinje cells, which were identified as anti-calbindin-D-immunopositive cells, and in other neurons in cerebellar cultures (Fig. 6*A*). Preabsorbtion of anti-$cPLA_2$ IgG by $cPLA_2$ antigen (Fig. 6*B*) or the deletion of anti-$cPLA_2$ IgG (Fig. 6*C*) from the immunohistochemical procedure abolished the positive signals for $cPLA_2$.

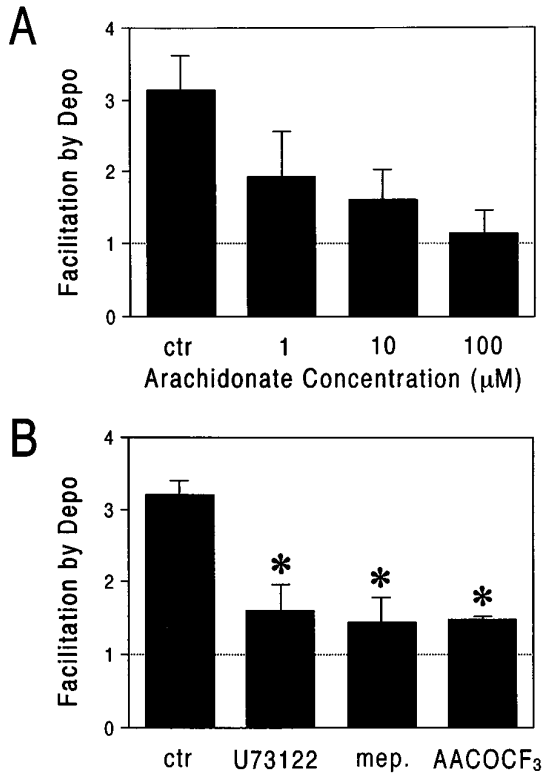

Figure 5. Suppression of the depolarization-induced enhancement of transporter-mediated current by preincubation with arachidonate or application of inhibitors of PLA_2. *A*, Amplitude of the current enhancement at 30 sec after the depolarization (−20 mV, 5 sec) was obtained from sister Purkinje cells in the same culture dish, bathed in the normal external solution (*ctr*; $n = 8$ cells), in the solution containing arachidonate at the given concentrations (1 μM, $n = 4$ cells; 10 μM, $n = 6$ cells; 100 μM, $n = 4$ cells). *B*, Amplitude of the current enhancement at 30 sec after the depolarization (−20 mV, 5 sec) was obtained from Purkinje cells bathed in the normal external solution (*ctr*; $n = 5$ cells), or in the solution containing 7–10 μM *U73122* ($n = 7$ cells), 100 μM mepacrine (*mep.*; $n = 7$ cells), or 35 μM arachidonyl trifluoroketone (*$AACOCF_3$*; $n = 4$ cells). In *A* and *B*, the data were plotted after normalization of the amplitude, with the value before depolarization taken as 1. Error bars indicate SDs. $^*p < 0.001$; significantly different from the control (*ctr*) value.

Western blot analysis of rat cerebellar tissue with the same antibody against $cPLA_2$ showed a single major band at ~100 kDa and several minor bands (Fig. 6*D*). The major band indicates the presence of $cPLA_2$ in the cerebellum. The minor bands having lower molecular weights may be caused by degradation of $cPLA_2$ protein because protease inhibitors prevented the production of those minor bands (H. Morii, personal communication). These findings suggest that arachidonate, released through the $cPLA_2$ activation, is likely the main mediator for the facilitation of postsynaptic transport system by the membrane depolarization in Purkinje cells.

DISCUSSION

Two types of Na^+-dependent EAA transporters were immunohistochemically demonstrated in rat or mouse cerebellar Purkinje cells, EAAC1 (Rothstein et al., 1994) and EAAT4 (Yamada et al., 1996). EAAC1 has a smaller anion conductance (Kanai et al., 1995) than EAAT4 (Fairman et al., 1995), and an EAA transport by EAAC1 was inhibited by extracellular arachidonate (H. Morii and Y. Watanabe, unpublished observations). These data suggest that the present transporter-mediated current in Purkinje cells

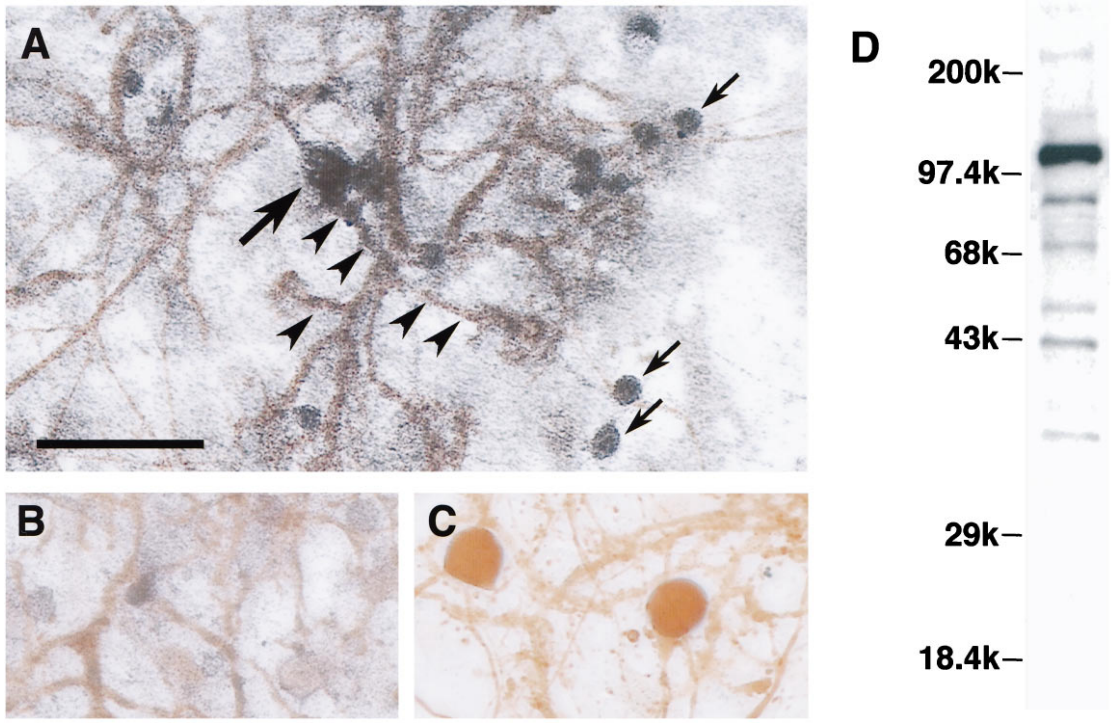

Figure 6. Immunohistochemical and Western blot analysis for $cPLA_2$ in the cerebellum. *A*, Immunopositive staining for $cPLA_2$, shown as *silver grains*, is observed in Purkinje cell soma (*large arrow*) and dendrites (*arrowheads*) and in other neurons including granule cells (*small arrows*) in this cerebellar culture. Scale bar, 50 μm. *B*, Antibody against $cPLA_2$ was preabsorbed by exogenous antigen before the immunohistochemical procedure. *C*, Antibody against $cPLA_2$ was omitted from the immunohistochemical procedure. *D*, Western blot analysis for $cPLA_2$ in cerebellar tissue. In *A–C*, Purkinje cells are identified as calbindin-D-immunopositive cells (*brown*). *A–C* are at the same magnification.

was mainly brought about by EAAT4, because the present current was carried dominantly by anion including Cl^- (Fig. 2) and was enhanced by an extracellular application of arachidonate (Fig. 4).

Three types of cloned Na^+-dependent EAA transporters [GLAST, GLT1, and EAAC1 (EAAT3)] have been reported to be dependent on the intracellular K^+ concentration (Barbour et al., 1988; Kanai and Hediger, 1992; Klöckner et al., 1993; Zerangue and Kavanaugh, 1996). The present transporter-mediated current in cultured Purkinje cells depended on Na^+ in the external solution (Fig. 1*B*) but not on cations (K^+, Cs^+, and choline$^+$) in the patch-pipette solution, although Szatkowski et al. (1991) pointed out a possibility of the incomplete removal of intracellular K^+ in the cells with Cs^+ or choline$^+$ in the patch pipette; however, Cs^+ or choline$^+$ in the patch pipette decreased the electrical noise level within a minute after the perforation of the patch membrane in Purkinje cells. In addition, when we applied GABA to Purkinje cells, which have the $GABA_A$ receptor, the reversal potential of $GABA_A$ receptor-mediated current shifted to near E_{Cl}, defined by the Cl^- concentration in the patch pipette, within a minute after the perforation of the patch membrane (data not shown). These observations suggest the considerable fast exchange of intracellular ions including K^+ for those in the patch pipette. Barbour et al. (1991) and Eliasof and Jahr (1996) reported a glial glutamate transporter-mediated current in salamander retina, using Cs^+ as a cation in the internal pipette solution, although Cs^+ may be able to substitute for K^+ for the transporter. Schwartz and Tachibana (1990), monitoring the intracellular K^+ concentration, reported that the glutamate transporter activity in the same glial cells was independent of intracellular cations, including K^+, Cs^+, TEA^+, and choline$^+$. Recently, however, Takahashi et al. (1996) isolated the EAA transporter-mediated current in Purkinje cells in rat cerebellar slices and found that the transporter could be run backwards by use of an internal solution containing sodium glutamate when the extracellular K^+ concentration was raised in the depolarized cells. Thus it has remained unclear whether the EAA uptake from the extracellular space requires the intracellular K^+ in intact Purkinje cells. Further study would be necessary to clarify the intracellular K^+ dependence of the EAA transport in Purkinje cells.

Mature cerebellar Purkinje cells have AMPA-type glutamate receptors, not the NMDA-type (Llano et al., 1991; Kataoka and Ohmori, 1996). The postsynaptic uptake system in Purkinje cells is activated at very low concentrations of EAA (<1 μM) in the extracellular space, and such concentrations can no longer activate AMPA-type glutamate receptors in Purkinje cells (Kataoka and Ohmori, 1996). The higher sensitivity of the uptake system to EAAs is thought to be necessary to maintain the resting EAA concentrations below the concentration that activates postsynaptic receptors. Furthermore, the postsynaptic Cl^- conductance in this transporter is extremely suitable for the maintenance of normal synaptic function: the postsynaptic membrane excitation in Purkinje cells can be terminated promptly, and the membrane can even be hyperpolarized. It has been reported that the transport systems in the excitatory synapse between parallel fiber or climbing fiber and Purkinje cell shorten the decay time and reduce the amplitude of excitatory postsynaptic current (Barbour et al., 1994; Takahashi et al., 1995, 1996). The postsynaptic transport system of EAA is thought to be responsible for the shape of the EPSP in Purkinje cells.

Membrane depolarization to -30 or -20 mV for >8 msec, which was accompanied by Ca^{2+} influx, enhanced the amplitude

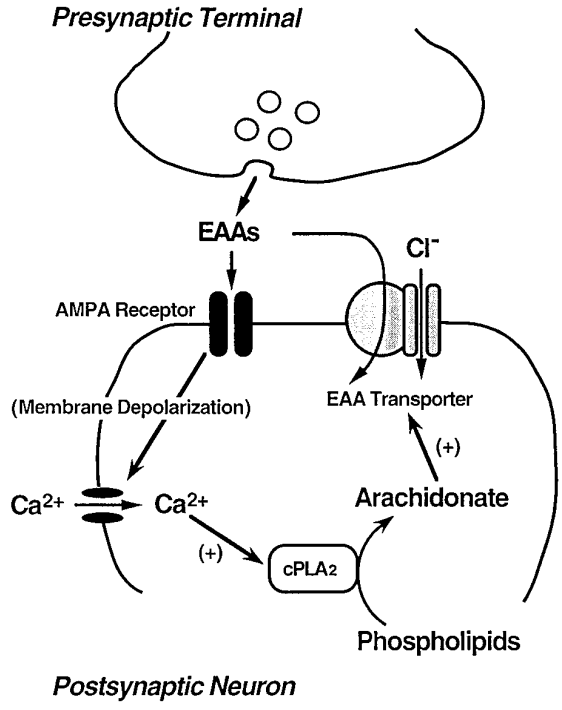

Figure 7. Schematic drawing of the expected regulatory mechanism of EAA transport system with Cl^- conductance in the postsynaptic Purkinje cell.

of the transporter-mediated current, and this enhancement was maintained for >20 min. THA, a substrate for EAA transporters, occluded the L-Asp-induced current even after the membrane depolarization, indicating that the enhanced current was also mediated by the EAA transport system. Does the membrane depolarization facilitate the EAA and Na^+ co-transport or increase only the anion conductance in the system? The reduced K_m value after the membrane depolarization in the concentration–response relationship for L-Asp (Fig. 3*D*) indicates that the depolarization facilitates both the EAA and Na^+ co-transport and also the anion conductance by the increase in affinity for EAA. The anion (NO_3^-) conductance-mediated current induced by L-Asp (100 μM) for maximal effect was also enhanced (1.6 times) by the membrane depolarization, indicating that the increase in I_{max} resulted, at least, from the increase in anion conductance in this system. It has remained unclear whether the EAA and Na^+ co-transport is concerned with the increase in I_{max}, because we could not get reliable data because the amplitude of the L-Asp and Na^+ co-transport-mediated current that was recorded at E_{Cl} was much smaller than the anion conductance-mediated current. Kinetic models indicating no thermodynamic coupling between the EAA transport and anion flux in the transport system were proposed using *Xenopus* oocytes expressing the human brain EAA transporters (Wadiche et al., 1995) or salamander retinal glial cells (Billups et al., 1996). If the EAA transport system in Purkinje cells has a similar kinetics in these reports, the membrane depolarization may increase the turnover rate of EAA transport or increase the anion flux in a transport cycle, as well as increase affinity for EAA on the transporter.

The enhancement of transporter-mediated current by the membrane depolarization with Ca^{2+} influx is most likely induced by arachidonate release via the activation of $cPLA_2$ in Purkinje cells (Fig. 7). This cytosolic enzyme is known to be activated by Ca^{2+} or ligands and to release arachidonate from membrane phospholipids (Clark et al., 1991). Protein kinase C has been reported to be activated by arachidonate (Blobe et al., 1995). In Purkinje cells, however, released arachidonate seems to directly facilitate the transport system without a mediation of other intracellular messenger-related enzymes, including protein kinase C and calcium/calmodulin-dependent protein kinase II, because the activation or inhibition of these protein kinases failed to affect the depolarization-induced facilitation.

Arachidonate application has been reported to suppress EAA uptake in brain cortical synaptosomes or cortical slices (Chan et al., 1983; Volterra et al., 1992) and also has been reported to suppress the transporter-mediated current in glial cells (Barbour et al., 1989). Zerangue et al. (1995), using *Xenopus* oocytes expressing cloned human brain EAA transporters, reported that L-Glu uptake and also the uptake-mediated current were suppressed with a decrease in the maximal current amplitude in EAAT1 and were enhanced with a decrease in K_m value in EAAT2 by an extracellular arachidonate application. EAAT3, which has a sequence similar to that of EAAC1 (Arriza et al., 1994), a neuronal transporter, was affected slightly by an arachidonate application in the external solution. Recently, H. Morii and Y. Watanabe (unpublished observations) showed that EAAC1 expressed in C6 glioma cells was suppressed by arachidonate applied in the external solution. On the other hand, it was facilitated through an activation of protein kinase C by endogenously released arachidonate. These findings reveal that arachidonate regulates the EAA transport system by complex mechanisms. As far as we know, the present study on Purkinje cells is the first demonstration suggesting that the EAA transport system is regulated by arachidonate endogenously released from the postsynaptic neuron in a neuronal activity-dependent manner (Fig. 7).

EAA transport systems in the excitatory synapse between parallel fiber or climbing fiber and Purkinje cell have been reported to shorten the decay time and reduce the amplitude of excitatory postsynaptic current (Barbour et al., 1994; Takahashi et al., 1995, 1996). The postsynaptic transport system in Purkinje cells would be responsible for those events. When excitatory inputs to Purkinje cells generate sufficient postsynaptic membrane depolarization or activate the metabotropic glutamate receptors, activation of which is accompanied by an increase in postsynaptic Ca^{2+} in Purkinje cells, the postsynaptic transporter with Cl^- conductance is facilitated for >20 min by the arachidonate release. The prolonged facilitation of the transport system may restrain the postsynaptic activity for that period by shortening the EPSP decay time and reducing the EPSP amplitude at each synapse on Purkinje cells. This event may be one of the factors that bring about the activity-dependent prolonged depression of EPSP in Purkinje cells. This transport system is thought to be a new postsynaptic modulatory mechanism for synaptic transmission, as well as a protective mediator against postneuronal excitotoxicity.

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