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Release of [^{3}H]-D-Aspartate from Primary Astrocyte Cultures in Response to Raised External Potassium

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There are significant Ca^{2+}-independent increases in extracellular glutamate and aspartate during various CNS insults such as ischemia and anoxia. However, the cellular sources of such presumed nonvesicular excitatory amino acid (EAA) release have not been established. To further explore potential mechanisms and sites for EAA release, we studied the release of preloaded [^{3}H]-D-aspartate from primary cultured astrocytes prepared from the cerebral cortices of rat pups. Two phases of release were seen in response to raised KCl. The first phase was small and transient, and the second phase was slower and increased progressively. The initial phase of [^{3}H]-D-aspartate release was greatly enhanced by ouabain pretreatment and was inhibited when astrocytes were preexposed to the EAA transport inhibitor threo-hydroxy β-aspartic acid (THBA). Neither of these manipulations affected the second release component. The second phase of release was inhibited by an anion channel blocker, L-644,711, which is known to inhibit hypotonic swelling-induced release of EAA. Ouabain also resulted in the first phase of release occurring at lower $[K^+]_o$. Omission of Ca^{2+} had no effect on either phase of [^{3}H]-D-aspartate release. These results support the hypothesis that the first component of release in cultured astrocytes is a reversal of the glutamate transporter, and the second component is a result of high KCl-induced swelling. Because marked increases in $[K^+]_o$ are well established in CNS pathologies such as ischemia, such release may represent a significant source for the increased extracellular EAAs seen in such conditions.

Key words: glutamate; transporter; astroglia; ischemia; potassium; reversal; swelling

Glutamate and aspartate are the major excitatory neurotransmitters in the mammalian CNS (Fonnum, 1984; Erecinska and Silver, 1990). There is tight regulation of extracellular glutamate levels, which are normally measured to be ~1-2 μM (Erecinska and Silver, 1990). High-affinity Na^+-dependent glutamate transporters are thought to be primarily responsible for maintaining this low extracellular glutamate concentration and are present on both neurons and astrocytes. There are now known to be at least three different subtypes of glutamate transporters (GLAST-1, GLT-1 and EAAC1) in the rat, as well as an EAAT4 isoform in human cerebellum (Pines et al., 1992; Kanai and Hediger, 1992; Storck et al., 1992; Wadiche et al., 1995). Recent work has suggested that GLAST-1 and GLT-1 are primarily responsible for maintaining low $[glu^-]_o$ (Nicholls and Attwell, 1990; Rothstein et al., 1994). Glutamate levels have been shown to increase during ischemia and other CNS insults (Benveniste et al., 1984; Wahl et al., 1994), and if *in vitro* extracellular glutamate levels increase $\geq$100 μM for longer than 5 min, neuronal death can occur (Choi et al., 1987).

It has been considered that there are at least three glutamate pools that can contribute to glutamate release during CNS insults. One is Ca^{2+}-dependent vesicular release from nerve terminals (Benveniste et al., 1984). The other two are cytosolic and can be released by Ca^{2+}-independent reversal of the glutamate transporter (Nicholls and Attwell, 1990; Szatkowski et al., 1990; Attwell et al., 1993). Another mechanism seen in primary astrocyte cultures is a swelling-induced, Ca^{2+}-independent release (Kimelberg et al., 1990). It has been proposed that inhibition of uptake and/or reversal of the glutamate transporter can occur when the electrochemical gradients for Na^+ and K^+ are disrupted during CNS insults; these effects can contribute significantly to the increased $[glu]_o$ seen during pathological states (Hansen, 1985; Ikeda et al., 1989; Attwell et al., 1993; Wahl et al., 1994).

This study focuses on showing how astrocytes might contribute to glutamate release during ischemia by measuring the efflux of preloaded [^{3}H]-D-aspartate under varying extracellular $[K^+]$ and extracellular and intracellular $[Na^+]$, using primary astrocyte cultures. A previous study from this laboratory has shown that when astrocytes were exposed to 100 mM KCl HEPES buffer not only was uptake inhibited, but [^{3}H]-D-aspartate was released in a biphasic manner (Kimelberg et al., 1995). We proposed then that the initial transient phase of [^{3}H]-D-aspartate release could be a result of reversal of the glutamate transporter, and the second phase was a KCl swelling-induced release process. In the present study, we used manipulations of the ion gradients and pharmacological treatments to further support this hypothesis. In addition, when we added ouabain to increase $[Na^+]_i$, we found that reversal of the glutamate transporter was the prominent form of glutamate release and that its sensitivity to varying $[KCl]_o$ was markedly increased.

MATERIALS AND METHODS

[^{3}H]-D-aspartate was obtained from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma (St. Louis, MO). Culture media and materials were from Gibco.

Cell culture. Primary astrocyte cultures were prepared from the cerebral cortex as described by Frangakis and Kimelberg (1984). In brief, the cerebral hemispheres of newborn rats (Sprague Dawley) were removed

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and the meninges carefully dissected away. The cortices were turned over from front to back exposing the hippocampus, which is removed along with the meninges from the underside of the hemispheres. The tissue was extracted with three to five 10-min dissociations with Dispase II in Joklik S-MEM (Boehringer Mannheim Biochemicals, neutral protease, Dispase Grade II). The first extraction was discarded, and DNase (3 drops of 4 mg/ml for 10 ml of S-MEM) was added for the second extraction. The dissociated cells were seeded and grown on poly D-lysine-coated 18 × 18 mm coverslips (Bellco Biotechnology, Vineland, NJ). Cultures were used after approximately 3–4 weeks when the cells reached a confluent monolayer. Immunocytochemistry showed >95% of the cells stained positively for the astrocytic marker glial fibrillary acidic protein.

Efflux measurements. Astrocytes grown on coverslips were incubated overnight in 2.5 ml of MEM containing 10% horse serum, together with 4 μCi/ml of [^{3}H]-D-aspartate (1 mCi/ml; specific activity, 86.4 mCi/mg aspartate). In some experiments, 8 μCi/ml $Na_2{}^{51}CrO_4$ was also added to the incubation medium (1 μCi/ml; specific activity, 50 mCi/mg Cr). The appearance of ^{51}Cr in the perfusate during release experiments can be used to determine whether an increase in [^{3}H]-D-aspartate release is a result of cell detachment or lysis (Kimelberg et al., 1993). Radiolabeled D-aspartate is used as a nonmetabolizable marker for the intracellular glutamate and aspartate pools. Both of these amino acids are transported on the same carrier protein and label the nonvesicular pool of EAAs (Erecinska and Silver, 1990; Barbour et al., 1993). D-glutamate is not taken up by the transporter. The loaded coverslips were inserted into a Lucite perfusion chamber with a cut out depression in the bottom for the 18 × 18 mm glass coverslips. The chamber has a screw top and when screwed down leaves a space above the cells of around 100 μm. This perfusion chamber is well suited for measuring the release of [^{3}H]-D-aspartate from astrocytes in response to various KCl buffers because the volume in the chamber is relatively small (18 × 18 × 0.1 mm = 32.4 μl). This chamber allows a complete change of the perfusing buffer within 2 min, as determined by removal of a trypan blue solution.

The cells were perfused with HEPES-buffered solution consisting of 140 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM (+)D-glucose, 25 mM HEPES. NaOH (10 N) was used to pH the buffers to 7.4. Increased KCl buffers were made by replacing NaCl with KCl. The osmolarity of all buffers were measured by a freezing point osmometer (Advanced Instruments, Needham Heights, MA); the osmolarities were 285–290 mOsm. Sucrose was added to make any adjustments in osmolarity to exactly 290.

The lucite chamber and a fraction collector were placed in an incubator set at 37°C, and the perfusate was collected in 1 min intervals. At the end of the experiment, the cells were digested off the coverslip with 1 N NaOH. The radioactivity was counted using a Packard Beckman LS 3801 Liquid Scintillation Analyzer (Beckman Instruments, Irvine, CA). Percent fractional release for each time point was calculated by summing the radioactive counts from the end time point to the beginning of each minute plus the radioactivity left in the cell digest and dividing the dpms released in each minute by these summed dpms. The number of release experiments for each condition ranged from two to four as indicated in each figure legend.

For Figure 1*B*, a paired *t* test was used to compare corresponding times for the different conditions, and all error bars are SEM. Two separate components of release were seen, and the initial eight points encompassing the first peak and the last eight points representing the second peak were used in analysis of the effects of varying [KCl]. The middle four points were not considered because of uncertainty of contribution of either component to this [^{3}H]-D-aspartate release. Basal release, which was the constant release rate before increasing KCl, was then subtracted from all values.

Intracellular Ca^{2+} measurements. Intracellular $[Ca^{2+}]_i$ was measured using a monochromator-based spectrophotofluorimetric system (Model RF D-4010 Deltascan, PTI, South Brunswick, NJ), with cells loaded with fura-2. The excitation wavelengths were set at 340 and 380 nm with a 2 nm bandwidth. The emission was measured at 505 nm. A 1 mM stock of fura-2 was made by dissolving the powder in a DMSO stock solution, which was then divided into 20 μl aliquots in Eppendorf tubes. The vials were kept frozen at −20°C until use. Three- to 4-week-old primary astrocyte cultures grown on 25 mm glass coverslips were incubated with 10 μM fura-2 for 30 min in normal HEPES buffer, after which the cells were washed to remove unloaded fura-2.

The coverslips were placed in a PDMI-2 open perfusion chamber (Medical Systems, Greenville, NY). Temperature was maintained at 36.5°C ± 0.5°C by a TC-202 bipolar temperature controller (Medical Systems, Greenville, NY). Buffers were changed by adding 1 ml of prewarmed buffers (approximately 36°C) by a pipette to 0.5 ml bath solution in the coverslip dish. The 0.5 ml volume was maintained by aspiration (LU-ASP, Medical Systems, Greenville, NY). The field being measured was a portion of a single cell that excluded the nucleus and any bright dots of fluorescence that might be a result of dye sequestration.

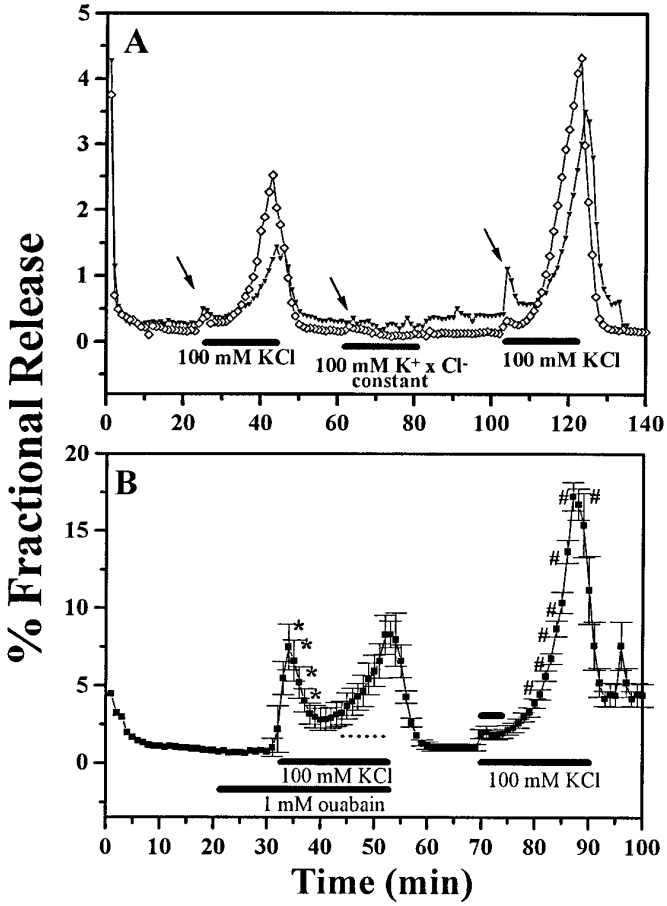

Figure 1. [^{3}H]-D-aspartate release from astrocytes exposed to high K^+. *A*, [^{3}H]-D-aspartate release induced by isotonic 100 mM KCl and isotonic 100 mM $K^+ \times Cl^-$ product constant buffer. K^+ replaces Na^+ in 100 mM KCl, and in the product constant buffer an impermeant anion, gluconate, replaces 122 mM Cl^- in addition to substituting Na^+ with K^+. *Arrows* mark small initial release peaks or their expected positions. Exposures to 100 mM KCl also shows a progressively increasing second phase of release. Results of two separate experiments are plotted. *B*, The effect of 1 mM ouabain on the release of [^{3}H]-D-aspartate. The initial peak is significantly increased with 1 mM ouabain present for 10 min before and during exposure to 100 mM KCl [n = 4 (±SEM) of four experiments]. The *asterisks* represent the time points that are significantly different from the *solid bar* above data points in the second 100 mM KCl exposure without ouabain. The *hatch marks* on the second phase of release indicate a significantly greater release than the *dotted bar* under the KCl + ouabain exposure (paired t test, $p < 0.05$, each pair of points independently compared).

RESULTS

Enhancement of the initial phase of release with ouabain

Understanding the effects of the varying K^+ and Na^+ gradients on the glutamate transporter has been facilitated by recent understanding that both of these ions contribute to the activity of the transporter, as shown in the model in Figure 9. For each glutamate transported inward there is cotransport of one positive charge, which also makes the transporter dependent on membrane potential (Nicholls and Attwell, 1990; Szatkowski et al.,

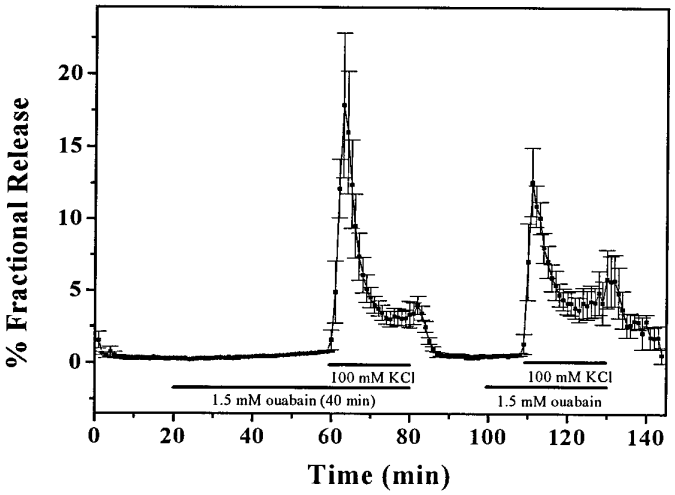

Figure 2. Extended ouabain pretreatment enhances the initial phase of release. The effect of 40 min exposure to 1.5 mM ouabain compared to the standard exposure of 10 min ($n = 3$; error bars show SEM).

1990; Attwell et al., 1993; Wadiche et al., 1995). For reversal, the gradients are simply switched.

Figure 1*A* shows the release pattern of [^{3}H]-D-aspartate from astrocyte cultures when exposed to 100 mM KCl buffer. The initial release component (first peak or shoulder) is small and transient, followed by a slower but progressively increasing phase of release. When the cells were exposed to 100 mM K^+ but with reduced Cl^- to keep the $K^+ \times Cl^-$ product constant, different release characteristics were observed with only the initial component being barely detectable (Fig. 1*A*, middle exposure). In the product constant buffer Cl^- was replaced with the impermeant anion gluconate, which cannot enter the cell with K^+ to cause swelling (Boyle and Conway, 1941; Hodgkin and Horowicz, 1959).

If the initial peak of release is reversal of the glutamate transporter, then increasing intracellular Na^+ concentration in conjunction with exposure to high extracellular K^+ should increase the initial phase of release. Therefore, we used ouabain, which has been shown to increase $[Na^+]_i$ in primary astrocyte cultures (Kimelberg et al., 1979; Rose and Ransom, 1996). This increase was also verified in experiments using the Na^+-sensitive dye SBFI-AM (data not shown), as reported by others (Rose and Ransom, 1996). Ouabain was used at concentrations of 1 to 1.5 mM, because rat astrocyte cells need ~1 mM ouabain for complete inhibition of the pump (Kimelberg et al., 1979). In rat hippocampal astrocyte cultures, $[Na^+]_i$ was 42 mM after 10 min of ouabain exposure (Rose and Ransom, 1996). In Figure 1*B*, it can be seen that the initial phase of release is significantly increased with a 10 min, 1 mM ouabain pretreatment, and then exposure to 100 mM KCl + 1 mM ouabain, as compared to 100 mM KCl without ouabain (second exposure). These data are the mean ± SEM of four separate experiments.

Because ouabain increased the first peak of release, it enabled us to more clearly examine the effect of different experimental manipulations on the first component of release. If the size of the first component depends on $[Na^+]_i$, then increasing ouabain pretreatment time from 10 to 40 min should cause an increase in the initial release component as intracellular Na^+ progressively increases (Kimelberg et al., 1979; Rose and Ransom, 1996). This occurred as shown in Figure 2.

We also reexamined the effect of constant $K^+ \times Cl^-$ product

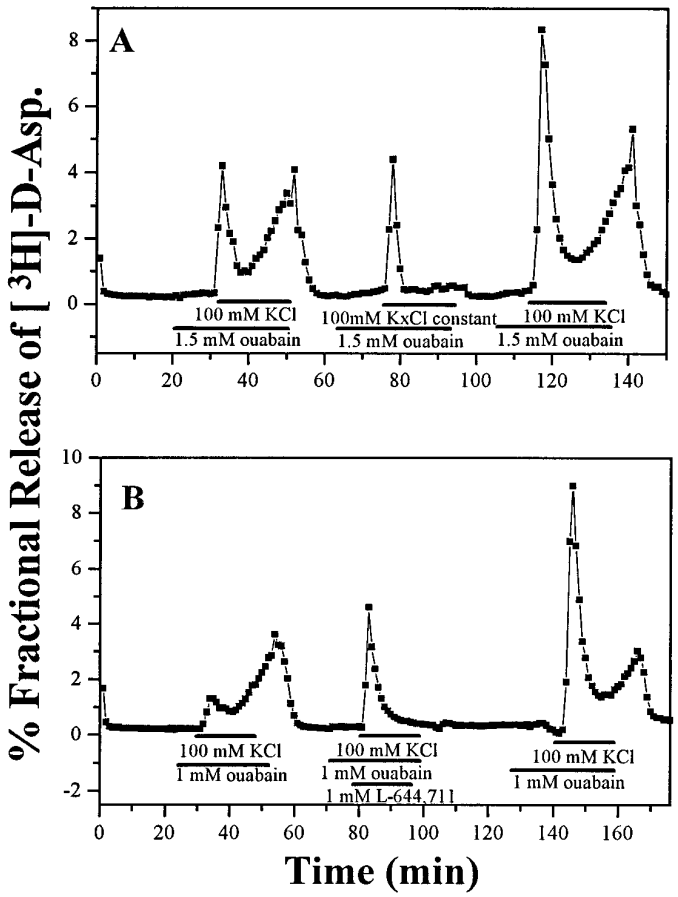

Figure 3. Inhibition of the second phase of [^{3}H]-D-aspartate release by keeping the $K^+ \times Cl^-$ product constant or by the anion transport inhibitor, L-644,711. *A*, [^{3}H]-D-aspartate release induced by 100 mM KCl and 100 mM $K^+ \times Cl^-$ product constant buffers in the presence of 1.5 mM ouabain to increase the first phase of release. *B*, The addition of 1 mM L-644,711 inhibits the second phase of [^{3}H]-D-aspartate release.

exposure in the presence of 1.5 mM ouabain. In Figure 3*A* (middle exposure), there is a sharp increase in release, followed by a rapid return to baseline while still in 100 mM $K^+ \times Cl^-$ buffer. This shows that keeping the $K^+ \times Cl^-$ product constant prevents the second K^+-induced swelling release. The initial component is thought to be influenced by $[K^+]_o$, $[Na^+]_i$, and the membrane potential, but not by swelling (Nicholls and Attwell, 1990). In our previous study (Kimelberg et al., 1995), we found also that L-644,711 inhibited the second component of K^+-induced release while leaving the initial component unaffected in the absence of ouabain. In Figure 3*B*, we show this more clearly because the augmented first component in the presence of ouabain was unaffected by exposure to L-644,711, whereas the second component was completely inhibited.

Inhibition of initial phase of [^{3}H]-D-aspartate release by an amino acid transport inhibitor

In Figure 4, we show an example of experiments designed to determine whether the glutamate/aspartate transport inhibitor THBA (DL-threo-β-hydroxyaspartate) could inhibit the initial phase of [^{3}H]-D-aspartate release without affecting the second component. A 40 min preincubation period was used to load THBA into astrocytes (Fig. 4*B*). Because THBA is a competitive inhibitor, it needs to act from the same side as the intracellularly

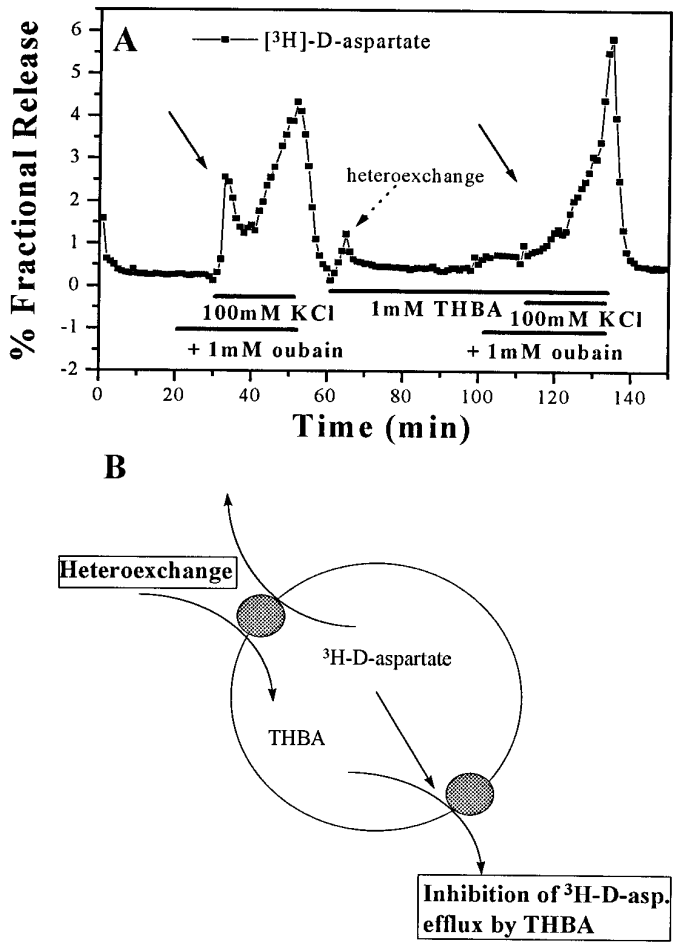

Figure 4. Inhibition of the initial phase of [^{3}H]-D-aspartate release by the glutamate uptake inhibitor THBA. *A*, Astrocytes exposed to THBA for 40 min to load THBA inside the cell by heteroexchange on the transporter causes an initial increase of [^{3}H]-D-aspartate release (*dashed arrow*), followed by a higher steady-state release. The initial phase of release (*solid arrows*) is present in the first exposure to ouabain plus 100 mM KCl but not after THBA loading ($n = 4$). *B,* Design of experiment using THBA. The loading of THBA inside the cell leads to competition with internal [^{3}H]-D-aspartate for the glutamate transporter, thereby inhibiting the initial phase of [^{3}H]-D-aspartate release seen before THBA pretreatment.

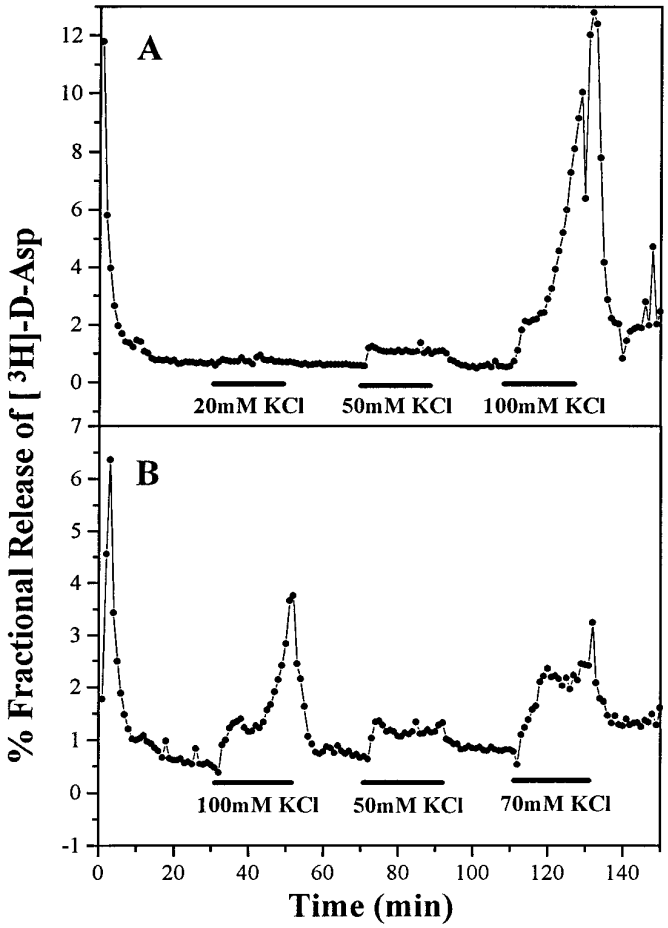

Figure 5. [^{3}H]-D-aspartate release as a function of varying $[K^+]_o$ concentration. *A*, *B* (separate experiments), The second component is only apparent at [KCl] > 50 mM. The initial peak begins to appear at [KCl] > 20 mM.

loaded [^{3}H]-D-aspartate. Experiments where cells preloaded with [^{3}H]-D-aspartate were simultaneously exposed to high K^+ and THBA did not show inhibition (data not shown). The solid arrows in Figure 4*A* indicate the presence of the initial phase of release caused by 100 mM KCl + ouabain before, and its expected position after, exposure for 40 min to 1 mM THBA. The small peak seen when THBA is first added (*dashed arrow*) and the subsequent elevated [^{3}H]-D-aspartate release is likely a result of heteroexchange on the EAA transporter (McMahon et al., 1989). The second phase of [^{3}H]-D-aspartate release was unaffected by the presence of THBA. The augmentation of this peak upon a second exposure to 100 mM KCl was seen both with and without THBA (see Discussion).

Sensitivity of [^{3}H]-D-aspartate release to increasing [KCl]

To assess the potential relevance of high K^+ induced release, it is important to determine its sensitivity to varying [KCl]. Figure 5, *A* and *B*, shows the dependency of release on KCl concentration in the absence of ouabain. Figure 5, *A* and *B*, represents two different cultures. The second component became prominent only above 70 mM KCl. The small initial release component appears at lower concentrations of KCl (20–50 mM) when no second phase of release was apparent. Because pretreatment with ouabain increases the first component of [^{3}H]-D-aspartate release, then the sensitivity of the first component to increasing $[K^+]$ can be better examined in the presence of ouabain. Figure 6*A–E* shows the results with a 10 min, 1.5 mM ouabain pretreatment, followed by various KCl concentrations in the continued presence of ouabain (middle or initial exposures as indicated), compared to release without ouabain pretreatment in the same experiment. Each panel shows two individual experiments for the KCl concentrations indicated. In Figure 6*F*, we have plotted the data from Figures 5 and 6*A–E*. The initial eight time points of release were plotted as the first component in the presence of ouabain (*solid circles*). The maximum release occurred at ~50 mM KCl and the half-maximal release at ~25 mM K^+. The second release component was tested at 100 mM KCl and was not significantly affected by ouabain (*large black square*). Clearly, there was an increased sensitivity to $[K^+]_o$, as well as an increase in magnitude of the initial response, so that the sensitivity to K^+ is now greater than in the absence of ouabain. We attribute this to increased $[Na^+]_i$.

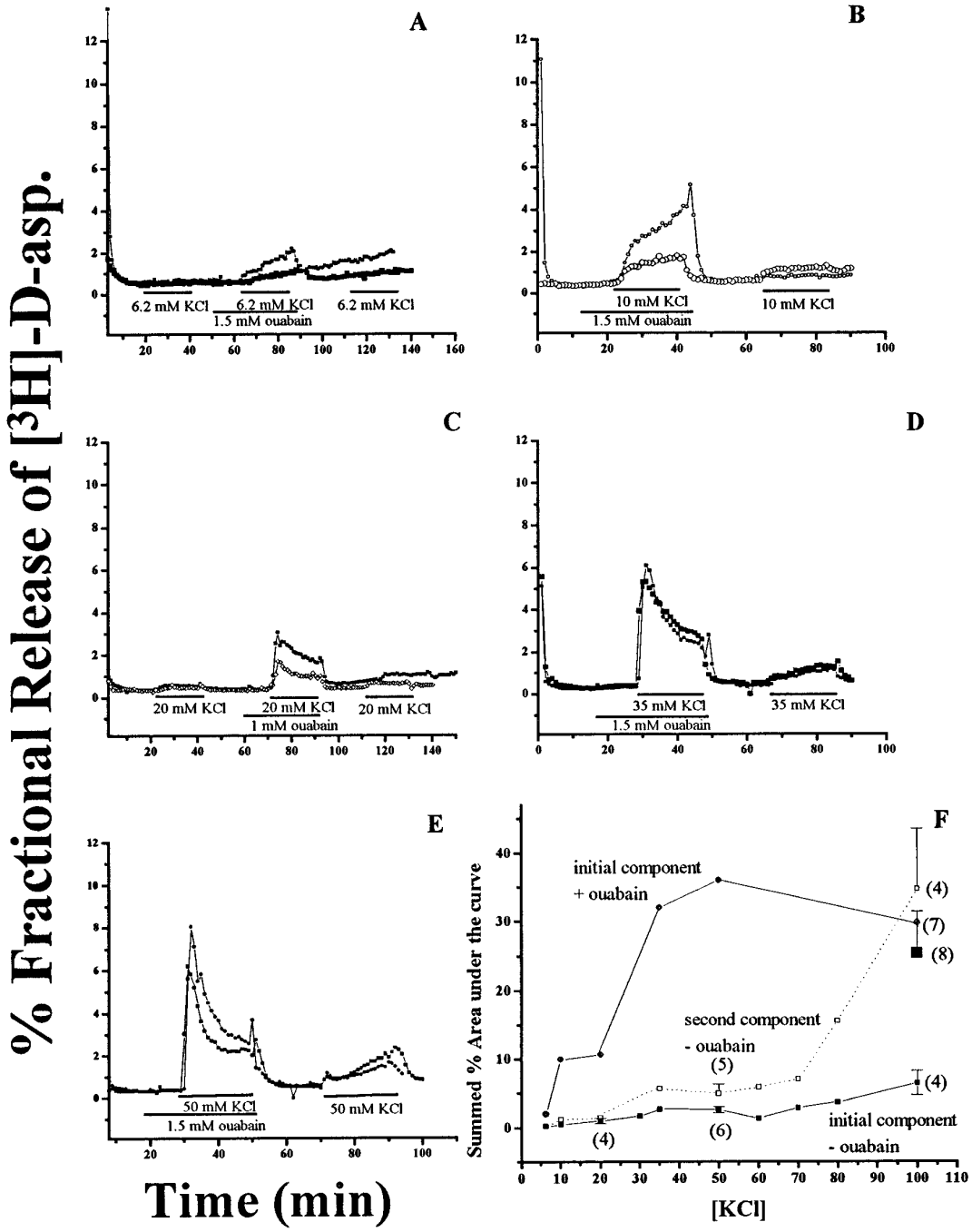

Figure 6. [^{3}H]-D-aspartate release as a function of varying $[K^+]_o$ in the presence of ouabain. *A–E,* Similar experiments as in Figure 5, but the initial or *middle trace* shows the effect of a 10 min 1.5 mM ouabain pretreatment, followed by various KCl concentrations + ouabain on [^{3}H]-D-aspartate release compared with no ouabain treatment. Two separate experiments are always shown. *F* is a graph of [^{3}H]-D-aspartate release by the initial phases of release versus varying [KCl] in the presence (*solid circles*) and absence (*solid squares*) of ouabain. Release by the second component without ouabain is shown as *open squares*. The effect of ouabain on the second phase of [^{3}H]-D-aspartate release is only for 100 mM KCl (*large black square*). The data points were calculated by summing the first and last eight release data points of a KCl exposure, and the basal release was then subtracted as described in Materials and Methods. The *numbers* in *parentheses* represent the number of experiments performed for those data points shown ± SEM. The rest of the data points represent means of two experiments.

Release not a result of low extracellular Na^+

As a control it is important to determine that the reduction in extracellular $[Na^+]$ when replaced with K^+ does not by itself cause [^{3}H]-D-aspartate release. Figure 7*A* shows that when 100 mM Na^+ was replaced by NMDG.Cl the [^{3}H]-D-aspartate release from the first component was less than when Na^+ in the media was replaced by 100 mM KCl. This experiment was in the absence of ouabain. In Figure 7*B*, the lack of any stimulation of the first component with a reduction in $[Na^+]_o$ is shown more clearly when ouabain was present. It can also be seen that the second phase of [^{3}H]-D-aspartate release (swelling-induced release) was also not seen during exposure to low Na^+-containing

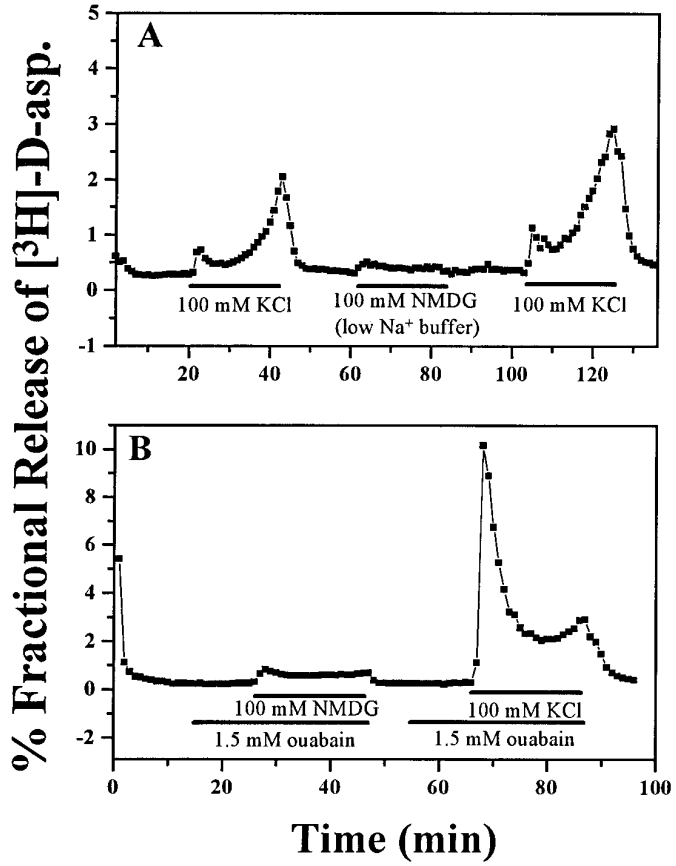

Figure 7. Release is not a result of a reduction in $[Na^+]_o$. *A*, 100 mM Na^+ is replaced by an equal molar NMDG.Cl, thereby reducing extracellular Na^+ concentration but with all other ions kept constant. *B*, The same experiment as *A*, but in the presence of ouabain. Representative of three experiments.

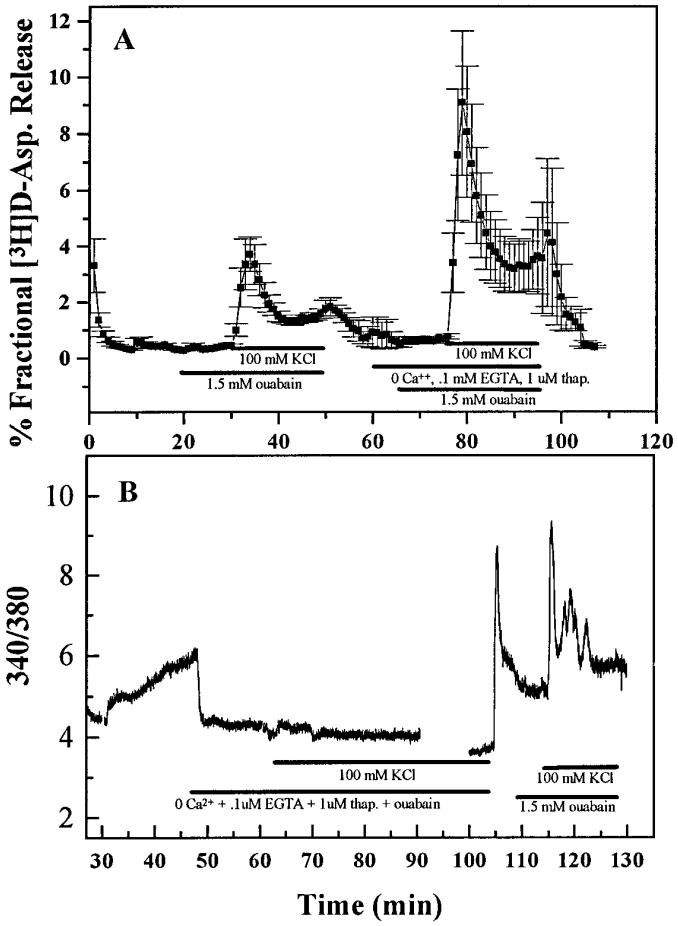

Figure 8. Release is independent of Ca^{2+}. *A,* The effect of Ca^{2+}-free media + 0.1 mM EGTA + 1 μM thapsigargin on [^{3}H]-D-aspartate release as indicated (n = 4, ±SEM). *B*, Intracellular Ca^{2+} measurements showing no Ca^{2+} transient during exposure to Ca^{2+}-free media and high K^+ exposure in the presence of thapsigargin. After 30 min equilibration at t = 31 min, fresh Ca^{2+}-containing media was added. The initial slow rise in signal was a result of temperature differences between the buffers. There was a drop in $[Ca^{2+}]_i$ after exposure to Ca^{2+}-free media. After reexposure to Ca^{2+}-containing media, a sharp increase in $[Ca^{2+}]_i$ occurred. Subsequent exposure to ouabain caused no change, whereas 100 mM KCl now caused a sharp rise in $[Ca^{2+}]_i$, followed by oscillations.

buffers. This is to be expected because no Donnan swelling should occur when K^+ is not increased (Hodgkin and Horowicz, 1959).

Ca^{2+} dependency

In terms of the potential relevance of these processes to Ca^{2+}-independent release of amino acids in ischemia and other pathological states, it is important to determine whether any of these release mechanisms are sensitive to the omission of extracellular Ca^{2+}. Figure 8*A* shows the results of four efflux experiments, where exposures to increased K^+ was done in the presence and absence of Ca^{2+}, respectively. It can be seen that there was increased [^{3}H]-D-aspartate release during the first phase of high K^+ exposure when the cells were exposed to a nominally Ca^{2+}-free solution plus 0.1 mM EGTA + 1 μM thapsigargin. Thapsigargin was present to eliminate any contribution from Ca^{2+} released from intracellular stores; this was verified by fura-2 experiments to measure changes in $[Ca^{2+}]_i$ as shown in Figure 8*B*. The cells were first equilibrated for 30 min, and fresh buffer added at t = 30 min. The increasing baseline after 30 min was a result of a temperature difference between the Iso HEPES buffer added and the bath buffer. As can be seen, there was no Ca^{2+} response to 100 mM KCl in Ca^{2+}-free media also containing EGTA, ouabain, and thapsigargin. Reexposure to Ca^{2+}-containing medium caused a sharp transient increase. When the cells were then exposed to Ca^{2+}-containing media and then to ouabain, followed by 100 mM KCl + ouabain, an initial sharp Ca^{2+} transient with subsequent oscillations was seen. This behavior was representative of three experiments.

DISCUSSION

Release of EAAs from glial cells

In ischemia and hypoxia, extracellular $[K^+]$ has been shown to increase to 80 mM or higher, and extracellular $[Na^+]$ decreases concomitant with an increased $[Na^+]_i$ (Somjen, 1979; Hansen, 1985). This is presumed to be mainly a result of the inactivation of the Na^+/K^+ ATPase pump caused by the depletion of ATP (Shimizu et al., 1993). Thus, the manipulation of ion gradients in our experiments model the ionic changes that occur during these CNS pathologies. Szatkowski et al. (1990) have shown that reversal of a current due to glutamate transport occurs in Müller cells when extracellular $[K^+]$ was increased. Mammalian astrocytes have also been shown to release [^{3}H]-D-aspartate when toxins were used to induce anoxia in primary astrocyte cultures (Gembra

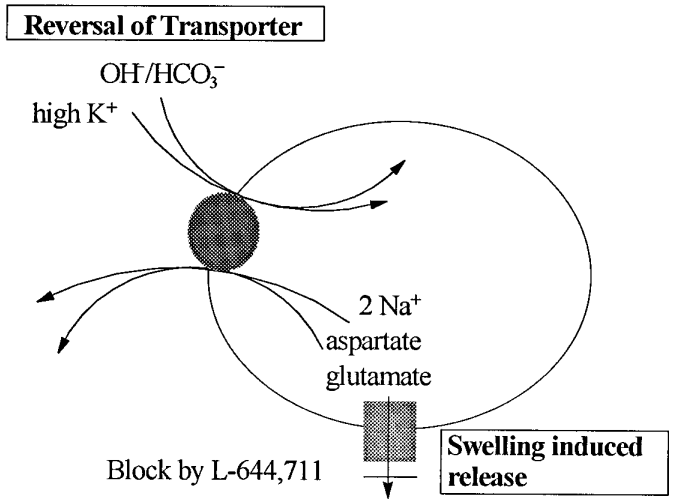

Figure 9. Model of [^{3}H]-D-aspartate release mechanisms from cultured astrocytes (see Discussion).

et al., 1994; Longuemare and Swanson, 1995). Our data show that in rat primary astrocyte cultures preloaded with [^{3}H]-D-aspartate, there are two phases of release in response to raised extracellular [K^+]. We propose that the first phase of [^{3}H]-D-aspartate release is through reversal of the glutamate transporter, and the second phase of [^{3}H]-D-aspartate release is activated by astrocytic swelling (Fig. 9). In support of this model, when the cells were pretreated with ouabain to raise [Na^+]$_i$, there was a marked increase in the first without any effect on the second component. Ouabain pretreatment also increased the sensitivity of release of the first component to extracellular [K^+] exposure compared to without ouabain pretreatment. A rise to 6.0–10 mM KCl, which is in the range of [K^+]$_o$ surrounding epileptic neurons (Somjen, 1979), caused an increase in basal [^{3}H]-D-aspartate release when cells were pretreated for 10 min with 1 mM ouabain.

Identification of the first peak as reversal of the EAA transporter was supported by the use of a potent competitive blocker of glutamate uptake, THBA. THBA was first loaded inside the cell utilizing the EAA transporter operating in its heteroexchange mode (McMahon et al., 1989). Once inside the cell, THBA competes with and reduces the initial phase of [^{3}H]-D-aspartate release (Fig. 4).

Critical role of [Na^+]$_i$

After exposure to high K^+, there are three driving forces that can cause reversal of the glutamate transporter: the reciprocal reduction in [Na^+]$_o$, membrane potential depolarization, and increased [K^+]$_o$. The results in Figure 7, *A* and *B*, suggest that K^+ is absolutely required for release to occur, and there is no effect from the reduction in [Na^+]$_o$ per se.

One reason why the initial phase of release is transient may be because [Na^+]$_o$ was reciprocally decreased when KCl concentration is increased to maintain isotonicity. This could result in a failure to adequately replenish [Na^+]$_i$ as it is depleted from the cells because of reversal of the EAA transporter. The modes of sodium entry into astrocytes could be by the $Na^+ + K^+ + 2Cl^-$ cotransporters or neurotransmitter-activated Na^+ channels such as the AMPA/KA or voltage-sensitive Na^+ channels, the functions of which have, in part, been proposed to be to maintain [Na^+]$_i$ in astrocytes for the continued action of the Na^+/K^+ ATPase pump (Walz, 1987; Sontheimer, 1995). However, a recent study by Rose and Ransom (1996) using cultured hippocampal astrocytes has shown that TTX had only a small effect on [Na^+]$_i$, and in only 15% of the cells studied.

It will be important to determine which of the two currently known astrocytic glutamate transporter subtypes, GLAST-1 or GLT-1, is expressed in primary cultures. Kondo et al. (1995) have recently shown that primary astrocyte cultures prepared from the cerebral cortices of rat pups express GLAST-1 and not GLT-1, which is the primary transporter expressed in astrocytes of the adult rat cortex (Rothstein et al., 1994). However, there is no evidence thus far to suggest that there would be any difference in reversal characteristics between the different isoforms. This would imply that neurons can also release EAAs by reversal of the EAAC1 transporter. However, there is no direct evidence for this as yet, and also no evidence that neurons can release EAAs by a swelling mechanism.

Swelling-induced release

The second phase of release seems to be independent of the first. It is likely to be a result of cell swelling because high KCl causes swelling of astrocytes (Walz, 1987), as it does in other cells (Hodgkin and Horowicz, 1959). L-644,711, an anion channel blocker that inhibits hypotonic media-induced swelling release of EAAs (Kimelberg et al., 1990), completely inhibits the second phase of [^{3}H]-D-aspartate release during 100 mM KCl exposure. Keeping the $K^+ \times Cl^-$ product constant by replacing Cl^- with gluconate also inhibited the second phase of [^{3}H]-D-aspartate release. Also, in complete contrast to the first component, the magnitude of the second component is not affected by ouabain. The second phase of release may be important because astrocytic swelling is prominent and occurs rapidly after the induction of anoxia, ischemia, hypoglycemia, and head trauma (Kimelberg, 1992) and seems detrimental to survival because when swelling was inhibited with L-644,711, an anion channel inhibitor (Barron et al., 1988), there was decreased mortality in an animal head injury model (Kimelberg et al., 1989). There are at least two mechanisms of action that might explain the effect of L-644,711 on the second release component. One is that being a Cl^- channel blocker that inhibits Cl^- influx, swelling because of Donnan forces is prevented. The second mechanism would be that L-644,711 blocks the release of [^{3}H]-D-aspartate via a proposed swelling-activated anion channel that allows passage of amino acids (Jalonen, 1993). Although the second phase of release seems to contribute little to overall [^{3}H]-D-aspartate with KCl concentrations <50 mM *in vitro,* other processes might occur *in vivo* that augment astrocytic swelling at relatively low [K^+]$_o$ concentrations (Kimelberg, 1992).

Role of Ca^{2+} and potentiation of [^{3}H]-D-aspartate release during high K^+ exposures

Ca^{2+}-free media with and without thapsigargin (see Fig. 8*A*, data not shown for latter condition) showed no inhibition of either the first or second release component. Indeed, the initial phase of release seems to be potentiated compared to Ca^{2+} containing 100 mM KCl. It supports the view that Ca^{2+}-independent release mechanisms can increase glutamate levels by both reversal of the EAA transporter and swelling-induced release in pathological states.

In most experiments, potentiation of both components occurred during identical second or third KCl exposures. The mechanisms of these are unknown, although the enhanced initial phase of release could be caused by phosphorylation of the EAA transporter, which increases the V_{max} in glial cells (Casado et al., 1991).

Conclusions

Since the initial studies of Drejer et al. (1985), it has been assumed that the increased $[EAA]_o$ seen in ischemia and other pathological studies is mainly a result of release from nerve terminals. There are two glutamate pools found in neurons. One is vesicular, located in the nerve terminals and is dependent on Ca^{2+} and ATP for release (Nicholls and Attwell, 1990). The second is free glutamate in the cytosol, and this can be released by reversal of the glutamate transporter. Recent studies have shown that with ischemia >20 min duration EAA release is independent of Ca^{2+} and even within the first 10 min about half of the release is independent of Ca^{2+} (Wahl et al., 1994). Using immunocytochemistry, Aas et al. (1995) and Torp et al. (1991) have shown that the neuronal cytosolic glutamate pool, but not the vesicular glutamate pool, decreases in neurons in ischemia supporting the hypothesis that vesicular release is inhibited because of the lack of ATP. There was a concomitant increase in glutamate labeling in astrocytes and, therefore, astrocytes could be acting as a sink for the increased extracellular glutamate. This increase could be a result of the fact that glutamine synthetase, which normally breaks down glutamate to glutamine, is inhibited by the reduction in ATP. However, these histological studies do not address the question of flux. If the increased glutamate levels in astrocytes is available for release, then astrocytes could play a significant role in increasing $[glu^-]_o$ by either of the two mechanisms described in this paper. In addition, our method of measuring preloaded [^{3}H]-D-aspartate release does not enable a quantitative measurement of EAA release. This is important in terms of functional significance and can best be addressed by studying actual glutamate release *in vivo*. *In vivo* microdialysis studies using manipulations to alter the relative amounts of terminal versus astrocytic release and pharmacological manipulations to identify the different mechanisms should help resolve these important issues.

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