Regulation of mitochondrial glutamine/glutamate metabolism by glutamate transport: studies with \(^{15}N\)

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We focused on the role of plasma membrane glutamate uptake in modulating the intracellular glutaminase (GA) and glutamate dehydrogenase (GDH) flux and in determining the fate of the intracellular glutamate in the proximal tubule-like LLC-PK\(_1\)-F\(^{\text{-}}\) cell line. We used high-affinity glutamate transport inhibitors \(\text{D-Asp}\) and \(\text{DL-threo-\(\beta\)-hydroxyaspartate (THA)}\) to block extraacellular uptake and then used \(\text{L-[15N]}\)glutamate or \(\text{[2-15N]}\)glutamine to follow the metabolic fate and distribution of glutamine and glutamate. In monolayers incubated with \(\text{[2-15N]}\)glutamine (99 atom % excess), glutamine and glutamate equilibrated throughout the intra- and extracellular compartments. In the presence of 5 mM \(\text{D-Asp}\) and 0.5 mM THA, glutamine distribution remained unchanged, but the intracellular glutamate enrichment decreased by 33% (\(P < 0.05\)) as the extracellular enrichment increased by 39% (\(P < 0.005\)). With glutamate uptake blocked, intracellular glutamate concentration decreased by 37% (\(P < 0.0001\)), in contrast to intracellular glutamine concentration, which remained unchanged. Both glutamine disappearance from the media and the estimated intracellular GA flux increased with the fall in the intracellular glutamate concentration. The labeled glutamate and \(\text{NH}_4^+\) formed from \(\text{[2-15N]}\)glutamine and recovered in the media increased 12- and 3-fold, respectively, consistent with accelerated GA and GDH flux. However, labeled alanine formation was reduced by 37%, indicating inhibition of transamination. Although both \(\text{D-Asp}\) and THA alone accelerated the GA and GDH flux, only THA inhibited transamination. These results are consistent with glutamate transport both regulating and being regulated by glutamine and glutamate metabolism in epithelial cells.

Glutamine is an essential amino acid for growth of cells in culture (10) as well as an important oxidative fuel for cells grown in culture (16, 30) and in intact functioning organs (28, 37). The \(\text{NH}_4^+\) nitrogen supports both hepatic ureagenesis (24) and renal ammoniagenesis in vivo (32). The rate-limiting step in intracellular glutamine utilization via the oxidative pathway is the deamination reaction (Fig. 1A, reaction 4), catalyzed by phosphate-dependent glutaminase (GA) localized to the inner mitochondrial membrane (7, 16, 30, 32). Recent studies in pig kidney mitochondria have demonstrated that the functional GA activity is expressed on the cytosolic surface of the inner membrane (17) and represents the dimeric form of the enzyme (27). Consequently, the products formed in this reaction, glutamate and \(\text{NH}_4^+\), are released directly into the cytosolic compartment (Fig. 1A). To further metabolize glutamate, it must undergo transamination (reaction 6) to alanine (32) and \(\alpha\)-ketoglutarate and/or deamination (reaction 7) to \(\text{NH}_4^+\) (32) and \(\alpha\)-ketoglutarate. The deamination reaction is localized to the mitochondrial matrix compartment (16, 30), while the transamination reaction can occur in both the cytosolic and mitochondrial compartments (19). Transport of cytosolic glutamate into the mitochondrial matrix is mediated by two inner membrane glutamate transporters operating as \(\text{Glu}^-/\text{OH}^-\) and \(\text{Glu}^-/\text{Asp}^-\) exchangers (reaction 3) coupled to glutamate dehydrogenase (GDH; reaction 7) and \(\text{NH}_4^+\) formation and alanine aminotransferase (ALT) and alanine (reaction 6) formation, respectively (16, 30). The cytosolic glutamate may also be transported out of the cell through plasma membrane system \(\text{X}_{\text{AG}}\) (3) or possibly by the neutral amino acid carrier \(\text{ASC}\) (18, 33). Thus plasma membrane (reaction 5) and mitochondrial membrane glutamate transporters (reaction 3) compete for the cytosolic glutamate generated from glutamine via the GA, with alanine, \(\text{NH}_4^+\), or glutamate appearing in the media as products of intracellular glutamine/glutamate metabolism (Fig. 1A).

Regulation of glutamine flux through the functional GA is modulated by the intracellular glutamate concentration (13), which competes with glutamine (31) for the enzyme's reactive site. Because the functional GA reacts with glutamine and glutamate in the cytosolic compartment, transport of glutamate into as well as out of this compartment regulates the GA flux and, hence, glutamine utilization. The uptake of glutamate...

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Fig. 1. Glutamate (Glu) fluxes in control LLC-PK1-F⁺ monolayers (A) and in the presence of D-Asp and DL-threo-β-hydroxyaspartate (THA, B). A: extracellular glutamate (Gln) hydrolyzed by γ-glutamyltransferase (γ-GT) (reaction 1) to Glu and NH₄⁺ with the Glu equilibrating with the extracellular Glu pool. System Xₐg-mediated Glu uptake into the cell (reaction 2) and efflux from the cell (reaction 5) contribute to the extracellular Glu pool turnover. Intracellular glutaminase (GA, reaction 4) is suppressed by high cytosolic Glu concentration ([Glu]) as the result of uptake. Cytosolic Glu equilibrates with intramitochondrial Glu via the inner membrane exchangers (reactions 3a and 3b) and with the extracellular pool via the plasma membrane exchanger (reaction 5). Cytosolic as well as intramitochondrial Glu can undergo transamination to yield Ala (reaction 6). Cytosolic Glu can also be effluxed into the media (reaction 5). B: effect of inhibiting plasma and mitochondrial membrane (Mito) Glu uptake with D-Asp and L-THA, respectively. Extracellular Glu is blocked from entering the cell, resulting in reduced cytosolic (Cyto) [Glu] and accelerated GA flux. The intracellular Glu formed from Gln is effluxed into the media, undergoes transamination in the cytosol, or enters the mitochondrial deamination pathway but is blocked by L-THA from entering the mitochondrial transamination pathway.

from the extracellular media (Fig. 1A, reaction 2) has been shown to play an important role in determining the intracellular glutamate concentration ([Glu]; Fig. 1A) (36). Because the translocated glutamate and GA share the cytosolic compartment, changes in the availability and uptake of extracellular glutamate play an important role in regulating intracellular glutamine metabolism. The source of extracellular glutamate is that preformed and present in the media (plasma) plus that generated by γ-glutamyltransferase (γ-GT) hydrolysis of extracellular glutamine (reaction 1) (20, 36).

Under physiological conditions, the extracellular [Glu] ranges from 20 to 50 μM (9), which is within the range of Michaelis-Menten constants for the cloned Xₐg subtypes expressed in mammalian cells (1). However, 10-fold elevations in plasma [Glu] have been reported in several pathophysiological conditions (9). Note that de novo generation of glutamate from extracellular glutamine mediated by γ-GT contributes to the extracellular glutamate pool and ensures continuous glutamate uptake. The contribution of extracellular glutamate to the cytosolic pool can be disrupted by blocking the extracellular glutamate uptake (Fig. 1A, reaction 2) using high-affinity inhibitors of system Xₐg, D-aspartate and DL-threo-β-hydroxyaspartate (THA), which competitively inhibit L-glutamate transport across the plasma membrane (1, 12). Accordingly, blocking glutamate uptake into the cell should decrease cytosolic [Glu] (Fig. 1B) and accelerate GA with an increased glutamate formation from cytosolic glutamine. The glutamate formed in the cytosol should be transaminated to alanine (reaction 6), transported into the mitochondrial matrix (reaction 3) forming NH₄⁺ (reaction 7) or alanine (reaction 6), transported from the cell (reaction 5), or accumulated with suppression of the GA flux.

To assess the fate of the cytosolic glutamate generated from glutamine, we incubated LLC-PK1-F⁺ monolayers in DMEM containing either 1.8 mM L-[2-¹⁵N]glutamine and 0.5 mM L-glutamate or 1.8 mM L-glutamine and 0.5 mM L-[¹⁵N]glutamate. Under these conditions, extracellular glutamate uptake was large, with labeled glutamate derived from glutamine equilibrating throughout the extra- and intracellular compartments; a high intracellular [Glu] maintained a low GA flux. However, in the presence of D-Asp and THA, labeled glutamate formed from glutamine via the accelerated GA flux did not equilibrate. Rather, intracellular glutamate decreased, despite a large increase in GA flux, with the glutamate generated transported out of the cell, where it accumulated, or into the mitochondria coupled to deamination and NH₄⁺ formation, while transamination was inhibited.

MATERIALS AND METHODS

LLC-PK₁-F⁺ cells were grown to confluence on 60-mm plastic dishes in DMEM containing (in mM) 1.8 L-glutamine, 0.034 L-glutamate, 28 bicarbonate, 10 pyruvate, and 5 D-glucose, as well as 10% fetal bovine serum (Hyclone, Ogden, UT), in 5% CO₂-95% atmosphere. The F⁺ strain resembles more closely the proximal tubule than the parental LLC-PK₁ cell line, in that the F⁺ strain exhibits gluconeogenesis and expresses an increased γ-GT and phosphate-dependent GA activities (14) (Fig. 1A, reactions 1 and 4). All experiments were performed 5–9 days after seeding, with fresh media exchanged daily. The seed plates were routinely screened for Mycoplasma, with contamination monitored by the use of a Mycoplasma PCR primer kit (Stratagene Cloning Systems, La Jolla, CA). Individual seed plates were split into six to eight plates, with three to four used as controls and three to four used as test plates.

Experimental design. To assess the distribution of labeled glutamate throughout the intracellular fluid, monolayers were incubated in either [¹⁵N]glutamate and unlabeled glutamine or [2-¹⁵N]glutamine and unlabeled glutamate. The enrichment of labeled glutamate in the intracellular fluid was then compared with that in the extracellular fluid. Similiar enrichments in the two compartments were taken to indicate equilibration of the labeled glutamate throughout the intracellular compartment. To block the extracellular glutamate uptake (Fig. 1A, reaction 2) as well as to limit mitochondrial glutamate uptake (reaction 3b), D-Asp (competitively inhibits plasma membrane glutamate uptake by Xₐg, reaction 1) plus THA (L-THA putatively inhibits mitochondrial glutamate uptake, reaction 2) was added to the media. To confirm that most of the extracellular glutamate uptake was inhibited, the incorporation of [¹⁵N]glutamate...
into alanine was monitored (reaction 6). To monitor the fate of extracellular glutamate and glutamate formed intracellularly from glutamine hydrolysis, monolayers were incubated in DMEM plus 0.5 mM L-[15N]glutamate (99 atom % excess; Cambridge Isotope Laboratories, Andover, MA) and 1.8 mM L-glutamine or DMEM plus 0.5 mM L-glutamate and 1.8 mM L-[2,15N]glutamine (99 atom % excess; Cambridge Isotope Laboratories). To test the effect of blocking the plasma membrane glutamate uptake, monolayers were incubated in the above media plus 5 mM D-aspartate and 0.5 mM THA. According to the model, labeled glutamate formed from [2-15N]glutamine (reaction 4) should be transaminated to alanine (reaction 6), accumulate in the cytosol, be transported out of the cell (reaction 5) and appear in the extracellular media behind the D-Asp block. Inhibiting the γ-GT (reaction 1) by adding 0.7 mM AT-125 (acivicin; kindly provided by Dr. Michaela Christian, National Cancer Institute) to the DMEM containing D-Asp and THA can differentiate the glutamate contributions of the γ-GT-GA reaction (reaction 1) (20) from the glutamate transported out of the cell (reaction 5).

To test whether system X-[A] plays a role in the efflux of intracellular anionic amino acids, monolayers were loaded with radiolabeled D-Asp, which is a specific substrate for system X-[A] (12). Besides being a specific substrate for system X-[A] (12), D-Asp also has the advantage that it is not metabolized by the LLC-PK1 cell line (29). Thus we used radiolabeled D-Asp in place of glutamate to determine whether D-Asp in combination with THA would have a effect on radiolabeled D-Asp retention different from that of D-Asp alone. D-[3H]Asp (12 Ci/mmol; NEN, Boston, MA) was loaded into monolayers from DMEM (1 μCi/ml media) over 20 min. After 20 min, the plates were washed three times with ice-cold PBS and then DMEM, DMEM + 5 mM D-Asp, or DMEM + 5 mM D-Asp + 0.5 mM THA were added. The plates were then returned to the incubator for 45 min. After 45 min the media were harvested, the monolayers were washed three times with ice-cold PBS, and then 1 ml of ice-cold 5% TCA was added. The monolayers were promptly scraped free, transferred to a homogenizing tube, and homogenized by use of a Polytron (half-speed for 30 s). The TCA homogenates were centrifuged (10,000 g for 10 min), the supernatant was retained to monitor [3H] and glutamate content by liquid scintillation spectrometry, and the pellet was dissolved to measure protein content. Protein content of the various fractions was determined by the dye-binding assay (5) after the TCA-precipitated pellet was dissolved in 0.2 N NaOH.

Analysis. After 16 h of incubation in the prescribed media, media samples were taken and the monolayers were rapidly washed three times with ice-cold PBS. The washed monolayers and aliquots of the medium were treated with ice-cold 40% perchloric acid. The concentration of glutamine, glutamate, alanine, and aspartate and their 15N enrichment were determined on the neutralized supernatants as previously described (24, 25). Briefly, the amino acids underwent precolumn derivatization with o-phthalaldehyde and separation by HPLC and fluorescent detection (20). Analysis of 15N in the amino acids was done by gas chromatography-mass spectrometry, as previously described (24, 25). Formation of 15NH4+ was determined after conversion of NH4+ to glutamate (25). NH4+ concentration was measured by the previously described microdiffusion method (36).

Calculations. The net uptake or release of glutamate and alanine was determined from the difference in the media glutamate present at the beginning (preformed) and at the end of incubation. The concentration difference multiplied by the media volume (4 ml) gave the net amount taken up or produced and expressed per milligram of protein. The net NH4+ production was obtained by subtracting the NH4+ formed by the monolayers over the 16-h time course from DMEM without glutamine from that formed in the presence of glutamine. Aspartate was not produced or taken up in net amounts. To calculate the conversion of [15N]glutamine to glutamate and alanine, the isotopic enrichment (atom % excess) of 15N in the particular metabolite was multiplied by the amount present and expressed as nanomoles per milligram of protein (25). The sum of the glutamine converted to alanine and to glutamate was added to the net NH4+ formation to obtain the glutamate metabolized through the two GAs and the transamination and deamination pathways (32). To obtain the glutamate metabolized via the intracellular pathways, the sum of glutamate and corresponding NH4+ formed by γ-GT (Fig. 1A, reaction 1) was subtracted from the total (i.e., NH4+ + [15N]Ala + [15N]Glu). This difference was then divided by 2 to obtain the estimated GA flux (reaction 4). Comparisons were made between pairs of control and the glutamate uptake-blocked monolayers (total 12–18 pairs). Significant differences were obtained using the Student’s t-test or ANOVA for multiple group comparisons and a one- or two-tailed t-table depending on whether a priori directional changes were postulated on the basis of the model shown in Fig. 1.

RESULTS

In control monolayers incubated for 16 h with [2-15N]glutamate, the extra- and intracellular glutamate enrichments were similar (79 ± 1 and 75 ± 1 atom % excess, respectively), indicating that glutamine equilibrates throughout the extra- and intracellular compartments. The intracellular fluid enrichment of [15N]glutamate (Fig. 2), formed from labeled glutamine, was equal to the extracellular glutamate enrichment (23 ± 1 and 21 ± 1 atom % excess, respectively) and approximately one-third of that of [2-15N]glutamine. In the presence of D-Asp + THA, [2-15N]glutamine enrichment is unchanged and simi-
lar within the two compartments (81 ± 1 and 74 ± 2 atom %excess, respectively). However, the equilibration of metabolically generated labeled glutamate no longer exists (Fig. 2). Compared with the control monolayers, enrichment of the intracellular glutamate decreases (P < 0.05) by −33% (from 21 ± 1 to 14 ± 0.5 atom %excess), while that of the extracellular fluid increases 1.4-fold (from 23 ± 1 to 32 ± 2 atoms %excess, P < 0.005). These results are consistent with transporter-mediated equilibration of glutamate throughout the intra- and extracellular compartments. More curious is the decline in intracellular glutamate enrichment in the presence of D-Asp + THA, despite glutamate generated by the GA (Fig. 1A, reaction 4; Fig. 1B) from labeled glutamine within the cell. This finding suggests that the extracellular GA (reaction 1) is responsible for the enrichment of labeled glutamate in the extracellular compartment, the labeled glutamate formed within the cells is exported (reaction 5) and accumulates behind the block in uptake, or both extracellular production and efflux are occurring.

In control monolayers incubated with media containing [15N]glutamate (Fig. 3), the intracellular enrichment was 38% (P < 0.02) higher than the extracellular enrichment (18 ± 1 vs. 13 ± 1 atom %excess). In the presence of D-Asp and THA, [15N]glutamate was 3.8-fold enriched in the extracellular compartment, while intracellular enrichment decreased 70% (P < 0.0005) compared with the control. These results show that when the source of labeled glutamate is the extracellular compartment, inhibiting high-affinity glutamate transport prevents equilibration between the two compartments with the expected enrichment of the extracellular [15N]glutamate and a marked decrease in the intracellular [15N]glutamate. Furthermore, the decrease in the extracellular [15N]glutamate enrichment from 99 to 49 ± 3 atom %excess represents the addition of 969 nmol of unlabeled glutamate. To confirm that the extracellular glutamate uptake was effectively blocked by 5 mM D-Asp and 0.5 mM THA, the formation of alanine from the [15N]glutamate was determined after 16 h of incubation. Under these conditions, the alanine formation from labeled glutamate decreased 87 ± 6% (P < 0.0001) from 236 ± 19 nmol/16 h in the control monolayers to 31 ± 11 nmol/16 h in the presence of D-Asp + THA (n = 6 pairs of plates). Thus, most, if not all, of the extracellular glutamate uptake is blocked, with the decline in extracellular enrichment representing addition of unlabeled glutamate.

To determine the contribution of the extracellular GA to the glutamine hydrolysis occurring with plasma membrane glutamate uptake blocked, γ-GT activity was inhibited and the accumulated glutamate was compared with that occurring with γ-GT active (Fig. 4). In the control monolayers, there is a net uptake of extracellular glutamate that reverses to a large accumulation in the presence of D-Asp + THA (from −991 ± 22 to 683 ± 76 nmol·mg⁻¹·16 h⁻¹, P < 0.0001). Inhibiting the γ-GT with acivicin reduced (P < 0.004) the glutamate accumulating in the presence of D-Asp + THA by 48% (from 683 ± 76 to 358 ± 32 nmol·mg⁻¹·16 h⁻¹). This indicates that 52% of the glutamate accumulating in the media with the glutamate uptake blocked is derived from an intracellular source.

To demonstrate that blocking glutamate uptake specifically accelerated intracellular GA flux, monolayers were incubated with [2,15N]glutamine and the 15N-labeled products were determined as presented in Table 1. After 16 h of incubation with D-Asp + THA, total cellular glutamate was decreased 37% (from 182 ± 10 to 114 ± 8 nmol/mg protein, P < 0.0001, n = 18 pairs), The intracellular glutamine content remained unchanged (48 ± 2 vs. 48 ± 3 nmol/mg protein). Consequently, the intracellular ratio of glutamine to glutamate increases, favoring accelerated glutamine breakdown. The sum of glutamate and alanine pro-

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**Fig. 3.** [15N]Glu distribution between media and cells in control and in the presence of D-Asp + THA. Values are means ± SE from 6 pairs of plates incubated for 16 h in the designated media containing an initial 99 atom %excess [15N]Glu. *Different from media, P < 0.05; **different from control, P < 0.001.

**Fig. 4.** Media Glu uptake (bottom) or accumulation (top) in control and with D-Asp + THA or D-Asp + THA + acivicin. Values are means ± SE from 12 control and 6 pairs of D-Asp + THA plates with or without 0.75 mM acivicin. *Different from control; **different from D-Asp + THA, P < 0.05.
duced from the $^{15}$N-labeled amino nitrogen of glutamine plus the total NH$_4^+$ formed increased 61% ($P < 0.001$) in the presence of d-Asp + THA (from 1,357 ± 52 to 2,179 ± 57 nmol/mg protein). The increase in these products resulting from glutamine hydrolysis was associated with enhanced ($P < 0.001$) glutamine disappearance from the media (1,329 ± 83 vs. 1,526 ± 86 nmol·mg$^{-1}$·16 h$^{-1}$), consistent with an accelerated GA flux. Recovery of the labeled [2-$^{15}$N]glutamine as glutamate increased ~12-fold (from 65 ± 6 to 745 ± 60 nmol/16 h, $P < 0.0001$). In addition, the total NH$_4^+$ production also increased ($P < 0.0002$) from 95 ± 68 to 1,186 ± 96 nmol·mg$^{-1}$·16 h$^{-1}$. However, alanine formation from [2-$^{15}$N]glutamine decreased (from 298 ± 40 to 252 ± 27 nmol·mg$^{-1}$·16 h$^{-1}$, $P < 0.05$). Nevertheless, the sum of the GA products clearly increased, although the relative contribution of the extracellular pathways requires further analysis. The contribution of the extracellular γ-GT activity determined above (Fig. 4) as 325 nmol/mg was doubled to include the NH$_4^+$ and then subtracted from the control and d-Asp + THA product total. The total of NH$_4^+$, alanine, and glutamate formed from the intracellular pathways was then divided by 2 to obtain the GA flux in nanomoles per milligram of protein (707 ± 53 ± 2 = 354 ± 26 and 1,530 ± 57 ± 2 = 764 ± 29 nmol/mg). In the control monolayers (Fig. 5), the estimated intracellular GA flux (Fig. 1A, reaction 4) was approximately equal to the flux through the extracellular GA (reaction 1): 354 ± 26 and 325 ± 105 nmol glutamine hydrolyzed/mg protein, respectively. However, after the uptake of the extracellular glutamate was blocked, the estimated intracellular GA flux increased 2.2-fold (from 354 ± 26 to 764 ± 29 nmol glutamine/mg protein, $P < 0.001$). Note that total NH$_4^+$ production, rather than $^{15}$NH$_4^+$ derived from the [2-$^{15}$N]glutamine, was used to estimate the GA flux in these studies.

To determine the metabolic fate of intracellular glutamate and to differentiate the action of d-Asp from that of THA, we monitored the formation of $^{15}$NH$_4^+$ and $^{15}$Nalanine from $^{15}$N-labeled amino nitrogen of glutamine in the presence of each inhibitor alone and in combination (Fig. 6). In the presence of d-Asp + THA, NH$_4^+$ formed from glutamate deamination was increased 3.1-fold ($P < 0.01$). Furthermore, THA added to d-Asp increased ($P < 0.03$) the deamination rate compared with d-Asp alone (575 ± 40 vs. 348 ± 41 nmol/mg). The alanine formation from $^{15}$N-labeled amino nitrogen of glutamine was reduced ($P < 0.03$) by THA, indicating that THA impedes the ALT flux; this

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**Table 1. [2-$^{15}$N]glutamine utilization and [15N]glutamate, [15N]alanine, and NH$_4^+$ formation**

<table>
<thead>
<tr>
<th></th>
<th>Gln</th>
<th>$^{13}$Glu</th>
<th>$^{13}$Ala</th>
<th>NH$_4^+$</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,329 ± 83</td>
<td>65 ± 6</td>
<td>298 ± 40</td>
<td>995 ± 68</td>
<td>1,557 ± 52</td>
</tr>
<tr>
<td>d-Asp + THA</td>
<td>1,516 ± 86</td>
<td>745 ± 60</td>
<td>252 ± 27</td>
<td>1,186 ± 96</td>
<td>2,179 ± 57</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol/mg protein for 16 h of incubation from 9 pairs of plates. Glu and Ala, formation of $^{15}$N-labeled Glu and Ala from [2-$^{15}$N]glutamine (see MATERIALS AND METHODS); Sum, combined total of Glu and Ala formed from glutamine plus total NH$_4^+$. $P$ values were obtained from comparison with control.

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![Fig. 5. Extra- and intracellular Gln hydrolysis in control monolayers and monolayers treated with d-Asp + THA. Extracellular hydrolysis was taken as the acivicin-inhibitable Glu formation (γ-GT) phosphate-independent glutaminase (γ-GT-PIG) in the presence of d-Asp (d-A) + THA. Intracellular Gln hydrolysis by phosphate-dependent GA (PDG) is Glu + Ala formed from labeled Gln + NH$_4^+$ 2 (see MATERIALS AND METHODS for calculation). Values are means ± SE from 9 pairs of plates. *Different from control, $P < 0.001$.](http://ajpcell.physiology.org/)

![Fig. 6. Effect of THA or D-Asp alone and in combination on [15N]Glu, [15N]NH$_4^+$ and [15N]Ala formation from the [2-$^{15}$N]Gln. Monolayers were incubated for 16 h in DMEM, DMEM + 0.5 mM THA, DMEM + 5 mM d-Asp, or DMEM + 0.5 mM THA + 5 mM d-Asp (DMEM contains 0.05 mM L-Glu). Values [isotopic enrichment (atom % excess) × concentration (nmol/mg protein)] are means ± SE. *Different from controls, $P < 0.05$; **different from d-Asp, $P < 0.05$.](http://ajpcell.physiology.org/)
The effect of THA was also in evidence in combination with d-Asp (357 ± 36 vs. 590 ± 67 nmol/mg for d-Asp alone, \( P < 0.05 \)). Both d-Asp and THA increased the accumulation of \( ^{15} \text{N} \) glutamate in the media, although THA reduced (\( P = 0.05 \)) the d-Asp-induced accumulation (400 ± 60 vs. 575 ± 27 nmol/mg). These results show that both THA and d-Asp alone increase the GDH flux but that THA decreases the ALT flux whether alone or in combination with d-Asp; additionally, THA apparently acts to slow the d-Asp-accelerated glutamate efflux.

The acute effect of d-Asp and THA on efflux pathway(s) was studied in monolayers preloaded with the nonmetabolizable d-[\(^{3} \text{H}\)]Asp and compared with glutamate efflux. After the monolayers were preloaded with d-[\(^{3} \text{H}\)]Asp, they were placed in control DMEM, DMEM containing 5 mM d-Asp, or DMEM containing 5 mM d-Asp + 0.5 mM THA for 45 min; then the d-[\(^{3} \text{H}\)]Asp retained in the monolayer was determined (Fig. 7). Compared with the control monolayers, those exposed to 5 mM d-Asp or 5 mM d-Asp + 0.5 mM THA retained 31 and 36% less d-[\(^{3} \text{H}\)]Asp than the controls (82 ± 16 × 10\(^{3} \) vs. 57 ± 20 × 10\(^{3} \) and 52 ± 19 × 10\(^{3} \) cpm/mg protein after 45 min, \( n = 4 \) pairs, \( P < 0.01 \)). Compared with monolayers taken at time 0, the control monolayers taken at 45 min had already lost 43 ± 10% of their d-[\(^{3} \text{H}\)]Asp. These findings indicate that d-Asp and d-Asp + THA accelerate the efflux of intracellular substrates for system X\(_{AG} \) and that a significant efflux occurs even in the normal mix of media amino acids. In this acute 45-min study, glutamate accumulated in the media with d-Asp and d-Asp + THA (Fig. 8), in contrast to the controls, which did not show an accumulation (4.4 ± 0.2 and 2.8 ± 0.4 nmol·mg\(^{-1}\)·min\(^{-1} \) for d-Asp and d-Asp + THA, respectively, vs. −0.2 ± 0.3 nmol·mg\(^{-1}\)·min\(^{-1} \) for control, \( P < 0.0001 \)). The presence of THA reduced (\( P < 0.05 \)) the media glutamate accumulation compared with the presence of d-Asp alone (2.8 ± 0.4 vs. 4.4 ± 0.2 nmol·mg\(^{-1}\)·min\(^{-1} \)) and also decreased (\( P < 0.01 \)) the formation of alanine compared with d-Asp (11.7 ± 0.7 vs. 16.2 ± 0.3 nmol·mg\(^{-1}\)·min\(^{-1} \), \( P < 0.01 \)). As expected from the longer term study above, THA + d-Asp increased (\( P < 0.05 \)) the NH\(_{4} \) formation over that produced with d-Asp alone. d-Asp alone and d-Asp + THA reduced the monolayer glutamate content (134 ± 10 and 131 ± 12 vs. 198 ± 3 nmol/mg protein, both \( P < 0.02 \) vs. control), indicating that THA did not back up the glutamate in the cytosol. These results suggest that THA does not reduce glutamate efflux by system X\(_{AG} \) but acts on a second stereoselective system that also contributes to extracellular glutamate accumulation under these conditions.

**DISCUSSION**

The objectives of this study were to determine the role of glutamate transporter activity in intracellular glutamine hydrolysis and in the fate of the intracellular glutamate generated (Fig. 1A). We chose inhibitors of the high-affinity plasma membrane glutamate transporters to block uptake of extracellular glutamate and to lower the intracellular glutamate concentration and, thereby, activate the intracellular GA (Fig. 1B). The inhibitors chosen did block extracellular glutamate uptake and produced a prompt and sustained reduction in the intracellular glutamate content. As a consequence of reducing this potent functional GA inhibitor (17), hydrolysis of glutamine increased more than twofold (Fig. 5). These results are consistent with the system X\(_{AG} \)-transported glutamate setting the cytosolic glutamate concentration, which, in turn, acts as a signal (35) in regulating flux through the functional GA. Accordingly, plasma membrane glutamate transport regulation of the functional GA is consistent with localization at the cytosolic (17), rather than the ma-
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...pathway. Accordingly, system XAG (20), driving the cytosolic glutamate into the GDH. It seems likely that the intracellular pH is depressed or directly inhibiting the reaction of glutamate with the enzyme (11). There are two forms of ALT as described in Fig. 1: one is present in the cytosol, and the other is localized to the mitochondrial matrix (19). Because L-THA is a competitive inhibitor of mitochondrial glutamate uptake via the Glu/Asp-exchanger (2), it could limit the available glutamate for intramitochondrial alanine formation. The presence of the cytosolic ALT activity might explain the reduced alanine formation in the presence of THA. Alternatively, the reduced alanine formation may reflect L-THA displacing glutamate from the ALT-reactive site (11). The effect of THA to reduce alanine formation was previously noted (34), while the overall energy generated by the mitochondria is maintained by the increased flux through the deamination pathway (Fig. 6). Further studies comparing the effects of D- and L-THA are required to confirm the active isomer and the mechanism of action in inhibiting transamination.

Glutamate metabolism is highly compartmentalized and, therefore, dependent on translocation between intra- and extracellular compartments (6, 16, 30). In the control monolayers, labeled glutamate, produced in both compartments, equilibrated throughout the extra- and intracellular compartments (Fig. 2), indicating that glutamate transporter activities in the plasma (6) and mitochondrial (16, 30) membranes are able to keep pace with the localized glutamate formation. This reveals a high bidirectional flux of glutamate across the plasma membrane under these conditions (Fig. 1). Furthermore, essentially all the glutamate uptake and a significant part of the efflux is dependent on the plasma membrane system XAG activity, since D-Asp and THA prevented equilibration of labeled glutamate added to the media (Fig. 3). System XAG has been shown to function in both modes (22), and countertransport activity plays important homeostatic roles (6). Our results show that the media labeled glutamate decreased 50%, indicating an influx of nonlabeled glutamate, since uptake was blocked. The sources of this nonlabeled glutamate were subsequently shown to be glutamine hydrolyzed by the extracellular γ-GT and glutamate transported out of the cell (Fig. 4). The intracellular source was further shown through the 15N label to be glutamine hydrolyzed by the functional GA. Inhibiting system XAG-mediated plasma membrane glutamate uptake in the brain led to >30% of labeled extracellular glutamine recovered as labeled extracellular glutamate (21). These findings suggest that the model presented in Fig. 1 may have a wider application than epithelial cells.

The surprising large fluxes of glutamate into and out of these cells indicate a significant cycling of glutamate across the plasma membrane, with system XAG acting as a homoexchanger (Fig. 1). The large influx was supported by the preformed and the γ-GT-generated glutamate as well as by a significant efflux of intracellular glutamate. Evidence for the efflux pathway in the control monolayers was the equilibration of labeled glutamate formed from labeled glutamine throughout both compartments, equilibrated throughout the extra- and intracellular compartments (Fig. 2), indicating that glutamate transporter activities in the plasma (6) and mitochondrial (16, 30) membranes are able to keep pace with the localized glutamate formation. This reveals a high bidirectional flux of glutamate across the plasma membrane under these conditions (Fig. 1). Furthermore, essentially all the glutamate uptake and a significant part of the efflux is dependent on the plasma membrane system XAG activity, since D-Asp and THA prevented equilibration of labeled glutamate added to the media (Fig. 3). System XAG has been shown to function in both modes (22), and countertransport activity plays important homeostatic roles (6). Our results show that the media labeled glutamate decreased 50%, indicating an influx of nonlabeled glutamate, since uptake was blocked. The sources of this nonlabeled glutamate were subsequently shown to be glutamine hydrolyzed by the extracellular γ-GT and glutamate transported out of the cell (Fig. 4). The intracellular source was further shown through the 15N label to be glutamine hydrolyzed by the functional GA. Inhibiting system XAG-mediated plasma membrane glutamate uptake in the brain led to >30% of labeled extracellular glutamine recovered as labeled extracellular glutamate (21). These findings suggest that the model presented in Fig. 1 may have a wider application than epithelial cells.
from preloaded monolayers (Fig. 7) and the disruption of the influx and acceleration of the efflux by D-Asp + THA (system X\textsubscript{AG} acting as a heteroexchanger) (22). The apparent function of this putative membrane cycling is to maintain a high X\textsubscript{AG} activity. The carrier turnover and, more specifically, the extravagent movement of ions (3 Na\textsuperscript{+}/1 K\textsuperscript{+}) may act as a regulator of glycolysis as they do in astrocytes (8). Accordingly, in epithelia, the formation of ADP, as a by-product of restoring the ionic gradients, may act to stimulate glycolysis and ATP formation, in turn coupled to cellular processes, e.g., paracellular permeability (34, 35). An excess of cycled glutamate is drawn off into the transamination or deamination pathways depending on the acid/base conditions. A deficit, on the other hand, accelerates glutamine hydrolysis supplying the cycled glutamate. Although metabolic acidosis decreases the extracellular glutamine hydrolysis (35), a fall in intracellular pH enhances the efflux of glutamate (26), ensuring a continuous supply for sustained glutamate uptake. From this perspective, glutamate/glutamate metabolism is regulated by glutamate transport and, in turn, plays a role in maintaining an adequate supply of glutamate dedicated to plasma membrane cycling and its function in cellular processes.

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