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Precursors of Glutamic Acid Nitrogen in Primary Neuronal Cultures: Studies with ^{15}N

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We utilized gas chromatography-mass spectrometry to study the transfer of ^{15}N from [2-^{15}N]glutamine, [^{15}N]leucine, [^{15}N]alanine, or $^{15}NH_4Cl$ to [^{15}N]glutamate and [^{15}N]aspartate in cultured cerebrocortical GABA-ergic neurons from the mouse. Initial rates of ^{15}N appearance (atom % excess) were somewhat higher with 2mM [2-^{15}N]glutamine as a precursor than with 1mM [^{15}N]leucine or 1mM [^{15}N]alanine, but initial net formation (nmol [^{15}N]glutamate/mg protein.min^{-1}) was roughly comparable with all precursors. At steady-state ^{15}N labeling was about two times greater with 2mM [2-^{15}N]glutamine as precursor. The subsequent transfer of ^{15}N from glutamate to aspartate was extremely rapid, the labelling pattern of these two amino acid pools being virtually indistinguishable. We observed little reductive amination of 2-oxo-glutarate to yield [^{15}N]glutamate in the presence of 0.3mM $^{15}NH_4Cl$. Reductive amination through glutamate dehydrogenase was much more prominent at a concentration of 3.0mM $^{15}NH_4Cl$. Glutamate formation via reductive amination was unaffected by inclusion of 1 mM 2-oxo-glutarate in the incubation medium. These results indicate that glutamate synthesis in cultured GABA-ergic neurons is derived not only from the glutaminase reaction, but also from transamination reactions in which both leucine and alanine are efficient N donors. Reductive amination of 2-oxo-glutarate in the glutamate dehydrogenase pathway plays a relatively minor role at lower concentrations of extracellular ammonia but becomes quite active at 3mM ammonia.

KEY WORDS: Glutamate; nitrogen; nitrogen-15; mass spectrometry.

INTRODUCTION

The release of transmitter glutamate or GABA from synaptic terminals imposes on neurons a constant need to replenish the pool of glutamate. Neuronal glutamate is thought to be derived from glutamine through the action of phosphate-dependent glutaminase, a mitochondrial enzyme present in nerve endings (1-7). The precursor glutamine is formed primarily in astrocytes, which are enriched in glutamine synthetase (8,9) activity and which rapidly remove glutamate from the extracellular space (reviewed in refs. 4,10). Conversion of glutamate to glutamine in astrocytes and the subsequent export of the latter to neurons, where it is re-converted to glutamate, constitutes the glutamate-glutamine cycle between astrocytes and neurons (11).

Considerable evidence suggests that brain glutamate metabolism is more varied than would be suggested by the glutamate-glutamine cycle. Thus, the rate of astrocyte glutamate uptake is greater than the rate of conversion to glutamine (12). Available evidence also indicates that astrocytes are capable of consuming as well as producing glutamine, with the rate of utilization being as high or even higher than that observed in neu-

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rons (13-16). The uptake of glutamine into cultured astrocytes may exceed that noted in neurons (17,18). In addition, the rate of neuronal glutamine uptake may not be fast enough to support the intracellular glutamate pool if the glutaminase pathway were the sole mechanism abetting glutamate synthesis.

If the hydrolysis of glutamine is not the sole source of neuronal glutamate, what other mechanisms could help support the intra-neuronal pool of this amino acid? The likely alternatives would include transamination reactions and the reductive amination of 2-oxo-glutarate via the glutamate dehydrogenase (GDH) reaction. The specific activities of various transaminases and GDH are relatively high in the brain (15, 19-21). Indirect evidence suggests that transamination is important for glutamate formation in both cultured cerebellar granule cells and in the medulla and cerebellum, since inhibition of transamination with aminooxyacetic acid sharply reduces glutamate release from these glutamatergic cells upon depolarization (22-24). Brain glutamate dehydrogenase activity is high in areas associated with glutamatergic transmission (25, 26). Whether this enzyme plays a prominent role in the synthesis of transmitter glutamate is problematic, however, since the K_m for the binding of ammonia to the enzyme is very high (10-30mM) (27,28).

In the current study we have utilized ^{15}N as a metabolic tracer with which to describe certain source(s) of glutamate nitrogen in primary cultures of GABA-ergic neurons. We previously applied this technique, using gas chromatography-mass spectrometry to measure ^{15}N isotopic enrichment, to the study of N metabolism in cultured astrocytes and synaptosomes (6, 7, 29). Our purpose was to compare the relative contributions of glutamine, leucine, alanine and NH_3 to the glutamate nitrogen in these neurons. The results show that although glutamine is a prominent source of glutamate N, it is not the sole source. Transamination reactions appear to be very important in maintaining intra-neuronal glutamate. Indeed, it is quite possible that transamination from all possible precursors constitutes a more significant mechanism for the replenishment of the amino group in brain glutamate than does the phosphate-dependent glutaminase pathway.

EXPERIMENTAL PROCEDURE

Materials. L-[^{15}N]Leucine, L-[2-^{15}N]glutamine, L[^{15}N]alanine and $^{15}NH_4Cl$ (each 98 atom% excess) were from MSD, Ltd. (Montreal, Quebec, Canada). All ion-exchange resins were from Bio-Rad Laboratories (Richmond, CA, USA). Reagents used for formation of derivatives were from Regis Chemical Co. (Chicago, IL, USA). o-phthalaldehyde was from Pierce Chemical Co. (Rockford, IL, USA). All other reagents were of the highest available grade and were from Fisher Chemical Co. (Silver Springs, MD, USA).

Preparation of Neuronal Cultures. Cortical neurons were cultured from cerebral cortices of 15 day-old mouse embryos, in principle as described by Dichter (30), but with several minor modifications. The meninges were removed and the hemispheres cut into small cubes and trypsinized for 2 min in 0.2% trypsin in Puck's solution at room temperature. After inhibition of the trypsin by addition of modified MEM with 20% horse serum, the tissue was triturated with a Pasteur pipette and centrifuged for 2 min at 900 *g*. The pellet was resuspended in a similar medium without serum and with 30 (instead of 7.5) mM glucose. A cell suspension corresponding to one-and-one-half brains per dish was seeded in 60-mm Falcon plastic tissue culture dishes that had been coated with polylysine by exposure overnight to 12.5 μg/ml of polylysine in water. After 15 min incubation at 37°C, unattached cells (non-neuronal cells) were removed together with the medium, which was replaced with a similar, fresh medium containing 5% horse serum. After 3 days of culturing, cytosine arabinoside was added to a final concentration of 40 μM, which led to the disappearance of most astrocytes (30). The cultures were refed 24 h later with fresh medium (with 5% serum) without the mitotic inhibitor and used for metabolic studies at day 14. Such cultures appear to contain mainly GABA-ergic neurons and to reach maximum functional development (characterized by a high glutamic acid decarboxylase (GAD) activity, an intense GABA uptake, and a potassium-induced GABA release) after 11-14 days in culture (31).

Methods of Incubation. For the studies of transamination reactions incubations were started by changing the steady-state incubation medium to a fresh solution containing the same amino acid composition as the prior medium except that either [^{15}N]leucine (1 mM), [2-^{15}N]glutamine (2 mM) or [^{15}N]alanine (1 mM) was substituted for the unlabelled species (0.8 mM leucine, 2 mM glutamine, no alanine in original medium). The cells then were incubated at 37°C for 15, 30, 60, 120, or 180 minutes. After washing with a phosphate-buffered saline solution, cold 10mM HCl was added to each cultured plate and the bottom of the plate was scraped vigorously with a piece of plastic. After freezing and thawing once, glutamate and aspartate were separated from the cell extract with a column of AG-1 (Cl^-; 100-200 mesh; X-8; 0.5 x 5 cm).

In order to study the role of a possible reductive amination in glutamate formation, the steady-state culture medium was replaced with fresh medium containing one of the following combinations of $^{15}NH_4Cl$ and 2-oxo-glutarate: (1) 0.3mM $^{15}NH_4Cl$ and 1 mM 2-oxo-glutarate; (2) 0.3mM $^{15}NH_4Cl$ only (3) 3mM $^{15}NH_4Cl$ and 1 mM 2-oxo-glutarate; (4) 3mM $^{15}NH_4Cl$ only. At 15, 30, 60, 120, 180 and 240 minutes this medium was removed and the cell monolayer was harvested and processed as described above.

Analytic Methods. Amino acids were measured as the o-phthalaldehyde derivatives (32) with reverse phase liquid chromatography.

^{15}N enrichment in glutamate and aspartate were determined following formation of the t-butyl-dimethylsilyl derivative. Labeling in [^{15}N]glutamate was measured from the m/z 433/432 ratio and that in aspartate from the m/z 419/418 ratio. Determinations were performed using selected ion monitoring on a Hewlett-Packard 5990A Mass Selective Detector.

Methods of Calculation. Isotopic abundance (atom % excess) was calculated according to Millard (33). The absolute ^{15}N concentration (nmol ^{15}N/mg protein) was calculated from the product of isotopic abundance/100 and metabolite concentration.

RESULTS

Isotopic abundance (atom % excess) in intracellular glutamate is shown in Figure 1A. Formation of [^{15}N]glutamate (initial rate of accumulation of ^{15}N during the first 15-30 min) was rapid after addition of either 2 mM [2-^{15}N]glutamine, 1 mM [^{15}N]leucine, or 1 mM [^{15}N]alanine. Enrichment (equilibrated level of ^{15}N during the 1-2 hr period) was greater with [2-^{15}N]glutamine as precursor, attaining a steady-state value of ~ 20-25 atom % excess, than with either 1mM [^{15}N]alanine or 1 mM [^{15}N]leucine, which reached an enrichment of 10-20 atom % excess. Leucine seemed to be a slightly more efficient donor of nitrogen than was alanine.

Transamination of [^{15}N]glutamate to form [^{15}N]aspartate was extremely fast. As illustrated in Figure 1B, the ^{15}N label appeared in aspartate within minutes of its appearance in [^{15}N]glutamate.

The intracellular glutamate level remained fairly constant during the course of the incubation, ranging between 40-50 nmol/mg protein. The intracellular aspartate also did not vary much, remaining between 20-30 nmol/mg protein.

The absolute amount (nmol ^{15}N/mg protein) of [^{15}N]glutamate produced is shown in Figure 2A. At 15 minutes, a total of 3.5-4 nmol of ^{15}N was resident in intracellular glutamic acid with either [2-^{15}N]glutamine or [^{15}N]alanine as precursor. Assuming a linear rate of formation, this would correspond to a net rate of [^{15}N]glutamate production of ~ 0.25 nmol/min.mg protein^{-1}. The rate of [^{15}N]aspartate synthesis at 15 minutes was 1.6 nmol, or ~ .11 nmol/min.mg protein^{-1}. The initial rate of incorporation was about half as great when [^{15}N]leucine was precursor.

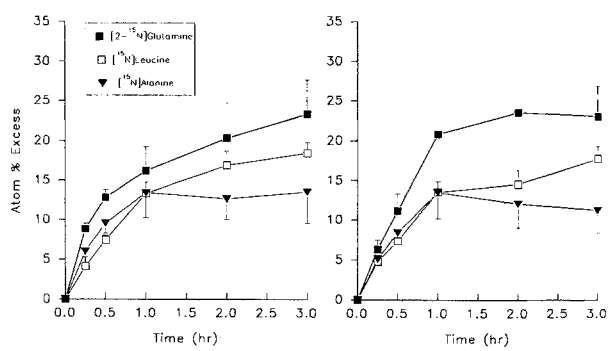

Fig. 1. Isotopic abundance (atom % excess) in intracellular [^{15}N]glutamate and [^{15}N]aspartate following exposure of GABA-ergic neurons to 2mM [2-^{15}N]glutamine, 1mM [^{15}N]leucine or 1mM [^{15}N]alanine. A: [^{15}N]glutamate. B: [^{15}N]aspartate. Each point represents the mean ± SEM of 4 experiments.

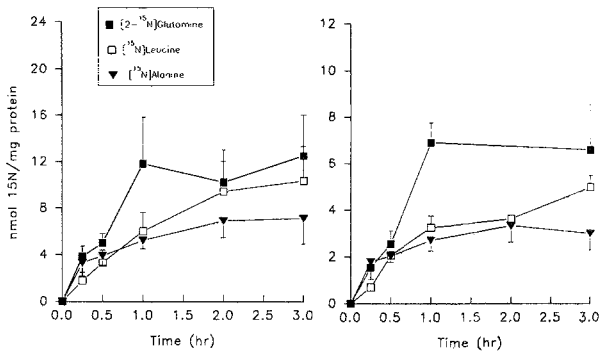

Fig. 2. Absolute concentration (nmol ^{15}N/mg protein) in intracellular [^{15}N]glutamate or [^{15}N]aspartate following incubation of GABA-ergic neurons with either 2 mM [2-^{15}N]glutamine, 1 mM [^{15}N]leucine or 1 mM [^{15}N]alanine. The calculation reflects the product of isotopic abundance/100 x intracellular concentration of glutamate and aspartate. A: [^{15}N]glutamate. B: [^{15}N]aspartate. Each point is the mean ± SEM of 4 experiments.

These data suggested that both glutaminase activity and transamination reactions were important for supplying the nitrogen group of glutamate in these neurons. In order to assess the extent to which glutamate could be produced by reductive amination of 2-oxo-glutarate in the glutamate dehydrogenase reaction, the neurons were incubated with 0.3 mM or 3.0 mM $^{15}NH_4Cl$ in the presence of absence of 1mM 2-oxo-glutarate. As shown in Figure 3A, isotopic abundance (atom % excess) in [^{15}N]glutamate was directly related to the $^{15}NH_4Cl$ concentration in the medium, increasing rapidly with 3mM $^{15}NH_4Cl$ but relatively slowly with 0.3mM $^{15}NH_4Cl$. Thus, at 15 minutes, ^{15}N labeling in glutamate was 10.4 vs. 1.3 atom % excess with 3 mM vs 0.3 mM $^{15}NH_4Cl$. Addition of 1 mM 2-oxo-glutarate to the medium together with $^{15}NH_4Cl$ did not augment either the rate of [^{15}N]glutamate production or the equilibrated level.

As shown in Figure 3B, labeling of [^{15}N]aspartate occurred virtually simultaneously with that of [^{15}N]glutamate.

The absolute concentration of intracellular [^{15}N]glutamate (nmol/mg protein) is illustrated in Figure 4A. With either 0.3 mM or 3.0 mM $^{15}NH_4Cl$ as precursor, the initial rate of synthesis appeared to be largely independent of the presence or absence of 2-oxo-glutarate in the incubation medium. At 15 minutes the concentration was either 0.3 (0.3 mM $^{15}NH_4Cl$) or 3.5-4.2 nmol (3.0 mM $^{15}NH_4Cl$) [^{15}N]glutamate/mg protein. Assuming a linear rate of incorporation of ^{15}N, this corresponds to ~ 0.02 or 0.27 nmol/min.mg protein^{-1}.

The concentration of [^{15}N]aspartate in the neurons is shown in Figure 4B. The levels were lower than those

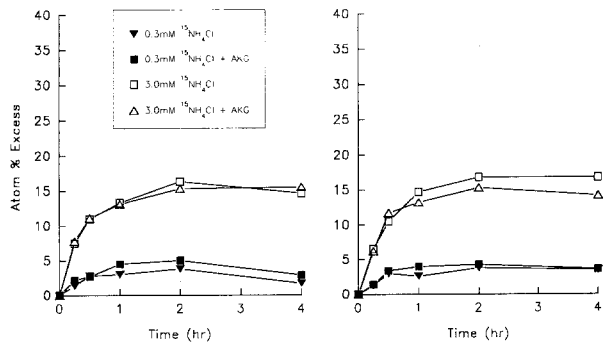

Fig. 3. Isotopic abundance (atom % excess) in intracellular [^{15}N]glutamate and [^{15}N]aspartate following incubation of GABA-ergic neurons with 0.3 mM or 3.0 mM $^{15}NH_4Cl$ in the presence or absence of 1 mM 2-oxo-glutarate. A: [^{15}N]glutamate. B: [^{15}N]aspartate. The 3 mM data is the mean of 3 experiments and the 0.3 mM data is the mean of 2 experiments.

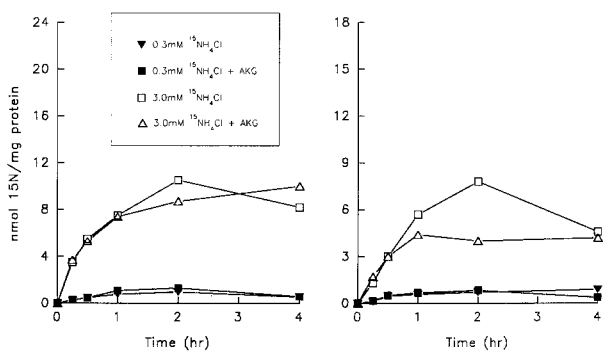

Fig. 4. Absolute concentration (nmol ^{15}N/mg protein) in intracellular [^{15}N]glutamate or [^{15}N]aspartate following incubation of GABA-ergic neurons with 0.3 mM or 3.0 mM $^{15}NH_4Cl$ in the presence or absence of 1 mM 2-oxo-glutarate. A: [^{15}N]glutamate. B: [^{15}N]aspartate. The 3 mM data is the mean of 3 experiments and the 0.3 mM data is the mean of 2 experiments.

of [^{15}N]glutamate, reflecting the relatively lower concentration of aspartate in the neurons. At the 2 hour time point the formation of [^{15}N]aspartate with 3 mM $^{15}NH_4Cl$ as precursor was greater in the absence of 2-oxo-glutarate, but the difference was not statistically significant ($p > .05$).

DISCUSSION

The current data indicate that cultured GABA-ergic neurons derive glutamate N via the hydrolysis of glutamine, the transamination of both leucine and alanine, and the reductive amination of 2-oxo-glutarate. Other amino acids presumably also are N donors via transamination. Of these three pathways the glutaminase pathway appeared to be somewhat more active (Figure 1A and 2A), but this conclusion is based on a study design in which only a single precursor was labelled with ^{15}N. It is possible that more glutamate N is formed via transamination from all potential nitrogen donors than would be derived from 2-N of glutamine in the glutaminase reaction. The fact that inhibition of transamination with aminooxyacetate also diminishes the release of glutamate from glutamatergic neurons upon depolarization is consistent with this formulation (22-24), as is the coincidence of high levels of aspartate aminotransferase activity in regions of high glutamatergic activity (34,35).

It must be stressed that the experimental design used in this study does not necessarily measure a net flux via transamination from a given precursor, e.g., [^{15}N]leucine or [^{15}N]alanine, to [^{15}N]glutamate. This nitrogen could be shuttled back from glutamate to the parent compound. The fact that labeling of [^{15}N]aspartate occurred almost simultaneously with that of [^{15}N]glutamate (Figure 1A) indicates a very fast articulation between these two pools. The possibility of such exchange reactions, however, should not diminish the significance of transamination to the maintenance of intra-neuronal glutamate. The reamination of 2-oxo-glutarate derived from glutamate by transamination with either leucine or alanine would help support the nitrogen of the glutamate pool as efficiently as would the derivation of "new" glutamate from glutamine via the glutaminase pathway. Otherwise, the 2-oxo-glutarate would be oxidized in the tricarboxylic acid cycle, thereby allowing the system to become depleted also of the carbon skeleton of glutamate.

Furthermore, the transamination of 2-oxo-glutarate with alanine, leucine and other amino acids affords a mechanism for the importation of nitrogen into the CNS from the periphery. Glutamine, which may cross the blood-brain barrier more slowly than other neutral amino acids (36), would not be a good vehicle for the carriage of "fresh" nitrogen into the system. Replenishment of nitrogen stores is of obvious urgency, since otherwise the brain inevitably would become depleted of vital stores of glutamate and other key amino acids. We have demonstrated previously that the branched-chain amino acids are crucial N donors in organotypic cerebellar explants (37), in which approximately 30% of glutamate N appeared to have been derived from leucine and valine. The current study indicates that the transamination of branched-chain amino acids is a very active process in neurons, in which these compounds and alanine are potential precursors to glutamate N.

The data also suggest that the reductive amination of 2-oxo-glutarate in the glutamate dehydrogenase re-

action, although clearly present in this experimental system, at physiologic levels of ammonia was a less active process than either transamination or the glutaminase pathway. At a relatively low (0.3 mM) concentration of $^{15}NH_4Cl$, ^{15}N resident in glutamate was only 10-20% of that transferred with labeled glutamine, alanine or leucine as a precursor. Reductive amination clearly was stimulated by the presence of a high (3mM) concentration of $^{15}NH_4Cl$ (Figure 4 vs Figure 2). Even though the equilibrium constant of GDH would favor reductive amination over oxidative deamination, the K_m of the enzyme for ammonia may be as high as 20mM (27,28). Our data therefore are consistent with the conception that reductive amination of 2-oxo-glutarate is not a prominent route of glutamate synthesis, except perhaps in hyperammonemic conditions. Recent data suggest that synaptosomal GDH functions primarily in the direction of oxidative deamination of glutamate rather than reductive amination (38).

A facile extrapolation of these data to physiologic reality should be avoided. The concentrations of amino acids in the culture medium are considerably higher than those commonly observed in the extracellular fluid. Furthermore, the production of glutamate undoubtedly is regulated by a variety of control mechanisms which would not be operative in the culture system. An important regulatory factor is depolarization of neurons, which we have found to be a potent modulator of flux through the phosphate-dependent glutaminase pathway (7). We also found transamination pathways to be responsive to the availability of metabolic substrates, with transamination from glutamate to aspartate in synaptosomes being much greater when glucose is omitted from the incubation medium (6). Similar studies must be performed in the cultured neurons, in order to ascertain how glutamate synthesis and utilization are regulated. It also must be emphasized that the present study has determined formation of total glutamate within the cells. Formation of releasable transmitter might be different (23) (see below).

Interpretation of the data also requires consideration of the compartmentation of glutamate into various intraneuronal pools. In the current study, we measured only the ^{15}N resident in glutamate of the integral system. However, it is very likely that the pool released upon depolarization may be segregated from the "metabolic" pool(s). Thus, a recent study showed that the release of glutamate from guinea pig synaptosomes is not strongly influenced by the intracellular concentration (39), suggesting a compartmentation of the transmitter pool. Indeed, it is not even certain that the pool(s) of [^{15}N]glutamate formed via the action of glutaminase is identical to that formed by transamination or by reductive amination. Such compartmentation may be inevitable, given the fact that both the glutaminase and GDH pathways are predominantly mitochondrial, but the transamination pathways exist in both mitochondria and cytosol (40).

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