Acute Insult of Ammonia Leads to Calcium-dependent Glutamate Release from Cultured Astrocytes, an Effect of pH*

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Hyperammonemia is a key factor in the pathogenesis of hepatic encephalopathy (HE) as well as other metabolic encephalopathies, such as those associated with inherited disorders of urea cycle enzymes and in Reye’s syndrome. Acute HE results in increased brain ammonia (up to 5 mM), astrocytic swelling, and altered glutamatergic function. In the present study, using fluorescence imaging techniques, acute exposure (10 min) of ammonia (NH₃/NH₄⁺) to cultured astrocytes resulted in a concentration-dependent, transient increase in [Ca²⁺]. This calcium transient was due to release from intracellular calcium stores, since the response was thapsigargin-sensitive and was still observed in calcium-free buffer. Using an enzyme-linked fluorescence assay, glutamate release was measured indirectly via the production of NADH (a naturally fluorescent product when excited with UV light). NH₃/NH₄⁺ (5 mM) stimulated a calcium-dependent glutamate release from cultured astrocytes, which was inhibited after preincubation with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester but unaffected after preincubation with glutamate transport inhibitors dihydrokainate and DL-threo-benzylxoxaspartate. NH₃/NH₄⁺ (5 mM) also induced a transient intracellular alkaline shift. To investigate whether the effects of NH₃/NH₄⁺ were mediated by an increase in pH, we applied trimethylamine (TMA⁺/TMA) as another weak base. TMA⁺/TMA (5 mM) induced a similar transient increase in both pH and [Ca²⁺]. This calcium transient was due to mobilization from intracellular calcium stores and resulted in calcium-dependent release of glutamate. These results indicate that an acute exposure to ammonia, resulting in cytosolic alkalization, leads to calcium-dependent glutamate release from astrocytes. A deregulation of glutamate release from astrocytes by ammonia could contribute to glutamate dysfunction consistently observed in acute HE.

Hyperammonemia consequently leads to increased concentrations of ammonia, up to 5 mM, in the brain. This high level of brain ammonia is a key factor in the pathogenesis of central nervous system dysfunction in acute and chronic liver failure. The nature and severity of the central nervous system disorder mainly depend upon the degree and acuteness of the onset of hyperammonemia (1). Acute liver failure (ALF)† resulting from viral infections or toxic liver injury is a life-threatening condition where hepatic encephalopathy (HE) develops rapidly and mortality rates are high due to brain stem herniation caused by increased intracranial pressure, a fatal consequence of cytotoxic brain edema. Excess ammonia is toxic to the brain resulting in deleterious effects, by both direct and indirect mechanisms, on cerebral metabolism and neurotransmission.

Over the past 10 years, there has been an increasing body of evidence demonstrating that ammonia toxicity is involved in alterations of glutamatergic synaptic regulation which is implicated in the pathophysiology of HE in ALF. Several reports have consistently described increased extracellular concentrations of brain glutamate in different models of experimental ALF (2–5); however, neither the cell type nor the underlying release mechanisms have been identified. One possible explanation for the increased extracellular glutamate may be ammonia’s inhibitory effects on the glutamate transporter system in astrocytes. It has been shown that ammonia inhibits glutamate uptake into astrocytes in vitro (6) and decreases protein and gene expression of the glutamate transporter GLT-1 (EAAT-2) in the frontal cortex of rats with ALF (7). The role of ammonia in the glutamatergic dysfunction demonstrated in HE is supported with a positive correlation between extracellular brain concentrations of glutamate and arterial ammonia concentrations in ALF in rats (4). In addition, using mild hypothermia as a treatment in rats with ALF, extracellular brain glutamate concentrations were normalized concomitantly with a lowering of brain ammonia (8).

Glutamate has been demonstrated to be an important signaling molecule for neuron-glia communication. Astrocytes express receptors and transporters for glutamate and recently have also been demonstrated to contain the protein machinery necessary to release glutamate by exocytosis through vesicles (9) and a fusion-related mechanism (10, 11). Overall, astrocytes have many characteristics that were previously considered exclusive for neurons and are therefore actively involved in cell signaling by releasing glutamate. Astrocytic glutamate release is calcium-dependent and can be triggered by any ligand that stimulates an increase in [Ca²⁺], such as bradykinins (12), prostaglandins (13), and ATP (14, 15). Even a spontaneous [Ca²⁺] increase leads to glutamate release from astrocytes (16).

A rapid increase in ammonia results in an increase in pH, (intracellular alkalinization) in all cell types, including astrocytes (17). It has been also demonstrated that intracellular alkalinization is accompanied with an increase in [Ca²⁺], in

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‡ The abbreviations used are: ALF, acute liver failure; HE, hepatic encephalopathy; GPT, glutamate pyruvate transaminase; AM, acetoxymethylster; BCECF, 2',7'-bis(carboxyethyl)-carboxyfluorescein; TBOA, DL-threo-benzylxoxaspartate; BAPTA, 1,2-bis(2-aminophenoxo)ethane-N,N,N',N'-tetracetic acid; ER, endoplasmic reticulum; TMA, trimethylamine; DHK, dihydrokainate.
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cultured aicin cells (18), in enthothelial cells (19), in pituatory cells (20), and in neurons (21). Furthermore, ammonia-induced intracellular alkalinization has been demonstrated to increase [Ca$^{2+}$], in microglia initiating Ca$^{2+}$ release from thapsigargin-sensitive stores (22).

The purpose of our study was to investigate whether ammonia-induced intracellular alkalinization could have an effect on [Ca$^{2+}$], signaling in astrocytes and furthermore study whether the effects of ammonia could play a pathophysiological role in glutamate release from astrocytes.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cultured Astrocytes**—Astrocytes were prepared from cortex of newborn NMRI mice as described previously (23). Briefly, cortical tissue was carefully dissected from blood vessels and meninges, rinsed with basal medium Eagle’s 10% fetal calf serum. One day later, cultures were washed and Hanks’ balanced salt solution to remove debris and maintained for 4 days. After reaching subconfluent state, cellular debris, microglia cells, oligodendrocytes as well as their early precursor cells were disabled by manual shaking and removed by washing with Hanks’ balanced salt solution. The purity of the astrocytes was routinely determined by immunofluorescence using a polyclonal antibody against glial fibrillary acidic protein (DAKO, Hamburg, Germany), a specific astrocyte marker. The cultures showed more than 90% cells positive for glial fibrillary acidic protein. Measurements were made from cells between days 11 and 15.

**Solutions**—All solutions were freshly prepared from refrigerated stock solutions. The standard bath solution was composed of 150 mM NaCl, 5.4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 10 mM glucose, pH adjusted to 7.4 by NaOH. To obtain calcium-free solution, CaCl$_2$ was omitted, replaced with an equivalent amount of MgCl$_2$. To obtain calcium-free solution, CaCl$_2$ was omitted, replaced with an equivalent amount of MgCl$_2$. For ammonia and trimethylamine solutions, NH$_4$Cl or TMACl were added to the solution by replacing an equivalent amount of NaCl.

**Fluorescence Imaging System**—Cells were visualized under water immersion with a ×40 objective (numerical aperture 0.9) from a perfusion chamber mounted on a stage with an upright microscope (Axioskop FS, FS, Oberschleißh.”

For measurements of pH in cultured astrocytes a ratiometric dye was measured at 360 nm and 530 nm, and the emission was measured at 530 ± 10 nm. The [Ca$^{2+}$], was calculated from the ratio of fluorescence recorded at 340 and 380 nm excitation wavelengths using the equation of Grynkiewicz et al. (26), [Ca$^{2+}$], = K$_b$R/R$_{min}$, where R$_{min}$, is the fluorescence ratio of calcium-free/fura-2 and R$_{max}$ is calcium-bound fura-2. The constant K$_b$, the calcium-bound dye, was determined empirically. The system was calibrated in situ by employing an ion-mycobased intracellular calibration procedure as described previously (27). The parameters K$_b$, R$_{min}$, and R$_{max}$ characterizing the system were 1.6, 0.2, and 2.1 µM, respectively.

**Measurement of Intracellular pH**—For measurements of pH, the H-sensitive fluorophore 2,7′-bis(carboxyethyl)-carboxyfluorescein (BCECF) was used. Cultured astrocytes on coverslips were incubated at room temperature in the dark with a 5 µM concentration of the membrane-permeant BCECF/AM in physiological buffer for 15 min and then washed and stored in the dark for an additional 15 min to ensure BCECF/AM hydrolysis. BCECF, a ratiometric dye was measured at excitation wavelengths 440 and 488 nm. Emission was measured at 530 ± 10 nm. An acid pH shift was demonstrated with a decrease in BCECF fluorescence. Calibrations of pH, with different pH solutions (6.0, 6.5, 7.0, 7.5, and 8.0) were measured in the presence of high K$^+$ (105 mM) solution containing nigericin (10 µM) (28). Each calibration was repeated three times with 20–30 cells from different cultures.

**Glutamate Transporter Inhibitors**—To block glutamate release, cells were pretreated, for 5 and 10 min prior to the experiments, with n-3,3-dimethyl-3-benzoyloxyxysartate (TBOA), an EAAT-1 glutamate transporter inhibitor, and dihydrokainate (DHK), an EAAT-2 glutamate transporter inhibitor at a final concentration of 10 and 100 µM, respectively. TBOA and DHK are nonsubstrate glutamate transporter inhibitors. An important functional distinction between substrate and nonsubstrate inhibitors is that substrate inhibitors can induce release of excitatory amino acids by heteroexchange or exchange with an intracellular substrate. Nonsubstrate inhibitors block uptake reversal through transporters by binding to the extracellular surface and therefore are not transported (29), preventing glutamate from being transported into and out of the cell.

**Materials**—Fura-2/AM, BCECF/AM, and BAPTA/AM were obtained from Molecular Probes, Inc. (Eugene, OR). NH$_3$, TMA/Cl, GDH (G26526), BPT (G7000), NAD$^+$ (N7004), alanine, nicotine, iomomycin, thapsigargin, TBOA, and DHK were obtained from Sigma.

**RESULTS**

$NH_3/NH_4^+$ Triggers a Transient Increase in [Ca$^{2+}$]—Extracellular application of ammonia chloride (NH$_4^+$/NH$_3$) (5 mM) at pH 7.4 triggered a transient increase in [Ca$^{2+}$], in cultured mouse astrocytes measured with the cell permeant calcium-sensitive fluorophore fura-2/AM (Fig. 1).

An increase in [Ca$^{2+}$], peaked within ~1 min and returned to baseline within 5–8 min in the continuous presence of NH$_4^+$/NH$_3$ (Fig. 2A). The absolute amplitude increase was, on average, $66.3 ± 4.4$ nM ($n = 217$) (Fig. 2D). Higher concentrations of NH$_4^+$/NH$_3$ (10 and 20 mM) elicited a response with similar time course but with a higher amplitude, whereas NH$_4^+$/NH$_3$, at 1 mM did not stimulate a significant increase in [Ca$^{2+}$], (Fig. 2D). To test whether NH$_4^+$/NH$_3$-stimulated [Ca$^{2+}$], transients could occur from an already elevated level of NH$_3$/NH$_4^+$, astrocytes...
FIG. 1. NH$_4^+$/NH$_3$ stimulates a transient increase in [Ca$^{2+}$], in cultured astrocytes. Astrocytes were loaded with the cell-permeant calcium-sensitive fluorophore fura-2/AM. The fluorescence image on the left shows the dye-loaded astrocytes. A region of interest (ROI) was selected from each astrocyte (typical example shown from one astrocyte). The corresponding trace to the right is the response from that ROI upon application of NH$_4^+$/NH$_3$ (10 min, 5 mM).

FIG. 2. NH$_4^+$/NH$_3$ stimulates a concentration-dependent transient increase in [Ca$^{2+}$], in cultured astrocytes in both physiological and calcium-free buffer. Typical traces of fura-2 fluorescence calibrated as changes [Ca$^{2+}$], during application of NH$_4^+$/NH$_3$ at 5, 10, and 20 mM. NH$_4^+$/NH$_3$ induced a concentration-dependent transient increase in [Ca$^{2+}$], in both physiological (control) (A) and calcium-free buffer (B). No significant increase in [Ca$^{2+}$], was observed with 1 mM NH$_4^+$/NH$_3$ (trace not shown) in both buffers. C, a transient increase in [Ca$^{2+}$], was also observed upon stimulation with 6 mM NH$_4^+$/NH$_3$, following a 1 mM NH$_4^+$/NH$_3$ application. D, bar graph demonstrating ammonia-induced concentration-dependent increase in [Ca$^{2+}$], in physiological buffer (0 mM NH$_4^+$/NH$_3$ control), 3.2 ± 1.3 nM (n = 176); 1 mM NH$_4^+$/NH$_3$, 6.7 ± 2.2 nM (n = 165); 5 mM NH$_4^+$/NH$_3$, 66.3 ± 4.4 nM (n = 217); **, p < 0.01); 10 mM NH$_4^+$/NH$_3$, 84.5 ± 5.8 nM (n = 198); **, p < 0.001); 20 mM NH$_4^+$/NH$_3$, 106.6 ± 6.5 nM (n = 156); **, p < 0.001) and in calcium-free buffer (0 mM NH$_4^+$/NH$_3$ control), 3.8 ± 2.0 nM (n = 179); 1 mM NH$_4^+$/NH$_3$, 6.9 ± 1.8 nM (n = 187); 5 mM NH$_4^+$/NH$_3$, 62.9 ± 5.4 nM (n = 224); **, p < 0.01); 10 mM NH$_4^+$/NH$_3$, 77.6 ± 5.5 nM (n = 191); **, p < 0.001); 20 mM NH$_4^+$/NH$_3$, 100.5 ± 5.7 nM (n = 173); **, p < 0.001). No significant difference in Δ[Ca$^{2+}$], values was found with the same ammonia concentration treatment between physiological and calcium-free buffer. Data are expressed as means ± S.E. Significant difference between groups was calculated using a one-way analysis of variance and post hoc Tukey’s test. Differences were considered when p < 0.05.

were bathed in 1 mM NH$_4^+$/NH$_3$ and then stimulated with 6 mM NH$_4^+$/NH$_3$ (Fig. 2C); this resulted in a similar [Ca$^{2+}$], transient as observed when NH$_4^+$/NH$_3$ (5 mM) was applied to a physiological bathing solution.

NH$_4^+$/NH$_3$-triggered Increase in [Ca$^{2+}$], Is Due to Release from Internal Stores—To test for the source of calcium, we compared [Ca$^{2+}$], responses to applications of NH$_4^+$/NH$_3$ in physiological and calcium-free bathing solutions. NH$_4^+$/NH$_3$ triggered similar calcium responses in the calcium-free bathing solution as compared with physiological (control) solution with respect to amplitude and time course and was effective at 5, 10, and 20 mM (Fig. 2B) but not 1 mM (Fig. 2D). To support the view that NH$_4^+$/NH$_3$ triggered calcium release from internal calcium stores, we used a paradigm to deplete endoplasmic reticulum (ER) stores; in the presence of thapsigargin (500 nM), a blocker for calcium transport into the ER stores, we applied ATP (100 μM) to further deplete ER stores. As shown before (30), ATP triggered a large increase in [Ca$^{2+}$], and typically all subsequent metabolic responses were abolished in the continuous presence of thapsigargin. When NH$_4^+$/NH$_3$ (5 mM) was applied following this paradigm and still in the presence of thapsigargin, it failed to elicit a calcium response (n = 91) (Fig. 3). We conclude that NH$_4^+$/NH$_3$ triggers calcium release from thapsigargin-sensitive intracellular stores.

TMA+/TMA, Another Weak Base, Mimics NH$_4^+$/NH$_3$-triggered [Ca$^{2+}$], Increase and Alkaline Shift—An increase of NH$_4^+$/NH$_3$ has been described to result in an alkalization of the cell plasma in most cells, including astrocytes (31). We therefore used another weak base, trimethylamine chloride (TMA+/TMA), to mimic the alkaline shift and test the impact of this alkalization on [Ca$^{2+}$], signaling. We measured intracellular pH using the cell-permeant pH-sensitive fluorophore BCECF/AM. NH$_4^+$/NH$_3$ (5 mM) triggered an intracellular alkalization by 0.44 ± 0.06 pH units (n = 173), and TMA+/TMA (5 mM) triggered one of 0.47 ± 0.05 pH units (n = 151) (Fig. 4, A and B). While in the continuous presence of NH$_4^+$/NH$_3$ (5 mM), pH returned to basal level within 4–6 min (Fig. 4A), whereas pH remained elevated for at least 10 min in the continuous presence of TMA+/TMA (5 mM) (Fig. 4B). Both NH$_4^+$/NH$_3$ (5 mM) and TMA+/TMA (5 mM) triggered a similar alkaline shift in calcium-free bathing solution. NH$_4^+$/NH$_3$ (5 mM) increased pH, by 0.41 ± 0.07 pH units (n = 146), and TMA+/TMA (5 mM) increased pH by 0.43 ± 0.06 pH units (n = 138). In conclusion, both agents trigger a similar alkaline shift.

FIG. 3. NH$_4^+$/NH$_3$, triggers calcium release from thapsigargin-sensitive intracellular calcium stores. To investigate the source of NH$_4^+$/NH$_3$ (5 mM) stimulated increase in [Ca$^{2+}$], a paradigm was used to deplete the ER calcium stores. In calcium-free buffer, thapsigargin (500 nM), a blocker for Ca$^{2+}$ transport into the ER stores, was applied, resulting in a transient increase in [Ca$^{2+}$]. To further stimulate the efflux of calcium from the ER stores, ATP (100 μM) was additionally applied, which resulted in another transient increase in [Ca$^{2+}$],. After [Ca$^{2+}$], had reached a new base line, NH$_4^+$/NH$_3$ (5 mM) was applied and did not result in a change in [Ca$^{2+}$].
TMA\(^+\)/TMA\(^-\) (5 mM) triggered a transient increase in \([\text{Ca}^{2+}]\), with a similar time course as recorded for NH\(^+\)/NH\(^-\) (5 mM) (Fig. 5A). The amplitude was comparable with that of the NH\(^+\)/NH\(^-\)-triggered response, namely 69.9 ± 5.3 nM (n = 132) in physiological (control) and 65.9 ± 4.8 nM (n = 147) in calcium-free buffer (Fig. 5A). We then applied the above described paradigm to depleted ER stores, namely by adding thapsigargin to the bath (500 nM) and briefly applying ATP (100 \(\mu\)M). When TMA\(^+\)/TMA\(^-\) (5 mM) was added (still in the presence of thapsigargin), it did not trigger a significant increase in \([\text{Ca}^{2+}]\), (Fig. 5B). Taken together, these data indicate that alkaline shifts trigger the release of calcium from cytoplasmic thapsigargin-sensitive stores.

An Enzyme-linked Fluorescence Assay Can Record Low Levels of Glutamate in the Presence of NH\(^+\)/NH\(^-\).—Glutamate released from cultured astrocytes has previously been recorded using an enzyme-linked fluorescence assay (13, 24, 25). With the enzyme GDH and the substrate NAD\(^+\) in the extracellular medium, glutamate produces \(\alpha\)-ketoglutarate, NH\(^+\), and NADH (Fig. 6 (inset), Reaction 1). Formation of NADH was recorded as a fluorescence signal when excited at 360 nm, and therefore changes in fluorescence reflect changes in glutamate levels in the bath. However, application of NH\(^+\)/NH\(^-\) results in product inhibition, decreasing the formation of NADH. Testing the degree of inhibition, different concentrations of NH\(^+\)/NH\(^-\) were applied with various concentrations of glutamate in a medium (without cells) containing NAD\(^+\) and GDH. NH\(^+\)/NH\(^-\) inhibited the production of NADH in a concentration-dependent manner, at a given (known) concentration of glutamate (Fig. 6A). At 5 \(\mu\)M glutamate, NADH formation was barely detectable.

To increase the sensitivity to detect NADH, an enzymatic loop was produced to amplify the NADH production. This was accomplished by adding a second enzyme GPT and alanine (Fig. 6 (inset), Reaction 2).

To demonstrate the difference between a nonamplified (GDH only) and an amplified (GDH + GPT) response, typical traces are shown in Fig. 6B with 50 \(\mu\)M of glutamate given in the absence and presence of NH\(^+\)/NH\(^-\) (5 mM). In the absence of NH\(^+\)/NH\(^-\), a significant increase in NADH formation with GDH + GPT (102%) was found compared with GDH alone (33%). NH\(^+\)/NH\(^-\) (5 mM) inhibited NADH formation in both assays, GDH and GDH + GPT; however, a significant rise in NADH formation was demonstrated with GDH + GPT (58%) compared with GDH (10%) (Fig. 6B). In the presence of GDH alone, NADH formation reached a plateau within 3 min with or without NH\(^+\)/NH\(^-\) (5 mM). Since NAD\(^+\) is present in excess, this plateau is most probably due to the limited amount of glutamate (i.e., all glutamate given has been converted to \(\alpha\)-ketoglutarate). With GDH + GPT, a continuous increase in NADH formation is demonstrated within the observation time of up to 20 min. Eventually, a plateau will be reached when all NAD\(^+\) or alanine is converted, but this obviously did not occur within 20 min. This implies that NADH formation must be measured with respect to time, and we chose 10 min as our temporal end point. NH\(^+\)/NH\(^-\) at concentrations of 1, 5, 10, 20 mM again caused a dose-dependent inhibition; however, the increase in NADH fluorescence was clearly detectable (Fig. 6A). Thus, with the combined enzyme system, GDH + GPT, the level of NADH formation can be measured in response to application of glutamate as low as 500 nM in the presence of NH\(^+\)/NH\(^-\) at 1 and 5 mM. A logarithmic standard curve with
which fluoresces when excited at 360 nm (reaction 1; see inset). NADH formation recorded as fluorescence indirectly reflects released glutamate concentrations. In the presence of GDH and NAD+, NH4+/NH3 (1, 5, 10, and 20 mM) concentration-dependently inhibits (through product inhibition) the production of NADH with different concentrations of applied glutamate. Detectability of NADH formation is very low with 5 mM glutamate. NADH formation can be amplified with the addition of GPT (10 min) and alanine (1 mM) (reaction 2; see inset). With amplification, quantification of NADH formation is measured in relation to time. In all experiments, 10 min was the standard time used. Amplifying NADH formation with the addition of GDH + GPT, in the presence of NAD+ and alanine, NH4+/NH3 (1, 5, 10, and 20 mM) again concentration-dependently inhibits the production of NADH with different concentrations of applied glutamate; however, levels of NADH are much higher as compared with levels without GPT and alanine. Detectability of glutamate increased to as low as 500 nM in the presence of GDH and NAD+ (reaction 2). With GDH in the absence (control) or the presence of NH4+/NH3 (5 mM), it did not stimulate an increase in NADH formation. Astrocytes preincubated with the calcium chelator BAPTA/AM (5 μM for 15 min) did not demonstrate any significant increase in NADH formation upon NH4+/NH3 (5 mM) stimulation in both physiological (NH4+/NH3 (5 mM); 23.7 ± 2.5% versus control; 9.3 ± 1.5%; *, p < 0.01) and calcium-free buffer (NH4+/NH3 (5 mM); 29.2 ± 2.6% versus control; 10.7 ± 1.9%; *, p < 0.01). NH4+/NH3 (1 mM) did not stimulate an increase in NADH formation. Application to cultured astrocytes resulted in an increase in NADH formation as a measure of glutamate release. In physiological buffer, the basal NADH formation within the 10-min recording period was 9.3 ± 1.5% (n = 19 coverslips). In the presence of NH4+/NH3 (5 mM), it was significantly higher (23.7 ± 2.5%, n = 26 coverslips, p < 0.01). NADH formation occurred with a small delay after application of NH4+/NH3 (5 mM). We assume that, in the beginning, NADH levels are too low to be detected by our system, but with further amplification, NADH levels surpassed the threshold of our detection system (Fig. 7A). No significant increase in NADH was seen when a similar volume of physiological buffer (without NH4+/NH3) was applied as a control (n = 11 coverslips). Furthermore, NH4+/NH3 (5 mM) did not increase NADH production in the absence of an enzyme-linked fluorescence system (n = 5 coverslips) in the extracellular medium.
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In contrast to NH$_4^+$/NH$_3^-$, TMA$^+$ (5 mM) did not stimulate a significantly higher increase in NADH formation (40.4 ± 3.8% vs. control; 8.9 ± 1.1%, n = 18 coverslips, p < 0.01) as compared with control (TMA$^+$ (5 mM); 8.9 ± 1.1%, n = 18 coverslips). In conclusion, both weak bases, NH$_4^+/NH_3^-$ (5 mM) and TMA$^+/TMA$ (5 mM), stimulated a significantly higher increase in NADH formation (40.4 ± 3.8%, n = 28 coverslips, p < 0.01) as compared with control (TMA$^+/TMA$ (5 mM); 9.6 ± 2.8%, n = 22 coverslips). In the absence of GDH + GPT, there was no significant difference in glutamate release within the TMA$^+/TMA$ (5 mM) stimulated (42.4 ± 2.7%, n = 18 coverslips) compared with control (43.0 ± 2.8%, n = 18 coverslips). No significant difference was found when astrocytes were preincubated with TBOA + DHK-pretreated and nonpretreated (40.4 ± 3.8%, n = 18 coverslips) upon stimulation of NAD$^+$/NADH (5 mM). This suggests that NH$_4^+/NH_3^-$ (5 mM) stimulated NADH release is not due to the reversal of glutamate transporters.

Similar results were obtained with TMA$^+/TMA$ (5 mM). With astrocytes pretreated with TBOA and DHK, TMA$^+/TMA$ (5 mM) stimulated a significantly high increase in NADH formation (45.8 ± 3.2%, n = 19 coverslips, p < 0.01) as compared with controls (13.6 ± 2.8%, n = 14 coverslips). No significant difference was found between TBOA + DHK-pretreated and nonpretreated (45.8 ± 3.2%, n = 19 coverslips) upon stimulation of TMA$^+/TMA$ (5 mM) (Fig. 8B).

Approximate Concentrations of Glutamate Released upon Stimulation with TMA$^+$ and NH$_4^+/NH_3^-$—Using the standard curves as shown in Fig. 6 for NH$_4^+/NH_3^-$ and Fig. 8 for TMA$^+/TMA$, the approximate glutamate levels in the bathing solution after a 10-min stimulation were calculated. As shown in Fig. 9, a 10-min NH$_4^+/NH_3^-$ stimulation resulted in an increase in glutamate between 4 and 8 μM. For TMA$^+/TMA$, we estimated a similar increase in the bathing solution. With both weak bases, glutamate release was not affected when astrocytes were pretreated with TBOA + DHK. However, glutamate release was inhibited when astrocytes were preincubated with BAPTA/AM. There were no significant differences in the amount of released glutamate between the NH$_4^+/NH_3^-$ (5 mM) and TMA$^+/TMA$ (5 mM) groups (Fig. 9).

**DISCUSSION**

Ammonia-triggered Release of Ca$^{2+}$ from Intracellular ER Stores Is Due to a pH Shift—Our data indicate that NH$_4^+/NH_3^-$ triggers a calcium release from intracellular ER stores, since 1) the response was similar in calcium-free buffer solution as compared with physiological (control) buffer and 2) the response was blocked after depleting internal calcium stores with thapsigargin, a potent inhibitor of the Ca$^{2+}$-ATPase of...
Our Assay Detects Glutamate Release in the Presence of NH$_4^+$—Glutamate release can be measured with techniques involving preloading cells with radiolabeled glutamate and subsequently measuring radiolabeled glutamate release after stimulus or with high performance liquid chromatography. Both techniques have the disadvantage that temporal resolution is lost and glutamate reuptake affects quantitative results. Alternatively, glutamate levels can be determined by an enzymatic assay; GDH deaminates glutamate to form α-ketoglutarate in concert with a conversion of NAD$^+$ to NADH. Here any glutamate released from the astrocytes will be deaminated, and glutamate reuptake will be prevented. The concentration of the naturally fluorescent NADH is therefore proportional to the released glutamate. However, in this reaction, NH$_2$ is a by-product, and therefore when applying NH$_4^+$/NH$_3$, product inhibition occurs concentration-dependently and consequently inhibits NADH production. To overcome this inhibition, another enzyme, GPT, along with alanine, was added to amplify the NADH production, allowing for increased sensitivity for glutamate. With this amplifying system, glutamate release was measured quantitatively with respect to time and could be compared at a given NH$_4^+$/NH$_3$ concentration. We used 5 mM NH$_4^+$ throughout the study, since 5 mM ammonium 1) demonstrates a significant increase in [Ca$^{2+}$], 2) is the pathophysiological concentration found in HE, and 3) does not display strong inhibition in the detection of glutamate (measured as low as 500 nm) with the amplifying enzymatic assay.

TMA$^-$/TMA (5 mM) did not have any inhibitory effects on the amplifying enzymatic assay and therefore resulted in higher NADH formation as compared with NH$_4^+$/NH$_3$ (5 mM). When calculating the glutamate concentrations from the measured NADH levels using the respective NADH standard curves for NH$_4^+$, TMA$^-$/TMA, similar concentrations of glutamate were released upon NH$_4^+$/NH$_3$ and TMA$^-$/TMA application (Fig. 8).

Potential Mechanism of Glutamate Release—Glutamate can be released from astrocytes, and several mechanisms have been proposed. Swelling induced opening of ion channels or reversal of glutamate transporters can occur, and these mechanisms are independent of an increase in [Ca$^{2+}$]. An increase in cytosolic [Ca$^{2+}$] has also been shown to result in glutamate release, and this release activity was proposed to occur as a vesicular or fusion-mediated release (9–11). Several ligands acting on receptors that trigger an increase in [Ca$^{2+}$], in astrocytes initiate the release of glutamate from the cells. These include bradykinin (12), ATP (14, 15), and glutamate (13, 35) and even spontaneous increases in cytosolic [Ca$^{2+}$] (16).

In our experiments, a 10-min application of NH$_4^+$/NH$_3$ (5 mM) triggered an efflux of glutamate in both physiological and calcium-free medium. However, increasing the intracellular buffering capacity with BAPTA/AM, the NH$_4^+$/NH$_3$ (5 mM)-stimulated glutamate release was attenuated, strongly suggesting that ammonia stimulates glutamate release from astrocytes in a calcium-dependent manner.

Cell swelling-induced glutamate release has been demonstrated in cortical astrocytes exposed to a hypo-osmotic medium (36). Since astrocytic swelling is a pathological characteristic observed in acute HE, it was important to test whether glutamate release was due to cell swelling. In our study, using the isosbestic point of fura-2 (where the probe is Ca$^{2+}$-insensitive and the emitted fluorescence is independent of intracellular calcium concentration), NH$_4^+$/NH$_3$ (5 mM) application did not lead to astrocytic swelling (data not shown). Furthermore, with BAPTA/AM having no effect on cell swelling, if NH$_4^+$/NH$_3$ (5 mM)-stimulated glutamate release was a result of cell swelling, glutamate release would have persisted when astrocytes were pretreated with BAPTA/AM, which was not the case. Therefore, the ammonia-induced glutamate release is not due to cell swelling. In support, Albrecht's group has shown that a 10-min treatment with 5 mM ammonia did not produce cell swelling in cultured rabbit Muller cells (37) or cultured cortical astrocytes (38). This further suggests that ammonia-induced astrocytic swelling may appear to develop with longer treatments of ammonia but not during a 10-min insult.
Astrocytes contain high affinity Na+-dependent glutamate transporters to regulate the concentration of extracellular glutamate. GLT-1 and GLAST, more commonly referred to as EAAT-2 and EAAT-1 respectively, are located in rat forebrain and cerebellum at birth. Szatrowski et al. (39) demonstrated that glutamate transporters reverse when insufficient energy is available to regulate the membrane potential. There is increasing evidence that an energy impairment develops in acute HE (40). We therefore tested the effect of glutamate transport inhibitors on NH4+/NH3 (5 mM)-triggered glutamate release. TBOA, a nonsubstrate EAAT-1 inhibitor, and DHK, an effective nonsubstrate EAAT-2 inhibitor, did not affect the amount of glutamate release following NH4+/NH3 (5 mM) application, suggesting that ammonia stimulated glutamate release is not due to the reversal of glutamate transporters.

NH4+/NH3 (5 mM) induced an increase in [Ca2+]i (−65 nM; Fig. 2D), which, according to Parpura and Hayden (41), is sufficient to trigger glutamate release. In their study, a Δ[Ca2+]i of 56 nM released enough glutamate to result in a slow inward current on neighboring neurons. In addition, further increased [Ca2+]i leads to more glutamate release, suggesting a dose-response relationship (16). Taken together, our data point to an exocytotic glutamate release from astrocytes in response to NH4+/NH3.

Astrocytes Release a Substantial Amount of Glutamate in Response to NH4+/NH3 or TMA+/TMA—We estimated the glutamate levels in the bathing solution after release from astrocytes to between 4 and 8 μM for NH4+/NH3 (5 mM) and TMA+/TMA (5 mM). If we assume that the astrocytes on our coverslip have a volume of 300 × 10−12 m3 (assuming a continuous layer on the coverslip with an average thickness of 5 μm) and a given bathing volume of 300 μl (equal to 300 × 10−9 m3), the intracellular astrocyte concentration prior to release would be 4–8 mM. This is about in the range as previously estimated, namely at 1–10 mM (42). It also implies that in the central nervous system extracellular space, glutamate would increase substantially. Assuming that astrocytes make up 30% of the brain volume and the extracellular space is about 20% (43), the same amount of glutamate released would lead to an extracellular glutamate concentration of 6–12 mM even if glutamate transporter activity might attenuate this high level. Millimolar glutamate levels are realistic to assume, and at this concentration glutamate can result in hyperexcitability.

Significance of NH4+/NH3-triggered Glutamate Release for HE in ALF—Astrocyte dysfunction has been assumed to be an important event in the pathologic cascade of HE. Swelling of the astrocytes is a major consequence that leads to brain edema, intracranial pressure, and fatal brain stem herniation. Increased extracellular brain glutamate has been consistently observed in different animal models of ALF where brain edema and increased intracranial pressure prevails. Our data indicate that NH4+/NH3 could trigger the release of glutamate from astrocytes, possibly leading to an increase in extracellular brain glutamate and consequently resulting in glutamatergic dysfunction and an overstimulation of NMDA receptors on neurons (44). This would result in hyperexcitability, and it is known that seizures are not uncommon in patients with ALF. The astrocytic end feet contact the blood capillaries and are thus the first elements to be exposed to elevated NH4+/NH3 from the blood. The level of NH4+/NH3 used in our study is in the range observed after ALF (32). For this to occur in vivo, NH4+/NH3 fluctuations at the astrocyte membrane in the millimolar range would have to arise; however, precise temporal NH4+/NH3 transients in vivo data are not available. Ammonia fluctuations and subsequently cytosolic alkaline shifts are larger at the onset of ALF, suggesting that a deregulation of glutamate release by ammonia from astrocytes may be an early phenomenon and in addition one of the sources leading to this increased extracellular brain glutamate/glutamatergic dysfunction consistently found in different models of ALF (45).

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