Effect of Acute Exposure to Ammonia on Glutamate Transport in Glial Cells Isolated From the Salamander Retina

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Mort, Dominic, Paikan Marcaggi, James Grant, and David Attwell. Effect of acute exposure to ammonia on glutamate transport in glial cells isolated from the salamander retina. J Neurophysiol 86: 836–844, 2001. A rise of brain ammonia level, as occurs in liver failure, initially increases glutamate accumulation in neurons and glial cells. We investigated the effect of acute exposure to ammonia on glutamate transporter currents in whole cell clamped glial cells from the salamander retina. Ammonia potentiated the current evoked by a saturating concentration of L-glutamate, and decreased the apparent affinity of the transporter for glutamate. The potentiation had a Michaelis-Menten dependence on ammonia concentration, with a \( K_m \) of 1.4 mM and a maximum potentiation of 31%. Ammonia also potentiated the transporter current produced by D-aspartate. Potentiation of the glutamate transport current was seen even with glutamine synthetase inhibited, so ammonia does not act by speeding glutamine synthesis, contrary to a suggestion in the literature. The potentiation was unchanged in the absence of Cl\(^-\) ions, showing that it is not an effect on the anion current gated by the glutamate transporter. Ammonium ions were unable to substitute for Na\(^+\) in driving glutamate transport. Although they can partially substitute for K\(^+\) at the cation counter-transport site of the transporter, their occupancy of these sites would produce a potentiation of \(<1\%\). Ammonium, and the weak bases methylamine and trimethylamine, increased the intracellular pH by similar amounts, and intracellular alkalinization is known to increase glutamate uptake. Methylamine and trimethylamine potentiated the uptake current by the amount expected from the known pH dependence of uptake, but ammonia gave a potentiation that was larger than could be explained by the pH change, and some potentiation of uptake by ammonia was still seen when the internal pH was 8.8, at which pH further alkalinization does not increase uptake. These data suggest that ammonia speeds glutamate uptake both by increasing cytoplasmic pH and by a separate effect on the glutamate transporter. Approximately two-thirds of the speeding is due to the pH change.

INTRODUCTION

In acute and chronic liver disease, failure of ammonia detoxification is strongly implicated in the etiology of hepatic encephalopathy (Mousseau and Butterworth 1994). Sudden deterioration into hepatic encephalopathy is frequently precipitated by nitrogen loading, for example after a high protein meal or during the digestion of blood proteins in the gut following gastrointestinal hemorrhage. Brain levels of ammonia can then rise to millimolar concentrations (Mousseau and Butterworth 1994; Zieve et al. 1984).

Ammonia can exert diverse effects on nervous tissue, including membrane depolarization (Fan and Szerb 1993; Raabe 1990), alteration of intracellular pH (Gillette 1983), increased conversion of glutamate to glutamine (Huang et al. 1994; Waniek 1992), and inhibition of glutaminase and respiratory enzymes (Cooper and Plum 1987). Particularly important may be a disturbance of excitatory glutamatergic neurotransmission (Albrecth 1998; Fan and Szerb 1993; Michalak and Butterworth 1997; Raabe 1992). The basal extracellular glutamate concentration is two to three times normal in animal models of hepatic encephalopathy (De Knejt et al. 1994; Michalak et al. 1996; Moroni et al. 1983), although the total brain glutamate level is reduced in such animals and in post-mortem brains of patients dying in hepatic encephalopathy (Bosman et al. 1992; Lavoie et al. 1987; Record et al. 1976). The increased extracellular glutamate level could reflect increased presynaptic release of glutamate or decreased uptake of released transmitter by Na\(^+\)-dependent glutamate transporters, located predominantly in astroglial cells. Studies that suggest an increased glutamate release (Butterworth et al. 1991; Moroni et al. 1983) have to be interpreted with caution since apparent increases in release may actually reflect reduced efficiency of the uptake mechanism.

A reduction in glutamate uptake when ammonia levels are persistently raised has been seen in cultured astrocytes after prolonged exposure to ammonium (Bender and Norenberg 1996), in synaptosomes from animals with experimental liver failure (Oppong et al. 1995), and in brain slices from patients dying in hepatic encephalopathy (Schmidt et al. 1990). In rats with liver failure, and in cultured astrocytes exposed to ammonia, there is reduced expression of the glial glutamate transporters GLT-1 (the main glutamate transporter in the brain) (Haugeto et al. 1996) and GLAST, suggesting that the increased extracellular glutamate level may result from decreased transporter expression (Knecht et al. 1997; Norenberg et al. 1997; Zhou and Norenberg 1999).

By contrast, acute ammonium exposure enhances glutamate uptake (Bender and Norenberg 1996; Rao and Murthy 1991). A better understanding of this may explain why downregulation of uptake occurs after more prolonged ammonium exposure. Rao and Murthy (1991) found that Na\(^+\)-dependent uptake of both \( ^3 \)H]-L-glutamate and its nonmetabolizable analogue \( ^3 \)H]-D-aspartate was increased in rat cerebellar astrocyte, neu-
ronal and synaptosome preparations exposed to 5 mM ammonium, and also in the same preparations from rats made acutely hyperammonaemic. The increase was due to an increase in $V_{\text{max}}$, with $K_{\text{m}}$ remaining unchanged. However, another study (Bender and Norenberg 1996) reported that 5–10 mM ammonium chloride increased $[^{3}H]_1$-glutamate accumulation in rat cortical astrocytes in culture, while $[^{3}H]_2$-aspartate accumulation was unchanged. This differential response was attributed to the fact that glutamate, but not aspartate, undergoes increased intracellular conversion to glutamine in the presence of ammonium, catalyzed by glutamine synthetase, which is expressed highly in astrocytes and radial glial cells (Hertz et al. 1999; Poityr et al. 2000). This is expected to increase the driving force for glutamate uptake (although, since both L-glutamate and D-aspartate are transported by the same carriers, a fall of intracellular glutamate concentration might increase the uptake of both external substrates) and also to increase the fraction of labeled glutamate (but not D-aspartate) taken up, which is retained within the cell as glutamine rather than leaving the cell again by exchange on the uptake carriers.

To clarify how acute ammonia exposure potentiates glutamate accumulation, we studied L-glutamate uptake in whole cell voltage-clamped salamander retinal glial cells, which mainly express a glutamate transporter homologous to the mammalian GLAST/EAA1 (Eliasof et al. 1998; Spiridon et al. 1998). Three Na$^{+}$ ions and a proton are carried into the cell with glutamate, in exchange for one K$^{+}$ ion (Levy et al. 1998; Zerangue and Kavanaugh 1996), so glutamate transport can be monitored as a net inward current using the whole cell patch-clamp technique (Brew and Attwell 1987).

METHODS

Salamander retinal glial cells

Tiger salamanders were killed in accordance with United Kingdom regulations (brain concussion followed by immediate destruction of the brain). Glial (Müller) cells were isolated from their retinae using papain (Barbour et al. 1991), and whole cell clamped with pipettes of series resistance (in whole cell mode) $\sim$3 M$\Omega$, giving series resistance voltage errors $<2$ mV.

Solutions

NH$_4$Cl, methylamine hydrochloride, and trimethylamine hydrochloride were prepared freshly as 1-M stock solutions. Adding these to the experimental solutions did not alter the pH significantly. Unless otherwise stated, the extracellular solution contained (in mM) 105 NaCl, 2.5 KCl, 3 CaCl$_2$, 0.5 MgCl$_2$, 15 glucose, 5 HEPES, and 6 BaCl$_2$ (to block inward rectifier K$^+$ channels), with pH adjusted to 7.4 with NaOH. Control experiments (described in RESULTS) showed that Ba$^{2+}$ had no effect on intracellular pH changes produced by ammonia, implying that there is no detectable flux of NH$_4^+$ through the K$^+$ channels blocked by barium. The standard pipette solution contained (in mM) 95 KCl, 5 NaCl, 1 CaCl$_2$, 7 MgCl$_2$, 5 NaATP, 5 K$_2$EGTA, and 5 HEPES, with pH adjusted to 7.0 with KOH. To block glutamine synthesis, 2 mM methionine sulfoximine was added to the pipette solution. In Cl$^{-}$-free solutions, the Cl$^{-}$ was replaced by gluconate. Na$^{+}$-free and K$^{+}$-free extracellular solutions contained choline chloride instead of NaCl and KCl, respectively. When replacing internal K$^{+}$ with Na$^{+}$, external K$^{+}$ was removed to prevent [K$^{+}$] $\downarrow$, rising due to K$^{-}$ entry through K$^{+}$ channels or on the Na$^{+}$/K$^{+}$ pump (Sztakowski et al. 1991). High buffering power internal solution contained (in mM) 50 KCl, 71 HEPES, 26 KOH, 5 NaCl, 5 K$_2$EGTA, 1 CaCl$_2$, 7 MgCl$_2$, and 5 NaATP, pH adjusted to 7.0 with KOH. For experiments with pH$_1$ (pipette pH) of 8.8 or 6.0, 95 mM KCl and 5 mM HEPES in the standard intracellular solution were replaced with 50 mM KCl, 30 mM KOH, and either 66 mM $N$-tris(hydroxymethyl)amino propane sulfonic acid (TAPS) or 66 mM MES, respectively (the pH inside the cell was within 0.2 units of 8.8 or 6.0) (Billups and Attwell 1996). Extracellular solutions for reversed uptake contained (in mM) 90 NaCl, 3 CaCl$_2$, 0.5 MgCl$_2$, 15 glucose, 5 HEPES, 6 BaCl$_2$, and 0.1 ouabain (to block any current contribution from the Na$^{+}$/K$^{+}$ pump), plus either 20 choline-Cl (for the K$^{+}$-free and NH$_4$Cl-free solution) or 10 choline-Cl and 10 KCl or 10 NH$_4$Cl (to evoke reversed uptake); pH was adjusted to 7.4 with NaOH. Intracellular solutions for reversed uptake contained (in mM) 92 choline-Cl, 10 Na-glutamate, 1 CaCl$_2$, 2 MgCl$_2$, 5 HEPES, 5 Na$_2$EGTA, and 5 NaATP, with pH set to 7.0 using NaOH. Experiments were at 23–26$^\circ$C.

Data analysis

Once in whole cell mode, cells were left for 2 min before applying glutamate to ensure complete dialysis with the patch pipette solution. Glutamate uptake currents in the presence of ammonium chloride (or other drugs) were “bracketed” by preceding and following control responses. The potentiation of the uptake current by ammonium chloride was calculated by dividing the amplitude of the uptake current in ammonium chloride by the average of the amplitudes of the preceding and following control responses. When comparing results between different cells (e.g., with and without Cl$^{-}$ present), cells studied in the two conditions were interleaved to reduce variability (note that specimen traces shown for different cells cannot be compared in absolute amplitude because of differences in cell size). Data are presented as means $\pm$ SE and statistical $P$ values are from two-tailed t-tests.

Measurement of intracellular pH

Cells were whole cell clamped with the pH-sensitive fluorescent dye BCECF [2',7'-bis-(carboxyethyl)-carboxy-fluorescein, 96 $\mu$M] added to the normal pipette solution for forward uptake. Cell autofluorescence was negligible compared with the BCECF signal. Fluorescence was excited at 490 nm, for which an acid pH shift decreases BCECF fluorescence, and emission at 530 nm was measured with a photomultiplier. Glutamate and amine-evoked pH changes cause no fluorescence change except for excitation at the isosbestic wavelength of 440 nm (Bouvier et al. 1992). Calibrations using high K$^+$ solution containing nigericin (Boyarsky et al. 1993) showed a fractional change in the fluorescence (F) of BCECF (excited at 490 nm), when changing the pH from 6.76 to 7.11 of $\Delta F/F_{\text{pH}=7.11} = 25.9 \pm 13\%$ (mean $\pm$ SE) in three cells, which is not significantly different from the 27.2% predicted by the in vitro calibration of Rink et al. (1982) and the 25.1% predicted by the Boyarsky et al. (1993) calibration in single astrocytes ($P = 0.41$ and 0.6, respectively). We therefore estimated pH changes using the calibration of Rink et al. (1982) assuming an initial pH of 7.0 (the pH of the pipette solution), for which a typical ammonia-evoked fluorescence increase of 30% implies an alkalinization of 0.3 units.

RESULTS

Unless otherwise stated, results are given from experiments conducted with pH$_1$ = 7.0 and pH$_0$ = 7.4, at a holding potential between $-60$ and $-70$ mV. We use the terms ammonia and ammonium chloride interchangeably: at pH 7.4 most (99%) of the ammonia present is in the form of NH$_4^+$, and 1% is in the form of NH$_3$ (from the Henderson-Hasselbalch equation below, using a pK of 9.4).

\[ \text{pH} = \frac{\text{pK} + \text{pOH}}{2} = 7.4 \]
Acute exposure to ammonia potentiates L-glutamate and d-aspartate uptake

Applying L-glutamate or D-aspartate evoked an inward transporter-mediated current in salamander retinal glial cells, as characterized previously (Barbour et al. 1991; Brew and Attwell 1987). Ammonium chloride potentiated the current associated with uptake of both glutamate (Fig. 1A) and D-aspartate (Fig. 1B). The potentiation had a rapid onset, apparently as fast as the kinetics of the bath solution changes, which occur within about 3 s in our system. The offset of the potentiating effect was somewhat slower, and varied between cells, taking up to 60 s to occur.

The dependence of the potentiation of L-glutamate uptake on the dose of NH₄Cl could be roughly fitted by a Michaelis-Menten curve, with an EC₅₀ of 1.4 mM and a maximum potentiation of 31% (Fig. 1C). At 5 mM concentration, ammonium chloride potentiated the current produced by a near-saturating glutamate dose (200 μM) by 26 ± 2% (n = 61) and that produced by a near-saturating dose of D-aspartate (100 μM) by 21 ± 2% (n = 12, P = 0.38 compared with the potentiation for glutamate). In subsequent experiments this concentration of ammonia was used, unless otherwise stated.

Although previous work has found ammonia to change only the maximum rate of uptake (Bender and Norenberg 1996; Rao and Murthy 1991), we found that it also significantly (P = 0.0035) decreased the apparent affinity of the transporters, increasing the Kₘ for glutamate from 11 to 15 μM (Fig. 2). The combination of the decreased affinity with the increased Vₘₐₓ results in ammonia having little effect on uptake at low glutamate doses, but an increase at doses above 5 μM (Fig. 2).

Potentiating effect does not depend on glutamine synthetase activity

When 2 mM methionine sulfoximine (MSO), an inhibitor of glutamine synthetase, was included in the internal solution (Fig. 3) the potentiating effect of 5 mM ammonia on the 200 μM L-glutamate uptake current was not reduced (31 ± 5%, n = 10, P = 0.75 on 2-tailed t-test comparing with interleaved cells in the absence of MSO). Thus ammonia does not act by speeding the conversion of glutamate to glutamine. We therefore investigated other possible causes of the ammonia-induced increase in glutamate- and aspartate-evoked currents.

Ammonia does not act just by potentiating the transporter-gated anion conductance

In addition to generating an inward current by co-transporting an excess of Na⁺ and H⁺ into the cell with each glutamate anion, glutamate transporters also generate some current by activating an anion conductance when the transporter cycles (Billups et al. 1996; Eliasof and Jahr 1996; Wadiche et al. 1995). In our experiments the chloride reversal potential is around 0 mV, so an inward current is generated by Cl⁻ efflux at −70 mV (lowering internal [Cl⁻] to 21 mM decreases the glutamate-evoked current by 26%) (Billups at al. 1996). A potentiation of this conductance could in principle generate an increased inward current, without any associated increase in glutamate transport. To test this we replaced both internal and
external chloride ions with the large nonpermeant anion gluconate. Abolition of the anion conductance in this way did not alter the ability of ammonium chloride to potentiate the glutamate (200 \( \text{mM} \)) uptake current (Fig. 4): in the absence of \( \text{Cl}^- \) the potentiation was 23 ± 3% \((n = 6)\), as compared with 23 ± 3% \((n = 7, P = 0.91)\) in interleaved cells in the presence of \( \text{Cl}^- \). Thus ammonia potentiates glutamate transport and not just the anion conductance activated during transport.

\( \text{NH}_4^+ \) ions do not substitute for \( \text{Na}^+ \) ions on the transporter

We considered the possibility that \( \text{NH}_4^+ \) ions might be able to replace \( \text{Na}^+ \) or \( \text{K}^+ \) at the cation transport sites of the glutamate transporter, so that when \( \text{NH}_4^+ \) is present these sites would become more saturated, speeding uptake. Replacing all external \( \text{Na}^+ \) with \( \text{NH}_4^+ \) completely abolished the glutamate uptake current (5 cells, Fig. 5A), consistent with the high cation selectivity observed previously for the \( \text{Na}^+ \)-binding site (Barbour et al. 1991).

\( \text{NH}_4^+ \) ions substitute poorly for \( \text{K}^+ \) ions on the transporter

Replacement of internal \( \text{K}^+ \) ions with \( \text{NH}_4^+ \) ions abolished the glutamate uptake current (8 cells, Fig. 5B), which was surprising because the \( \text{K}^+ \)-binding site is less specific than that for \( \text{Na}^+ \) and can also bind \( \text{Cs}^+ \) and \( \text{Rb}^+ \) (Barbour et al. 1991). We wondered, therefore, whether the inhibition by internal \( \text{NH}_4^+ \) might partly reflect an acidification of the cell (which is known to inhibit uptake: see Fig. 6B) caused by \( \text{NH}_3 \) passing out across the cell membrane, causing internal \( \text{H}^+ \) to dissociate to form more \( \text{NH}_3 \) and \( \text{H}^+ \). To investigate the possible activation of the transporter’s \( \text{K}^+ \) site by \( \text{NH}_4^+ \) further, we recorded reversed uptake currents evoked by raising \( [\text{K}^+]_o \) or \( [\text{NH}_4^+]_o \) from 0 to 10 mM (replacing choline) around cells clamped with pipettes filled with a solution containing 10 mM sodium glutamate. As reported by Szatkowski et al. (1990), raising \( [\text{K}^+]_o \) evokes an outward current at depolarized potentials that is reduced by the presence of 200 \( \mu \text{M} \) external glutamate (which stops the transporter losing glutamate at the outer membrane surface). A similar but smaller reversed uptake current was produced by raising \( [\text{NH}_4^+]_o \) (Fig. 5C). In 13 cells this current was 53 ± 6% of that evoked by bracketing applications of \( \text{K}^+ \). We attribute the lack of an uptake current in cells filled with \( \text{NH}_4^+ \) therefore to intracellular acidification produced by \( \text{NH}_3 \) passing out continuously across the cell membrane.

Could the potentiation of glutamate uptake by external ammonia be due to internal \( \text{NH}_4^+ \) binding to the \( \text{K}^+ \) site? When ammonia is applied to the outside of the cell during experiments on forward glutamate uptake, some \( \text{NH}_3 \) will permeate the cell and be converted to \( \text{NH}_4^+ \) inside, where it could potentiate uptake by adding to the activation of the \( \text{K}^+ \) counter-transport site by intracellular \( \text{K}^+ \). An upper limit to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Inhibiting glutamine synthetase does not reduce the potentiation produced by ammonia. A: potentiation of the 200-\( \mu \text{M} \) glutamate uptake current at \(-70 \text{ mV} \), as in Fig. 1A, with 2 mM of the glutamine synthetase blocker methionine sulfoximine (MSO) included in the whole cell pipette. B: fractional potentiation produced by 5 mM \( \text{NH}_4^+ \) with pipette solution lacking (control, 12 cells) or containing MSO (10 cells).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Ammonia potentiates the glutamate uptake current even with internal and external \( \text{Cl}^- \) removed to abolish the transporter’s anion conductance. A: potentiation of the 200-\( \mu \text{M} \) glutamate uptake current at \(-70 \text{ mV} \), as in Fig. 1A, with internal and external \( \text{Cl}^- \) replaced by gluconate. B: fractional potentiation produced by 5 mM \( \text{NH}_4^+ \) with intra- and extracellular \( \text{Cl}^- \) present or absent (6 cells with \( \text{Cl}^- \) absent interleaved with 7 cells with \( \text{Cl}^- \) present).}
\end{figure}
this potentiation can be estimated as follows. The concentration of \(\text{NH}_3\) in equilibrium with the \(\text{NH}_4^+\) in the external solution is given by the Henderson-Hasselbalch equation as

\[
\frac{[\text{NH}_3]}{[\text{NH}_4^+]} = 10^{pK - p\text{Hi}} \tag{1}
\]

where \(pK = 9.4\) and \(p\text{Hi} = 7.4\). To obtain an upper estimate for the internal \([\text{NH}_3]\), we assume that sufficient \(\text{NH}_3\) crosses the membrane to make \([\text{NH}_3]^\text{i}\) the same as \([\text{NH}_3]^\text{o}\). From the Henderson-Hasselbalch equation again

\[
\frac{[\text{NH}_3]}{[\text{NH}_4^+]} = 10^{pK - p\text{Hi}} \tag{2}
\]

Thus from Eqs. 1 and 2

\[
[\text{NH}_3]^\text{i} = [\text{NH}_3]^\text{o}, 10^{p\text{Hi} - p\text{Hi}} \tag{3}
\]

and, for \([\text{NH}_4^+]^\text{i} = 5\text{mM}, \text{pH}_i = 7.4\) and \(\text{pHi} = 7.0\), \([\text{NH}_4^+]^\text{i} = 12.6\text{mM}\) (in fact the amount of \(\text{NH}_4^+\) formed will be less than this, because the binding of \(\text{H}^+\) by \(\text{NH}_4\) entering raises \(\text{pHi}\) above 7.0, as described below). The reversed uptake experiments described above can be used to estimate the extra forward uptake current this \(\text{NH}_4^+\) will produce, if one assumes that the \(K_m\) values for transporter activation by internal and external \(\text{NH}_4^+\) are similar, as is approximately the case for \(K^+\) (~15 mM) (Barbour et al. 1988; Szatkowski et al. 1990). The smaller reversed uptake current activated by \(\text{NH}_4^+\) compared with \(K^+\) might reflect either 1) a higher \(K_m\) for activation by \(\text{NH}_4^+\) (calculated to be 37 mM, assuming the same maximum transport rate for transporters activated by \(\text{NH}_4^+\) and \(K^+\)), or 2) a maximum transport rate that is only 53% of that for transporters activated by \(K^+\) (assuming the same \(K_m\) for activation by \(\text{NH}_4^+\) and \(K^+\)). Using these numbers, and a first-order reaction scheme (Barbour et al. 1988) for activation of forward uptake by internal ions (105 mM \(K^+\), or 105 mM \(K^+\) plus 12.6 mM \(\text{NH}_4^+\)), one can calculate that if the smaller activation of reversed uptake by \(\text{NH}_4^+\) is attributed to a higher \(K_m\) for activation by \(\text{NH}_4^+\) and a lower \(V_{\text{max}}\), then the extra 12.6 mM \(\text{NH}_4^+\) will either increase the uptake current by \(<1\%\), or decrease it by \(4\%\), respectively. We conclude that the potentiation does not result from \(\text{NH}_4^+\) acting at the transporter’s \(K^+\) counter-transport site.

**Role of intracellular pH changes in the potentiating effect**

The amines methylamine and trimethylamine (5 mM) also increased the glutamate (200 \(\mu\text{M}\)) uptake current, but by less than bracketing applications of ammonia (Fig. 6, A and D). The potentiation produced by 5 mM methylamine and trimethylamine was 12.9 \(\pm\) 1.0% (5 cells) and 17.8 \(\pm\) 3.6% (9 cells), respectively. A possible mechanism by which the amines and ammonia might potentiate uptake is via an intracellular alkalinization, which is known to increase the uptake current (Billups and Attwell 1996) probably by increasing the electrochemical gradient for co-transported \(\text{H}^+\) ions [Fig. 6B, redrawn from Billups and Attwell (1996)]. The neutral form of the amine or \(\text{NH}_3\) enters (as described above for \(\text{NH}_4\)) and binds intracellular \(\text{H}^+\) to reform charged amine or \(\text{NH}_4^+\) inside the cell. From Eq. 3, for 5 mM extracellular \(\text{NH}_4^+\), the amount of \(\text{NH}_4^+\) formed within the cell at equilibrium, and hence the amount of \(\text{H}^+\) bound is given by

\[
\Delta[H^+] = [\text{NH}_4^+] = [\text{NH}_3^+] = 10^{p\text{Hi} - p\text{Hi}} = 5\text{mM} \times 10^{1.4 - 2p\text{Hi}} \tag{4}
\]

where \(\Delta[H^+]\) is the fall in total intracellular proton concentration, and \(p\text{Hi}\) is the change of internal \(\text{pH}\) from its initial value. From the definition of intracellular buffering power, \(\beta_i\), the resulting \(\text{pH}\) change will be

\[
\Delta p\text{Hi} = \Delta[H^+] / \beta_i \tag{5}
\]

where \(\beta_i\) is \(\approx 20\text{mM per pH unit}\) for these cells (Bouvier et al. 1992). From Eqs. 4 and 5

\[
\Delta p\text{Hi} = 0.25 \times 10^{1.4 - 2p\text{Hi}} \tag{6}
\]

Solving this gives a predicted \(\Delta p\text{Hi}\) of 0.31 units.

We used the \(\text{pH}\)-sensitive fluorescent dye BCECF, loaded via the patch pipette, to measure the \(\text{pH}\) changes that were actually produced by ammonia and the amines (Fig. 6C). Ammonia (5 mM) increased the BCECF fluorescence by 33 \(\pm\) 2% in five cells, similar to the ~31% increase predicted (see
METHODS) for a 0.31 unit pH increase. Without Ba\(^{2+}\) in the external solution to block inward rectifier K\(^{+}\) channels, a similar NH\(_4^+\)-induced pH change (32 ± 2% in 6 cells) was seen, with no rebound acidification on removal of NH\(_4^+\), showing that ammonia mainly crosses the cell membrane in the form of NH\(_3\), unlike in some glia (Marcaggi et al. 1999; Nagaraja and Brookes 1998) where NH\(_4^+\) can also enter via inward rectifier K\(^{+}\) channels and Cl\(^{-}\)-dependent transport and produce an acidification. Methylamine and trimethylamine gave alkaline pH shifts that were slightly smaller than (−0.94 of) those produced by ammonia in the same cells (Fig. 6, C and D), possibly because the extracellular concentration of the neutral form of these molecules is lower, due to their higher pK\(_a\), so they enter the cell slower than NH\(_3\) and equilibrate less fully.

In contrast to the similar pH changes produced by the amines and ammonia, the potentiation of the uptake current produced by 5 mM methylamine and trimethylamine was only 50–60% of that produced by ammonia in the same cells (Fig. 6D). An intracellular alkalization of 0.31 units is expected to increase the uptake current by 18% (from Fig. 6B), which is similar to the potentiation produced by methylamine and trimethylamine, but only about two-thirds of the potentiation produced by ammonia. This suggests that intracellular alkalization can account for all of the potentiation produced by the amines, but for only approximately two-thirds of that produced by ammonia.

To try to test this further, we increased the buffering power of the cell from its normal value of around 20 mM/pH unit (Bouvier et al. 1992) to around 50 mM/pH unit, by raising the pipette solution [HEPES] to 71 mM. This reduced by 37% the intracellular pH change produced by ammonia (Fig. 6E). If all of the potentiation were due to the pH change, the nonlinearity of Fig. 6B predicts that a 37% decrease of a 0.31 unit pH change would reduce the potentiation by 32%. However, a smaller reduction than this (−22%) is expected if only approximately two-thirds of the potentiation is due to the pH change. Experimentally (Fig. 6E) raising the buffering power reduced the potentiation by 24% (not significant, \(P = 0.23\)) in 11 pairs of interleaved cells studied with high and low buffering power.

When the intracellular (pipette) pH was lowered to 6.0, the potentiation by ammonia increased to 128 ± 8% in seven cells (Fig. 7, A and C). This is consistent with part of the potentiation reflecting an ammonia-evoked alkalization, first because this alkalization is predicted to be larger when the internal pH is more acid (because more of the NH\(_3\) entering is converted to NH\(_2^+\) at a more acid pH), and second because the pH\(_i\) dependence of the uptake current predicts a larger fractional increase per pH change at lower pH\(_i\) (Fig. 6B). Conversely, when the pipette solution pH was set to 8.8, at which value further alkalization is not expected to increase uptake (Fig. 6B) and the intracellular alkalization predicted to be produced by 5 mM NH\(_4^+\) is only 0.01 units (from a calculation like that for Eq. 6, assuming 20 mM/pH unit buffering power), a potentiation of 7 ± 2% (\(P = 0.01\)) was still seen in 19 cells (Fig. 7, B and C). This residual potentiation, not mediated by intracellular alkalization, reinforces the notion that part of the potentiation produced by ammonia under normal conditions is not via an internal pH change.

**DISCUSSION**

We observe a stimulation of glutamate uptake by acute exposure to ammonia in voltage-clamped glial cells, which is similar in magnitude to that reported previously in radiotracing experiments (Bender and Norenberg 1996; Rao and Murthy 1991). An early potentiation of glutamate accumulation by ammonia has been attributed to ammonia stimulating the conversion of glutamate to glutamine by glutamine synthetase (Bender and Norenberg 1996; Poitry et al. 2000). This would lower the intracellular glutamate concentration and thus pro...
NH₄⁺ to explain the potentiation of uptake current produced by ammonia. Since we find a potentiation of glutamate uptake even with glutamine synthetase inhibited, we conclude that, at least in salamander Müller cells, ammonia can substitute for extracellular Na⁺ by substituting for extracellular K⁺ as the transporters’ ion-binding sites. Replacing external Na⁺ by NH₄⁺ abolished the uptake current, suggesting that NH₄⁺ ions are not active at the external Na⁺-binding sites on the transporter (although we cannot rule out the possibility that only 1 of the 3 Na⁺-binding sites on the transporter cannot bind NH₄⁺ ions). Similarly, although NH₄⁺ can substitute for K⁺ as the counter-transported cation on the transporter, its efficacy in this role and concentration achieved within the cell are far too low to explain the potentiation of uptake current produced by ammonia.

The glutamate uptake current is strongly increased by intracellular alkalinization (Billups and Attwell 1996), probably because alkalinization increases the driving force for entry of co-transported H⁺ into the cell. Acute exposure to ammonia raised the intracellular pH (Fig. 6C), as seen previously in a number of cell types. This results from NH₃ rapidly crossing the cell membrane and binding intracellular protons to form NH₄⁺ (Boron and De Weer 1976; Thomas 1974). Can the alkalinizing effect of ammonia explain the potentiation of the uptake current? At 5 mM, ammonia potentiates the L-glutamate uptake current by 26%. From the data of Billups and Attwell (1996; replotted in Fig. 6B) such a potentiation could be produced by an alkaline shift of 0.5 of a pH unit. This is much larger than the shift of about 0.25 units measured in other preparations on application of 5 mM NH₄⁺ (Goldsmith and Hilton 1992; Thomas 1974), and larger than the 0.31 unit shift, which we expect theoretically (see Eq. 6) and measure experimentally (Fig. 6C). Thus, although a large fraction (~2/3) of the potentiation is attributable to a pH change, a significant fraction cannot be. This is also demonstrated by the results of experiments that do not depend on knowing the exact pH dependence of the uptake current. First, we found that methylvamine and trimethylamine, which produced a similar pH change to NH₄⁺, only produced 0.5–0.6 of the potentiation (Fig. 6D). Furthermore, raising the buffering power of the pipette solution reduced the potentiation less than the pH change, as predicted if only two-thirds of the potentiation was pH-related (Fig. 6E). Finally, at an internal pH of 8.8, where ammonia should not change the intracellular pH significantly and where further alkalinization in any case does not increase the uptake current, ammonia still produced some potentiation (Fig. 7). Taken together, these data suggest that approximately two-thirds of the potentiation reflects ammonia-induced alkalinization of the cell interior, while the remaining potentiation results from an effect of NH₃/NH₄⁺ that is not produced by the larger amines. This could be a direct action of NH₃/NH₄⁺ at a modulatory site on the transporter, or conceivably NH₄⁺ might donate protons to the H⁺ co-transport site on the uptake carrier, and the larger amines cannot do this because they are too large to access this site.

What are the limitations of extrapolating these data to the effects of ammonia in liver failure? Our experiments are on GLAST transporters, and the properties of the more abundant glial GLT-1 transporter could differ; in particular the dependence of its uptake rate on intracellular pH has not been reported. Nevertheless, since GLT-1 has a homologous structure and is known to co-transport protons (Levy et al. 1998), its uptake rate is also likely to be increased by intracellular alkalinization. During the application of ammonia, cultured rat astrocytes show an alkalinization (a 0.66 unit pH change with 20 mM ammonia) (Boyarsky et al. 1993), that slowly reduces as NH₄⁺ enters the cell. [By contrast in cultured mouse astrocytes a very high NH₄⁺ permeability has been reported to result in ammonia acidifying the cell (Nagaraja and Brookes 1998).] Smaller alkalin shifts will occur in prolonged liver failure, both because of NH₄⁺ accumulation, and because the increase of ammonia concentration is usually less than the 5 mM we use in most of our experiments. Very few data are available on the effect of ammonia on glial pH in the brain. In rats with portacaval shunts, to mimic liver failure, a steady-state alkalin shift of just 0.11 units was found by Swain et al. (1991). This would only produce a 7% increase in uptake current from the cellular alkalalinization (Billups and Attwell 1996), probably because alkalinization increases the driving force for entry of co-transported H⁺ into the cell.
pH dependence in Fig. 6B (and this will be reduced by an astrocyte depolarization observed by Swain et al. in the shunted animals). However, the non-pH component of ammonia’s action will produce an extra potentiation, and the alkalization is probably significantly larger at the onset of the liver failure and after meals.

For hepatic encephalopathy, the decrease in glutamate uptake at late times, caused by reduced transporter expression, is more important than the initial increase of uptake. Transporter expression can be regulated by cAMP and several growth factors (Zeleniaia et al. 2000). Conceivably modulation of the latter pathways by NH4 + or altered pH might lead to down-regulation. Alternatively, it is possible that cells have a mechanism for sensing the amount of intracellular glutamate and regulating transporter expression accordingly. In this case, the increased uptake at early times that we have characterized, which presumably leads, initially, to a rise of intracellular glutamate concentration, could be a trigger for a long-lasting down-regulation of transporter expression.

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