Original Paper

Parallel Changes in Intracellular Water Volume and pH Induced by $\text{NH}_3/\text{NH}_4^+$ Exposure in Single Neuroblastoma Cells

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Key Words

Ammonium • Ammonia • Isosmotic cell swelling • Isosmotic cell shrinkage • Isosmotic regulatory volume decrease • Isosmotic regulatory volume increase • Hyperammonemia • Brain edema

Abstract

Background: Increased blood levels of ammonia (NH_3) and ammonium (NH_4^+), i.e. hyperammonemia, leads to cellular brain edema in humans with acute liver failure. The pathophysiology of this edema is poorly understood. This is partly due to incomplete understanding of the osmotic effects of the pair $\text{NH}_3/\text{NH}_4^+$ at the cellular and molecular levels. Cell exposure to solutions containing $\text{NH}_3/\text{NH}_4^+$ elicits changes in intracellular pH (pH_i), which can in turn affect cell water volume (CWV) by activating transport mechanisms that produce net gain or loss of solutes and water. The occurrence of CWV changes caused by $\text{NH}_3/\text{NH}_4^+$ has long been suspected, but the mechanisms, magnitude and kinetics of these changes remain unknown. **Methods:** Using fluorescence imaging microscopy we measured, in real time, parallel changes in pH_i and CWV caused by brief exposure to $\text{NH}_3/\text{NH}_4^+$ of single cells (N1E-115 neuroblastoma or NG-108 neuroblastoma X glioma) loaded with the fluorescent indicator BCECF. Changes in CWV were measured by exciting BCECF at its intracellular isosbestic wavelength (~438 nm), and pH_i was measured ratiometrically. **Results:** Brief exposure to *isosmotic* solutions (i.e. having the same osmolality as that of control solutions) containing NH_4Cl (0.5–30 mM) resulted in a rapid, dose-dependent swelling, followed by *isosmotic* regulatory volume decrease (iRVD). NH_4Cl solutions in which either extracellular $[\text{NH}_3]$ or $[\text{NH}_4^+]$ was kept constant while the other was changed by varying the pH of the solution, demonstrated that $[\text{NH}_3]_o$ rather than $[\text{NH}_4^+]_o$ is the main determinant of the NH_4Cl -induced swelling. The iRVD response was sensitive to the anion channel blocker NPPB, and partly dependent on external Ca^{2+} . Upon removal of NH_4Cl , cells shrank and displayed *isosmotic* regulatory volume increase (iRVI). Regulatory volume responses could not be activated by comparable CWV changes

produced by anisotonic solutions, suggesting that membrane stretch or contraction by themselves are not sufficient to trigger these responses. Inhibition of glutamine synthetase partially blocked the NH_4Cl -induced swelling. **Conclusions:** A quantitative description of the osmotic changes produced by exposure to $\text{NH}_3/\text{NH}_4^+$ in single neurons and glial cells shows that ~35 to 45% of the initial cell swelling can be explained by intracellular accumulation of NH_4^+ due to rapid permeation and protonation of NH_3 . Another ~23% of the swelling can be accounted for by rapid glutamine accumulation. The results are discussed in terms of basic cell physiology and their potential relevance to the pathophysiology of hyperammonemic cellular brain edema.

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Introduction

The transport of the neutral base ammonia (NH_3) and its conjugated acid, ammonium (NH_4^+) across cell membranes is of high biological significance. In mammals, besides its importance in nitrogen metabolism, transport of NH_3 and NH_4^+ plays a key role in the regulation of systemic pH [1, 2]. Increased blood levels of NH_3 and NH_4^+ are implicated in the pathogenesis of various neurological disorders, collectively termed hyperammonemic encephalopathies [3, 4]. Hyperammonemia produces cellular brain edema, which is a leading cause of mortality in humans with acute liver failure [5-9]. The pathophysiology of this edema is poorly understood [4, 10]. This is partly due to our lack of understanding of the osmotic effects of the pair $\text{NH}_3/\text{NH}_4^+$ at the cellular and molecular levels, particularly in neurons and glial cells. Cell exposure to $\text{NH}_3/\text{NH}_4^+$ produces changes in intracellular pH (pH_i) that are determined by the relative permeability of the plasma membrane to NH_3 and NH_4^+ , and the specific pH regulating transport systems present in each cell type. These changes in pH_i and their underlying mechanisms have been extensively studied and are well understood [11-13], but relatively little is known about how $\text{NH}_3/\text{NH}_4^+$ and the pH_i changes it produces affect cell osmotic control, a basic problem of cell physiology that has been addressed but not solved for more than half a century. Osmotic changes in cell volume are expected to occur upon changes in pH_i because regulation of these two functional processes often involves common membrane ion transporters and channels.

With a few notable exceptions [14-16], most cells are highly permeable to NH_3 , and to a variable extent to NH_4^+ . Early investigations addressing the cell membrane permeability to weak acids and bases correctly attributed the alkalization observed in plant and animal cells exposed to ammonium salts to rapid permeation of the non-electrolyte NH_3 [17, 18]. Inside the cells, protonation of NH_3 determines an increase in pH_i that will proceed until NH_3 achieves equilibrium across the plasma membrane. During this process, NH_4^+ is generated and can be accumulated intracellularly in millimolar concentrations [11, 19, 20]. Thus, a net intracellular accumulation of osmotically active particles (NH_4^+) proceeds concomitantly with the change in pH_i . This accumulation may be enhanced by direct NH_4^+ influx via membrane channels or transporters. Thus, exposure to $\text{NH}_3/\text{NH}_4^+$ results in an increase in intracellular osmotic pressure leading to water influx and cell swelling. Unless counteracted by volume regulatory mechanisms, the osmotic swelling can culminate in cell lysis. A similar reasoning was invoked several decades ago to explain the osmotic effects of ammonium salts on erythrocytes and sea urchin eggs [21, 22]. Remarkably, apart from these early studies, few recent investigations address the biophysical mechanisms and kinetics of the osmotic effects of short-term exposure to $\text{NH}_3/\text{NH}_4^+$ of animal cells [23, 24].

In the present study, we used a method to measure, in real-time (resolution of <1 s), parallel changes in cell water volume (CWV) and pH_i in response to brief exposure to solutions containing $\text{NH}_3/\text{NH}_4^+$, in single cells loaded with the fluorescent dye BCECF [25]. As model systems, we used two murine cell lines, N1E-115 neuroblastoma and NG-108 neuroblastoma X glioma. The cells were briefly exposed to isosmotic solutions containing various concentrations of NH_4Cl (0.5-20 mM). With BCECF, changes in CWV are assessed from the emitted fluorescence upon exciting the fluorophore at its pH-insensitive intracellular

isosbestic wavelength (~ 438 nm), while changes in pH_i are assessed ratiometrically. In addition to the expected changes in pH_i , exposure to NH_4Cl -containing solutions isosmotic with extracellular media induced a highly reproducible pattern of CWV changes consisting of a rapid cell swelling followed by regulatory volume decrease (isosmotic RVD, or iRVD). Removal of the NH_4Cl solution caused cell shrinkage, after which CWV was restored by regulatory volume increase (isosmotic RVI, or iRVI). Experiments in which either $[\text{NH}_3]$ or $[\text{NH}_4^+]$ were kept fixed showed that the initial increase in both pH_i and CWV depended on the external $[\text{NH}_3]$, and not on external $[\text{NH}_4^+]$. Thus, it is concluded that a rapid entry and protonation of NH_3 leads to a rapid increase in intracellular NH_4^+ concentration ($[\text{NH}_4^+]_i$), exerting an increase in intracellular osmotic pressure that results in cell swelling. From the concomitant pH_i measurements and using the Henderson-Hasselbalch formalism, changes in $[\text{NH}_4^+]_i$ were quantified and their relative contribution to the raise in intracellular osmolality and cell water volume were modeled. It was found that about 35 to 45 % of the initial cell swelling could be explained by intracellular accumulation of NH_4^+ . Interestingly, after the initial peak swelling caused by $\text{NH}_3/\text{NH}_4^+$ application, in spite of a gradual, sustained increase in $[\text{NH}_4^+]_i$ (and hence in intracellular osmolality) now caused by slow NH_4^+ influx, the cells displayed isosmotic RVD (iRVD). These results provide clear evidence that neuronal or glial swelling can result from direct osmotic effects of intracellular NH_4^+ accumulation, and establish for the first time that initial NH_4^+ accumulation is due primarily to NH_3 influx followed by delayed NH_4^+ influx. Further, the present study establishes the validity of this *in vitro* model to study, at the single cell level, the consequences of $\text{NH}_3/\text{NH}_4^+$ on cell volume and its interdependence with pH_i . The results are discussed in terms of their potential relevance to the pathophysiology of hyperammonemic cellular brain edema.

Materials and Methods

Cell Culture

N1E-115 mouse neuroblastoma [26] and NG108-15 mouse neuroblastoma x rat glioma [27] cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco-Invitrogen No. 12430) supplemented with 10% fetal calf serum (FCS, HyClone), 1% hypoxanthine-aminopterin-thymidine (HAT; Sigma-Aldrich) and 1% L-glutamine (Gibco-Invitrogen), in a 5% CO_2 /95% air atmosphere. N1E-115 cells are an adrenergic clone of mouse neuroblastoma C1300 cells and possess acetylcholinesterase and tyrosine hydroxylase activity [26]. NG108-15 are a fusion of the mouse neuroblastoma clone N18TG-2 and the rat glioma clone C6 BV-1, express characteristics of both glial and neuronal cells and have both cholinergic and GABAergic properties [28]. Cells from passages 16-25 were plated on 25-mm-diameter glass coverslips previously treated with poly-D-lysine (BD Biosciences). Differentiation was induced 24 h after plating by supplying the cells with a differentiation medium composed of 98% DMEM, 2% FCS, 1% HAT, 1% L-glutamine, 1 mM theophylline, and 10 μM prostaglandin E1 [29]. Theophylline and prostaglandin E1 came from Sigma-Aldrich. Cells were used for experiments 3 to 7 days after the differentiation treatment.

Solutions

The control isosmotic solution (ISO) contained (in mM): 120 NaCl, 5.5 KCl, 2.5 CaCl_2 , 1.25 MgCl_2 , 20 HEPES and 10 D-glucose. The final osmolality was adjusted with sucrose (~ 32 mM) to ~ 312 mosmol/kg water, to match the osmolality of the differentiation media. Anisomotic calibration solutions were prepared by sucrose addition or removal to keep the ionic concentrations constant and at the value of the control ISO; they were expressed as percentage decrement or increment with respect to the control ISO (i.e. $\pm 10\%$ with respect to the osmolality of the ISO). NH_4Cl -containing solutions (0.5 to 30 mM) were prepared by equimolar replacement of NaCl by NH_4Cl in the ISO. The zero- Ca^{2+} solution was made by substituting CaCl_2 for MgCl_2 and adding 1 mM EGTA. The pH of all these solutions was adjusted to 7.3 with NaOH. The isosmotic solutions with fixed $[\text{NH}_3]$ (0.23 mM) and various $[\text{NH}_4^+]$ (4.77, 9.77, 19.77 and 29.77 mM) were made from solutions containing 5, 10, 20 and 30 mM NH_4Cl in which the pH was adjusted to 7.91, 7.61, 7.31 and 7.13, respectively. The solutions having fixed $[\text{NH}_4^+]$ (10 mM) and various $[\text{NH}_3]$ (0.05, 0.11, 0.22 and 0.33 mM) were prepared from solutions with 10.05, 10.11, 10.22 and 10.33 mM NH_4Cl , and the pH was set to 7.0, 7.3, 7.6 and 7.77,

respectively. 5-nitro-2-(3-phenylpropylamine) benzoic acid (NPPB) from Sigma-Aldrich was dissolved in DMSO. The final concentration of DMSO in the experimental solutions was $\leq 0.1\%$. Experimental solutions were perfused into the recording chamber at a rate of 6.0 ml/min using an electric valve-controlled system (Warner Instruments). The fluid at the recording area of the chamber was exchanged by 90% in 12.6 ± 0.6 s ($n = 6$).

Dye Loading

A coverslip with the cells attached and equilibrated with ISO was mounted in an imaging chamber (Warner Instruments RC-21BRW) and placed on the stage of an epifluorescence inverted microscope (Olympus IX81). After recording the background fluorescence from each cell, they were loaded with ISO containing the acetoxymethyl (AM) esters of either BCECF (5 μM) or calcein (2 μM ; both from Invitrogen-Molecular Probes) dissolved in DMSO. Dye loading progress was monitored from each of the cells in the field of view until reaching the desired levels of fluorescence (~ 10 -30 min). The loading solution was washed-out with ISO and the cells were equilibrated for ~ 15 -30 min in this solution before starting recording. For measurement of changes in intracellular free- Ca^{2+} concentration, cells were loaded by incubation for ~ 30 min with ISO containing 4 μM Fura-2-AM (TEF Labs) and 10% (wt/wt) pluronic F-127 (Invitrogen-Molecular Probes) dissolved in DMSO.

Fluorescence Measurements

Total fluorescence from a small circular digital pinhole region placed at the image plane of each fluorophore-loaded single cell was measured with an imaging system described in detail elsewhere [25]. Each pinhole region was placed at the soma of each neuron. The size of each pinhole region was 3 to 10 % of the total area of the soma at the image plane. The imaging system included an epifluorescence inverted microscope equipped with a 40 X oil immersion objective lens ($\text{NA} = 1.35$; Olympus America, Melville, NY). The excitation light coming from a 75W xenon arc lamp passed through the input slit of a monochromator (Optoscan, Cairn Research Limited, Faversham, UK) that allowed independent slit width control for each wavelength. BCECF was excited with light pulses (40-80 ms duration) at its pH-sensitive wavelength (495 ± 2 -3 nm), and at 438 ± 2 -4 nm, its intracellular isosbestic wavelength (IW). The interval between these two excitation wavelengths was 15 ms. Calcein was excited at 495 ± 2 nm and Fura-2 at 340 ± 3 nm and 380 ± 3 nm. Emitted fluorescence (detected at 535 nm for BCECF and calcein, and at 510 nm for Fura-2) was monitored on-line using a cooled CCD camera (ORCA-ER C4742-95, Hamamatsu). MetaFluor imaging software (Molecular Devices, Sunnyvale, CA) was used for image acquisition, digital pinhole size and positioning and fluorescence recording. Fluorescence was sampled at 0.2 Hz. All experiments were done at room temperature (21 - 24°C).

Cell Water Volume Measurements

The validation of the method and procedures for measuring cell water volume (CWV) changes using fluorescent dyes has been explained in detail elsewhere [25, 30]. In brief, CWV changes (V_t/V_0) are computed from monitored changes in relative fluorescence (F_t/F_0) resulting from exciting BCECF at the intracellular IW (~ 438 nm) or calcein at 495 nm, according to the following equation:

$$[(F_0/F_t) - F_{\text{bkg}}] / (1 - F_{\text{bkg}}) = V_t/V_0 \quad (\text{Eq.1})$$

where F_0 is the fluorescence from a pinhole region of the cell equilibrated with an ISO control solution at $t = 0$; F_t is the fluorescence of the same region at $t = t$; F_{bkg} is the fraction of intracellularly trapped and osmotically-insensitive fluorescence of the dye-loaded cell (see below); V_0 is the baseline CWV in ISO ($V_0 = 1$) and V_t is the CWV at time t .

One of the advantages of this method is that the fluorescence signals can be calibrated for defined changes in extracellular osmolality in each individual cell, so that each cell is used as its own osmometer. Accordingly, osmotic calibrations were done by exposing each cell to two pulses of anisotonic solutions (5 to 7 min duration) having nominal osmolalities of $\pm 10\%$ with respect to the ISO. Within this range of osmolalities, the cells do not show any signs of regulatory responses and exhibit osmometric behavior. An example of the changes in BCECF relative fluorescence in response to pulses of anisotonic calibration

Fig. 1. Calibration of fluorescence signals to measure CWV changes in single cells. (A) Changes in BCECF relative fluorescence (F_t/F_0) recorded by exciting the dye at its intracellular isosbestic wavelength (438 ± 2.5 nm) in response to two pulses of calibration solutions ($\pm 10\%$ anisotonic), as indicated by the bars at the bottom of panel C. (B) Plot of the reciprocal of the apparent steady state changes in fluorescence (F_0/F_t) as a function of the reciprocal of the relative osmotic pressure of the medium (π_0/π_t) for the calibration pulses shown in A. The y intercept of the linear regression fitted to the observed data points (solid line) corresponds to the fraction of intracellularly trapped and osmotically-insensitive fluorescence (F_{bkg}) that in this case was 0.58. Subtraction of F_{bkg} yields the dotted line indicating ideal osmometric behavior. (C) Changes in CWV (V_t/V_0) resulting from transformation of the fluorescence signals shown in A, using Eq. 1. (D) Plot of the apparent steady-state changes in V_t/V_0 as a function of π_0/π_t . The solid line fitted to the data points has a slope ≈ 1 which corresponds to ideal osmometric behavior.

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solutions when exciting the dye at its intracellular IW (438 ± 2.5 nm) is shown in Fig. 1 A. Relative fluorescence is expressed as F_t/F_0 , where F_0 is the fluorescence measured at the pinhole region in an ISO control solution having osmotic pressure π_0 and F_t is the fluorescence of the same region upon exposure to a calibration solution having osmotic pressure π_t . Plotting the reciprocal of the apparent steady state changes in fluorescence (F_0/F_t) for each calibration pulse as a function of the reciprocal of the relative osmotic pressure of the medium (π_0/π_t) yields a linear relationship (Fig. 1 B), indicating that the changes in fluorescence reflect changes in intracellular concentration of BCECF that in turn reflect changes in CWV. Before subtraction of F_{bkg} , the slope of the regression line fitted to the data points (solid line in Fig. 1B) is always less than 1, the slope expected for ideal osmometric behavior (dotted line Fig. 1B). F_{bkg} was estimated for each cell from the y intercept of plots of F_0/F_t versus π_0/π_t , like the one shown in Fig. 1B. Subtraction of F_{bkg} yields the dotted line shown in Fig. 1B, indicating ideal osmometric behavior. The nature of F_{bkg} , as well as alternative methods to determine its value, has been extensively discussed in previous publications [25, 30, 31]. Changes in CWV (V_t/V_0) resulting from transformation of the fluorescence signals shown in Fig. 1A, using Eq 1 are shown in Fig. 1C. A plot of the apparent steady state changes in V_t/V_0 as a function of π_0/π_t is shown in Fig. 1D. The solid line fitted to the data points has a slope ≈ 1 , which corresponds to ideal osmometric behavior, as discussed in detail in a previous publication [25].

Analysis of Cell Water Volume Changes

When drift of the fluorescence signal occurred as a result of dye leakage and/or photobleaching, it was corrected by fitting a linear regression to the base line and multiplying the slope of this regression line by the time at which each data point was sampled. This process yields point by point drift values that in turn are subtracted from the fluorescence record yielding the corrected trace. The nomenclature used to refer to the measured variables of CWV changes in response to transient exposure to isosmotic solutions containing NH_4Cl is shown in Fig. 3A. The extent of the iRVD response was measured 4 min after its onset (f in Fig. 3A) and represents the percentage of cell volume recovery from the initial normalized peak swelling (100%), according to the following expression:

$$\% \text{ iRVD} = [(peak V_t/V_0 - reg V_t/V_0) / (peak V_t/V_0 - 1)] \times 100 \quad (\text{Eq. 2})$$

where 'peak V_t/V_0 ' is the peak swelling value and 'reg V_t/V_0 ' is the regulated relative volume 4 min after the onset of the iRVD response.

Measurement of Intracellular pH

Intracellular pH changes measured in parallel with CWV were estimated from the ratio of emitted fluorescence upon excitation of BCECF at 495 and 438 nm, using the high K^+ -nigericin *in vivo* calibration technique as described by Boyarski's group [32, 33]. Only cells having an initial pH_i between 6.8 and 7.4 were analyzed. Fig 3B illustrates the nomenclature used to refer to the measured variables of the pH_i transient in response to NH_4Cl exposure and removal.

Statistical analyses

Two-tailed t-test analysis was used to compare differences between means. A p value of <0.05 was considered as significant. Results are expressed as the mean $\pm$ SEM (standard error of the mean)

Results

Parallel Changes in Cell Water Volume and Intracellular pH Induced by $\text{NH}_3/\text{NH}_4^+$ in Neuroblastoma Cells

Changes in CWV and pH_i produced by brief (7 to 20 min) exposure to isosmotic solutions containing NH_4Cl (0.5-30 mM) were recorded simultaneously in single neuroblastoma cells loaded with BCECF. Fig. 2 shows a representative example of these responses. The upper panel (A) shows the CWV changes (V_t/V_0 ; black trace), and the lower panel (B) shows the concomitant pH_i transients produced by exposure and removal of an isosmotic solution that, in this case, contained 20 mM NH_4Cl . Upon exposure to the NH_4Cl solution the cell swelled (phase 1v) and underwent regulatory volume decrease (phase 2v). Since the latter response occurs in isosmotic media, we denote it as "isosmotic regulatory volume decrease" (iRVD) to distinguish it from the much studied "regulatory volume decrease" (RVD) elicited by exposure to hyposmotic solutions. Upon removal of the NH_4Cl solution the cell shrank (phase 3v) and slowly recovered its initial volume (phase 4v). We call this response "isosmotic regulatory volume increase" (iRVI) to distinguish it from the RVI that occurs during exposure to a hyperosmotic solution or the RVI that occurs after a cell that was challenged with an hyposmotic solution is placed back into isosmotic solution.

Concomitant with the changes in CWV, the cell responded to the NH_4Cl pulse with the pH_i transients shown in Fig. 2B. These are the pH_i changes expected for a cell permeable to both NH_3 and NH_4^+ [11, 13]. They consisted of an initial rapid alkalization (phase 1p) followed by a slow decrease in pH_i (phase 2p) known as plateau acidification. Removal of the NH_4Cl pulse resulted in a marked and rapid acidification (phase 3p) followed by a relatively slow pH_i recovery (phase 4p). The inset in Fig. 2A shows the initial changes in CWV and pH_i upon exposure to the NH_4Cl pulse, displayed at a faster time base. As expected, the changes in pH_i precede those in CWV.

The results obtained from 60 N1E-115 cells exposed to 20 mM NH_4Cl are summarized in Table 1. The changes in CWV and pH_i were quantified following the nomenclature described in Fig. 3. In these experiments, the NH_4Cl pulse had an average duration of 7.45 ± 0.4 min. The number of observations for some parameters describing the late parts of the response (e.g. phases 3v and 4v) is less than 60 because upon removal of the NH_4Cl pulse a small number of cells (7% of the population) returned to their initial volume without presenting shrinkage. For the cells that shrank (i.e. 93% of the population), the latency of the peak shrinkage (g in Fig. 3A) and the time course of the subsequent iRVI were variable, and in a few occasions, iRVI was absent.

The basic pattern of CWV changes described above was observed also in NG108 cells ($n=13$), a hybrid of neuroblastoma and glioma (see below). Further, the basic pattern of CWV

Fig. 2. Changes in CWV and pH_i recorded simultaneously in a single BCECF-loaded N1E-115 cell exposed to an isosmotic solution containing NH_4Cl . (A) Changes in CWV (V_t/V_0 , black trace). Upon exposure to the NH_4Cl solution (20 mM), the cell swelled to a maximum of 8.3% above its initial volume (phase 1v) in ~21 s, at an initial rate of ~56% min^{-1} . The cell remained swollen at its maximum value for ~120 s and then displayed isosmotic regulatory volume decrease (iRVD; phase 2v) at an initial rate of -8.2% min^{-1} . The extent of cell volume recovery, measured 4 min after the onset of iRVD as defined by Eq. 2, was 36.7%. Upon termination of the NH_4Cl pulse and readmission of the isosmotic control solution, CWV decreased (phase 3v) to a maximum of ~3% below the baseline in ~30 s. This cell shrinkage was followed by isosmotic regulatory volume increase (iRVI; phase 4v) that proceeded at an initial rate of ~3.2% min^{-1} . *Inset in (A)* shows the initial changes in CWV (dark blue trace) and pH_i (orange trace) at an expanded time base. The green trace superimposed on the CWV transient in (A) corresponds to the $[\text{NH}_4^+]_i$ calculated from Eq. 3 using the pH_i data recorded during phase 2p in panel B. The values of $[\text{NH}_4^+]_i$ were corrected for the recorded changes in CWV for each instant. The red trace corresponds to the “predicted” changes in CWV (V_t/V_0) calculated from the increase in intracellular osmolality resulting from NH_4^+ accumulation (Eq. 4) assuming osmometric behavior (i.e. lack of regulatory responses). (B) pH_i changes occurring simultaneously with those in CWV. The initial basal pH_i was 7.1. Upon exposure to NH_4Cl there was a rapid alkalinization (phase 1p) that peaked at pH_i 7.53, followed by a recovery phase (plateau acidification or phase 2p) that proceeded at an initial rate of -0.022 pH_i units min^{-1} . Upon NH_4Cl removal, there was a rapid decrease in pH_i that peaked at 6.8 (phase 3p) followed by a recovery phase (phase 4p) at an initial rate of 0.02 pH_i units min^{-1} .

changes in cells loaded with BCECF could also be reproduced in cells loaded with calcein (Fig. 4), a fluorescent dye that has spectral properties different from those of BCECF and is well characterized as an independent CWV marker [25, 30, 31]. These results demonstrate that the observed CWV changes were not dye- or cell line type- dependent.

Mechanisms of the Cell Swelling Induced by $\text{NH}_3/\text{NH}_4^+$

The initial pH_i increase and the subsequent time course of the pH_i response upon NH_4Cl exposure and removal indicate that N1E-115 and NG 108 cells, like most animal cell types, have a higher permeability to NH_3 than to NH_4^+ [12, 34, 35]. Consequently, when they are challenged with NH_4Cl , NH_3 rapidly enters through the plasma membrane and combines with intracellular protons, forming NH_4^+ and shifting the pH_i in the alkaline direction. Since NH_4^+ , but not NH_3 , is the osmotically active species, we hypothesize that the net gain in osmotically active particles represented by NH_4^+ accumulation increases the intracellular osmolality, thereby inducing net osmotic water influx and cell swelling. To test this hypothesis, we calculated the magnitude and time course of intracellular NH_4^+ accumulation during the NH_4Cl pulse. To this end, we measured for each individual cell the values of pH_i at the peak alkalinization and throughout the plateau acidification. At the peak alkalinization and during the slowly developing plateau phase, NH_3 is expected to be at or near equilibrium

Table 1. CWV and pH_i Changes in N1E-115 Cells Exposed to 20 mM NH_4Cl . All values are means $\pm$ SEM. The nomenclature for CWV ($a - i$) and pH_i parameters is as in Fig. 3

Parameter		n
a , peak swelling, (%)	14 ± 1	60
b , initial rate of swelling, (% min^{-1})	63 ± 5	60
c , time to peak swelling, (s)	36 ± 2	60
d , latency of iRVD onset, (s)	69 ± 5	60
e , initial rate of iRVD, (% min^{-1})	-9.2 ± 1	60
f , extent of iRVD at $t = 4$ min after onset, (%)	34 ± 3	60
g , time to peak shrinkage upon NH_4Cl removal, (s)	75 ± 8	56
h , peak shrinkage, (%)	-7 ± 0.6	56
i , initial rate of iRVI, (% min^{-1})	12.3 ± 2	54
pH_i $t=0$	7.15 ± 0.02	60
pH_i $t=1$	7.55 ± 0.03	60
ΔpH_i (pH_i $t=1 - \text{pH}_i$ $t=0$)	0.39 ± 0.01	60
pH_i $t=2$	7.41 ± 0.02	59
pH_i $t=3$	6.82 ± 0.02	59
pH_i $t=4$	7.05 ± 0.05	60
$-\text{dpH}_i/\text{dt}$, initial rate of plateau acidification (pH_i units $\cdot \text{min}^{-1}$)	-0.024 ± 0.001	60

Fig. 3. Schematic drawing illustrating measured variables of CWV and pH_i changes elicited by NH_4Cl exposure and removal. (A) CWV changes. Ordinate: relative cell volume (V_t/V_0). Abscissa: time. (a) Maximum (peak) swelling, (b) initial rate of swelling, (c) time to peak swelling, (d) latency of iRVD onset, (e) initial rate of iRVD, (f) extent of iRVD at $t = 4$ min, (g) time to peak shrinkage upon NH_4Cl removal, (h) peak shrinkage, (i) initial rate of iRVI. (B) Points at which pH_i was measured. $t = 0$, basal; $t = 1$, at peak alkalization; $t = 2$, before NH_4Cl removal; $t = 3$, at peak acidification; $t = 4$, after recovery from acidification; $-\text{dpH}_i/\text{dt}$, initial rate of plateau acidification.

across the plasma membrane, and $[\text{NH}_4^+]_i$ can be calculated using the following expression, derived from the Henderson-Hasselbalch equation:

$$[\text{NH}_4^+]_i = [\text{NH}_4^+]_o \times 10^{\text{pH}_o - \text{pH}_i} \quad (\text{Eq. 3})$$

where $[\text{NH}_4^+]_i$ is the intracellular NH_4^+ concentration and $[\text{NH}_4^+]_o$ is the extracellular NH_4^+ concentration, which for a 20 mM NH_4Cl solution at 25°C has a value of 19.78 mM

Fig. 4. Changes in CWV produced by $\text{NH}_3/\text{NH}_4^+$ in a single N1E-115 cell loaded with calcein. The cell was exposed to a pulse (7 min) of an isosmotic solution containing 20 mM NH_4Cl . The observed CWV changes had the same basic pattern observed in cells loaded with BCECF.

Fig. 5. Peak cell swelling amplitude as a function of $[\text{NH}_4^+]_i$ and basal pH_i in response to $\text{NH}_3/\text{NH}_4^+$ exposure. Peak increase in cell volume (V_t/V_0) plotted as a function of the corresponding $[\text{NH}_4^+]_i$ (A) and initial ($t=0$) basal pH_i (B) in response to a 20 mM NH_4Cl pulse. Each point represents a single cell ($n=60$). The correlation coefficient of the regression lines was 0.78 ($p < 0.0001$) in A, and 0.79 ($p < 0.0001$) in B.

($\text{pK}=9.25$; $\text{pH}_0=7.3$). The values of $[\text{NH}_4^+]_i$ were corrected point by point for the measured changes in CWV, to take into account intracellular dilution caused by such changes. For the cell in Fig. 2, the estimated $[\text{NH}_4^+]_i$ with respect to time is shown in the green trace in (A). At the peak alkalization, $[\text{NH}_4^+]_i$ reached ~ 12.5 mM, and thereafter it continued increasing gradually throughout the duration of the NH_4Cl exposure, reaching a value of ~ 15.7 mM just before the end of the NH_4Cl pulse (i.e. at $t \approx 7$ min). The $[\text{NH}_4^+]_i$ at the peak swelling is likely to result mostly from rapid entry of NH_3 and formation of NH_4^+ . The continued buildup of $[\text{NH}_4^+]_i$ during NH_4Cl exposure is probably due to influx of NH_4^+ as suggested by the *plateau* acidification of the pH_i response. To evaluate if the increase in $[\text{NH}_4^+]_i$ could explain the magnitude of the observed peak swelling, we calculated the change in CWV that would result from such accumulation, according to the following equation:

$$V_t/V_0 = (\text{mosmol/kg water})_{t=t} / (\text{mosmol/kg water})_{t=0} \quad (\text{Eq. 4})$$

where V_t and V_0 have been defined, and the intracellular osmolality is expressed in mosmol/kg water. For this calculation, it is assumed that prior to NH_4Cl exposure the cell is in osmotic equilibrium with the extracellular solution, and hence the intracellular and extracellular osmolalities are equal (311 mosmol/kg water in this case). For the cell in Fig. 2, a net increase in intracellular osmolality resulting from accumulation of 12.5 mosmol/kg water should produce a peak swelling of $\sim 4\%$ ($V_t/V_0 = (311 + 12.5) / 311$). However, the observed peak swelling was 8.3%, thus $\sim 48\%$ of the *peak* swelling can be explained, in this example, by intracellular accumulation of NH_4^+ . We also calculated the theoretical time course of the CWV changes that would occur if there were no regulatory volume adjustments, using Eq. 4. This is shown in Fig. 2A (V_t/V_0 , red trace). Note that under these assumptions, CWV progressively increases in parallel with the net gain in intracellular NH_4^+ . However, the cell responds with iRVD, thereby counteracting the progressive increase in intracellular osmolality and the volume expansion that would otherwise occur.

In the 60 N1E-115 cells analyzed, the mean $[\text{NH}_4^+]_i$ estimated at the peak alkalization was 14.2 ± 0.8 mM. The predicted mean peak swelling for this $[\text{NH}_4^+]_i$ was 4.6 ± 0.3 % (range 1.7 to 9.6%). The observed mean peak swelling (a in Fig. 3) was 14 ± 1 % (range 3 to 29%, Table 1). Thus, ~35% of the peak swelling can be explained by intracellular accumulation of NH_4^+ . Similarly, in NG-108 cells the peak swelling was 15.1 ± 1.7 %, and the predicted volume 5.9 ± 0.8 %. Thus, ~46% of the peak swelling amplitude can be accounted for intracellular NH_4^+ accumulation. Although NH_4^+ accumulation by itself cannot fully account for the observed swelling, the strong correlation ($r = 0.78$; $p < 0.0001$) between the magnitude of the observed cell swelling and the $[\text{NH}_4^+]_i$ (Fig. 5A) is consistent with the hypothesis that NH_4^+ accumulation is a significant determinant of the increase in CWV. Coherent with this hypothesis is also the observation that the peak swelling amplitude was inversely related to the initial pH_i (i.e. $\text{pH}_{i,t=0}$), as shown in Fig. 5B. This was expected since lowering the pH_i favors the protonation of NH_3 .

The above mentioned results indicate that in addition to NH_4^+ , other osmolytes, likely derived from metabolic reactions that utilize NH_4^+ , contribute to the initial cell swelling. We addressed the question of whether one of these osmolytes could be glutamine, a main product of the detoxification of $\text{NH}_3/\text{NH}_4^+$, that has been found to accumulate in the brain during hyperammonemia [36]. First, we confirmed the presence of glutamine synthetase (GS) in N1E-115 and NG108 cells by Western blot analysis (data not shown). Next, N1E-115 cells were exposed to control and test pulses with 20 mM NH_4Cl , in the absence and in the presence of the GS inhibitor methionine sulfoximine (MSO), respectively. Peak swelling amplitude was 23% lower in the presence of MSO ($n=6$; $p<0.05$), suggesting that glutamine synthesis contributes to the initial volume change mediated by $\text{NH}_3/\text{NH}_4^+$ (data not shown). These results indicate that in addition to NH_4^+ , glutamine accumulation partly contributes to the initial increase in CWV mediated by $\text{NH}_3/\text{NH}_4^+$.

The Amplitude of the Peak Swelling Varies with the External Concentration of NH_4Cl

It has long been known that the amplitude of the intracellular alkalization produced by NH_4Cl exposure depends on the external concentration of this salt, and that this effect is due to rapid NH_3 permeation and its subsequent intracellular protonation [34]. Our findings are consistent with the hypothesis that an important part of the initial swelling induced by NH_4Cl is due to rapid NH_3 permeation and intracellular accumulation of the osmotically active species NH_4^+ . Accordingly, the peak amplitude of the swelling and the magnitude of the initial intracellular alkalization ($\Delta\text{pH}_i = \text{pH}_{i,t=1} - \text{pH}_{i,t=0}$) should be a function of the extracellular concentration of NH_4Cl ($[\text{NH}_4\text{Cl}]_o$). Fig. 6A shows the relationship between the observed (filled circles) and predicted (open circles) values of the peak swelling amplitude as a function of $[\text{NH}_4\text{Cl}]_o$. The inset shows typical CWV traces from a cell exposed to two pulses of low $[\text{NH}_4\text{Cl}]_o$ (0.5 and 1 mM, respectively). The latter solutions have NH_3 concentrations similar to those present in acute liver failure, as well as in some congenital hyperammonemias [6, 8]. As predicted by our model (Fig. 2), the amplitude of the peak swelling increased with $[\text{NH}_4\text{Cl}]_o$. Consistent with the results presented in the previous section, there was a significant difference between predicted and observed values of the peak swelling amplitudes for the entire range of $[\text{NH}_4\text{Cl}]_o$ tested. Also, as predicted, both the ΔpH_i and the $[\text{NH}_4^+]_i$ were a function of $[\text{NH}_4\text{Cl}]_o$ (Fig 6B).

The External Concentration of Ammonia (NH_3) but not Ammonium (NH_4^+) Determines the Peak Amplitude of Both Cell Swelling and Intracellular Alkalization

The results so far presented are consistent with intracellular NH_4^+ accumulation being a major determinant of the NH_4Cl -induced cell swelling. Given that N1E-115 and NG108 cells are permeable to both NH_3 and NH_4^+ , accumulation of intracellular NH_4^+ could result not only from protonation of entering NH_3 , but also from direct NH_4^+ influx. In NH_4Cl -containing solutions, NH_3 is in chemical equilibrium with NH_4^+ , and the relative concentration of each species is determined by the pH of the solution. To investigate the relative contribution of each of these species to the magnitude of the peak swelling, we tested the effects on CWV

Fig. 6. Peak cell swelling amplitude, $[\text{NH}_4^+]_i$ and ΔpH_i in cells exposed to various NH_4Cl concentrations. (A) Observed (filled circles) and predicted (open circles) mean peak increase in CWV (V_i/V_o), plotted as a function of external $[\text{NH}_4\text{Cl}]$. The predicted peak increase in CWV (peak cell swelling) was calculated from the increase in intracellular osmolality that would result from $[\text{NH}_4^+]_i$ estimated from pH_i values concomitant with the peak cell swelling, applying Eqs. 3 and 4. Inset shows that volume responses could already be elicited with low NH_4Cl concentrations, i.e. 0.5 mM and 1 mM, respectively. (B) Magnitude of the initial change in pH_i [ΔpH_i ; (filled circles)] and $[\text{NH}_4^+]_i$ (open circles) corresponding to peak alkalization and peak cell swelling values plotted as a function of external $[\text{NH}_4\text{Cl}]$. Symbols are mean $\pm$ SEM. The number of cells analyzed for each $[\text{NH}_4\text{Cl}]$ is shown in parentheses in A. *, $p < 0.05$; ***, $p < 0.001$.

Fig. 7. Influence of external $[\text{NH}_3]$ and $[\text{NH}_4^+]$ on CWV and pH_i . (A) Changes in CWV (V_i/V_o ; top) and pH_i (bottom) recorded in a single BCECF-loaded cell exposed to two NH_4Cl pulses (P_1 and P_2) in which $[\text{NH}_4^+]_o$ was kept constant (10 mM), while $[\text{NH}_3]_o$ was varied from 0.11 mM (P_1) to 0.33 mM (P_2). Note that the amplitude of both the cell swelling and ΔpH_i increased with $[\text{NH}_3]_o$. (B) Changes in CWV (V_i/V_o) and pH_i produced by two NH_4Cl pulses in which $[\text{NH}_3]_o$ was kept constant (0.23 mM), while $[\text{NH}_4^+]_o$ was varied from 19.77 (P_1) to 4.77 mM (P_2). Note that the amplitude of both the swelling and the pH_i change was similar in both pulses despite a 4-fold difference in $[\text{NH}_4^+]_o$.

and pH_i of isosmotic solutions containing NH_4Cl in which either $[\text{NH}_3]_o$ or $[\text{NH}_4^+]_o$ was kept constant while the other was changed by varying the pH of the solution. Fig. 7A shows an example of changes in CWV (V_i/V_o ; upper traces) and pH_i (lower traces) elicited in a single cell by two consecutive NH_4Cl pulses (P_1 and P_2) in which $[\text{NH}_3]_o$ was varied from 0.11 mM (P_1) to 0.33 mM (P_2), while $[\text{NH}_4^+]_o$ was kept constant (10 mM). The amplitude of both the initial swelling and the intracellular alkalization were ostensibly larger in P_2 , i.e. the pulse in which $[\text{NH}_3]_o$ was higher. Fig. 7B shows CWV and pH_i changes recorded from a

Fig. 8. Peak swelling and ΔpH_i as a function of external $[\text{NH}_3]$ and $[\text{NH}_4^+]$. Data was collected from experiments like those shown in Fig. 7. (A) Amplitude of the peak swelling (V_t/V_0) and (C) ΔpH_i plotted as a function of $[\text{NH}_3]_o$ keeping $[\text{NH}_4^+]_o$ constant (10 mM). (B) Amplitude of the peak swelling and (D) ΔpH_i plotted as a function of $[\text{NH}_4^+]_o$ keeping $[\text{NH}_3]_o$ constant (0.23 mM). Symbols are mean $\pm$ SEM. The number of cells for each data point is shown in parentheses. Lines are linear regressions.

single cell exposed to two NH_4Cl pulses in which the $[\text{NH}_3]_o$ was now fixed (0.23 mM), and $[\text{NH}_4^+]_o$ varied from 19.77 mM (P_1) to 4.77 mM (P_2). Note that although the $[\text{NH}_4^+]_o$ in P_1 was ~ 4 times higher than that in P_2 , the amplitude of the swelling and the pH_i increase were similar for both pulses.

Data obtained from experiments like those shown in Fig. 7 are summarized in Fig. 8. Note that both peak swelling and ΔpH_i increased with $[\text{NH}_3]_o$ (Figs. 8A and C) when $[\text{NH}_4^+]_o$ was kept constant (10 mM). In contrast, in cells exposed to solutions in which $[\text{NH}_3]_o$ was kept constant (0.23 mM) but $[\text{NH}_4^+]_o$ varied, the amplitude of the cell volume and the ΔpH_i did not change significantly with $[\text{NH}_4^+]_o$ (Figs. 8B and D). These results demonstrate that $[\text{NH}_3]_o$ rather than $[\text{NH}_4^+]_o$ is the main determinant of the NH_4Cl -induced swelling.

Membrane Permeability of N1E-115 cells to NH_3

The permeability to NH_3 (P_{NH_3}) of mammalian neuronal membranes has not yet been reported. We calculated the P_{NH_3} of N1E-115 cells from the transmembrane flux of NH_3 (J_{NH_3}) upon NH_4Cl exposure, defined as

$$J_{\text{NH}_3} = d\text{pH}_i/dt \times \beta_i \times v/s \quad (\text{Eq. 5})$$

where $d\text{pH}_i/dt$ is the initial rate of intracellular alkalinization, β_i is the intrinsic buffering power of the cells (in mM pH_i units $^{-1}$) and v/s is their volume to surface ratio. $d\text{pH}_i/dt$ was measured in cells exposed to 0.5 to 5 mM NH_4Cl , having a basal pH_i of ~ 6.7 to 7.0. Since the pK for $\text{NH}_3/\text{NH}_4^+$ at 25°C is ~ 9.25 , less than 1% of the NH_4^+ entering the cells will release their protons at this pH_i range, and hence the initial rate of the intracellular alkalinization will not be substantially affected by concurrent NH_4^+ -born protons. In this group of cells, cell volume (V) and membrane surface area (S) were measured from images obtained using differential interference optics. Since the cells were attached to coverslips, we considered them as hemispheres, with $S = 3\pi r^2$ and $V = \frac{2}{3}\pi r^3$. The mean radius of cell's soma was $16 \pm 0.4 \mu\text{m}$ ($n = 26$), and $v/s \approx 3.6 \mu\text{m}$. β_i is defined as $[\text{NH}_3 + \text{NH}_4^+]_i/\Delta\text{pH}_i$ and was calculated from the "off" pH_i response measured upon removal of NH_4Cl pulses in two sets of experiments. The first set included short (~ 2 min) NH_4Cl pulses (0.5–20 mM) in which pH_i returned to the baseline, i.e. there was no rebound acidification. In the second group, NH_4Cl (20 mM) exposure and removal were done in isosmotic Na^+ -free solution, a maneuver that blocked pH_i

Fig. 9. Intrinsic buffering power and transmembrane flux of NH_3 in N1E-115 cells. (A) Intrinsic buffering power (β_i) as a function of pH_i . β_i was estimated from ΔpH_i measured upon removal of short (~ 2 min) NH_4Cl (0.5–20 mM) pulses (open circles), or long (~ 7 min) NH_4Cl (20 mM) pulses in Na^+ -free solutions (filled circles). (B) Transmembrane flux of NH_3 (J_{NH_3}) as a function of the external $[\text{NH}_3]_o$, calculated using Eq. 5. The slope of the best-fit line corresponds to the apparent membrane permeability to NH_3 ($3.8 \times 10^{-3} \text{ cm s}^{-1}$).

recovery (not shown) by inhibition of Na^+/H^+ exchange [37, 38]. Blocking Na^+/H^+ exchange permits a more accurate estimate of β_i ; activation of this antiporter upon cell acidification in the “off” response decreases the amplitude of the acidification and causes overestimations of β_i [39]. Fig. 9A shows a plot of β_i as a function of pH_i , the latter being the midpoint of ΔpH_i for the “off” response. As in other cell types [39–41], β_i was inversely related to pH_i . The slope of a linear regression (solid line) fitting the data points was $-39.8 \text{ mM pH}_i \text{ unit}^{-1}$. The β_i used for calculation of J_{NH_3} was obtained for each cell by linear interpolation from the data shown in Fig. 9A.

Figure 9B shows J_{NH_3} plotted as a function of $[\text{NH}_3]_o$. The slope of the straight line represents the approximate P_{NH_3} of these cells, which was $38 \pm 2 \mu\text{m s}^{-1}$ ($n = 28$), a value that falls within the range reported for other animal cell membranes [35].

Isosmotic Regulatory Volume Decrease

The mechanisms mediating volume regulation in anisomotic media have been extensively studied in various cell types. In contrast, little is known about regulatory volume responses in isosmotic media. Given that the osmolality of the extracellular fluids is tightly regulated and maintained constant within narrow limits, isosmotic volume regulatory responses are likely to predominate under physiological conditions and under pathological conditions that do not seriously compromise renal function. We observed two isosmotic regulatory responses during exposure and removal of NH_4Cl , iRVD and iRVI, respectively (Fig. 2, phases 2v and 4v). A salient feature of these regulatory volume responses is that they occurred upon relatively small or moderate changes in CWV elicited in isosmotic media containing $\text{NH}_3/\text{NH}_4^+$, but not upon changes in CWV of similar amplitude produced by exposure to anisomotic solutions (Fig. 10A). This suggests that the mechanism(s) that trigger volume regulatory responses in isosmotic and anisomotic conditions are different, and demonstrates that changes in membrane mechanical tension (i.e., expansion or contraction) are not by themselves a sufficient condition for volume regulation to ensue.

We began to study the mechanisms underlying the iRVD response triggered during $\text{NH}_3/\text{NH}_4^+$ exposure. Anion channels are known to play a central role in the RVD produced by relatively large cell expansions elicited by hyposmotic media ($\geq 40\%$) in different cell types [42–44]. To determine if anion channels are also involved in iRVD elicited by NH_4Cl exposure, we evaluated the effects of NPPB (10 μM), an anion channel blocker known to inhibit anisomotic RVD. NPPB inhibited or even reversed the regulatory response (Figs. 10B and C). These results suggest that activation of NPPB-sensitive anion channels is a key component of iRVD elicited during $\text{NH}_3/\text{NH}_4^+$ exposure.

Fig. 10. Properties of iRVD elicited during exposure to $\text{NH}_3/\text{NH}_4^+$. (A) Superimposed traces of CWV (V_t/V_0) and pH_i elicited in the same cell by exposure to anisotonic ($\sim \pm 11\%$) solutions (gray traces) or to 20 mM NH_4Cl (black trace). Note that changes in cell volume produced by the anisotonic solutions were neither followed by regulatory responses nor were they accompanied by changes in pH_i . In contrast, the NH_4Cl solution and its removal elicited isotonic regulatory volume responses (iRVD and iRVI respectively) and the characteristic changes in pH_i . (B) Effect of NPPB on iRVD: superimposed traces of V_t/V_0 elicited by exposure to NH_4Cl (20 mM) in the absence (gray trace) and in the presence (black trace) of NPPB (10 μM). Note that in the presence of NPPB the regulatory response was abolished and the cell continued increasing its volume during the duration of the NH_4Cl pulse. (C) Percent inhibition of iRVD produced by NPPB (10 μM) in cells exposed to either 20 or 5 mM NH_4Cl pulses. The number of cells analyzed in each case is shown in parentheses. ***, $p < 0.001$. (D) Extent of iRVD measured during exposure to a first (control) pulse of 20 mM NH_4Cl , and a second (zero Cl^-) pulse of 20 mM NH_4^+ -gluconate. (E) Superimposed traces of V_t/V_0 in a cell exposed to NH_4Cl in the presence (control) and in the absence of external Ca^{2+} . (F) Effects of removal of external Ca^{2+} on the initial rate ($-d\text{CWV}/dt$) and extent of iRVD. The number of cells analyzed is shown in parentheses. ***, $p < 0.001$. (G) Changes in fura-2 fluorescence in a single cell exposed to NH_4Cl in the presence and in the absence of external Ca^{2+} . Exposure to NH_4Cl induced a transient increase in intracellular Ca^{2+} that was attenuated in the absence of external Ca^{2+} .

Among the intracellular anions that could be exiting the cells through NPPB-sensitive channels during iRVD are Cl^- and organic osmolytes. As shown in Fig. 10 D, the extent of iRVD measured during exposure to Cl^- -free, NH_4^+ -gluconate pulses ($23.7 \pm 5.6\%$) was in fact slightly larger, but not significantly different, than the control response ($17.6 \pm 3.2\%$; $n = 8$). This suggests that osmolytes other than Cl^- , probably organic anions, contribute to this regulatory response.

It has been previously shown that an increase in intracellular Ca^{2+} occurs in N1E-115 cells upon exposure to large ($\sim 40\%$) hyposmotic solutions. This increase, however, was not necessary for RVD to occur [45]. To investigate the possible Ca^{2+} dependency of iRVD, we exposed the cells to isosmotic solutions containing NH_4Cl (20 mM) in the presence (control pulse) and in the virtual absence of external Ca^{2+} (test pulse). Both the initial rate ($-d\text{CWV}/dt$) and the extent of iRVD were significantly reduced in Ca^{2+} -free- NH_4Cl solutions (Fig. 10E-F). This effect was independent of the order in which the control and the test pulses were applied. Similar results were obtained in cells loaded with calcein (not shown). In parallel experiments, changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were recorded in cells loaded with Fura-2 and exposed to NH_4Cl solutions with or without Ca^{2+} . NH_4Cl exposure

consistently elicited an increase in $[\text{Ca}^{2+}]_i$ that was attenuated in the virtual absence of external Ca^{2+} (Fig. 10G). These results suggest that unlike anisotonic RVD, the iRVD elicited by $\text{NH}_3/\text{NH}_4^+$ depends, to a large extent, on the presence of external Ca^{2+} .

Discussion

Classical observations on hemolysis of erythrocytes produced by ammonium salts, and theoretical considerations on the relative permeability and equilibrium distribution of NH_3 and NH_4^+ across cell membranes, led Merkel H. Jacobs (1884–1974) to propose several decades ago, that exposure to isosmotic solutions containing the pair $\text{NH}_3/\text{NH}_4^+$ should result in cell swelling [18, 21, 22]. In spite of its fundamental importance for cell biophysics of pH and volume regulation, and its possible clinical implications in the pathophysiology of hyperammonemic disorders, this hypothesis has remained largely untested. The present paper provides a kinetic description, at the single cell level, of the changes in cell water volume associated with exposure and removal of $\text{NH}_3/\text{NH}_4^+$ and their mechanistic relationship with concurrent pH_i changes. The method used here allows real time recording of parallel changes in pH_i and CWV in single cells, with time resolution of <1 second and sensitivity to osmotic changes of ~1% [25]. Sudden exposure of neuroblastoma cells (N1E-115 and NG-108) to isosmotic solutions containing the pair $\text{NH}_3/\text{NH}_4^+$ (NH_4Cl concentrations ranging from 0.5 to 20 mM) produces a rapid cell swelling followed by iRVD. Removal of NH_4Cl and readmission of the isosmotic control solution causes cell shrinkage, followed by iRVI. These volume changes are accompanied by the changes in pH_i expected for a cell significantly more permeable to NH_3 than to NH_4^+ , i.e. an initial alkalization followed by a plateau acidification, and upon removal of the NH_4Cl pulse, a rebound acidification followed by pH_i recovery [11, 13].

Mechanisms of Cell Swelling Elicited by Isosmotic Solutions Containing $\text{NH}_3/\text{NH}_4^+$

In isosmotic media, any increase in cell volume results from a primary increase in intracellular osmolyte contents. The present results suggest that an increase in intracellular osmolality brought about by rapid intracellular NH_4^+ accumulation is an important determinant of the initial cell swelling observed upon exposure to $\text{NH}_3/\text{NH}_4^+$ -containing solutions. The NH_4^+ accumulation could result from protonation of entering NH_3 , from direct influx of NH_4^+ via channels or transporters, or from a combination of these processes. Our results show that, like most cell types [35], the permeability of N1E-115- and NG108-cells to NH_3 is much higher than that to NH_4^+ . Thus, exposure to $\text{NH}_3/\text{NH}_4^+$ produces an intracellular alkalization due to rapid permeation of NH_3 followed by its intracellular protonation. Consequently, osmotically active NH_4^+ is formed and accumulated in the cytosol, thus increasing intracellular osmotic pressure and promoting water influx and cell swelling. By varying the concentration of either NH_3 or NH_4^+ in the isosmotic solution, while keeping the concentration of the remaining species constant, we demonstrated that intracellular NH_4^+ generation due to rapid NH_3 influx and protonation, rather than direct influx of NH_4^+ , is the main determinant of the observed cell swelling. After both pH_i and swelling peaks are reached, a gradual acidification ensues, due in part to slow entry of NH_4^+ . In spite of this progressive NH_4^+ accumulation, cells decrease their volume by activation of compensatory mechanisms i.e., iRVD.

The magnitude of the NH_4^+ accumulation during the peak swelling accounted for ~35% of its measured amplitude (~46% in NG108 cells). Since NH_4^+ accumulation imposes a net gain in intracellular cations, an equimolar increase in intracellular negative charges must occur to preserve macroscopic electroneutrality [46]. Such an increase in intracellular negative charges, however, is unlikely to promote a further rise in intracellular osmolality and cell volume, as it should result primarily from deprotonation of intrinsic fixed and mobile buffers [47, 48], whose concentration is expected to remain unaltered. In other words, as NH_3 is protonated, NH_4^+ is formed, cytoplasmic pH rises, and the net charge on the cytoplasmic

buffers becomes more negative, exactly balancing the positive charge on the NH_4^+ that is formed. Part of the initial swelling, however, could also result from Cl^- influx, driven by the NH_4^+ -induced depolarization and/or via cotransport of NH_4^+ through the K^+ binding site of the Na^+ - K^+ - 2Cl^- -cotransporter 1, i.e. NKCC1 [49] or that of the K^+ - Cl^- cotransporters, e.g. KCC2 [50]. Cl^- influx via channels is unlikely inasmuch as N1E-115 cells are derived from sympathetic ganglion neurons, in which the equilibrium potential for Cl^- (E_{Cl}) is 10 to 20 mV more positive than the resting membrane potential (E_{m}), under basal conditions [51] and hence the driving force for Cl^- is outward. Consistent with this hypothesis, we observed no significant differences in the magnitude of the initial swelling upon replacement of external Cl^- with gluconate. However, experiments performed in the presence of 10 μM bumetanide to block NKCC1 in cells challenged with 20 mM NH_4Cl produced a slight but significant increase in the peak intracellular alkalinization (ΔpH_i control 0.37 ± 0.4 to 0.43 ± 0.05 $n=9$, $p<0.001$). Likewise, the initial rate of plateau acidification decreased in the presence of bumetanide from -0.023 ± 0.003 to -0.016 ± 0.002 pH_i units/min ($p<0.0001$). These observations suggest that part of the NH_4^+ entry may be mediated by NKCC1. Expression of NKCC1 was ascertained in these cells by Western blot and immunolabeling (data not shown). Interestingly, changes in CWV were unaffected by bumetanide, suggesting that NH_4^+ influx mediated by NKCC1 may be balanced by efflux of osmolytes involved in iRVD. These results are in agreement with recent observations using 3-D image reconstructions of rat astrocytes, showing that bumetanide has no measurable effect on NH_4Cl -induced swelling [52].

As mentioned above, another potential NH_4^+ influx pathway is K^+ - Cl^- cotransport; NH_4^+ is a transport substrate of KCC2 when this cotransporter is exogenously expressed in mutant MDCK LK-C1 cells challenged with 30 mM NH_4Cl pulses [50]. This transport pathway, however, can be ruled out in our experiments; Western blot analysis revealed that N1E-115 cells do not express KCC2 (not shown). Nevertheless, NH_4^+ influx could occur through isoforms of K^+ - Cl^- cotransporter other than KCC2. However, we found that variations in external $[\text{NH}_4^+]$ in the range between ~ 5 to ~ 20 mM while keeping constant extracellular $[\text{NH}_3]$ did not affect the extent of cell swelling induced by NH_4Cl , which is rather a function of the external $[\text{NH}_3]$. Thus, any potential effects on cell volume due to NH_4^+ entry via K^+ - Cl^- cotransport are negligible in our experimental models. Moreover, furosemide, at a concentration expected to block all known isoforms of K^+ - Cl^- cotransporters (1mM), had similar effects to those of bumetanide (not shown).

We hypothesized that part of the unaccounted fraction of the swelling induced by $\text{NH}_3/\text{NH}_4^+$ was due to accumulation of organic osmolytes. The neutral aminoacid glutamine, a main product of the detoxification of $\text{NH}_3/\text{NH}_4^+$, has been implicated in brain cell edema in animal and *in vitro* models of liver failure [9, 36, 52], and could be one of the osmolytes that accumulates besides NH_4^+ . The enzyme glutamine synthetase (GS) catalyzes the production of glutamine from glutamate and NH_3 . The presence of GS in murine neuroblastoma cells N2a has been reported [53, 54]. Using Western blot analyses we demonstrated the expression of this enzyme in both N1E-115 and NG108 cells (data not shown). In the presence of the GS inhibitor MSO, the amplitude of the peak swelling induced by 20 mM NH_4Cl was reduced by $\sim 23\%$. Taken together these results suggest that a significant fraction of the swelling induced by $\text{NH}_3/\text{NH}_4^+$ is due to intracellular accumulation of glutamine.

The results discussed so far suggest that intracellular accumulation of NH_4^+ and glutamine can explain ~ 60 to 70% of the swelling; the question arises as to which are the other osmolytes that are accumulated. Besides glutamine, other organic osmolytes like lactate, which has been shown to accumulate in neuroblastoma cells exposed to NH_4Cl [55], could also contribute to some of the swelling. Further, a number of metabolic pathways that utilize NH_4^+ as a nitrogen donor are likely to intervene in the generation of organic anions in neuroblastoma cells. For instance, glutamate and aspartate can be produced by activation of glutamate dehydrogenase and subsequent glutamate transamination. In future studies, measurement of metabolite levels during exposure to $\text{NH}_3/\text{NH}_4^+$ should provide a better understanding of the resulting alterations in intracellular osmolality due to metabolic factors.

Isosmotic Volume Regulatory Responses in the Continuous Presence of $\text{NH}_3/\text{NH}_4^+$

The mechanisms by which animal cells respond to alterations in their water volume in *isosmotic* media are poorly understood. Another novel observation reported here is the occurrence of *isosmotic* regulatory volume decrease (iRVD) and *isosmotic* regulatory volume increase (iRVI) following exposure and removal of *isosmotic* solutions containing $\text{NH}_3/\text{NH}_4^+$. A noteworthy characteristic of these responses is that they are triggered by increments and reductions in CWV as small as ~3%. The observation that CWV changes of similar magnitude elicited by hypo- or hyper-osmotic solutions, that is, *anisomotic* solutions, do not result in regulatory responses suggests that the mechanisms sensing cell size are different in each case, and stresses the fact that volume expansion or contraction are not sufficient to trigger regulatory volume responses.

We began characterizing the mechanisms underlying iRVD and found that NPPB (10 μM) powerfully inhibited this response, suggesting that anion channels are importantly involved. We also show here that the iRVD was strongly attenuated by removal of extracellular Ca^{2+} . This effect indicates that in N1E-115 cells, RVD and iRVD entail different mechanisms, as hypotonic RVD has been shown to be Ca^{2+} -independent [45]. Although the molecular identity of the anion channels mediating iRVD remains unknown, a likely candidate is the volume-sensitive organic osmolyte/anion channel (VSOAC) that was described in N1E-115 cells [56]. In a recent study performed in cultured rat astrocytes, it was found that exposure to NH_4Cl (5mM) produces a progressive increase in cell volume without regulatory volume responses [52]. The lack of iRVD in their study could be due to a difference in cell type and/or insufficient time and amplitude resolution of the method they used to measure cell volume changes.

Relevance for the Pathophysiology of Hyperammonemic Brain Edema

Brain edema is a dreaded complication of hyperammonemic syndromes and an important cause of death in acute liver failure [6, 57]. It has been proposed that this edema results from astrocyte swelling secondary to accumulation of glutamine, derived from metabolism of NH_3 and glutamate [58, 59]. However, direct proof of a causal relationship between glutamine accumulation and development of brain edema is lacking [60], and long term exposure of cultured astrocytes to NH_4Cl results in cell swelling without a concurrent increase in glutamine levels [61]. Other studies, however, show that glutamine and molecular mechanisms involved in NH_3 -induced reactive oxygen and nitrogen species (RNOS) formation are related to astrocyte swelling [52]. Although the specific mechanism of cellular and brain edema remain elusive, numerous cell culture studies in glial cells have implicated mitochondrial dysfunction and oxidative stress, leading to nuclear factor kappaB activation and nitric oxide production, as key features of the cell swelling resulting from chronic $\text{NH}_3/\text{NH}_4^+$ exposure [52, 62]. Surprisingly, neither in astrocytes nor in neurons the possibility that intracellular accumulation of NH_4^+ may contribute to the brain edema resulting from hyperammonemia has been hitherto tested. Our results show that upon exposure to $\text{NH}_3/\text{NH}_4^+$, NH_4^+ accumulate rapidly inside the cells and contribute to significant cell swelling.

Conflict of Interests

The authors declare that there are no conflicts of interest.

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