A Cl⁻ Cotransporter Selective for NH₄⁺ Over K⁺ in Glial Cells of Bee Retina

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abstract There appears to be a flux of ammonium (NH₄⁺/NH₃) from neurons to glial cells in most nervous tissues. In bee retinal glial cells, NH₄⁺/NH₃ uptake is at least partly by chloride-dependant transport of the ionic form NH₄⁺. Transmembrane transport of NH₄⁺ has been described previously on transporters which NH₄⁺ replaces K⁺, or, more rarely, Na⁺ or H⁺, but no transport system in animal cells has been shown to be selective for NH₄⁺ over these other ions. To see if the NH₄⁺-Cl⁻ cotransporter on bee retinal glial cells is selective for NH₄⁺ over K⁺ we measured ammonium-induced changes in intracellular pH (pHᵢ) in isolated bundles of glial cells using a fluorescent indicator. These changes in pHᵢ result from transmembrane fluxes not only of NH₄⁺, but also of NH₃. To estimate transmembrane fluxes of NH₄⁺, it was necessary to measure several parameters. Intracellular pH buffering power was found to be 12 mM. Regulatory mechanisms tended to restore intracellular [H⁺]ᵢ after its displacement with a time constant of 3 min. Membrane permeability to NH₃ was 13 μM s⁻¹. A numerical model was used to deduce the NH₄⁺ flux through the transporter that would account for the pHᵢ changes induced by a 30-s application of ammonium. This flux saturated with increasing [NH₄⁺]ᵢ; the relation was fitted with a Michaelis-Menten equation with Kₘ = 7 mM. The inhibition of NH₄⁺ flux by extracellular K⁺ appeared to be competitive, with an apparent Kᵢ of ~15 mM. A simple standard model of the transport process satisfactorily described the pHᵢ changes caused by various experimental manipulations when the transporter bound NH₄⁺ with greater affinity than K⁺. We conclude that this transporter is functionally selective for NH₄⁺ over K⁺ and that the transporter molecule probably has a greater affinity for NH₄⁺ than for K⁺.

key words: ammonia • K-Cl cotransporter • neuroglia • pH • Apis

INTRODUCTION

Although transmembrane transport of ammonium in animals has been studied, mainly in the mammalian kidney, there are two well-established cases of fluxes of ammonium from neurons to glial cells in nervous tissue. In vertebrate brain, where glutamate is the main neurotransmitter, the uptake of glutamate by astrocytes followed by its amination to glutamine, which is returned to the neurons and deaminated, implies a flux of ammonium (Benjamin and Quastel, 1975; Hassel et al., 1997). In bee retina, the main metabolic substrate of the neurons (photoreceptors) is alanine formed by amination of pyruvate in the predominant glial cells (outer pigment cells). The alanine is transferred to the photoreceptors and deaminated to pyruvate and the tissue releases ammonium (Tsacopoulos et al., 1994, 1997b; Coles et al., 1996).

Uptake of ammonium into cells can be monitored continuously, but indirectly, by measuring the changes in intracellular pH (pHᵢ) that it causes. Ammonium has a pKₐ of ~9.2 in water (Sillén, 1964) so that at physiological pH (in the range 6.5–7.5) a fraction in the order of 1% is in the neutral NH₃ form. Nearly all cell membranes are permeable to NH₃ (but see Singh et al., 1995), so, when ammonium is applied outside a cell, NH₃ diffuses into it, combines with H⁺, and tends to raise pHᵢ (Jacobs, 1940). In contrast, in astrocytes cultured from neonatal mouse, application of ammonium lowers pHᵢ because there is an influx of NH₄⁺ whose effect on pHᵢ outweighs the effects of NH₃ fluxes (Nagaraja and Brookes, 1998). The glial cells in slices of bee retina also take up NH₄⁺ (Coles et al., 1996), an observation that has been confirmed and extended on bundles of glial cells freshly dissociated from adult retinas (Marcaggi et al., 1999). Application of ammonium causes a fall in pHᵢ that requires the presence of external Cl⁻ and is blocked by loop diuretics such as bumetanide (Marcaggi et al., 1999). These observations suggest that NH₄⁺ enters the glial cells by cotransport with Cl⁻ on a transporter with functional similarities to the cation-chloride cotransporters present on many types of cells. The transport on the bee glial cells is not blocked in the absence of Na⁺ (Marcaggi et al., 1999), indicating that the transport is of the K⁺-Cl⁻ class rather than the Na⁺-K⁺-2Cl⁻ class (see Race et al., 1999).

Several cases have been described of cation-chloride cotransporters.
cotransporters, particularly in kidney, being able to transport NH$_4^+$ in the place of K$^+$, although with a lower affinity (Kinne et al., 1986). However, in plant roots, transporters are known that are selective for NH$_4^+$ over K$^+$ (e.g., Kaiser et al., 1998) so such selectivity is a demonstrated biological possibility. We have found that uptake of NH$_4^+$ by the transporter in bee retinal cells is only moderately affected by external [K$^+$]. This suggested that the transporter might be the first to be described in an animal cell that is selective for NH$_4^+$ over K$^+$ and prompted us to make a quantitative estimate of its selectivity.

Influx of NH$_4^+$ into a cell is generally associated with transmembrane fluxes of NH$_3$ (Boron and De Weer, 1976; see Fig. 2 C), so the relation between changes in pH$_i$ (ΔpH$_i$) and NH$_4^+$ flux (F$_{NH_4}$)$^2$ is complex. We tackled the question of the NH$_4^+/K^+$ selectivity in two stages. First, we deduced F$_{NH_4}$ from ΔpH$_i$ for relatively brief applications of ammonium. This required accurate absolute measurements of pH$_i$ and measurement of several other parameters: membrane permeability to NH$_3$, intracellular buffering power, and the kinetics of pH$_i$ regulation. Use of this “cell model” showed a functional selectivity for NH$_4^+$ over K$^+$. We then recorded pH$_i$ responses to longer and more complex NH$_4^+$ application protocols. By simulating these responses with a standard minimal model for a cotransport process, to which we added competitive inhibition, we estimated the NH$_4^+$ and K$^+$ affinities of the transporter molecule.

**Materials and Methods**

Intracellular pH (pH$_i$) in bundles of glial cells dissociated from the retina of the drone (male) Apis mellifera was measured by techniques developed from those described in Marcaggi et al. (1999). One record is shown (see Fig. 9 D) from an intracellular microelectrode recording of glial membrane potential in a slice of retina prepared and superfused with oxygenated Cardinaud solution, as described previously (e.g., Coles et al., 1996). Unless otherwise stated, results are given as mean ± SD and the two-tailed paired t test was used to determine P values. Errors of quotients were estimated by the calculus of errors (Abramowitz and Stegun, 1965).

**Dissociation Procedure and Loading of the Cells**

Bees were obtained from A. Dittlo (Villandraut) or J. Kefuss (Toulouse, France) and maintained on sugar water. A slice of drone head ~500-μm thick was cut with a razor blade. The slice was incubated for 40 min in a 1.5 ml Eppendorf tube containing 1 ml oxygenated Cardinaud solution (see below) to which had been added 2 mg trypsin (T-4665; Sigma-Aldrich). The slice was washed in Cardinaud solution lacking Ca$^{2+}$ and Mg$^{2+}$ and the retinal tissue dissected out and triturated. 150 μl of cell suspension was placed in the perfusion chamber (see below) whose floor consisted of a microscope cover slip coated with polylysine. The cells were allowed to settle for 10 min and then exposed to the acetoxymethyl ester of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) (Molecular Probes, Inc.) at a concentration of 10 μM for 40 min.

**Measurement of Fluorescence**

The chamber was placed on the stage of an inverted microscope (Diaphot; Nikon) equipped with a 40× objective, photomultiplier detection, and dual wavelength excitation at 440 and 495 nm switched by liquid crystal shutters, as described in Coles et al. (1999). The stimulating light intensity was attenuated so that fluorescence from a bundle of loaded glial cells excited at 440 nm gave a signal of ~10,000 photon counts s$^{-1}$ for both excitation wavelengths and it was checked that this fluorescence was not affected by ammonium superfusion (n = 4). This background fluorescence was automatically taken into account in the in situ pH calibration of each cell bundle (see below). The excitation pattern was usually 440 nm, 100 ms off, 20 ms, 495 nm, 600 ms, off 20 ms. To minimize the noise of the ratio, the signal resulting from excitation near the isosbestic point (440 nm) was averaged over several minutes before the PC computer calculated the ratio using a program available from Jean-Louis Lavie (University Bordeaux, Bordeaux, France).

**Solutions**

The standard perfusion solution contained (mM): 200 NaCl, 10 KCl, 4 MgCl$_2$, 2 CaCl$_2$, pH was buffered with 10 mM MOPS hemisodium salt and set to 6.90 with HCl. Osmolality was adjusted to 685 mOsm with mannitol (~240 mM). The salt components, the pH, and the osmolarity of this solution are similar to those measured in vivo (Cardinaud et al., 1994). For other pHs, PIPES, MOPS, or HEPES were used for solutions of pH 6.20–6.50, 6.90–7.50, or 7.70, respectively. Most other variants were obtained by equimolar replacement of NaCl (or by increasing [NaCl] when [KCl] was reduced). Chloride-free solutions were made by replacing Cl$^-$ by an equivalent quantity of gluconate and increasing Ca$^{2+}$ to 8 mM to counteract the chelating effect of gluconate (Kenyon and Gibbons, 1977). To test the sensitivity of the responses to ammonium to changes in the osmolarities of solutions, osmolarity was intentionally increased or decreased by 5% (~34 mOsm) by changing the concentration of mannitol. Such a change in osmolarity had in itself a barely detectable effect on the emission ratio and no detectable effect on the pH response to 2 mM ammonium (n = 3). Therefore, in some experiments, salts such as NH$_4$Cl were simply added to solutions to final concentrations up to 5 mM without a compensatory reduction in [NaCl].

**Perfusion System**

To be able to make sufficiently rapid solution changes without detaching the cells from the floor of the chamber, we developed a perfusion chamber with no eddy currents. A factor that appeared to be important was the presence of a curved junction between the floor and the wall of the channel (Fig. 1 A). Solutions were gravity fed and selected by computer-controlled solenoid valves whose outflows passed through fine tubes at ~30 μl s$^{-1}$ into a common pathway to the chamber. It was found that mixing of solutions was negligible. We obtained a measure of the speed of the solution change in the chamber by recording the change in fluorescence during a switch from standard solution to one containing 1 μg liter$^{-1}$ fluorescein (Fig. 1 B). The change in pH, measured with BCECF in response to propionate or trimethyl-
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Calibration of pH Measurements

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Figure 1. Speed of the solution changes. (A) The perfusion chamber. A channel was milled in a polymethacrylate slab and a glass cover slip was glued on the bottom. Note the rounded edges of the channel. (B) Speed of solution change. The data points show the change in fluorescence observed through the 40× microscope objective focused on cells on the floor of the chamber during a 120-s perfusion change from standard solution to one containing 1 μM liter⁻¹ fluorescein. The time scale was displaced so that zero coincides with the beginning of the fluorescence change. The solid lines represent the best fits of data points obtained by regressions with simple exponentials \( \exp(-t/\tau_1) \) and \( \exp(-(t-120)/\tau_2) \) whose time constants, \( \tau_1 \) and \( \tau_2 \), are given in the figure.

Lamin (TMA) was nearly as fast (see Figs. 4 B and 5 A). The change in fluorescein fluorescence was well described by an exponential; for flow rates used in experiments with cells, the mean time constant was 5.4 ± 1.9 s (± SD, n = 16), and this exponential was used to describe the changes in extracellular concentration in our numerical models.

Comparison of the Permeabilities of the Neutral Forms of a Weak Base and a Weak Acid

We choose a weak acid AH/A⁻ whose \( K_a = \) pK \(_a\) < 5 so that at pH \(_a\) in [6; 8] its total concentration \( C_a = [AH]_o + [A⁻]_o = [AH]_a \). We choose a weak base BH⁻/B whose \( K_b = \) pK \(_b\) > 9 so that at pH \(_b\) in [6; 8] its total concentration \( C_b = [BH⁻]_o + [B]_o = [BH⁻]_a \). We set \( C_a = C_b \) and find the pH \(_a\) (or \( [H+]_o \)) for which the initial inward transmembrane flux of B (\( F_B = F_{AH} \times [AH]_a \)) is equal to that of AH (\( F_{AH} = F_{AH} \times [AH]_a \)):

\[ F_B = F_{AH} \Rightarrow [AH]_a = (P_B/P_{AH}) \times [B]_o \]

\[ \Rightarrow \frac{[H⁺]_o}{K_a} \times \frac{P_B}{P_{AH}} \times [B]_o \Rightarrow 0.5 \times (\frac{pK_a}{pK_a} - 0.5 \times \log(P_B/P_{AH}). (5) \]
For \( pH_i \in [6; 8] \), the initial rate of \( pH_i \) change induced by the weak acid is \( -F_{NH_4}/\beta \), since, in the cell, most of \( AH \) dissociates to form \( A^- + H^+ \); similarly, the initial rate of \( pH_i \) change induced by the weak base is \( F_\beta/\beta \). Thus, if one of the permeabilities is known, the other permeability can be deduced from the value of \( pH_i \) for which the initial direction of the \( pH_i \) change during the application of the mixture of the weak base and the weak acid reverses \( (F_\beta = F_{NH_4}) \).

Online Supplemental Material

The arguments leading from the observed changes in \( pH_i \) to the properties of the transporter molecule involve a model of transmembrane fluxes in the cell (essentially that used by Marcaggi et al., 1999) and a multistate model of a hypothetical \( NH_4^-\) Cl- cotransporter with \( K^+ \) inhibition. Details of these models and their analysis are available online at http://www.jgp.org/cgi/content/full/116/2/125/DC1

RESULTS

In agreement with Marcaggi et al. (1999), \( pH_i \) in bundles of glial cells superfused with solution at the physiological \( pH \) of 6.90 had values up to \( \sim 7.55 \) (e.g., see Fig. 7). More acid \( \Delta pH < 7.0 \) were encountered in bundles that were visibly damaged or whose \( pH_i \) recovered only slowly from an acid load. In slices of bee retina, mean \( pH_i \) measured in glial cells selected for their negative membrane potentials has been reported as 7.31 (Coles et al., 1996). For this reason, and also because the amplitude of \( pH_i \) responses of isolated bundles to \( NH_4^+ \) application correlated positively with \( pH_i \) (Marcaggi et al., 1999), bundles with \( pH_i > 7.1 \) were usually selected, except for some experiments on \( NH_3 \) permeability for which a more acid baseline \( pH_i \) was advantageous (e.g., see Fig. 4).

The Ammonium-induced Decrease in \( pH_i \) Is Inhibited by a High Concentration of \( K^+ \)

Fig. 2 A illustrates how 2 mM ammonium applied for 30 s to an isolated bundle of bee retinal glial cells at the measured physiological \( pH_o \) of 6.90 (Cardinaud et al., 1994) causes a decrease in \( pH_i \), indicating entry of \( NH_4^+ \). Marcaggi et al. (1999) have reported that this acidification requires external \( Cl^- \) (but not \( Na^+ \)) and is inhibited by bumetanide (at 100 \( \mu M \)) and by piretanide, properties of the family of \( K^+-Cl^- \) cotransporters. Between applications of \( NH_4^+ \) in Fig. 2 A, the external \( K^+ \) concentration \( ([K^+]_o) \) was at its normal physiological value of 10 mM (Cardinaud et al., 1994), and it was maintained at this value during the second and fifth applications of \( NH_4^+ \). For the first \( NH_4^+ \) application, \( [K^+]_o \) was reduced to 1 mM, which slightly increased the acidification, and for the third application it was increased to 50 mM. Although increasing \( [K^+]_o \) to 50 mM for up to 2 min in the absence of ammonium caused only negligible changes in \( pH_i \) \((n = 6; \text{not shown})\), 50 mM \( K^+ \) reduced the ammonium-induced acidification to about half that in the presence of 10 mM \( K^+ \). \( Rb^+ \), an ion that can replace \( K^+ \) in many transport processes, produced a greater inhibition that we did not investigate further (fourth ammonium application). Hence, Fig. 2 A suggests that inward transport of \( NH_4^+ \) is inhibited by \( K^+ \), and \( Rb^+ \) (as would be expected if \( NH_4^+ \) and \( K^+ \) (and \( Rb^+ \)) competed for the same transporting site, for example). More interestingly, the inhibition may be relatively weak: increasing \( [K^+]_o \) from 10 to 50 mM only halved the \( pH_i \) response to 2 mM ammonium, suggesting that the transport may be selective for \( NH_4^+ \) over \( K^+ \). To quantify this selectivity from measurements of \( pH_i \), it was necessary to have a scheme of the transmembrane fluxes of \( NH_4^+ \) and \( NH_3 \) and to determine parameters relating these fluxes to changes in \( pH_i \).

Parameters to be Determined

Fig. 2 B shows a response to a longer (5 min) application of ammonium on an expanded time scale. This response can be divided into five phases that can be explained by the schemes of Fig. 2 C (see also Boron and De Weer, 1976; Marcaggi et al., 1999). Initially, \( pH_i \) increases because of the predominant effect of the rapid entry of \( NH_3 \) which combines with \( H^+ \) (Phase 1). Since the equilibrium of the reaction \( NH_3 + H^+ \leftrightarrow NH_4^+ \) is so far to the right at \( pH \) near 7, it is sufficient that the inward flux of \( NH_3 \) exceeds \( \sim 1\% \) of the inward flux of \( NH_4^+ \). As the ratio \( [NH_4^+]_o/ [NH_3]_o \approx [H^+]_o \), Phase 1 is expected to be greater for cells with acid \( pH_i \). This was actually the case: Phase 1 was detected only for cells with baseline \( pH_i < 7.2 \). When the \( NH_3 \) concentrations approach equality on each side of the cell membrane, there is still an inward \( NH_4^+ \) gradient because \( [H^+]_o > [H^+]_i \). Then the \( NH_3 \) flux becomes outward while \( NH_4^+ \) continues to enter the cells and release \( H^+ \) ions (Phase 2). A steady state is reached (Phase 3) when the production of \( H^+ \) ions equals their extrusion by \( pH \) regulatory processes. When extracellular ammonium is suddenly removed, intracellular ammonium exits the cell faster in the \( NH_3 \) form than in the \( NH_4^+ \) form, so \( NH_4^+ \) dissociates to form \( NH_3 \) and there is a rebound acidification (Phase 4), followed by a slower return to baseline as proton equivalents are pumped out of the cell (Phase 5).

Marcaggi et al. (1999) showed that a simple mathematical model based on Fig. 2 D could simulate the main features of the experimental records, but used parameters that were only roughly estimated. We have now made more precise measurements of the following parameters required by the model: the absolute values of baseline \( pH_i \), the buffering power (\( \beta \)), the \( NH_3 \) permeability (\( P_{NH_3} \)), the \( pH_i \) regulation rate (characterized by a time constant \( \tau_{reg} \)), and the \( Cl^- \) concentration gradient that can help drive \( NH_4^+ \) into the cell.

Absolute Determination of \( pH_i \) During Application of \( NH_4^+ \)

The precise value of \( pH_o - pH_i \) in the presence of external ammonium (the plateau phase) is related to the force driving \( NH_4^+ \) across the membrane and is our main moti-
vation for seeking an accurate measure of $pHi$. Marcaggi et al. (1999) calibrated their measurements by applying nigericin, but it has been shown that this technique can give systematic errors (Nett and Deitmer, 1996; Boyarsky et al., 1996). To determine the absolute value of $pHi$ during NH$_4^+$ perfusion, we applied a weak acid and a weak base simultaneously as described by Eisner et al. (1989) (see materials and methods). Fig. 3 A shows a typical experiment. NH$_4^+$ was first applied at $pHi$ 6.90 and then at $pHo$ 7.30. At each plateau phase, $\Delta pHi$ (10 mM propionate, 10 mM TMA) and $\Delta pHo$ (10 mM propionate, 5 mM TMA) were in opposite directions, and we estimated by linear interpolation the concentration of TMA that would have given no change in $pHi$ when applied with 10 mM propionate (Eq. 4). The absolute value of $pHi$ was then calculated by Eq. 3. The method assumes that intracellular $pK_a$ equals extracellular $pK_a$ and that the membranes are relatively impermeable to the charged forms of the weak acid and base. This latter assumption was confirmed by the observation that, during applications of propionate ($n = 21$; not shown) or TMA (see Fig. 5 A), recovery of $pHi$ was slow and could be fully accounted for by $pH$ regulatory processes. Since $pHi$ during the plateau phase depends partly on $pHi$ regulatory processes (Fig. 2 C 3), short NH$_4^+$ applications at the beginning and end of the experiment were made to check that the rates of recovery remained approximately the same. From 12 experiments, as in Fig. 3 A, $pHi$ was calculated to be 6.844 ± 0.017 ($\pm SD$, $n = 12$) after an 8-min application of NH$_4^+$ with $pHo$ 6.90. This $pHi$ was significantly less than $pHo$.

Figure 2. Effect of extracellular ammonium application on $pHi$ of an isolated bundle of glial cells. (A) The ammonium-induced acidification was slightly inhibited by K$^+$ or Rb$^+$. For each application of 2 mM NH$_4^+$, [K$^+$]$_o$ was either maintained at its baseline value of 10 mM, changed as indicated, or replaced by 50 mM Rb$^+$. (B) The response to a 5-min application of 2 mM NH$_4^+$ can be divided into five phases, which correspond to different patterns of fluxes (C). This cell had a fairly acid baseline $pHi$, (≈7.17) so that Phase 1 was prominent. (C) Schemes of fluxes corresponding to four of the phases indicated in B. (Phase 1) Inward flux of NH$_3$ is greater than $\sim$1% of inward flux of NH$_4^+$. The maintenance of intracellular equilibrium NH$_4^+ \leftrightarrow$ NH$_3$ + H$^+$ consumes H$^+$ ions. (Phase 2) NH$_4^+$ transmembrane gradient is still inward, while [NH$_3$]$_i$ slightly exceeds [NH$_3$]$_o$. H$^+$ ions are shuttled into the cell. (Phase 3) Extrusion of H$^+$ ions by $pH$ regulatory mechanisms equals inward flux of NH$_4^+$. (Phase 4) This phase is approximately the inverse of Phase 1. Phase 5 (not shown) consists almost entirely of H$^+$ efflux. (D) Scheme of transmembrane fluxes during ammonium exposure. $pHi$ changes result from three transmembrane fluxes, $F_{NH4}$, $F_{NH3}$, and net H$^+$ flux through pH regulatory processes ($F_{reg}$) (see text).
Selectivity for NH₄⁺ of a Cl⁻ Cotransporter

To see whether the result depended on the specific weak acid and weak base used, we used other weak acid/weak base couples. In experiments similar to that of Fig. 3 A, the estimated difference pHₒ₂pHi after 10 min of perfusion with 2 mM ammonium was not significantly different when the following couples were used: propionate/TMA; propionate/MA; acetate/TMA and caproate/TMA (Fig. 3 C). To see whether the value of (pHₒ – pHᵢ) reached during the plateau phase was related to the baseline pHᵢ, we compared cells with a baseline pHᵢ < 7.1 with those with pHᵢ > 7.1 (Fig. 3 C, first two columns). The difference in the mean values of (pHₒ – pHᵢ) during the plateau phase was not significant. In contrast, as illustrated in Fig. 3 A, the level of the plateau did indeed depend strongly on the pHₒ at which the NH₄⁺ was applied. Absolute values of pHᵢ estimated during superfusion with 2 mM NH₄⁺ at pHₒ 6.50 ± 0.005, 6.900 ± 0.005, 7.300 ± 0.005, and 7.700 ± 0.005 are plotted in Fig. 3 D and show a very precise linear correlation with pHₒ such that (pHᵢ = 6.142) = 0.9264 × (pHₒ – 6.142). In later experiments, we calibrated the measurements of pHᵢ simply by superfusing the cells with 2 mM ammonium for at least 8 min and using this relation.

Intracellular Buffering Power

The H⁺ ions released into (or taken up from) the cytoplasm as a consequence of the transmembrane fluxes of NH₄⁺ and NH₃ affect pHᵢ, according to the relation...
\[ \Delta p Hi = \Delta Q / \beta_i \]
where \( \Delta Q \) is the quantity of H\(^+\) ions/\( \text{U} \) volume and \( \beta_i \) is the intracellular buffering power (see Roos and Boron, 1981). We estimated \( \beta_i \) by applying the weak acid propionate (as in Figure 1 of Marcaggi et al., 1996); the change in \( p Hi \) reached its maximum very rapidly compared with the time course of \( pHi \) recovery in the presence of propionate, and we did not attempt to block \( p Hi \) regulation (compare Szatkowski and Thomas, 1989). Mean \( \beta_i \) was 12.2 \( \pm \) 2.9 mM \((n = 11)\) and we took 12 mM for the model.

To see if \( \beta_i \) varied markedly with \( pHi \), we shifted \( pHi \) by applying NH\(_4\)\(^+\) at various pH\(_i\)s. The results and the analysis, which is complicated by the effects of the NH\(_3\)/NH\(_4\)\(^+\) system, are given in Marcaggi (1999); the conclusion is that \( \beta_i \) is effectively constant in the range 6.7–7.3.

**Permeability to NH\(_3\)**

We estimated NH\(_3\) permeability (\( P_{NH3} \)) from measurements of \( pHi \) under conditions in which entry of NH\(_4\)\(^+\) was blocked so that changes in \( pHi \) were due only to the inward flux of NH\(_3\). We have previously shown that NH\(_4\)\(^+\) does not enter through barium-sensitive K\(^+\) channels, the major cationic conductance in these cells, and also that NH\(_4\)\(^+\) entry is totally blocked by bumetanide or by removal of external chloride (Marcaggi et al., 1999). We therefore applied ammonium in Cl\(^-\) free solutions to which, in some cases, bumetanide had been added, and measured the rate of change of \( pHi \) (in the alkaline direction) induced by NH\(_3\) entry into the cells.

With the cell bundles adhering to the floor of the perfusion chamber, we failed to find a molecule causing a 10–90% \( pHi \) change faster than the one produced by ammonium: perhaps the change of solution at the cell membrane (0–20 \( \mu \text{m} \) from the floor of the chamber) was not fast enough for this measurement. To expose cells to faster solution changes, we caught hold of bundles of cells with a 3-\( \mu \text{m} \) tip diameter pipette and carried them 50–100 \( \mu \text{m} \) up from the floor of the chamber. To increase the time resolution of the rapid initial slope of the \( pHi \) change, we measured the fluorescence ratio with faster switching of the excitation wavelengths (\( \sim 3 \text{Hz} \)). To reduce delays due to diffusion, we applied ammonium at a high concentration (10 mM) but at an acid pH (6.50) so that \([NH3]_i\) was low but benefited from facilitated diffusion (Engasser and Horvath, 1974). To increase the NH\(_3\)-induced \( \Delta pHi \), baseline \( pHi \) was reduced (to \( \sim 6.80 \)) by perfusing the cells for 30–60 min with solution buffered at pH\(_o\) 6.20. After a 1-min perfusion with 0 Cl\(^-\) + 0.5 mM bumetanide, 10 mM ammonium was applied at pH\(_o\) 6.50 (Fig. 4 A). In these conditions, the 10–90%-\( pHi \) change induced by 10 mM propionate (Fig. 4 B) was twice as fast as the one induced by ammonium, showing that the speed of solution change at the cell membrane did not significantly limit the influx of NH\(_3\).

Although \( pHi \) was \( \sim 7.00 \) during the ammonium application, while \( pHi \) was 6.50, no slow \( pHi \) decrease was observed, as would have been the case if the membranes had had some permeability to NH\(_4\)\(^+\). This confirms that NH\(_4\)\(^+\) pathways were insignificant in these conditions.

The slope of the \( pHi \) change was measured at 50% of the \( \Delta pHi \) response, where it is known that \([NH3]_o\) has reached its final concentration, since the effect of propionate is 90% at this time (Fig. 4). Since, for \( pHi \) \( \sim 7.00 \), \([NH4^+] \), \( > 100 \times [NH3]_i \), then \( \beta_i \times \Delta pHi / \Delta t = \delta [NH4^+] / \Delta t \approx \delta ([NH4^+]_i + [NH3]_i) / \Delta t = F_{NH3} \times \Delta pHi / \Delta V \) where \( F_{NH3} \) is the NH\(_3\) transmembrane flux and \( \Delta pHi / \Delta V \) is the ratio of membrane surface to intracellular volume in which the ammonium is distributed. The ratio of the surface to the total cell volume has been estimated to be \( \approx 1.2 \mu \text{m}^{-1} \) (Marcaggi et al., 1999), but the ammonium will be present almost entirely in the water phase that occupies 0.775 of the total volume of the tissue (Coles and Rick, 1985). Taking this factor for the water content of the glial cells gives an estimated effective \( \Delta pHi / \Delta V \) of 1.55 \( \mu \text{m}^{-1} \). Knowing \( \beta_i \), we could then calculate the transmembrane flux of NH\(_3\) from the rate of change of \( pHi \); \( F_{NH3} \approx \Delta pHi / \Delta t \times \beta_i \). From this flux, we found \( P_{NH3} = 14.7 \pm 2.9 \mu \text{m} \cdot \text{s}^{-1} \) \((n = 6)\) in 0 Cl\(^-\) + 0.5 mM bumetanide, which was not significantly different from the value in 0 Cl\(^-\) only \((n = 5)\), showing that bumetanide did not further inhibit NH\(_4\)\(^+\) entry.

Holding up the cells with a pipette will have introduced some stress in the cell membrane, which may have modified its permeability. To check that NH\(_3\) permeability is the same for cells plated on the bottom of the chamber (the conditions used for the other experiments), we also determined \( P_{NH3} \) by an indirect method. Methylamine (MA; CH\(_3\)NH\(_3\)\(^+\)/CH\(_3\)NH\(_2\)) is a weak base with a \( pK_a \) that is high (\( \sim 10.6 \); Robinson and Stokes, 1959) compared with that of ammonium (\( \sim 9.2 \)). Because of this \( pK_a \) difference, at pH \( < 8 \) (at which charged forms are preponderant), if \([CH3NH3^+] + [CH3NH2] = [NH4^+] + [NH3]_i \), then \([CH3NH2] < 0.04 \times [NH3]_i \). It follows that if \( P_{CH3NH2} \) (\( P_{MA} \)) is not far different from \( P_{NH3} \), for equal concentrations of MA and ammonium applied, \( F_{CH3NH2} \ll F_{NH3} \). This is why \( P_{MA} \) can be measured directly even with a slow speed of solution change at the cell membrane. Fig. 5 A illustrates the \( pHi \) response to 10 mM MA compared with the \( pHi \) response to 10 mM TMA (\( pK_a \approx 9.6 \)). The speed of the \( pHi \) change induced by TMA was far faster than the one induced by MA, showing that the speed of the solution change was fast enough for measurement of \( P_{MA} \), which was found to be 27.4 \( \pm 8.1 \mu \text{m} \cdot \text{s}^{-1} \) \((n = 8)\). Once this permeability was known, it was possible to deduce the permeability of propionate by ascertaining...
Selectivity for NH$_4^+$ of a Cl$^-$ Cotransporter

The initial direction of the pH$_i$ change induced by a simultaneous application of 10 mM propionate and 10 mM MA. To avoid too great a variation of the net $\Delta$pH$_i$ during this simultaneous application, we used a condition in which pH$_i$ $<$ pH$_o$, which was obtained by including 2 mM ammonium in the superfusate (Fig. 3D). As illustrated in Fig. 5B, the initial direction of the pH$_i$ change reversed for 7.40 $<$ pH$_o$ $<$ 7.60 (n = 4). Taking the mean of this range (pH$_o$ = 7.50 ± 0.10) gives $x = P_{MA}/P_{prop} = 3.16 \pm 1.46$ according to Eq. 5. So, $P_{prop} = P_{MA}/x = 8.67 \pm 6.57$ $\mu$m s$^{-1}$.

The same protocol was used to estimate $P_{NH3}$ from the now known $P_{prop}$. The simultaneous application being done in 0 Cl$^-$ to prevent the entry of NH$_4^+$. As illustrated in Fig. 5C, the initial direction of the pH$_i$ change reversed for 7.00 $<$ pH$_o$ $<$ 7.10 (n = 4), which gives $x = P_{NH3}/P_{prop} = 1.02 \pm 0.24$ according to Eq. 5. So $P_{NH3} = x \times P_{prop} = 8.84 \pm 8.78$ $\mu$m s$^{-1}$.

In conclusion, the two methods of estimation of $P_{NH3}$ gave values not significantly different. The standard deviation obtained with the second method was increased by the successive approximations so we give more weight to the value obtained with the first method and conclude that $P_{NH3}$ is in the range of 7–19 $\mu$m s$^{-1}$; we take the value 13 $\mu$m s$^{-1}$ for the model.

pH Regulation

When pH$_i$ falls below its baseline value, pH regulatory mechanisms tend to restore it by extruding H$^+$ ions. To quantify the kinetics of this regulation, we acid loaded the cells by exposure to ammonium and analyzed the recovery (Roos and Boron, 1981; Thomas, 1984). Fig. 6
A shows the recoveries of pHᵢ in a single bundle of cells after initial displacements of various amplitudes induced by applications of ammonium at various concentrations. For each ammonium application, the recovery was analyzed for 8 min starting 45 s after the end of the application («t») to allow for the rebound acidification (Phase 4). The plot is semilogarithmic, the ordinate being \(\ln([H⁺]_i - [H⁺]_0)\), where \([H⁺]_0\) was the baseline \([H⁺]\) at rest. Linear regressions showed that the recoveries were exponential irrespective of the initial displacement, and had slopes \(= -1/\tau_{reg}\) that were not systematically different.

Values of the time constant \(\tau_{reg}\) for 17 bundles of cells for which at least three different ammonium concentrations were tested were plotted as a function of the initial pHᵢ displacement, \(\Delta pH_i(NH_4⁺)\) (Fig. 6 B). Linear regression of \(\tau_{reg}\{\Delta pH_i(NH_4⁺)\}\) confirmed that \(\tau_{reg}\) was independent of the pH [mean slope of 0.3 ± 1.8 min (pH unit)⁻¹; \(n = 17\)]. We conclude that despite considerable variability, \(\tau_{reg}\) was approximately constant irrespective of the initial pHᵢ displacement with a mean value of 3.0 ± 1.1 min (\(n = 17\)). We therefore described the pHᵢ regulation by Eq. 6:

\[
F_{reg} = (1/\tau_{reg}) \times ([H⁺]_i - [H⁺]_0),
\]

with \(\tau_{reg} = 3\) min.

Driving Force

The flux rate of a Cl⁻ cotransporter will depend in part on \([Cl⁻]_0\) and \([Cl⁻]_0\), and we will use values of these concentrations in the transporter model of Fig. 10 A (see online supplemental material). \([Cl⁻]_0\) being known, we attempted to estimate \([Cl⁻]_0\). Measurements in slices of bee retina with ion-selective microelectrodes have shown that in the glial cells Cl⁻ (and also K⁺) are at close to electrochemical equilibrium (Coles et al., 1986, 1989). Hence, \([Cl⁻]_0/\[Cl⁻]_0\) could be deduced approximately from \(V_m\). We did not succeed in measuring \(V_m\) in the isolated bundles of glial cells directly (by electrode techniques) and used an indirect method. We argued that if the membranes were made permeable to H⁺ then \([H⁺]_0\) would be determined by \(V_m\). We applied the H⁺/K⁺ exchanger, nigericin, in solutions with normal \([K⁺]_0\) and observed the resulting change in pHᵢ (Fig. 3 E). The minimum pHᵢ reached during nigericin was compared with pHᵢ at the plateau phase induced by 2 mM NH₄⁺, the mean difference being 0.02 ± 0.05 (\(n = 7\); Fig. 3 C). It follows that the mean value of \(pH₀ - pH₁\) at the maximum of the nigericin-induced pHᵢ change was ~0.07 (Fig. 3 C). On the assumption that H⁺, being now in equilibrium with K⁺, was distributed passively across the membrane, \(V_m\) was ~4 mV. Since nigericin is not perfectly selective for H⁺ (Pressman et al., 1967; Margolis et al., 1989), it probably depolarized the membranes somewhat, as suggested by the slow increase in pHᵢ during nigericin (Fig. 3 E). Thus, the true \(V_m\) is probably more negative than ~4 mV and \([Cl⁻]_0/[Cl⁻]_0 = \exp(-V_m/F/RT > 1.18\).

Concentration Dependence of the pHᵢ Changes Induced by 30-s Applications of Ammonium

To record the responses to increasing concentrations of NH₄⁺ in the absence of external K⁺, we superfused the cells in 0 K⁺ for 15 s before and during each NH₄⁺ application (Fig. 7 A). Repeated exposure to high \([NH₄⁺]_0\) appeared to lead to impairment of pHᵢ regulation and, for 7 of 11 experiments, pHᵢ did not recover from the acidification induced by 10 mM NH₄⁺. Measurements were therefore made only on the records from the four experiments for which pHᵢ recovered...
Figure 7. pH<sub>i</sub> changes as a function of ammonium concentration (for 30-s applications in 0 K<sup>+</sup>). (A) Typical recording of pH<sub>i</sub> responses to various NH<sub>4</sub><sup>+</sup> concentrations. Cells were normally superfused with 10 mM K<sup>+</sup> standard solution that was switched to a 0 K<sup>+</sup> solution 15 s before each NH<sub>4</sub><sup>+</sup> application. The response to 10 mM propionate (near the end of the experiment) gives a lower limit for the rapidity of the solution changes. On the same cells, after a delay of ~20 min ("Δt") removal of extracellular K<sup>+</sup> with no ammonium application had no detectable effect on pH<sub>i</sub>. (B) Superposition of pH<sub>i</sub> responses to NH<sub>4</sub><sup>+</sup> (from A) on a shorter time scale. Dotted lines for 0.5 and 1 mM NH<sub>4</sub><sup>+</sup> are the second (control) responses. The delay between activation of the solenoid valve (t = 0) and the start of the changes in pH<sub>i</sub> was ~10 s. Note that during the NH<sub>4</sub><sup>+</sup> application, pH<sub>i</sub> fell more rapidly for 10 mM NH<sub>4</sub><sup>+</sup> than for 20 mM, but that the peak reached after the application (Phase 4) was greater for 20 mM. (C) Calculation of Δ pH<sub>i</sub>/Δt(NH<sub>4</sub><sup>+</sup>) for each NH<sub>4</sub><sup>+</sup>-induced acidification. Mean slopes were measured between 15 and 35 s after the onset of the NH<sub>4</sub><sup>+</sup> application by linear regression. Taking into account the delay for arrival of solutions, this interval corresponds to the last 20 s of the NH<sub>4</sub><sup>+</sup> applications. (D) Mean values for ΔpH<sub>i</sub>([NH<sub>4</sub><sup>+</sup>]o) (peak change in pH<sub>i</sub>) and for which the response to subsequent control application of 0.5 or 1 mM NH<sub>4</sub><sup>+</sup> was closely similar to the initial response (in the record of Fig. 7 A, a final application of 20 mM NH<sub>4</sub><sup>+</sup> was made). To make sure that the effect of NH<sub>4</sub><sup>+</sup> was not rate limited by the speed of the solution change (as was probably the case in the previous study by Marcaggi et al., 1999), we checked that application of propionate gave a more rapid pH<sub>i</sub> change (Fig. 7 A). Data were analyzed only for experiments in which the time for the 10–90% propionate-induced pH<sub>i</sub> change was <15 s. The last part of Fig. 7 A shows that 0 K<sup>+</sup> alone did not affect pH<sub>i</sub> on the time and pH scales of these experiments.

Fig. 7 B shows the NH<sub>4</sub><sup>+</sup> responses from the record of Fig. 7 A on a shorter time scale. The time of onset of the response to propionate (not shown) indicated that in this experiment there was a dead time of ~5 s between the switching of the electromagnetic valves and the arrival of a new solution at the cell membrane. The slope of the NH<sub>4</sub><sup>+</sup>-induced pH<sub>i</sub> change (ΔpH<sub>i</sub>/Δt) was measured before the rebound (Phase 4), between 15 and 35 s after the valves were actuated. ΔpH<sub>i</sub>/Δt(NH<sub>4</sub><sup>+</sup>) was calculated by linear regression as shown in Fig. 7 C. ΔpH<sub>i</sub>/Δt(NH<sub>4</sub><sup>+</sup>) increased with [NH<sub>4</sub><sup>+</sup>]o in the range 0.5–10 mM NH<sub>4</sub><sup>+</sup> but for 20 mM NH<sub>4</sub><sup>+</sup>, although the total pH<sub>i</sub> change induced by NH<sub>4</sub><sup>+</sup> [Δ pH<sub>i</sub>([NH<sub>4</sub><sup>+</sup>]o)] continued in every case to increase, in three of the four experiments, ΔpH<sub>i</sub>/Δt for 20 mM NH<sub>4</sub><sup>+</sup> was less than for 10 mM, as in the example shown in Fig. 7 A–C. Mean data from the four experiments are shown in Fig. 7 D. ΔpH<sub>i</sub>([NH<sub>4</sub><sup>+</sup>]o) was well fitted by a Michaelis-Menten curve for [NH<sub>4</sub><sup>+</sup>]o with half saturation, b, for [NH<sub>4</sub><sup>+</sup>]o of 0.993 (R = 0.993) with half saturation, b, for [NH<sub>4</sub><sup>+</sup>]o of 6.62 ± 0.57 mM (n = 4). But ΔpH<sub>i</sub>/Δt([NH<sub>4</sub><sup>+</sup>]o) could only be fitted by a Michaelis-Menten curve for [NH<sub>4</sub><sup>+</sup>]o = 5 mM (R = 0.926), half saturation being at [NH<sub>4</sub><sup>+</sup>]o = 3.37 ± 0.98 mM (n = 6) (Fig. 7 D). Because the relation between NH<sub>4</sub><sup>+</sup> transport and pH<sub>i</sub> changes is indirect, the value of [NH<sub>4</sub><sup>+</sup>]o that half saturates pH<sub>i</sub> changes does not necessarily correspond to the one that half saturates transport of NH<sub>4</sub><sup>+</sup>. To deduce the flux of NH<sub>4</sub><sup>+</sup>, we had recourse to a mathematical model.
Dependence of NH$_4^+$ Flux on [NH$_4^+$]$_o$

Three transmembrane fluxes determine pH$_i$ during and after application of ammonium (Fig. 2 C). Of these, we have a phenomenological description of the pH$_i$ regulation (F$_{reg}$ in Fig. 2 D), and we assume that the flux of NH$_3$ (F$_{NH3}$ in Fig. 2 D) results from simple diffusion (Fick’s law). To deduce the flux of NH$_4^+$ through the cotransporter (F$_{NH4}$ in Fig. 2 D) from the changes in pH$_i$, we use the model of Fig. 2 D, expressed mathematically in the supplemental material. From the measurements described above, values for parameters of the model were: $\beta_i = 12 \text{ mM}, p\text{H}_i = 7.4, \tau_{reg} = 3 \text{ min}$, and $P_{NH3} = 13 \mu\text{m s}^{-1}$. The surface-to-volume ratio, $S/V$, with its attendant uncertainty, was used to calculate $P_{NH3}$ but cancels out in the calculations.

As a first step, a constant inward F$_{NH4}$ ($inF_{NH4}$) was imposed for 30 s, with [NH$_4^+$]$_o$ + [NH$_3$]$_o$ set to 2 mM. The resulting $\Delta pH/\Delta t$ was calculated 15 s after the onset of the imposed $inF_{NH4}$ and plotted against $inF_{NH4}$ for various $P_{NH3}$ (7, 13, and 19 $\mu\text{m s}^{-1}$; Fig. 8 A). Increasing $P_{NH3}$ increased $\Delta pH/\Delta t$, but only slightly, showing that $P_{NH3}$ is not a major rate-limiting factor.

A similar simulation, still using an imposed $inF_{NH4}$, was then performed in the presence of various [NH$_4^+$]$_o$ (+ [NH$_3$]$_o$) set to 2 mM. The resulting $\Delta pH/\Delta t$ was calculated 15 s after the onset of the imposed $inF_{NH4}$ and plotted against $inF_{NH4}$ (Fig. 8 B). It is clear that the experimental result in which $\Delta pH/\Delta t$ was smaller for an application of 20 mM ammonium than for 10 mM (Fig. 7) does not necessarily imply that inward F$_{NH4}$ (20 mM NH$_4^+$) < inward F$_{NH4}$ (10 mM NH$_4^+$). We also note that since the relation of $\Delta pH/\Delta t$ to $inF_{NH4}$ is curved (Fig. 8 B), $\Delta pH/\Delta t$ vs. [NH$_4^+$]$_o$ will saturate more rapidly than will $inF_{NH4}$ vs. [NH$_4^+$]$_o$.

The pH$_i$ peak reached after withdrawal of external ammonium must depend both on [NH$_4^+$]$_i$, at the end of the ammonium application, and on the effluxes of NH$_4^+$ and of NH$_3$ after withdrawal. To start modeling this, we considered the case of a 30-s application of 2 mM extracellular NH$_4^+$ with a constant $outF_{NH4}$ (6.65 mM min$^{-1}$ in Fig. 8 C). After removal of extracellular NH$_4^+$, the concentration gradient of NH$_4^+$ is outwards. We tested the simplest reasonable assumption, which is that outward F$_{NH4}$ = $outF_{NH4} \propto [NH_4^+]$. With no loss of generality, this can be written: $outF_{NH4} = \text{outF}_{NH4 \text{ max}} \times ([\text{NH}_4^+]/[\text{NH}_4^+ \text{ max}])$, where $[\text{NH}_4^+ \text{ max}]$ is the intracellular NH$_4^+$ concentration reached at t = 30 s and $\text{outF}_{NH4 \text{ max}}$ is an initially arbitrary constant corresponding to the maximum transient $outF_{NH4}$. As illustrated in Fig. 8 C, the rebound acidification on removal of extracellular NH$_4^+$ is maximal for zero $outF_{NH4}$ and decreases with increasing $outF_{NH4}$. Let $\Delta pH(inF_{NH4})$ be the total pH$_i$ change induced by a 30-s $inF_{NH4}$ followed by an $outF_{NH4}$ defined as above. From the experimental data, the mean ratio $\Delta pH/\delta [\text{NH}_4^+]_o$ measured from 30-s applications of 2 mM NH$_4^+$ in 0 K$^+$ was $0.60 \pm 0.12$ min ($n = 10$); the closest approach to this in

![Figure 8. Use of the cell model (Fig. 2 D) to derive inward F$_{NH4}$([NH$_4^+$]$_o$) from measured pH$_i$ changes induced by brief applications of NH$_4^+$. (A) Constant inward F$_{NH4}$ ($inF_{NH4}$) was imposed on the model for 30 s $\delta pH/\delta t$, calculated 15 s after onset, was plotted against $inF_{NH4}$. Data are shown for simulations with [NH$_4^+$]$_o$ set to 2 mM (to fix [NH$_3$]$_o$) and P$_{NH3}$ = 7, 13, and 19 $\mu$m s$^{-1}$. (B) As in A, $\delta pH/\delta t$ was plotted versus $inF_{NH4}$ for simulations with [NH$_4^+$]$_o$ = 0.5 mM (triangles), 1 mM (diamonds) and 5, 10, and 20 mM (circles). P$_{NH3}$ = 13 $\mu$m s$^{-1}$. Because increasing [NH$_4^+$]$_o$ increases [NH$_3$]$_o$, $\delta pH/\delta t$($inF_{NH4}$) is smaller for higher [NH$_4^+$]$_o$. (C) Simulations in which an $inF_{NH4}$ was imposed for 30 s and followed by an outward F$_{NH4}$ ($outF_{NH4}$). $inF_{NH4}$ was set to 6.65 mM min$^{-1}$ and [NH$_4^+$]$_o$ was fixed by setting [NH$_4^+$]$_o$ = 2 mM; this gave $\delta pH/\delta t = 0.46$ pH unit min$^{-1}$, equal to the mean measured pH$_i$ change induced by 2 mM NH$_4^+$ in 0 K$^+$ for cells with baseline pH$_i$ = 7.4. (D) Plot of $\Delta pH(inF_{NH4})$ measured as baseline pH$_i$ (7.4) minus the minimal pH$_i$ reached during the rebound acidification after 30 s of influx $inF_{NH4}$. Simulations for 0.5 mM (triangles) or 20 mM (circles) NH$_4^+$ (-NH$_3$) show that [NH$_3$]$_o$ has little effect on $\Delta pH(inF_{NH4})$. $outF_{NH4 \text{ max}}$ = 0, (E) $inF_{NH4}$([NH$_4^+$]$_o$) calculated from $\delta pH/\delta t$([NH$_4^+$]$_o$) (●) and $\Delta pH([NH_4^+])$ (○) of Fig. 7 D. The points were fitted by Michaelis-Menten curves (R = 0.963 and 0.994, respectively) with apparent constants $K_m$ equal to 5.9 ± 1.3 and 7.8 ± 0.7 mM.
Fig. 8 C is 0.56 min for outFNH4max = 0, which we accept as
an approximation. \( \Delta pH = (\text{inFNH}_\text{4}) \) is very little affected by
\([\text{NH}_4^+]_o \) (Fig. 8 D), much less so than is \( \Delta pHi / \Delta t \) (Fig. 8 B). Thus, the inverse operation
of estimating inward \( F_{\text{NH}_4}(\text{NH}_4^+) \) is better done from
\( \Delta pHi(\text{NH}_4^+) \) than from \( \Delta pHi / \Delta t(\text{NH}_4^+) \). By compar-
ing experimental \( \Delta pHi(\text{NH}_4^+) \) (Fig. 7 D) and simulated \( \Delta pHi(\text{inFNH}_4) \) (Fig. 8 D), we calculated
\( \text{inFNH}_4(\text{NH}_4^+) \) (Fig. 8 E). The points were well fitted by a Michaelis-Menten equation of the form (Eq. 7):

\[
\text{inFNH}_4(\text{NH}_4^+) = \frac{[\text{NH}_4^+]_o}{K_m^' + [\text{NH}_4^+]_o}. \tag{7}
\]

The constant, \( K_m^' \), corresponding to half saturation of
inward \( F_{\text{NH}_4} \), was 7.8 \( \pm \) 0.7 mM. Variant analyses from
\( \Delta pHi / \Delta t([\text{NH}_4^+]_o) \) or using Lineweaver-Burke plots all
gave lower values, down to 4.9 mM (Marcaggi, 1999). We conclude that \( K_m^' = 7.8 \) mM is a conservative estimate of the affinity (an upper limit for \( K_m^' \)) of the
transporter for \( \text{NH}_4^+ \) in these experimental conditions.

Functional Selectivity for \( \text{NH}_4^+ \) over \( K^+ \)

Having established the dependence of \( \text{inFNH}_4 \) on
\([\text{NH}_4^+]_o \) (for 30-s applications of ammonium), we then
extended the approach to analyze the inhibitory effect
of \( K^+ \). Fig. 9 A shows an experiment in which cells were
superfused for 30 s with \( \text{NH}_4^+ \) in 0 or 10 mM \( K^+ \). \( \Delta pHi(\text{NH}_4^+) \) from six such experiments is shown plotted with double inverse scales as a function of
\([\text{NH}_4^+]_o \) in Fig. 9 B. Using the model, as described
above, a value of \( \text{inFNH}_4(\text{NH}_4^+) \) was deduced for each
measurement of \( \Delta pHi(\text{NH}_4^+) \) and a second inverse
plot was made (Fig. 9 C). This plot suggests that the inhibi-
tion was competitive since straight lines passing
through the data points intersect near the ordinate axis
(same \( \text{inFNH}_4\text{max} \)).

Fig. 9 D illustrates how \( K^+ \) depolarizes these glial
cells. In this record, from a glial cell in a retinal slice, the
depolarization is greatly damped by electrical coupling
between the cells and the slowness of the increase in \( [K^+] \) in the extracellular clefts (Coles and Orkand,
1983). But in isolated cell bundles, the depolarization
might be greater and in some way affect \( \text{NH}_4^+ \) uptake. We therefore used Ba\(^2+\), which blocks the depolariza-
tion for at least 45 s after the application of \( K^+ \) (Fig. 9 D),
to study the effect of \( K^+ \) on \( \Delta pHi(\text{NH}_4^+) \) in the ab-

Figure 9. Inhibition of inward \( \text{NH}_4^+ \) flux by external \( K^+ \). (A) Comparison of responses to 2 and 5 mM \( \text{NH}_4^+ \) in 0 and 10 mM \( K^+ \). For
each response, \( \text{NH}_4^+ \) was applied for 30 s when applied in 0 \( K^+ \), \( K^+ \) was removed from the superfusate 15 s earlier. (B) Double inverse plot
of mean \( \Delta pHi \) vs. \([\text{NH}_4^+]_o \) (\( n = 6 \)). Straight lines passing through mean data points intersect the abscissa at \([\text{NH}_4^+]_o = 5.02 \) mM (0 \( K^+, \odot \))
and 6.90 mM (10 \( K^+, \bullet \)). (C) Double inverse plot of \( \text{inFNH}_4 \) vs. \([\text{NH}_4^+]_o \). \( \text{inFNH}_4 \) is the mean value during the \( \text{NH}_4^+ \) application calcu-
lated from \( \Delta pHi \) of the six experiments of B using the relation of Fig. 8 E. Straight lines passing through mean data points intersect the abscissa for \([\text{NH}_4^+]_o = 5.16 \) mM (0 \( K^+, \odot \)) and 7.09 mM (10 \( K^+, \bullet \)). (D) Intracellular recording from a retinal slice with a microelec-
trode in the glial compartment. Increasing \([K^+] \) from 10 to 20 mM in the superfusate induced a depolarization of the cell membranes. When the same increase of \([K^+] \) was made after superfusing the slice with 5 mM barium for a few minutes, the depolarization was undetectable for at least the first 45 s.
11; not shown). Hence, the depolarization is unlikely to
be responsible for the inhibition of NH$_4^+$ transport by extracellular K$^+$.

To quantify the inhibitory effect of K$^+$, we calculated
an apparent inhibitory constant, K'$_i$, defined by:

$$K'_m = \frac{1}{[K^+]_o / K'_i},$$

where K'_m is the Michaelis-Menten constant estimated above from responses to
NH$_4^+$ in 0 K$^+$ and K'_m is the constant estimated from the responses to NH$_4^+$ in 10 mM K$^+$. K'_i was found to be
26.7 mM, which is greater than K'_m (=7.8 mM). Variant analyses also gave K'_i > K'_m (see Marcaggi, 1999).

**Affinities of the Transporter Molecule for NH$_4^+$ and K$^+$**

In the previous two sections, we established the depen-
dence of the mean inward F$_{NH4}$ on [NH$_4^+$]$_o$ during 30-s (brief) applications of ammonium, and the inhibition
of this flux by [K$^+$]$_o$. We now describe the changes in
pH$_i$ under more varied conditions, notably, longer applications of NH$_4^+$. These more complex responses impose additional constraints on the interpretation of the underlying processes and allow us to test whether the transport can be described by a standard minimal kinetic model of membrane cotransport to which we add competition by K$^+$ for the NH$_4^+$ binding site (Fig. 10 A). As explained by Sanders et al. (1984), the kinetic behavior of a cotransporter can be accounted for by
models with different orders of binding of ions; we
have chosen to consider binding first of Cl$^-$, then K$^+$ or NH$_4^+$. This and other assumptions implicit in the model of Fig. 10 A, their justification where possible, and the techniques used to deduce the parameter values from experimental responses to NH$_4^+$ are given in the
online supplemental material. Using the experimental results obtained so far, the model suggested that K$_m$, the binding affinity for NH$_4^+$, was 6–8 mM and K$_i$, the binding affinity for K$^+$, was in the range 10–20 mM, values that we now refine.

In the experiment of Fig. 10 B, [NH$_4^+$]$_o$ was increased in steps, each lasting 8 min. The level of the plateau phase (Phase 3 in Fig. 2 B) rose no further for
[NH$_4^+$]$_o >$ 5 mM (n = 4). Fig. 10 C shows simulated responses to the same protocol of stepwise increases in
[NH$_4^+$]$_o$ for a cell containing the transporter model of
A with K$_i$ = 15 mM and K$_m$ = 5 mM (continuous trace)
or 20 mM (dashed trace). It is seen that the time course of pH$_i$, particularly for the step change [NH$_4^+$]$_o$ from 5
to 10 mM, is better simulated with K$_m$ = 5 mM; i.e., with K$_m < K_i$.

Increasing [K$^+$]$_o$ in the Presence of NH$_4^+$

Fig. 11 A illustrates the inhibitory effect on NH$_4^+$ trans-
port of increasing [K$^+$]$_o$ during the plateau phase in-
duced by a long application of NH$_4^+$. An increase in
[K$^+$]$_o$, from 10 to 50 mM rapidly increased pH$_i$ by 0.092 ± 0.012 pH unit in 2 min in 2 mM NH$_4^+$ (n = 5) and
by a greater amount, 0.115 ± 0.022 pH unit in 20 mM NH$_4^+$ (n = 5). The difference is significant with P = 0.01. This observation raised the question of whether the inhibition by [K$^+$]$_o$ was purely competitive.

Simulations were performed with the transporter model of Fig. 10 A and the protocol of Fig. 11 A. K$_m$ was set to 7 mM. Simulations with K$_i$ = 10, 15, and 20 mM (Fig. 11 B) show that inhibition by a 2-min increase in
[K$^+$]$_o$ from 10 to 50 mM differed from the experimental record in three aspects. First, the inhibition in 20
mM NH₄⁺, although larger than the inhibition in 2 mM NH₄⁺, was not as markedly larger as in the experiments. Second, the increases in pHₜ induced by rises in [K⁺]₀ were slower than the experimental ones. Third, after returning to 10 mM K⁺, the small rebound acidification present in the experimental records was not reproduced. A transporter model in which inhibition by extracellular K⁺ was noncompetitive (Fig. 11 C, legend) corrected these failings, but excessively so. We did not attempt to fit the experimental data more precisely since our transporter model is highly simplified, but these comparisons to simulations do suggest that inhibition by extracellular K⁺ may be partly noncompetitive.

**DISCUSSION**

Sensitivity to loop diuretics and external chloride (Marcaggi et al., 1999) indicate that the cotransporter studied belongs to the electroneutral cation-chloride cotransporter family (Haas and Forbush, 1998). Despite the electroneutrality of the process and the simultaneous flux of NH₃ across the membrane (Marcaggi et al., 1999), we have quantified the transport of NH₄⁺ on the cotransporter after first measuring several parameters (buffering power, P NH₃...) that link the transmembrane flux of NH₄⁺ to changes in pHₜ. Parameters for the Cell Model: pHₜ, β, P NH₃

The null method of pH measurement used on the isolated bundles of glial cells showed that many bundles had pHₜs at least as alkaline as those measured with pH microelectrodes in slices of retina (mean: 7.31; Coles et al., 1996), in agreement with the generally alkaline pH reported in many kinds of glial cells (see Deitmer and Rose, 1996). β [12.2 mequiv (pH unit · liter)⁻¹] is close to the value of 10.4 measured in snail neurons (Sztawkowski and Thomas, 1989). Our estimate of P NH₃ (13 μm s⁻¹) is well within the large range of values reported for biological membranes, which range from 108 μm s⁻¹ or higher in erythrocytes (Klocke et al., 1972; Labotka et al., 1995) to undetectably small at the apical membranes of colonic crypt cells (Singh et al., 1995). This variation appears in part to be correlated inversely with the density of proteins in the membrane: P NH₃ is high in protein-free artificial membranes (Antonenko et al., 1997) and low in membranes of urinary bladder, which are densely packed with uroplakins (Chang et al., 1994). P NH₃ has not, to our knowledge, been determined for cells of nervous tissue other than the bee retinal glial cells, so we do not know if our value is typical. From experiments similar to that of Fig. 6 B, we compared the permeabilities of the membrane to various neutral lipophilic compounds. We found that the permeabilities to the neutral forms of the amines TMA, MA, and ammonium or the carboxylic acids caproate, propionate, and acetate were greater the greater the hydrophobic part of the molecule (-CH₂- groups); i.e., P TMA > P MA > P NH₃ and P caproate > P propionate > P acetate. Thus, it appears that the relative permeability of the cell membrane to these nonelectrolytes depends more on the hydrophobicity of the molecule than on its size, in accordance with Overton’s rule (Overton, 1899).
Membrane Potential, Cl\textsuperscript{–} Gradient, and pH Regulation

A major difference, potentially important for certain cell functions, between the glial cells in the isolated bundles and those on which published results were obtained in slices of bee retina, is the apparent membrane potential. On the assumption that application of nigericin caused H\textsuperscript{+} to distribute across the membrane with the same passive distribution as K\textsuperscript{+}, we concluded that mean V\textsubscript{m} in the bundles was \(-4\) mV. Support for a small V\textsubscript{m} is given by the observation (Marcaggi et al., 1999) that in dissociations of the kind used here, rhodamine 123 selectively labeled photoreceptor cells. Since rhodamine 123 tends to partition preferentially into negatively charged compartments, this observation is compatible with the isolated bundles of glial cells having a membrane potential, V\textsubscript{m}, much smaller than that of the photoreceptors. Despite the smallness of V\textsubscript{m}, the glial cell bundles were able to regulate their pH\textsubscript{i} and to recover from repeated acid loads, although slightly more slowly than the recovery from a stimulus-induced acidification of glial cells in slices (Coles et al., 1996). And in electrically functioning retinal slices, a wide range of glial cell membrane potentials have been recorded (\(-10\) to \(-75\) mV) with little apparent consequence for homeostasis of extracellular ions or metabolism (Bertrand, 1974; Coles et al., 1986; our unpublished observations). The mechanism of pH regulation in the bee glial cells is unknown and a mechanism not dependent on an ionic gradient is conceivable, as reported in C6 glial cells (Volk et al., 1998).

Transporters of the cation-Cl\textsuperscript{–} family are normally electroneutral, and the effect of ammonium on glial cell V\textsubscript{m} in bee retinal slices is compatible with electroneutral transport (Coles et al., 1996), so V\textsubscript{m} is expected to have no direct effect on the thermodynamics of the NH\textsubscript{4}\textsuperscript{+} transport. However, since Cl\textsuperscript{–} is distributed approximately passively (Coles et al., 1989), the concentration gradient is much greater in glial cells in vivo and it is predicted that uptake of NH\textsubscript{4}\textsuperscript{+} would be more effective than in the isolated bundles.

NH\textsubscript{4}\textsuperscript{+}/K\textsuperscript{+} Selectivity of the Transporter

Until now, the few studies of competition between K\textsuperscript{+} and NH\textsubscript{4}\textsuperscript{+} for inward transport into animal cells on transporters have reported a selectivity for K\textsuperscript{+} (Kinne et al., 1986; Cougnon et al., 1999). It has, however, been proposed that NH\textsubscript{4}\textsuperscript{+} transport is a physiologically significant process, notably in kidney cells (Good, 1994) and in salivary acinar cells (Evans and Turner, 1998). We have shown that for brief applications of ammonium in the millimolar range, the Cl\textsuperscript{–}-dependent transport in bee retinal glial cells is functionally selective for NH\textsubscript{4}\textsuperscript{+} over K\textsuperscript{+}. Further, a minimal numerical model of the transport process in which NH\textsubscript{4}\textsuperscript{+} competes for a transporting site with an affinity approximately twice that for K\textsuperscript{+} accounted for the main features of the pH\textsubscript{i} responses not only for brief applications of ammonium but also for more complex protocols. Since K\textsuperscript{+} is the physiological cation whose ionic radius is closest to that of NH\textsubscript{4}\textsuperscript{+} (Robinson and Stokes, 1959) and whose permeation through channels is most similar to that of NH\textsubscript{4}\textsuperscript{+} (Hille, 1992), it is unlikely that the transporter has as high an affinity for any other major physiological ion, and we conclude that it is selective for NH\textsubscript{4}\textsuperscript{+}.

Reported values for K\textsubscript{m} (K\textsuperscript{+}) calculated for K\textsuperscript{+} influx by Cl\textsuperscript{–}-dependent transport into erythrocytes are 55 mM (sheep; Delpire and Lauf, 1991) and 140 mM (human; Kaji, 1989). These values are higher than the K\textsubscript{i} calculated in this study (10–27 mM) on the assumption (supported by the Lineweaver-Burke plots of Fig. 9, B and C) that the inhibition is purely competitive. However, if, as suggested by Fig. 11, the inhibition is partly noncompetitive, then the K\textsubscript{i} for the competitive component will be higher and closer to the values for erythrocytes. Not only does the transporter on the bee retinal glial cell have a lower affinity for K\textsuperscript{+} than for NH\textsubscript{4}\textsuperscript{+}, but preliminary results suggest that even the K\textsuperscript{+} that is bound may not be transported rapidly (Marcaggi and Coles, 1998).

Possible Advantages of Glial Uptake of Ammonium in the NH\textsubscript{4}\textsuperscript{+} Form

We have shown that ammonium enters bee retinal glial cells overwhelmingly in the NH\textsubscript{4}\textsuperscript{+} form. It is striking that this is also the case for mammalian astrocytes (at least those cultured from neonatal mice), although, in contrast to the bee glial cells, the NH\textsubscript{4}\textsuperscript{+} entry into cultured astrocytes appears to occur mainly through Ba\textsuperscript{2+}-sensitive channels (Nagaraja and Brookes, 1998; P. Sartor and P. Marcaggi, unpublished data). Entry of ammonium into cells is favored in two ways if it crosses the membrane as NH\textsubscript{4}\textsuperscript{+} instead of as NH\textsubscript{3}. First, at physiological pH\textsubscript{5}s, the majority of the ammonium is in the NH\textsubscript{4}\textsuperscript{+} form. Second, the entry can be coupled to a gradient. In the case of bee retinal cells in vivo, this is the Cl\textsuperscript{–} concentration gradient, and in cultured mouse astrocytes it is the electrical potential gradient.

A major ammonium-consuming process in bee retinal glial cells is the conversion of pyruvate to alanine (Tsacopoulos et al., 1994, 1997a). As a substrate, NH\textsubscript{4}\textsuperscript{+} will contribute to the regulation of the reactions (Tsacopoulos et al. 1997a,b). In addition, NH\textsubscript{4}\textsuperscript{+} allosterically activates phosphofructokinase (Lowry and Passos, 1975), an effect that, in mammals, may contribute to the coupling of glutamate release by neurons to glycolysis in astrocytes proposed by Pellerin and Magistretti (1994) (see also Magistretti et al., 1999). In the case of bee retinal glial cells, the ammonium...
consumption can be summarized by the reaction: CH₃-CO-CO⁻ + NH₄⁺ + NADH + H⁺ → CH₂CH₂NH₃⁺-COO⁻ + H₂O + NAD⁺.

Since this reaction consumes H⁺, pH is better conserved if ammonium is supplied in the NH₄⁺ form. In astrocytes, the pathways of energy metabolism are still a matter of debate (see, e.g., Demestre et al., 1997), but there, too, the proportion of ammonium that enters as NH₃ or NH₄⁺ will affect pH homeostasis in the brain.

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REFERENCES


**A Cl\textsuperscript{−} Cotransporter Selective for NH\textsubscript{4}\textsuperscript{+} over K\textsuperscript{+} in Glial Cells of Bee Retina**

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**SUMMARY**

A mathematical model is described for calculating fluxes of NH\textsubscript{4}\textsuperscript{+} into and out of a cell from changes in intracellular pH (pH\textsubscript{i}) for brief (30-s) applications of ammonium. A kinetic model is then described for a NH\textsubscript{4}\textsuperscript{+}–Cl\textsuperscript{−} cotransporter with competitive inhibition by K\textsuperscript{+} at the NH\textsubscript{4}\textsuperscript{+} site.

**Transmembrane Fluxes Associated with Application of Ammonium to a Cell**

Given the large diffusion coefficient of ammonium in saline solutions (≈2 × 10\textsuperscript{3} μm\textsuperscript{2} s\textsuperscript{−1}; Robinson and Stokes, 1959) and the size of the cells (≈5-μm diameter), ammonium is likely to reach equilibrium in the whole cytosol in <10 ms. The processes studied here were on a time scale of seconds, so concentrations can be considered uniform throughout the cytosol.

Fluxes illustrated in Fig. 2 D (NH\textsubscript{4}\textsuperscript{+}\textsubscript{o} → NH\textsubscript{4}\textsuperscript{+}\textsubscript{i}, NH\textsubscript{4}\textsuperscript{+}\textsubscript{i} → NH\textsubscript{3}\textsubscript{i}, and NH\textsubscript{3}\textsubscript{i} → NH\textsubscript{3}\textsubscript{o}) were represented by an electrical circuit diagram (Fig. S1), similar to that of Figure 9 C in Marcaggi et al. (1999), in which voltages, currents, and capacitances correspond to concentrations, fluxes, and volumes. Transport was initiated by imposing on the extracellular node, NH\textsubscript{4}\textsuperscript{+}\textsubscript{o}, a step potential ([NH\textsubscript{4}\textsuperscript{+}]\textsubscript{o}) that reached its full value either instantaneously, or exponentially with τ = 5.5 s (see Figs. S2 and S3, below). Each intracellular node was linked to a capacitance of 1 F (corresponding to a volume of 1 liter) so as to relate charges and voltages (i.e., quantities of molecules and intracellular concentrations). Nodes NH\textsubscript{4}\textsuperscript{+}\textsubscript{o} and NH\textsubscript{4}\textsuperscript{+}\textsubscript{i} were linked by F\textsubscript{NH4}, which was either an imposed step function (Figs. 8 and 9) or deduced from the transport scheme of Figs. 10 A and S2 B (Figs. S2, C and D, S3, 10 C, and 11, B and C). Nodes NH\textsubscript{4}\textsuperscript{+}\textsubscript{i} and NH\textsubscript{3}\textsubscript{i} were linked by a flux maintaining the equilibrium [NH\textsubscript{3}]\textsubscript{i}/[H\textsuperscript{+}]/[NH\textsubscript{4}\textsuperscript{+}]/10\textsuperscript{−9.2} and rapid enough to avoid slowing the other fluxes (idem for nodes NH\textsubscript{4}\textsuperscript{+}\textsubscript{o} and NH\textsubscript{3}\textsubscript{o}). Nodes NH\textsubscript{3}\textsubscript{i} and NH\textsubscript{3}\textsubscript{o} were linked by a constant conductance P\textsubscript{NH3} × 9/V. Finally, node H\textsuperscript{+} was bound to a unit capacitance whose initial potential was [H\textsuperscript{+}]\textsubscript{i} = 10\textsuperscript{−7.4} M. A net addition Δ[H\textsuperscript{+}]\textsubscript{tot} of H\textsuperscript{+} to the cytosol produces an increase in free H\textsuperscript{+}, [H\textsuperscript{+}]\textsubscript{o}, given by Eq. 1:

\[
\delta[H^+] / \delta[H^+]_{tot} = -[H^+]_i \times \ln 10 \times \delta p_H / \delta[H^+]_{tot} = [H^+]_i \times \ln 10 \times (1/\beta_i).
\] (1)
Hence, the net flux into node $H^+_i$ was (Eq. 2)

$$F_{H^+_i} - F_{\text{reg}} = [H^+_i] \times \ln(10) \times \text{(net flux } NH_4'^+_i \to NH_3g^i) / \beta_i - ([H^+]_i - [H^+]_o) / \tau_{\text{reg}},$$  \hspace{1cm} (2)

where $\beta_i = 12 \text{ mM}$ and $\tau_{\text{reg}} = 3 \text{ min}$. The simulations were performed with the program PSpice\textsuperscript{TM} (MicroSim Corp.).

**Multistate Model of a NH$_4'^+$-Cl$^-$ Cotransporter with Competitive Inhibition by K$^+$**

In simple standard models, a full description of the binding and cotransport of two ions (in the present case NH$_4'^+$ and Cl$^-$) requires at least 12 kinetic parameters (Sanders et al., 1984), and a further 6 parameters are needed to account for a competitive inhibition by K$^+$ on both sides of the membrane (Fig. S2 A). To express our experimental results quantitatively we consider a drastically simplified model. This model then defines apparent binding constants for NH$_4'^+$ and K$^+$ which we can estimate numerically by comparison with the data.

The simplification of the model (from Fig. S2 A to S2 B) is equivalent to making the following assumptions. (a) We assume that the binding of K$^+$ instead of NH$_4'^+$ does not affect the kinetics of the transit step of the loaded transporter. It follows that $g_1 = g_3$ and $g_2 = g_4$. (b) We make the arbitrary assumption that the free energy of the transporter and of the transporter plus ion pairs is the same on both sides of the membrane, so that $g_1 = g_2$, $g_3 = g_4$, and $k_1 = k_0$. Since the ion pairs are neutral and the estimated membrane potential is small, it is plausible that this assumption might be approximately true. (c) We assume that the cotransporter exists in equilibrium with transported ligands (Cl$^-$, K$^+$, and NH$_4'^+$) so that the three transit steps of the transporter from one side of the membrane to the other side are rate limiting. Each pair of kinetic parameters for the binding of the ligands to the transporter ($b$ and $b_{-i}$) can then be replaced by a single affinity constant $K_j = b_{-j} / b_j$. (d) The principle of detailed balance (see Stein, 1986) adds two constraints for the kinetic parameters of the general scheme of Fig. S2 A.

We set $[K^+]_o = [K^+]_i = 0$ and $[NH_4'^+]_o, [Cl^-]_o = [NH_4'^+]_i, [Cl^-]_i$, so that NH$_4'^+$ and Cl$^-$ are at equilibrium and the net flux $F_{NH_4}$ = 0. It follows that the cycling flux of transporter states in the negative direction of Fig. S2 A (Xo $\to$ XClo $\to$ XCINH$_3^+o$ $\to$ XCINH$_3^+i$ $\to$ XCl $\to$ Xi $\to$ Xo) equals the cycling flux in the positive direction. Thus, $b_1[Xo][Cl^-]_o \times b_3[XCl][NH_4'^+]_o \times g_1[XClNH_3^+o] \times b_4[XClNH_3^+i] \times b_5[XCl][Cl^-]_i \times b_6[XCl][NH_4'^+]_i \times g_2[XClNH_3^+i] \times b_3[XClNH_3^+o] \times b_4[XCl].$ Since $[NH_4'^+]_o[Cl^-]_o = [NH_4'^+]_i[Cl^-]_i$, it follows that:

$$g_1 k_2 b_1 b_3 b_{-6} = g_2 k_1 b_{-3} b_4 b_6.$$ \hspace{1cm} (3)

From assumptions a and b, $g_1 = g_2 = g_3 = g_4 = g$ and $k_1 = k_2 = k$, so Eq. 3 can be written

$$\frac{b_{-1}}{b_1} \times \frac{b_{-3}}{b_3} = \frac{b_{-4}}{b_4} \times \frac{b_{-6}}{b_6}; \text{ i.e., } K_1 \times K_3 = K_4 \times K_6.$$ \hspace{1cm} (4)

The complementary set of conditions with $[NH_4'^+]_o = [NH_4'^+]_i = 0$ and K$^+$ and Cl$^-$ at equilibrium, would lead to:

$$K_1 \times K_2 = K_4 \times K_5.$$ \hspace{1cm} (5)

Eqs. 4 and 5 are the two constraints for the kinetic parameters of the transporter that follow from the principle of detailed balance.

If we then assume that at least one of the outside binding constants is equal to the corresponding inside binding constant ($K_i = K_{i+j}$), it follows that all the binding constants are symmetric and we can write $K_1 = K_4$, $K_2 = K_3$, $K_3 = K_6$, and the scheme of Fig. S2 A reduces to that of B.

**Calculation of $F_{\text{NH}_4}$ through the NH$_4'^+$-Cl$^-$ Cotransporter Model**

**Calculation at steady state (for given binding ion concentrations).** By “steady state,” we mean that the distribution of the transporter molecules between the two faces of the membrane is constant. This cannot be true when the free concentrations of binding ions change, externally because of an applied change and internally as a consequence of the resulting change in $F_{\text{NH}_4}$. However, if the transit steps are rapid compared with the rates of change of the binding ion concentrations, the rate of change of the distribution of the transporter molecules between the two faces of the membrane can be negligible. The consequences of this approximation will be tested (Fig. S2 C and D).
Figure S2. Kinetic model of the cotransporter. (A) General kinetic scheme of the cotransport of Cl\(^-\) and NH\(_4\)^+ or K\(^+\). The unloaded transporter molecule is symbolized by X; o and i indicate the position of the transporter at the external and internal side of the cell membrane, respectively. Binding ions Cl\(^-\), NH\(_4\)^+, and K\(^+\) are not shown in this scheme for visual simplification. (B) Simplified kinetic scheme of the cotransport. By means of four assumptions (see text), the kinetic scheme was reduced to a simpler scheme with only five kinetic parameters: three binding constants \(K_c\), \(K_m\), and \(K_i\) for Cl\(^-\), NH\(_4\)^+, and K\(^+\) irrespective of the side to which the transporter faces, and two kinetic constants \(k\) and \(g\) for the transit step of the unloaded and loaded transporter. (C) Calculated changes in concentrations of two states of the transporter (XClNH\(_4\)o and XClNH\(_4\)i) during and after a 30-s application of 2 mM NH\(_4\)^+. \([\text{NH}_4^+]_o\) was transiently increased from 0 to 2 mM either instantaneously (dotted trace) or in accordance with the observed time course of solution changes (an exponential with \(\tau = 5.5\) s; see Fig. 1B) (continuous trace). The kinetic scheme of B (with parameters: \(K_c = 20\) mM, \(K_m = 7\) mM, \(K_i = 20\) mM, \(k = 1\) s\(^{-1}\), \(g = 0.0299\) s\(^{-1}\), \(n = 103.8 \times 10^{-18}\) mol \(\mu\)m\(^{-2}\)) was incorporated in the cell model (with parameters: pH \(_i\) = 7.4, \(\beta_i = 12\) mM, \(p_{\text{NH}_4} = 13\) μM s\(^{-1}\), \(\tau_{\text{reg}} = 3\) min) with concentrations of binding ions Cl\(^-\) and K\(^+\) as detailed in the text, and simulations gave the changes in [XClNH\(_4\)o] (thick traces) and [XClNH\(_4\)i] (thin traces) for the two patterns of increase of \([\text{NH}_4^+]_o\). (D) Calculated pH\(_i\) changes induced by 2 mM NH\(_4\)^+ applied for 30 s. All parameters are as in C. Thick continuous and dashed traces show pH\(_i\) changes (for the two patterns of increase of \([\text{NH}_4^+]_o\)) obtained from a simulation with the transport scheme of B included in the cell model; \(F_{\text{NH}_4} = g\{\text{XClNH}_4\}_o - g\{\text{XClNH}_4\}_i\). Thin trace shows pH\(_i\) change (for the exponential onset of the change in \([\text{NH}_4^+]_o\)) obtained from a simulation with \(F_{\text{NH}_4}\) of Eq. 9 (Table I).
The unidirectional outward component of the NH₄⁺ with [150x149]By combining Eqs. 6 and 7, \([XClNH₄₀]\) is obtained as a function of [150x415]and it follows that:

\[
K_m = \frac{[\text{NH}_4^+]_o[XClO]}{[XClNH₄₀]} = \frac{[\text{NH}_4^+]_o[XCl]}{[XClNH₄_i]};
\]

\[
K_i = \frac{[\text{K}^+]_o[XClO]}{[XClKo]} = \frac{[\text{K}^+]_i[XCl]}{[XClKi]};
\]

\[
K_c = \frac{[\text{Cl}^-]_o[XCl]}{[XCl]} = \frac{[\text{Cl}^-]_i[Xi]}{[XCl]};
\]

From the conservation of matter, the sum of the concentrations of the various states of the transporter X remains constant:

\[
[Xo] + [XClO] + [XClKo] + [XClNH₄₀] + [Xi] + [XCl] + [XClKi] + [XClNH₄_i] = n
\]

\[\Rightarrow \left( \frac{K_i K_m}{[\text{Cl}^-]_o[\text{NH}_4^+]_o} + \frac{K_m[K^+]_o}{K_m[\text{NH}_4^+]_o} + 1 \right) [XClNH₄₀] = \frac{K_i K_m}{[\text{Cl}^-]_o[\text{NH}_4^+]_o} + \frac{K_m[K^+]_o}{K_m[\text{NH}_4^+]_o} + 1
\]

\[\Rightarrow [XClNH₄₀] = \frac{[\text{NH}_4^+]_o[\text{Cl}^-]_o + [\text{NH}_4^+]_o[\text{Cl}^-]_o + [\text{K}^+]_o[\text{Cl}^-]_o + [\text{K}^+]_o[\text{Cl}^-]_o}{[\text{Cl}^-]_o[\text{NH}_4^+]_o + [\text{Cl}^-]_o[\text{NH}_4^+]_o}
\]

At steady state:

\[\delta([Xi] + [XCl] + [XClKi] + [XClNH₄_i]) = 0,
\]

and it follows that:

\[g[XClNH₄₀] + g[XClKo] + k[Xo] = g[XClNH₄_i] + g[XClKi] + k[Xi]
\]

\[\Rightarrow \left( g + \frac{K_i K_m}{K_m[\text{NH}_4^+]_o} + \frac{k K_i K_m}{K_m[\text{NH}_4^+]_o} \right) [XClNH₄₀] = \frac{g + k K_m}{K_m[\text{NH}_4^+]_o + [\text{Cl}^-]_o[\text{NH}_4^+]_o}
\]

\[\Rightarrow [XClNH₄₀] = \frac{[\text{NH}_4^+]_o[\text{Cl}^-]_o + [\text{NH}_4^+]_o[\text{Cl}^-]_o + [\text{K}^+]_o[\text{Cl}^-]_o}{[\text{Cl}^-]_o[\text{NH}_4^+]_o + [\text{Cl}^-]_o[\text{NH}_4^+]_o + [\text{K}^+]_o[\text{Cl}^-]_o + k K_i K_m}
\]

By combining Eqs. 6 and 7, \([XClNH₄₀]\) is obtained as a function of n and binding ion concentrations, and then the unidirectional inward flux of NH₄⁺ (\(F_i\)) through the transporter:

\[F_i = \frac{g[XClNH₄₀]}{n} \times \frac{(k/g) K_i K_m [\text{NH}_4^+]_o[\text{Cl}^-]_o + [\text{NH}_4^+]_o[\text{Cl}^-]_o}{D};\]

with

\[D = \left( 1/g \right) \{K_i K_m + K_m[\text{Cl}^-]_o \} + (K_m/K_i)[\text{K}^+]_o[C]_o + \left( [\text{K}^+]_o/K_i \right) \{[\text{NH}_4^+]_o[C]_o \}
\]

\[+ (K_m/K_i)[\text{K}^+]_o[C]_o + \left( k/g \right) K_i K_m + \left( K_m/K_i \right) [\text{K}^+]_o[C]_o + \left( K_m/K_i \right) [\text{K}^+]_o[C]_o + \left( k/g \right) K_i K_m.
\]

The unidirectional outward component of the NH₄⁺ flux (\(F_o\)) is obtained by inverting indices i and o in Eq. 8. The net flux of NH₄⁺ (\(F_{NH₄}\)) is the difference between \(F_i\) and \(F_o\). Development and factorization of D lead to the familiar looking Eq. 9 of Table I, which gives the net flux of NH₄⁺ through the transporter in a form that makes visible the experimentally accessible transport parameters \(K, K_m, R_m, R_o, R_c\).

**Calculation from flux equilibrium.** The transporter scheme of Fig. S2 B was represented by an equivalent electrical circuit in which the eight states of the transporter corresponded to nodes Xo, XClO, XClKo, XClNH₄₀, Xi, XCl, XClKi, and XClNH₄i, each of which connected to a unit capacitance. Initial potentials at nodes Xo and Xi were n/
treated by PSpice simultaneously with the circuit of the cell model (Fig. S1), the two circuits sharing potentials at
binding affinity of the transporter molecule for extracellular NH$_4^+$, ammonium-induced pHi changes in a cell in which the transmembrane flux of NH$_4^+$ changes in a cell model including the transporter scheme of B (thick traces) were not far different from the ammo-
diffusion delay (continuous trace). Calculated changes in concentration of the XClNH$_4^+$o and XClNH$_4^+$i forms of the trans-
was increased from zero for 30 s as illustrated in Fig. S2 C, either instantaneously (dotted trace) or with a diffusion

\[
\text{F}_{\text{NH}_4} = \frac{K([\text{NH}_4^+]_o,[\text{Cl}^-]_o - [\text{NH}_4^+]_i,[\text{Cl}^-]_i) + (K_m / K_i)([\text{Cl}^-]_o + [\text{Cl}^-]_i)([\text{NH}_4^+]_o - [\text{NH}_4^+]_i)([\text{K}^+]_o)}{K^2R + KR([\text{NH}_4^+]_o,[\text{Cl}^-]_o + KR([\text{NH}_4^+]_i,[\text{Cl}^-]_i) + R_i([\text{NH}_4^+]_o,[\text{Cl}^-]_o - [\text{NH}_4^+]_i)([\text{K}^+]_i)}
\]

This result is for the special case of the kinetic scheme of Fig. S2 B. $n$ is the total number of cotransporters per unit area of

2 (for [Xo] + [Xi] = n). Initial potentials at the other nodes were zero. Each binding step was expressed as a flux
rapid enough to avoid slowing the system, such that the binding equilibrium was maintained (for example,

\[
\text{F}_{\text{NH}_4} = \frac{K([\text{NH}_4^+]_o,[\text{Cl}^-]_o - [\text{NH}_4^+]_i,[\text{Cl}^-]_i) + (K_m / K_i)([\text{Cl}^-]_o + [\text{Cl}^-]_i)([\text{NH}_4^+]_o - [\text{NH}_4^+]_i)([\text{K}^+]_o)}{K^2R + KR([\text{NH}_4^+]_o,[\text{Cl}^-]_o + KR([\text{NH}_4^+]_i,[\text{Cl}^-]_i) + R_i([\text{NH}_4^+]_o,[\text{Cl}^-]_o - [\text{NH}_4^+]_i)([\text{K}^+]_i)}
\]

This result is for the special case of the kinetic scheme of Fig. S2 B. $n$ is the total number of cotransporters per unit area of

membrane. Note that for $[K^+]_o = [K^+]_i = 0$ or for $K_i \to \infty$ one can find again the solution for a kinetic scheme with no
competition for the binding site of the substrate (see Stein, 1986).

Testing the Steady State Approximation

Initial choice of parameter values. For an initial choice of parameter values for the transporter, $K_m$ and $K_i$ were made
equal to our preferred estimates for $K_m$ and $K_i$ for the functional transport ($K_m = 7 \text{ mM}$, $K_i = 20 \text{ mM}$). From Fig
S3, A–C, $n \times k = 103.8 \times 10^{-11} \text{ mol s}^{-1} \text{ nm}^{-2}$ and $n \times g = 2.165 \times 10^{-18} \text{ mol s}^{-1} \text{ μm}^{-2}$. We set $K_i = 20 \text{ mM}$. Parameters
determined from independent experiments were: $P_{\text{NH}_4} = 13 \text{ μm s}^{-1}, \beta_i = 12 \text{ mM}$, baseline pH$_2 = \text{pH}_2 = 7.4$, $\tau_{\text{reg}} =
3 \text{ min}$. $[K^+]_o$ and $[\text{Cl}^-]_o$ were the concentrations used in the perfusate, 10 and 222 mM. From the effect of nigerin
on pH$_2$, we deduced that a lower estimate of $[\text{Cl}^-]_o/[\text{Cl}^-]_i$ was 1.18 (Fig. S3 E); the best fit to the records of pH$_2$ was obtained

\[
\text{Is the steady state approximation valid during a 30-s application of NH}_4^+? \text{ To see if the steady state approximation}
(dXj/dt = 0) was satisfactory during brief 30-s NH$_4^+$ applications on cells, we used the cell model as previously
described (above), but allowed F$_{\text{NH}_4}$ to be determined by the transporter model instead of being imposed. [NH$_4^+$]$_o$
was increased from zero for 30 s as illustrated in Fig. S2 C, either instantaneously (dotted trace) or with a diffusion
delay (continuous trace). Calculated changes in concentration of the XClNH$_4$O and XClNH$_4$i forms of the transport-
er were shown in Fig. S2 C (bottom). This example shows that for this experimental protocol, the steady state assumption
is imperfectly valid. Indeed significant changes in the concentration of each state of the transporter are observed during the whole 30 s of the ammonium application, especially when [NH$_4^+$]$_o$ changes are modeled with a diffusion delay (continuous traces, Fig. S2 C). Nevertheless, Fig. S2 D shows that ammonium-induced pH$_2$ changes in a cell model including the transporter scheme of B (thick traces) were not far different from the ammonium-induced pH$_2$ changes in a cell in which the transmembrane flux of NH$_4^+$ was set equal to the steady state calculated F$_{\text{NH}_4}$ (Eq. 9 in Table I) (thin trace).

NH$_4^+$/K$^+$ Selectivity of the Transporter Model

The relation between the [NH$_4^+$]$_o$ which half saturates inward F$_{\text{NH}_4}$ in the experimental conditions used, and the binding affinity of the transporter molecule for extracellular NH$_4^+$ is indirect. We used the model transporter of
Figs. 10 A and S2 B to obtain an idea of how the effect on pHi of increasing [NH4+]o and the inhibition by extracellular K+ can be related to binding affinities for NH4+ (Km) and for K+ (Ki). Simulations were first performed for Kc = 20 mM, Ki = 100 mM, Km = 100 mM, and n = 1.2 fmol µm⁻² for the case of long applications of NH4+ at 2 mM on cells with baseline pHi = 7.40 and [K+]o = 10 mM. Parameters [Cl⁻], (and as a consequence [K+]i, see above), k, and g were varied until the two following conditions that correspond to mean values of experimental data were fulfilled: mean δpHi/δt between 10 and 30 s after the onset of the application of 2 mM NH4+ = 0.340 pH units
min\(^{-1}\) and pH\(_i\) = 6.844 after ammonium application lasting at least 10 min. Fig. S3 A shows that these conditions obtained for [Cl\(^-\)]\(_i\) = 151 mM, k = 1 s\(^{-1}\) and g = 0.015085 s\(^{-1}\). Parameters [Cl\(^-\)] and k were then maintained at these values. The procedure was performed with various values of Ki and the above two conditions were fulfilled by simultaneously varying g as indicated in Fig. S3 B, with no changes in the other parameters. From now on, values of Ki were linked to values of g according to the relation of Fig. S3 B. This procedure was again performed with various values of K\(_m\) and the second condition (plateau pH\(_i\) = 6.844 with [NH\(_4^+\)]\(_o\) = 2 mM and [K\(^+\)]\(_o\) = 10 mM) was fulfilled by simultaneously varying n, as indicated in Fig. S3 C. The bottom plot in Fig. S3 C shows that δpH\(_i\)/δt(NH\(_4^+\)), 2 mM in K\(^+\), 10 mM) remains fairly constant for these simultaneous changes of K\(_m\) and n. Also, since the plots of n versus K\(_m\) obtained for (Ki, g) = (1 mM, 0.1574 s\(^{-1}\)) and (100 mM, 0.015085 s\(^{-1}\)) were nearly superposable, the couples (K\(_m\), n) defined in this way were almost independent of the couples (Ki, g) defined in Fig. S3 B. To summarize, any couple (Ki, K\(_i\)) can fit the two conditions of Fig. S3 A provided that n and g have the corresponding values given by the plots in Fig. S3 B and C.

To estimate K\(_i\), we considered simulated pH\(_i\) responses to 30-s applications of 2 mM NH\(_4^+\) in 0 and 10 mM external K\(^+\). Fig. S3 D shows a simulation for (K\(_m\), K\(_i\)) = (10 mM, 20 mM), the responses being characterized by the slopes δpH\(_i\)/δt = p\(_1\), p\(_2\) and the peak changes ΔpH\(_i\) = Δ\(_1\), Δ\(_2\). K\(_i\) was then varied and for each value the corresponding Ki was calculated to give the ratios p\(_1\)/p\(_2\) = 1.353 and Δ\(_1\)/Δ\(_2\) = 1.264, which approximate the experimentally observed ones. The resulting pairs of values of K\(_i\) were plotted versus K\(_m\) in Fig. S3 E, showing that K\(_i\) estimated from the inhibition of δpH\(_i\)/δt by 10 mM K\(^+\) was in the range 10–15 mM, and estimated from inhibition of ΔpH\(_i\) by 10 mM K\(^+\), 15–20 mM, irrespective of the value taken for K\(_m\) in the range 2–100 mM.

On the assumption that these values (10–20 mM) bracket the true K\(_i\), the (modeled) [NH\(_4^+\)]\(_o\) that half saturated simulated δpH\(_i\)/δt(NH\(_4^+\)) was calculated from pH\(_i\) responses to 0.5, 1, 2, and 5 mM and plotted versus K\(_m\) of the model (Fig. S3 F, filled symbols). Similarly, the [NH\(_4^+\)]\(_o\) that half saturated simulated δpH\(_i\) was estimated from pH\(_i\) responses to 0.5, 1, 2, 5, 10, and 20 mM and plotted versus K\(_m\) of the model transporter (Fig. S3 F, open symbols). From comparison with the experimental data of Fig. 7 D, we conclude that K\(_m\) is in the range 6–8 mM, which is approximately half of K\(_i\) according to Fig. S3 E. This whole simulation procedure, illustrated by Fig. S3 for P\(_{\text{NH}_3}\) = 13 \(\mu\)m s\(^{-1}\), was also performed for P\(_{\text{NH}_3}\) = 7 and 19 \(\mu\)m s\(^{-1}\); the calculated values for K\(_m\) and K\(_i\) were little changed and K\(_m\)/K\(_i\) remained ~0.5.

*Limits of the Model for the Transport Process*

In our model of the transporter kinetics, we only considered the case of Cl\(^-\) binding before NH\(_4^+\) or K\(^+\) at the outer and inner faces of the membrane. The order of binding of substrates on a K\(^+\)-Cl\(^-\) cotransporter was deduced by Delpire and Lauf (1991), but only on the assumption that the rate limitation occurs in transmembrane transit, so that the ligand-binding reactions are at equilibrium (as for our model; see above). This assumption, as pointed out by Sanders et al. (1984), is physically dubious, and for simple models lacking this assumption, any transport kinetics can be accounted for by appropriate sets of kinetic parameters for the binding steps, irrespective of the order of binding of the substrates. We therefore chose not to investigate different orders of binding of Cl\(^-\) and NH\(_4^+\).

**REFERENCES**


