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 on 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⁺] 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⁺], 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, 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$_i$ ($\Delta$ pH$_i$) and NH$_4^+$ flux ($F_{NH_4}$) is complex. We tackled the question of the NH$_4^+$/K$^+$ selectivity in two stages. First, we deduced $F_{NH_4}$ from $\Delta$ pH$_i$ for relatively brief applications of ammonium. This required 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 poly-l-lysine. The cells were allowed to settle for 10 min and then exposed to the acetoxymethyl ester of 2',7'-bis(2-carboxylethyl)-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 trimethy-
lamine (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.

Calibration of pH \textsubscript{i} Measurements

We initially used two techniques for calibrating pH \textsubscript{i}. To estimate the shape of the curve that gives pH \textsubscript{i} as a function of I\textsubscript{495}/I\textsubscript{440}, the cell membranes were made permeable to H\textsuperscript{+} with nigericin so that pH \textsubscript{i} varied with pH \textsubscript{o} (Thomas et al., 1979). At the end of each of 11 experiments, the cells were superfused with 130 mM K\textsuperscript{+} Cardinaud solution. Perfusion was stopped and nigericin was added to the chamber to a final concentration of 10 \textmu M. After 10 min, cells were quickly superfused with 130 mM K\textsuperscript{+} Cardinaud solution at different pHs. It was found that the effect of the nigericin persisted so that it was unnecessary to include it in the calibration solutions; there was no significant difference between calibration curves obtained with solutions containing 5 \textmu M nigericin (n = 4) and those obtained without (n = 7). The value of I\textsubscript{495}/I\textsubscript{440} corresponding to pH 6.84 (I\textsubscript{495}/I\textsubscript{440} \approx 6.84) was estimated by linear interpolation for each of the 11 data sets. I\textsubscript{495}/I\textsubscript{440} was described by the equation of Boyarsky et al. (1988) (Eq. 1):

$$I_{495}/I_{440} = \left[ I_{495}^{(o)} \right]^{6.84} \times \left[ 1 + b \left( \frac{10^{(pH - pK)}}{1 + 10^{(pH - pK)}} - \frac{10^{(6.84-pK)}}{1 + 10^{(6.84-pK)}} \right) \right].$$ \hspace{1cm} (1)

The values obtained for the constants were 6.93 ± 0.03 for pK and 0.991 ± 0.022 for b (n = 11). The advantage of this procedure is that calibration for each experiment is reduced to obtaining the fluorescence ratio corresponding to pH 6.84. This ratio was obtained by superfusing the cells with 2 mM NH\textsubscript{4}\textsuperscript{+} at pH 6.90, a procedure that we found to give a pH\textsubscript{i} = 6.84 (see Fig. 3, A–D).

Absolute Measurement of pH \textsubscript{i}

The null method of Eisner et al. (1989) was used. Let \Delta \text{pH} \textsubscript{i} be the change in pH \textsubscript{i} that would have been produced by superfusion with a concentration aC of a weak acid AH\textsuperscript{−} + B, assuming that the diffusion of the neutral form (AH or B) and its re-equilibration with the charged form in the cell are rapid compared with pH \textsubscript{i} regulatory mechanisms, then:

$$\Delta \text{pH} \textsubscript{i} = -5 \times [([H^+]_o/([H^+]_o)] \times \beta_i,$$ \hspace{1cm} (2a)

$$\Delta \text{pH} \textsubscript{i} = \frac{\beta_i}{\beta_o} \times [([H^+]_o/([H^+]_o)] \times \beta_i,$$ \hspace{1cm} (2b)

where \beta is the buffering power. Let \Delta \text{pH} \textsubscript{i} be the net pH \textsubscript{i} change produced by a simultaneous application of a concentration cC of a weak base BH\textsuperscript{−} + B. Assuming that the diffusion of the neutral form (BH or B) and its re-equilibration with the charged form in the cell are rapid compared with pH \textsubscript{i} regulatory mechanisms, then:

$$\Delta \text{pH} \textsubscript{i} = -5 \times [([H^+]_o/([H^+]_o)] \times \beta_i,$$ \hspace{1cm} (3)

where \beta is the buffering power. Let \Delta \text{pH} \textsubscript{i} be the net pH \textsubscript{i} change produced by a simultaneous application of a concentration cC of a weak base BH\textsuperscript{−} + B, assuming that the diffusion of the neutral form (BH or B) and its re-equilibration with the charged form in the cell are rapid compared with pH \textsubscript{i} regulatory mechanisms, then:

$$\Delta \text{pH} \textsubscript{i} = -5 \times [([H^+]_o/([H^+]_o)] \times \beta_i,$$ \hspace{1cm} (4)

This method is most accurate when cC/ C = 1 and hence when pH\textsubscript{1} = pH\textsubscript{o}; we were able to bring pH\textsubscript{1} close to pH\textsubscript{o} by applying NH\textsubscript{4}\textsuperscript{+} (see Fig. 3; A–D).

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

We choose a weak acid AH/A\textsuperscript{−} whose pK\textsubscript{a} = 5\textsuperscript{o} pK\textsubscript{a} < 5 so that at pH\textsubscript{1} \epsilon [6; 8] its total concentration C\textsubscript{o} = [AH] o = A\textsubscript{o} = [A]\textsubscript{o}. We choose a weak base BH/B\textsuperscript{−} whose pK\textsubscript{b} = 5 so that at pH\textsubscript{1} \epsilon [6; 8] its total concentration C\textsubscript{o} = [BH] o = B\textsubscript{o} = [B]\textsubscript{o}. We set C\textsubscript{a} = C\textsubscript{o} and find the pH\textsubscript{1} < 6 so that the initial inward transmembrane flux of B (F\textsubscript{B} = F\textsubscript{OH} \times [B]\textsubscript{o}) is equal to that of AH (F\textsubscript{AH} = F\textsubscript{OH} \times [AH]\textsubscript{o}):
For $pH_i \in [6; 8]$, the initial rate of $pH_i$ change induced by the weak acid is $-F_{bgh}/\beta$, 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 $F_{bh}/\beta$. Thus, if one of the permeabilities is known, the other permeability can be deduced from the value of $pH_o$ 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 ($F_b = F_{bgh}$).

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 analyses 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 $pH_i$s ($<7.0$) 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; 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 $[NH_3]/[NH_4^+] \approx [H^+]$, 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 $[H^+] > [H^+]$, and the $NH_3$ flux continues 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 pH_i, 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 pH_i during NH4^+ 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. NH4^+ was first applied at pH_o 6.90 and then at pH_o 7.30. At each plateau phase, ΔpH_i (10 mM propionate, 10 mM TMA) and ΔpH_i (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 pH_i when applied with 10 mM propionate (Eq. 4). The absolute value of pH_i 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 pH_i was slow and could be fully accounted for by pH regulatory processes. Since pH_i during the plateau phase depends partly on pH_i regulatory processes (Fig. 2 C 3), short NH4^+ 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, pH_i was calculated to be 6.844 ± 0.017 (±SD, n = 12) after an 8-min application of NH4^+ with pH_o = 6.90. This pH_i was significantly less than pH_o.

Figure 2. Effect of extracellular ammonium application on pH_i 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 NH4^+, [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 NH4^+ can be divided into five phases, which correspond to different patterns of fluxes (C). This cell had a fairly acid baseline pH_i (≈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 NH3 is greater than ~1% of inward flux of NH4^+. The maintenance of intracellular equilibrium NH4^+ ↔ NH3 + H^+ consumes H^+ ions. (Phase 2) NH4^+ transmembrane gradient is still inward, while [NH3]_i slightly exceeds [NH3]_o. H^+ ions are shuttled into the cell. (Phase 3) Extrusion of H^+ ions by pH regulatory mechanisms equals inward flux of NH4^+. (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. pH_i changes result from three transmembrane fluxes, F_{NH4}, F_{NH3}, and net H^+ flux through pH regulatory processes (F_{reg}) (see text).
Selectivity for NH$_4$ of a Cl$^-$ Cotransporter

(P < 0.0001) and remained so for at least 35 min (Fig. 3 B). 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 p$_{H_0}$ - p$_{H_i}$ 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 (p$_{H_0}$ - p$_{H_i}$) reached during the plateau phase was related to the baseline pH$_i$, we compared cells with a baseline pH$_i$ < 7.1 with those with pH$_i$ > 7.1 (Fig. 3 C, first two columns). The difference in the mean values of (p$_{H_0}$ - p$_{H_i}$) 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 pH$_o$ at which the NH$_4^+$ was applied. Absolute values of pH$_i$ estimated during superfusion with 2 mM NH$_4^+$ at pH$_o$ 6.50 ± 0.005, 6.90 ± 0.005, 7.30 ± 0.005, and 7.70 ± 0.005 are plotted in Fig. 3 D and show a very precise linear correlation with pH$_o$ such that (pH$_i$ - 6.142) = 0.9264 × (pH$_o$ - 6.142). In later experiments, we calibrated the measurements of pH$_i$ 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 NH$_4^+$ and NH$_3$ affect pH$_i$, according to the relation
\( \Delta pHi = \Delta Q / \beta_i \), where \( \Delta Q \) is the quantity of \( H^+ \) ions/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 \( pH_i \) reached its maximum very rapidly compared with the time course of \( pH \), recovery in the presence of propionate, and we did not attempt to block \( pH \) 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 \( pH_i \), we shifted \( pH_i \) by applying \( NH_4^+ \) at various \( pH \)s. The results and the analysis, which is complicated by the effects of the \( NH_3/\text{NH}_4^+ \) 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 (\( P_{NH_3} \)) from measurements of \( pH_i \) under conditions in which entry of \( NH_4^+ \) was blocked so that changes in \( pH_i \) 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 \( pH_i \) (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% \( pH \) change faster than the one produced by ammonium: perhaps the change of solution at the cell membrane (0–20 \( \mu \)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-\( \mu \)m tip diameter pipette and carried them 50–100 \( \mu \)m up from the floor of the chamber. To increase the time resolution of the \( pH \) change, we measured the fluorescence ratio with faster switching of the excitation wavelengths (>3 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 \( [NH_3]_{o} \) was low but benefited from facilitated diffusion (Engasser and Horvath, 1974). To increase the \( NH_3 \)-induced \( \Delta pH \) baseline \( pH_i \) was reduced (to \( \sim 6.80 \)) by perfusing the cells for 30–60 min with solution buffered at \( pH_0 \) 6.20. After a 1-min perfusion with 0 Cl\(^-\) + 0.5 mM bumetanide, 10 mM ammonium was applied at \( pH_0 \) 6.50 (Fig. 4 A). In these conditions, the 10–90%–\( pH \) 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 \( pH_i \) was \( \sim 7.00 \) during the ammonium application, while \( pH_0 \) was 6.50, no slow \( pH \) 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 \( pH \) change was measured at 50% of the \( \Delta pH \), response, where it is known that \([NH_3]_{o}\) has reached its final concentration, since the effect of propionate is 90% at this time (Fig. 4). Since, for \( pH_i \sim 7.00, [NH_4^+] > 100 \times [NH_3], \) then \( \beta_i \times \Delta pH / \Delta t = \delta [NH_4^+] / \Delta t \approx \delta ([NH_4^+] + [NH_3]) / \Delta t = F_{NH3} \times S/V \), where \( F_{NH3} \) is the \( NH_3 \) transmembrane flux and \( S/V \) 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 \( \approx 1.2 \mu m^{-1} \) (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 \( S/V \) of 1.55 \( \mu m^{-1} \). Knowing \( \beta_i \), we could then calculate the transmembrane flux of \( NH_3 \) from the rate of change of \( pH_i \): \( F_{NH3} \approx V/S \times \beta_i \times \Delta pH / \Delta t \). From this flux, we found \( P_{NH3} = 14.7 \pm 2.9 \mu mol \cdot s^{-1} \) (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; \( CH_3NH_3^+ / CH_3NH_2 \)) is a weak base with a \( pK_a \) that is high (\( \approx 10.6 \); Robinson and Stokes, 1959) compared with that of ammonium (\( \approx 9.2 \)). Because of this \( pK_a \) difference, at \( pH < 8 \) (at which charged forms are preponderant), if \( [CH_3NH_3^+] + [CH_3NH_2] = [NH_4^+] + [NH_3], \) then \( [CH_3NH_2] < 0.04 \times [NH_3]. \) 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 \( pH_i \) response to 10 mM MA compared with the \( pH_i \) response to 10 mM TMA (\( pK_a \approx 9.6 \)). The speed of the \( pH \) 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 \( \mu mol \cdot s^{-1} \) (n = 8). Once this permeability was known, it was possible to deduce the permeability of propionate by ascertaining...
the initial direction of the pH change induced by a simultaneous application of 10 mM propionate and 10 mM MA. To avoid too great a variation of the net $\Delta pHi$ during this simultaneous application, we used a condition in which $pHi < pHo$, which was obtained by including 2 mM ammonium in the superfusate (Fig. 3 D). As illustrated in Fig. 5 B, the initial direction of the pH change reversed for $7.40 < pHo < 7.60$ ($n = 4$). Taking the mean of this range ($pHo = 7.50 \pm 0.10$) gives $x = P_{MA}/P_{prop} = 3.16 \pm 1.46$ according to Eq. 5. So, $P_{prop} = P_{MA}/x = 8.67 \pm 6.57$ $\mu m \cdot s^{-1}$.

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. 5 C, the initial direction of the pH change reversed for $7.00 < pHo < 7.10$ ($n = 4$), which gives $x = P_{NH3}/P_{prop} = 1.02 \pm 0.24$ according to Eq. 5. So $P_{NH3} = x \times P_{prop} = 8.84 \pm 8.78$ $\mu m \cdot s^{-1}$.

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 $\mu m \cdot s^{-1}$; we take the value 13 $\mu m \cdot s^{-1}$ for the model.

**pH Regulation**

When pH 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 pH$_i$ 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 ($t_{\text{end}}$) to allow for the rebound acidification (Phase 4). The plot is semilogarithmic, the ordinate being $\ln([H^+]_i - [H^+]_\infty)$, where $[H^+]_\infty$ was the baseline $[H^+]$ at rest. Linear regressions showed that the recoveries were exponential irrespective of the initial displacement, and had slopes ($=-1/\tau_{\text{reg}}$) that were not systematically different.

Values of the time constant $\tau_{\text{reg}}$ for 17 bundles of cells for which at least three different ammonium concentrations were tested were plotted as a function of the initial pH$_i$ displacement, $\Delta$H$_i$(NH$_4^+$) (Fig. 6 B). Linear regression of $\tau_{\text{reg}}$ against $\Delta$H$_i$(NH$_4^+$) confirmed that $\tau_{\text{reg}}$ was independent of the pH [mean slope of 0.3 ± 1.8 min (pH unit)$^{-1}$; $n = 17$]. We conclude that despite considerable variability, $\tau_{\text{reg}}$ was approximately constant irrespective of the initial pH$_i$ displacement with a mean value of 3.0 ± 1.1 min ($n = 17$). We therefore described the pH$_i$ regulation by Eq. 6:

$$F_{\text{reg}} = (1/\tau_{\text{reg}}) \times ([H^+]_i - [H^+]_\infty), \quad (6)$$

with $\tau_{\text{reg}}$ = 3 min.

Driving Force

The flux rate of a Cl$^-$ cotransporter will depend in part on [Cl$^-$]$_o$ and [Cl$^-$]$_i$, and we will use values of these concentrations in the transporter model of Fig. 10 A (see online supplemental material). [Cl$^-$]$_o$ being known, we attempted to estimate [Cl$^-$]$_i$. 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$^-$]$_i$/[Cl$^-$]$_o$ 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$^+$]$_i$ would be determined by V$_m$. We applied the H$^+$/K$^+$ exchanger, nigericin, in solutions with normal [K$^+$]$_o$ and observed the resulting change in pH$_i$ (Fig. 3 E). The minimum pH$_i$, reached during nigericin was compared with pH$_i$ at the plateau phase induced by 2 mM NH$_4^+$, the mean difference being 0.02 ± 0.05 (n = 7; Fig. 3 C). It follows that the mean value of pH$_o$ - pH$_i$ at the maximum of the nigericin-induced pH$_i$ 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 pH$_i$ during nigericin (Fig. 3 E). Thus, the true V$_m$ is probably more negative than ~4 mV and [Cl$^-$]$_o$/[Cl$^-$]$_i$ = exp($-V_m$/R/T) > 1.18.

Concentration Dependence of the pH$_i$ Changes Induced by 30-s Applications of Ammonium

To record the responses to increasing concentrations of NH$_4^+$ in the absence of external K$^+$, we superfused the cells in 0 K$^+$ for 15 s before and during each NH$_4^+$ application (Fig. 7 A). Repeated exposure to high [NH$_4^+$] appeared to lead to impairment of pH$_i$ regulation and, for 7 of 11 experiments, pH$_i$ did not recover from the acidification induced by 10 mM NH$_4^+$. Measurements were therefore made only on the records from the four experiments for which pH$_i$ recovered
from 10 mM NH₄⁺ and for which the response to subsequent control application of 0.5 or 1 mM NH₄⁺ was closely similar to the initial response (in the record of Fig. 7 A, a final application of 20 mM NH₄⁺ was made). To make sure that the effect of NH₄⁺ 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 change (Fig. 7 A). Data were analyzed only for experiments in which the time for the 10–90% propionate-induced pH change was <15 s. The last part of Fig. 7 A shows that 0 K⁺ alone did not affect pH on the time and pH scales of these experiments.

Fig. 7 B shows the NH₄⁺ 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 NH₄⁺-induced pH change (∆pH/Δt) was measured before the rebound (Phase 4), between 15 and 35 s after the valves were actuated. ∆pH/Δt(NH₄⁺) was calculated by linear regression as shown in Fig. 7 C. ∆pH/Δt(NH₄⁺) increased with [NH₄⁺]₀ in the range 0.5–10 mM NH₄⁺; but for 20 mM NH₄⁺, although the total pH change induced by NH₄⁺ [ΔpH(NH₄⁺)] continued in every case to increase, in three of the four experiments, ∆pH/Δt for 20 mM NH₄⁺ 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. Mean values for ∆pH([NH₄⁺]₀) and the start of the changes in pH were measured between 15 and 35 s after the valves were actuated. ∆pHi/Δt(NH₄⁺) was well fitted by a Michaelis-Menten curve for [NH₄⁺]₀ = 0.5, 1, 2, 5, 10, and 20 mM (solid line) and of ∆pHi/Δt([NH₄⁺]₀)[NH₄⁺]₀ for [NH₄⁺]₀ = 0.5, 1, 2, and 5 mM (dotted line) are shown.
Dependence of NH₄⁺ Flux on [NH₄⁺]₀

Three transmembrane fluxes determine pHᵢ during and after application of ammonium (Fig. 2 C). Of these, we have a phenomenological description of the pHᵢ regulation (F_reg in Fig. 2 D), and we assume that the flux of NH₃ (F_NH₃ in Fig. 2 D) results from simple diffusion (Fick's law). To deduce the flux of NH₄⁺ through the cotransporter (F_NH₄ in Fig. 2 D) from the changes in pHᵢ, 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: βᵢ = 12 mM, pHᵢ = 7.4, τ_reg = 3 min, and P_NH₃ = 13 µm s⁻¹. The surface-to-volume ratio, S/Vᵢ, 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 [NH₄⁺]₀ + [NH₃]₀ set to 2 mM. The resulting ΔpH / Δt 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 µm s⁻¹; Fig. 8 A). Increasing P_NH₃ increased ΔpH / Δt, 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 [NH₄⁺]₀ (7, 13, and 19 mM NH₄⁺) and of NH₃ after withdrawal. To start modeling this, we considered the case of a 30-s application of 2 mM extracellular NH₄⁺ with a constant inF_NH₄ (6.65 mM min⁻¹ in Fig. 8 C). After removal of extracellular NH₄⁺, the concentration gradient of NH₄⁺ is outwards. We tested the simplest reasonable assumption, which is that outward F_NH₄ = outF_NH₄ ≈ [NH₄⁺]ᵢ. With no loss of generality, this can be written: outF_NH₄ = outF_NH₄,max × ([NH₄⁺]ᵢ / [NH₄⁺]₀)ₘ₅₅, where [NH₄⁺]ₘ₅₅ is the intracellular NH₄⁺ concentration reached at t = 30 s and outF_NH₄,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 NH₄⁺ is maximal for zero outF_NH₄ and decreases with increasing outF_NH₄. Let ΔpH (inF_NH₄) be the total ΔpH change induced by a 30-s inF_NH₄ followed by an outF_NH₄ defined as above. From the experimental data, the mean ratio ΔpH / (ΔpH / Δt) measured from 30-s applications of 2 mM NH₄⁺ in 0 K⁺ was 0.60 ± 0.12 min (n = 10); the closest approach to this

Figure 8. Use of the cell model (Fig. 2 D) to derive inward F_NH₄([NH₄⁺]ᵢ) from measured pH, changes induced by brief applications of NH₄⁺. (A) Constant inward F_NH₄ (inF_NH₄) was imposed on the model for 30 s. ΔpH / Δt, calculated 15 s after onset, was plotted against inF_NH₄. Data are shown for simulations with [NH₄⁺]ᵢ set to 2 mM (to fix [NH₃]₀) and P_NH₃ = 7, 13, and 19 µm s⁻¹. (B) As in A, ΔpH / Δt was plotted versus inF_NH₄ for simulations with [NH₄⁺]ᵢ = 0.5 mM (triangles), 1 and 2 mM (diamonds), and 5, 10, and 20 mM (circles). P_NH₃ = 13 µm s⁻¹. Because increasing [NH₄⁺]ᵢ increases [NH₃]₀, ΔpH / Δt (inF_NH₄) is smaller for higher [NH₄⁺]ᵢ. (C) Simulations in which an inF_NH₄ was imposed for 30 s and followed by an outward F_NH₄ (outF_NH₄). [NH₄⁺]₀ was set to 6.65 mM min⁻¹ and [NH₃]₀ was fixed by setting [NH₃]₀ = 2 mM; this gave ΔpH / Δt = 0.44 pH unit min⁻¹, equal to the mean measured pH change induced by 2 mM NH₄⁺ in 0 K⁺ for cells with baseline pHᵢ = 7.4. At t = 30 s, F_NH₄ switched instantaneously from inF_NH₄ to maximum outF_NH₄. outF_NH₄,max, and decreased to zero as [NH₄⁺]ᵢ decreased to zero (see text). Simulations for outF_NH₄,max equal to 0, −6.65, and −20 mM min⁻¹ show that the rebound acidification after 30 s decreased when outF_NH₄,max increased. (D) Plot of ΔpH (inF_NH₄) measured as baseline pHᵢ (7.4) minus the minimal pHᵢ reached during the rebound acidification after 30 s of influx inF_NH₄. Simulations for 0.5 mM (triangles) or 20 mM (circles) NH₃ᵢ₀ (−[NH₃]₀) show that [NH₃]₀ has little effect on ΔpH (inF_NH₄). outF_NH₄,max = 0, (E) ΔpH (inF_NH₄) calculated from ΔpH / Δt([NH₄⁺]ᵢ) (●) and ΔpH (NH₃ᵢ₀) (○) of Fig. 7 D. The points were fitted by Michaelis-Menten curves (R = 0.963 and 0.994, respectively) with apparent constants Kᵢₘ equal to 5.9 ± 1.3 and 7.8 ± 0.7 mM.
Fig. 8 C is 0.56 min for \( q_{n+\text{max}}^\text{out} \), which we accept as an approximation. \( \Delta p_H \) \( f^{(n)\text{NH}_{4}^{+}} \) is very little affected by \([\text{NH}_{4}^{+}]_o\) (still for an imposed \( q_{n+\text{max}}^\text{in} \); Fig. 8 D), much less so than is \( \Delta t \) (Fig. 8 B). Thus, the inverse operation of estimating inward \( q_{n+\text{NH}_{4}^{+}} \) is better done from \( \Delta p_H \) \( ([\text{NH}_{4}^{+}]_o) \) than from \( \Delta t ([\text{NH}_{4}^{+}]_o) \). By comparing experimental \( \Delta p_H \) \( ([\text{NH}_{4}^{+}]_o) \) (Fig. 7 D) and simulated \( \Delta p_H \) \( ([\text{NH}_{4}^{+}]_o) \) (Fig. 8 D), we calculated \( q_{n+\text{NH}_{4}^{+}} ([\text{NH}_{4}^{+}]_o) \) (Fig. 8 E). The points were well fitted by a Michaelis-Menten equation of the form (Eq. 7):

\[
q_{n+\text{NH}_{4}^{+}} ([\text{NH}_{4}^{+}]_o) = q_{n+\text{NH}_{4}^{+}}^{\text{max}} \frac{[\text{NH}_{4}^{+}]_o}{K_m + [\text{NH}_{4}^{+}]_o} .
\]

The constant, \( K_m \), corresponding to half saturation of inward \( q_{n+\text{NH}_{4}^{+}} \) was 7.8 ± 0.7 mM. Variant analyses \( \Delta p_H / \Delta t ([\text{NH}_{4}^{+}]_o) \) 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 \( q_{n+\text{NH}_{4}^{+}} \) 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 \( K^+ \). \( \Delta p_H ([\text{NH}_{4}^{+}]_o) \) 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 \( q_{n+\text{NH}_{4}^{+}} ([\text{NH}_{4}^{+}]_o) \) was deduced for each measurement of \( \Delta p_H ([\text{NH}_{4}^{+}]_o) \) and a second inverse plot was made (Fig. 9 C). This plot suggests that the inhibition was competitive since straight lines passing through the data points intersect near the ordinate axis (same \( q_{n+\text{NH}_{4}^{+}}^{\text{max}} \)).

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 \([K^+]_o\) 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 \( \text{Ba}^{2+} \), which blocks the depolarization for at least 45 s after the application of \( K^+ \) (Fig. 9 D), to study the effect of \( K^+ \) on \( \Delta p_H ([\text{NH}_{4}^{+}]_o) \) in the absence of changes in membrane potential. In confirmation of Marcaggi et al. (1999), \( \text{Ba}^{2+} \) (at 5 mM) had in itself no effect on \( \Delta p_H ([\text{NH}_{4}^{+}]_o) \) (not shown). Nor did it have a significant effect on the inhibition of \( \Delta p_H ([\text{NH}_{4}^{+}]_o) \) produced by raising \( K^+ \) to 20 mM (n =

![Figure 9](image-url)
Hence, the depolarization is unlikely to be responsible for the inhibition of NH4+ transport by extracellular K+.

To quantify the inhibitory effect of K+, we calculated an apparent inhibitory constant, $K'_i$, defined by: $K'_i = K'_m (1 + [K^+]/K'_i)$, where $K'_m$ is the Michaelis-Menten constant estimated above from responses to NH4+ in 0 K+ and $K'_i$ is the constant estimated from the responses to NH4+ in 10 mM K+. $K'_i$ was found to be 26.7 mM, which is greater than $K'_m$ ($\approx$7.8 mM). Variant analyses also gave $K'_i > K'_m$ (see Marcaggi, 1999).

**Affinities of the Transporter Molecule for NH4+ and K+**

In the previous two sections, we established the dependence of the mean inward $F_{NH4}$ on [NH4+]o during 30-s (brief) applications of ammonium, and the inhibition of this flux by [K+]o. We now describe the changes in pH, under more varied conditions, notably, longer applications of NH4+. 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 transport can be described by a standard minimal kinetic model of membrane cotransport to which we add.

**Figure 10.** pH responses to stepwise increases in [NH4+]o are accounted for by a model of the transport process. (A) Kinetic scheme for visual simplicity. The three binding constants of the cotransport of Cl− and NH4+ or K+. The unloaded transporter molecule is symbolized by X; o or i indicate the position of the transporter at the external or internal side of the cell membrane. Binding ions Cl−, NH4+, and K+ are not shown in this scheme for visual simplicity. The three binding constants $K_o$, $K_m$, and $K_i$ for Cl−, NH4+, and K+ are unaffected by the side to which the transporter faces. Two kinetic constants, k and g, describe the transit steps of the unloaded and loaded transporter. (B) Experimental response to stepwise increases in [NH4+]o. (C) Simulated response of the cell model of Fig. 2D including the transporter model of A to stepwise increases in [NH4+]o with affinity for NH4+, $K_m = 5$ mM (continuous line) and 20 mM (dashed line). $K_i$ was 15 mM.

Increasing [K+]o in the presence of NH4+

Fig. 11 A illustrates the inhibitory effect on NH4+ transport of increasing [K+]o during the plateau phase induced by a long application of NH4+. An increase in [K+]o from 10 to 50 mM rapidly increased pH, by 0.092 ± 0.012 pH unit in 2 min in 2 mM NH4+ (n = 5) and by a greater amount, 0.115 ± 0.022 pH unit in 20 mM NH4+ (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
Selectivity for NH$_4^+$ of a Cl$^-$/Cotransporter

mM NH$_4^+$, although larger than the inhibition in 2 mM NH$_4^+$, was not as markedly larger as in the experiments. Second, the increases in pH$_i$ induced by rises in [K$^+$]$_o$ 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 NH$_3$ across the membrane (Marcaggi et al., 1999), we have quantified the transport of NH$_4^+$ on the cotransporter after first measuring several parameters (buffering power, P$_{NH3}$...) that link the transmembrane flux of NH$_4^+$ to changes in pH$_i$.

Parameters for the Cell Model: pH$_i$, $\beta$, P$_{NH3}$

The null method of pH measurement used on the isolated bundles of glial cells showed that many bundles had pH$_i$s 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). $\beta$, [12.2 mequiv (pH unit · liter)$^{-1}$] is close to the value of 10.4 measured in snail neurons (Sztakowski and Thomas, 1989). Our estimate of P$_{NH3}$ (13 mM s$^{-1}$) is well within the large range of values reported for biological membranes, which range from 108 mM 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 (-CH$_2$- 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 Overtton’s rule (Overtton, 1899).
Membrane Potential, Cl⁻ 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 $H^+$ to distribute across the membrane with the same passive distribution as $K^+$, we concluded that mean $V_m$ in the bundles was $-4$ mV. Support for a small $V_m$ 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, $V_m$, much smaller than that of the photoreceptors. Despite the smallness of $V_m$, the glial cell bundles were able to regulate their pH 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⁻ family are normally electroneutral, and the effect of ammonium on glial cell $V_m$ in bee retinal slices is compatible with electroneutral transport (Coles et al., 1996), so $V_m$ is expected to have no direct effect on the thermodynamics of the NH₄⁺ transport. However, since Cl⁻ 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 NH₄⁺ would be more effective than in the isolated bundles.

NH₄⁺/K⁺ Selectivity of the Transporter

Until now, the few studies of competition between $K^+$ and NH₄⁺ for inward transport into animal cells on transporters have reported a selectivity for $K^+$ (Kinne et al., 1986; Cougnon et al., 1999). It has, however, been proposed that NH₄⁺ 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⁻-dependent transport in bee retinal glial cells is functionally selective for NH₄⁺ over $K^+$. Further, a minimal numerical model of the transport process in which NH₄⁺ competes for a transporting site with an affinity approximately twice that for $K^+$ accounted for the main features of the pH responses not only for brief applications of ammonium but also for more complex protocols. Since $K^+$ is the physiological cation whose ionic radius is closest to that of NH₄⁺ (Robinson and Stokes, 1959) and whose permeation through channels is most similar to that of NH₄⁺ (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 NH₄⁺.

Reported values for $K_m$ (K⁺) calculated for K⁺ influx by Cl⁻-dependent transport into erythrocytes are 55 mM (sheep; Delpire and Lauf, 1991) and 140 mM (human; Kaji, 1989). These values are higher than the $K_i$ 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 $K_i$ 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 $K^+$ than for NH₄⁺, but preliminary results suggest that even the $K^+$ that is bound may not be transported rapidly (Marcaggi and Coles, 1998).

Possible Advantages of Glial Uptake of Ammonium in the NH₄⁺ Form

We have shown that ammonium enters bee retinal glial cells overwhelmingly in the NH₄⁺ 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 NH₄⁺ entry into cultured astrocytes appears to occur mainly through Ba²⁺-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 NH₄⁺ instead of as NH₃. First, at physiological pHs, the majority of the ammonium is in the NH₄⁺ form. Second, the entry can be coupled to a gradient. In the case of bee retinal cells in vivo, this is the Cl⁻ 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, NH₄⁺ will contribute to the regulation of the reactions (Tsacopoulos et al. 1997a,b). In addition, NH₄⁺ allosterically activates phosphofructokinase (Lowry and Passoneau, 1966; Sugden and Newsholme, 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: \( CH_3-CO-COO^- + NH_4^+ + NADH + H^+ \rightarrow CH_3CHNH_3^- COO^- + H_2O + NAD^+ \).

Since this reaction consumes \( H^+ \), \( pH \) is better conserved if ammonium is supplied in the \( NH_4^+ \) 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 \( NH_3 \) or \( NH_4^+ \) will affect \( pH \) homeostasis in the brain.

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