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⁺] 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⁺]o; 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 pKᵢ 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$_{NH4}$) is complex. We tackled the question of the NH$_4^+$/K$^+$ selectivity in two stages. First, we deduced F$_{NH4}$ 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 acetoxyethyl 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 Lawe (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. Osmolarity 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, osmolality 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-
calibration curves obtained with solutions containing 5 μM nigericin (n = 4) and those obtained without (n = 7). The value of \( I_{495}/I_{440} \) corresponding to pH 6.84 (\( I_{495}/I_{440} \) was estimated by linear interpolation for each of the 11 data sets. \( I_{495}/I_{440} \) was described by the equation of Boyarsky et al. (1988) (Eq. 1):

\[
I_{495} = I_{495}^{max} \times \left[ 1 + b \times \frac{10^{(pH_i - pK)}}{1 + 10^{(pH_i - pK)}} \right].
\]  

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 NH4+ at pH 6.90, a procedure that we found to give a pHi = 6.84 (see Fig. 3, A–D).

Absolute Measurement of pHi

The null method of Eisner et al. (1989) was used. Let \( \Delta pHi \) be the change of pHi that would have been produced by superfusion with a concentration \( c \) of a weak acid AH + A− and \( \Delta pHi \) that for a concentration \( c \) of a weak base BH− + B. Assuming that the diffusion of the neutral form (AH or B) and its re-equilibration with the changed form in the cell are rapid compared with pHi regulatory mechanisms, then:

\[
\Delta pHi = \frac{[H^+]_i/[H^+]_o}{\beta_i},
\]

(2a)

\[
\Delta pHi = \frac{[H^+]_i/[H^+]_o}{\beta_i},
\]

(2b)

where \( \beta_i \) is the buffering power. Let \( \Delta pHi \) be the net pH-change produced by a simultaneous application of a concentration \( c \) of AH + A− and \( c \) of BH− + B. We are able to determine \( \Delta pHi \) and \( \Delta pHi \) which gave rise to \( \Delta pHi \) and \( \Delta pHi \) applied successively. The desired \( \Delta pHi \) was then estimated from:

\[
bC = bC_1 + \left[ 1 - \left( (cC_2 - cC_1) \times \frac{\Delta pHi}{\Delta pHi - \Delta pHi} \right) \right]
\]

(4)

This method is most accurate when \( \Delta pHi \) is close to \( \Delta pHi \) and hence when pHi = pHo; we were able to bring pHi close to pHo by applying NH4+ (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− whose \( pK_a \) is small so that at pH7 in [6; 8] its total concentration \( C_0 = [AH]_o = [A]_o = [A]_o \). We choose a weak base BH+/B whose \( pK_b \) is large so that at pH7 in [6; 8] its total concentration \( C_0 = [BH^+]_o + [B]_o = [BH^+]_o \). We set \( C_0 = C_0 \) and find the pHo, (\( k_{a} \) in [6; 8]) for which the initial inward transmembrane flux of B (\( F_B = F_AH \times [AH]_o \)) is equal to that of AH (\( F_AH = F_AH \times [AH]_o \)):

\[
F_B = F_AH \Leftrightarrow [AH]_o = (P_B/P_AH) \times [B]_o.
\]

\[
eq [H^+]_o \times [K_k]_o = (P_B/P_AH) \times [K_k]_o \times [H^+]_o
\]

\[
eq \text{pH}_o = 0.5 \times ([K_k]_o + 5[K_k]) - 0.5 \times \log(pH_B/pH_AH).
\]

(5)
For $p\text{H}_i \in [6; 8]$, the initial rate of pH change induced by the weak acid is $-F_{\text{am}}/\beta$, since in the cell, most of AH dissociates to form $A^- + H^+$; similarly, the initial rate of pH change induced by the weak base is $F_{\text{am}}/\beta$. Thus, if one of the permeabilities is known, the other permeability can be deduced from the value of $p\text{H}_i$ for which the initial direction of the pH change during the application of the mixture of the weak base and the weak acid reverses ($F_{\text{am}} = F_{\text{am}}$).

Online Supplemental Material

The arguments leading from the observed changes in pH, 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 ~7.55 (e.g., see Fig. 7). More acid pH$_i$ (< 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$^+$.

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 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 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 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, pH_i was calculated to be 6.844 ± 0.017 (±SD, n = 12) after an 8-min application of NH_4^+ 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 NH_4^+ the [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 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 NH_3 is greater than ~1% of inward flux of NH_4^+. The maintenance of intracellular equilibrium NH_4^+ ↔ 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. pH_i changes result from three transmembrane fluxes, F_NH4, F_NH3, and net H^+ flux through pH regulatory processes (F_reg) (see text).
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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 \( \text{pH}_i - \text{pH}_o \) 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 \( \text{pH}_i - \text{pH}_o \) reached during the plateau phase was related to the baseline \( \text{pH}_o \) we compared cells with a baseline \( \text{pH}_o < 7.1 \) with those with \( \text{pH}_o > 7.1 \) (Fig. 3 C, first two columns). The difference in the mean values of \( \text{pH}_i - \text{pH}_o \) 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 \( \text{pH}_o \) at which the \( \text{NH}_4^+ \) was applied. Absolute values of \( \text{pH}_i \) estimated during superfusion with 2 mM \( \text{NH}_4^+ \) at \( \text{pH}_o = 6.50 \pm 0.005, 6.90 \pm 0.005, 7.30 \pm 0.005, 7.70 \pm 0.005 \) are plotted in Fig. 3 D and show a very precise linear correlation with \( \text{pH}_o \) such that \( \text{pH}_i = -6.142 \times \text{pH}_o + 6.9264 \). In later experiments, we calibrated the measurements of \( \text{pH}_i \) simply by superfusing the cells with 2 mM ammonium for at least 8 min and using this relation.

**Intracellular Buffering Power**

The \( \text{H}^+ \) ions released into (or taken up from) the cytoplasm as a consequence of the transmembrane fluxes of \( \text{NH}_4^+ \) and \( \text{NH}_3 \) affect \( \text{pH}_i \) according to the relation...
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\[ \frac{\Delta p_{\text{H}}}{} = \frac{\Delta Q}{\beta_i} \text{, where } \Delta Q \text{ is the quantity of } H^+ \text{ ions/ U volume and } \beta_i \text{ is the intracellular buffering power (see Roos and Boron, 1981). We estimated } \beta_i \text{ by applying the weak acid propionate (as in Figure 1 of Marcaggi et al., 1996); the change in } p_{\text{H}} \text{ reached its maximum very rapidly compared with the time course of } p_{\text{H}}, \text{ recovery in the presence of propionate, and we did not attempt to block p}_{\text{H}} \text{ regulation (compare Szatkowski and Thomas, 1989). Mean } \beta_i \text{ was } 12.2 \pm 2.9 \text{ mM (n = 11) and we took 12 mM for the model.}

To see if } \beta_i \text{ varied markedly with } p_{\text{H}}, \text{ we shifted } p_{\text{H}} \text{ by applying } NH_4^+ \text{ at various } p_{\text{H}}. \text{ The results and the analysis, which is complicated by the effects of the } NH_4^+/NH_3 \text{ system, are given in Marcaggi (1999); the conclusion is that } \beta_i \text{ is effectively constant in the range 6.7–7.3.}

**Permeability to NH}_3**

We estimated NH}_3 \text{ permeability (P}_{NH}_3 \text{) from measurements of } p_{\text{H}} \text{ under conditions in which entry of } NH_4^+ \text{ was blocked so that changes in } p_{\text{H}} \text{ were due only to the inward flux of } NH_3. \text{ We have previously shown that } NH_4^+ \text{ does not enter through barium-sensitive K}^+ \text{ channels, the major cationic conductance in these cells, and also that } NH_4^+ \text{ entry is totally blocked by bumetanide or by removal of external chloride (Marcaggi et al., 1999). We therefore applied ammonium in Cl}^- \text{ solutions to which, in some cases, bumetanide had been added, and measured the rate of change of } p_{\text{H}} \text{ (in the alkaline direction) induced by } NH_3 \text{ 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% } p_{\text{H}} \text{ 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 } p_{\text{H}} \text{ 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 } p_{\text{H}} \text{ (6.50) so that } [NH_3]_o \text{ was low but benefited from facilitated diffusion (Engasser and Horvath, 1974). To increase the } NH_3 \text{-induced } p_{\text{H}} \text{ baseline, } p_{\text{H}} \text{ was reduced (to ~6.80) by perfusing the cells for 30–60 min with solution buffered at } p_{\text{H}} 6.20. \text{ After a 1-min perfusion with } 0 \text{Cl}^- + 0.5 \text{mM bumetanide, } 10 \text{mM ammonium was applied at } p_{\text{H}} 6.50 \text{ (Fig. } 4 \text{ A). In these conditions, the 10–90%–} p_{\text{H}} \text{ change induced by } 10 \text{mM propionate (Fig. } 4 \text{ 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. \text{ Although } p_{\text{H}} \text{ was ~7.00 during the ammonium application, while } p_{\text{H}} \text{ was 6.50, no slow } p_{\text{H}} \text{ decrease was observed, as would have been the case if the membranes had had some permeability to } NH_4^+. \text{ This confirms that } NH_4^+ \text{ pathways were insignificant in these conditions.}

The slope of the } p_{\text{H}} \text{ change was measured at 50% of the } p_{\text{H}} \text{ response, where it is known that } [NH_3]_o \text{ has reached its final concentration, since the effect of propionate is 90% at this time (Fig. } 4 \text{). Since, for } p_{\text{H}} 7.00, [NH_4^+] > 100 \times [NH_3], \text{ then } \beta_i \times \delta p_{\text{H}} / \delta t = \delta [NH_4^+] / \delta t \approx \delta ([NH_4^+] + [NH_3]) / \delta t = F_{NH_3} S/V, \text{ where } F_{NH_3} \text{ is the } NH_3 \text{ transmembrane flux and } S/V \text{ 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 ~1.2 } \mu \text{m}^{-1} \text{ (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 \text{ of 1.55 } \mu \text{m}^{-1}. \text{ Knowing } \beta_i, \text{ we could then calculate the transmembrane flux of } NH_3 \text{ from the rate of change of } p_{\text{H}}: \text{ } F_{NH_3} \approx \sqrt{S/V} \times \beta_i \times \delta p_{\text{H}} / \delta t \text{. From this flux, we found } P_{NH_3} = 14.7 \pm 2.9 \text{ } \mu \text{m} \cdot \text{s}^{-1} (n = 6) \text{ in } 0 \text{Cl}^- + 0.5 \text{mM bumetanide, which was not significantly different from the value in } 0 \text{Cl}^- \text{ only } (n = 5), \text{ showing that bumetanide did not further inhibit } NH_4^+ \text{ 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 \text{ permeability is the same for cells plated on the bottom of the chamber (the conditions used for the other experiments), we also determined } P_{NH_3} \text{ by an indirect method. Methyamine (MA; } CH_3NH_3 \text{ / } CH_3NH_2 \text{) is a weak base with a } pK_a \text{ that is high (~10.6; Robinson and Stokes, 1959) compared with that of ammonium (~9.2). Because of this } pK_a \text{ difference, at } p < 8 \text{ (at which charged forms are preponderant), if } [CH_3NH_3]^+ + [CH_3NH_2] = [NH_4^+] + [NH_3], \text{ then } [CH_3NH_2] < 0.04 \times [NH_3]. \text{ It follows that if } P_{CH_3NH_2} (P_{MA}) \text{ is not far different from } P_{NH_3} \text{ for equal concentrations of MA and ammonium applied, } F_{CH_3NH_2} \ll F_{NH_3}. \text{ This is why } P_{MA} \text{ can be measured directly even with a slow speed of solution change at the cell membrane. Fig. } 5 \text{ A illustrates the } p_{\text{H}} \text{ response to } 10 \text{mM MA compared with the } p_{\text{H}} \text{ response to } 10 \text{mM TMA (} pK_a \approx 9.6). \text{ The speed of the } p_{\text{H}} \text{ 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}, \text{ which was found to be } 27.4 \pm 8.1 \text{ } \mu \text{m} \cdot \text{s}^{-1} (n = 8). \text{ Once this permeability was known, it was possible to deduce the permeability of propionate by ascertaining...
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the initial direction of the pHᵢ change induced by a simultaneous application of 10 mM propionate and 10 mM MA. To avoid too great a variation of the net ΔpHᵢ 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 pHᵢ change reversed for 7.40 < pHₒ < 7.60 (n = 4). Taking the mean of this range (pHₒ < 7.50 ± 0.10) gives x = Pₗ₅ₐ₄/Pₗ₅-prop = 3.16 ± 1.46 according to Eq. 5. So, Pₗ₅-prop = Pₗ₅ₐ₄/ x = 8.67 ± 6.57 μm s⁻¹.

The same protocol was used to estimate Pₗ₅₃NH₃ from the now known Pₗ₅₅ₐ₄. The pHᵢ change induced by 10 mM methylamine (MA) (thick line) was compared with that induced by 10 mM trimethylamine (TMA) (thin line) and to the change in fluorescence on switching to a solution containing 1 μg liter⁻¹ fluorescein (dotted line). The microscope was focused on the same bundle for the three recordings. (B) Relative cell membrane permeabilities to neutral forms of propionate (Prop) and MA. A bundle of cells was superfused with 2 mM NH₄⁺ so that pHᵢ was maintained at a value close to pHₒ (Fig. 3 D). When 10 mM Prop + 10 mM MA was applied at pHₒ 7.2, entry of the neutral form of Prop initially predominated, but as [Prop] increased, entry of MA began to predominate and pHᵢ started to increase. The initial change in pHᵢ reversed for a value of pHₒ between 7.40 and 7.60. (C) Relative cell membrane permeabilities to NH₃ and to the neutral form of Prop. A mixture of 5 mM NH₄⁺ and 5 mM Prop was applied to cells superfused with a 0 Cl⁻ solution at pHₒ 7.10 (thick trace) or 7.00 (thin trace). The two recordings were from the same bundle of cells. At pHₒ 7.10, the initial change in pHᵢ was an increase, while at pHₒ 7.00 it was a decrease.

In conclusion, the two methods of estimation of Pₗ₅₃NH₃ 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 Pₗ₅₃NH₃ is in the range of 7–19 μm s⁻¹; we take the value 13 μm s⁻¹ for the model.

pH Regulation

When pHᵢ 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\textsubscript{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 (\textit{t}_f) to allow for the rebound acidification (Phase 4). The plot is semilogarithmic, the ordinate being \(\ln([H^{+}]_{i} - [H^{+}]_{o})\), where \([H^{+}]_{o}\) 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\textsubscript{i} displacement, \(\Delta\text{pH}_i(\text{NH}_4^+)^*\) (Fig. 6 B). Linear regression of \(\tau_{\text{reg}}(\Delta\text{pH}_i(\text{NH}_4^+)^*)\) confirmed that \(\tau_{\text{reg}}\) was independent of the pH \(\text{[mean slope of 0.3 ± 1.8 min (pH unit)]}; n = 17\). We conclude that despite considerable variability, \(\tau_{\text{reg}}\) was approximately constant irrespective of the initial pH\textsubscript{i} displacement with a mean value of 3.0 ± 1.1 min \((n = 17)\). We therefore described the pH\textsubscript{i} regulation by Eq. 6:

\[
F_{\text{reg}} = (1/\tau_{\text{reg}}) \times ([H^{+}]_{i} - [H^{+}]_{o}),
\]

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

Driving Force

The flux rate of a Cl\textsuperscript{-} cotransporter will depend in part on \([\text{Cl}^-]_{o}\) and \([\text{Cl}^-]_{i}\), and we will use values of these concentrations in the transporter model of Fig. 10 A (see online supplemental material). \([\text{Cl}^-]_{o}\) being known, we attempted to estimate \([\text{Cl}^-]_{i}\). Measurements in slices of bee retina with ion-selective microelectrodes have shown that in the glial cells Cl\textsuperscript{-} (and also K\textsuperscript{+}) are at close to electrochemical equilibrium (Coles et al., 1986, 1989). Hence, \([\text{Cl}^-]_{o}/[\text{Cl}^-]_{i}\) 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\textsuperscript{+} then \([H^{+}]_{i}\) would be determined by \(V_m\). We applied the H\textsuperscript{+}/K\textsuperscript{+} exchanger, nigericin, in solutions with normal [K\textsuperscript{+}]\textsubscript{o} and observed the resulting change in pH\textsubscript{i} (Fig. 3 E). The minimum pH\textsubscript{k}, reached during nigericin was compared with pH\textsubscript{i} at the plateau phase induced by 2 mM NH\textsubscript{4}\textsuperscript{+}, the mean difference being 0.02 ± 0.05 \((n = 7);\) Fig. 3 C). It follows that the mean value of pH\textsubscript{k} − pH\textsubscript{i} at the maximum of the nigericin-induced pH\textsubscript{i} change was −0.07 (Fig. 3 C). On the assumption that H\textsuperscript{+}, being now in equilibrium with K\textsuperscript{+}, was distributed passively across the membrane, \(V_m\) was −4 mV. Since nigericin is not perfectly selective for H\textsuperscript{+} (Pressman et al., 1967; Margolis et al., 1989), it probably depolarized the membranes somewhat, as suggested by the slow increase in pH\textsubscript{i} during nigericin (Fig. 3 E). Thus, the true \(V_m\) is probably more negative than −4 mV and \([\text{Cl}^-]_{o}/[\text{Cl}^-]_{i} = \exp(-V_m/FRT) > 1.18\).

Concentration Dependence of the pH\textsubscript{i} Changes Induced by 30-s Applications of Ammonium

To record the responses to increasing concentrations of NH\textsubscript{4}\textsuperscript{+} in the absence of external K\textsuperscript{+}, we superfused the cells in 0 K\textsuperscript{+} for 15 s before and during each NH\textsubscript{4}\textsuperscript{+} application (Fig. 7 A). Repeated exposure to high [NH\textsubscript{4}\textsuperscript{+}] appeared to lead to impairment of pH\textsubscript{i} regulation and, for 7 of 11 experiments, pH\textsubscript{i} did not recover from the acidification induced by 10 mM NH\textsubscript{4}\textsuperscript{+}. Measurements were therefore made only on the records from the four experiments for which pH\textsubscript{i} recovered...
Fig. 7. pH changes as a function of ammonium concentration (for 30-s applications in 0 K\textsuperscript{+}). (A) Typical recording of pH\textsubscript{i} responses to various NH\textsubscript{4}\textsuperscript{+} concentrations. Cells were normally superfused with 10 mM K\textsuperscript{+} standard solution that was switched to a 0 K\textsuperscript{+} solution 15 s before each NH\textsubscript{4}\textsuperscript{+} 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 \approx 20 min ("\Delta t"), removal of extracellular K\textsuperscript{+} with no ammonium application had no detectable effect on pH\textsubscript{i}. (B) Superposition of pH\textsubscript{i} responses to NH\textsubscript{4}\textsuperscript{+} (from A) on a shorter time scale. Dotted lines for 0.5 and 1 mM NH\textsubscript{4}\textsuperscript{+} are the second (control) responses. The delay between activation of the electromagnetic valves and the solution changes can be attributed to the switching of the electromagnetic valves and the arrival of a new solution at the cell membrane. The NH\textsubscript{4}\textsuperscript{+}-induced pH\textsubscript{i} change (\delta pH/\delta t) was measured before the rebound (Phase 4), between 15 and 35 s after the valves were actuated. \delta pH/\delta t[(NH\textsubscript{4}\textsuperscript{+})\textsubscript{o}] was calculated by linear regression as shown in Fig. 7 C. 

The mean value of \delta pH/\delta t for [NH\textsubscript{4}\textsuperscript{+}]\textsubscript{o} that half saturates pH\textsubscript{i} changes does not necessarily correspond to the one that half saturates transport and pH\textsubscript{i} changes is indirect, the value of [NH\textsubscript{4}\textsuperscript{+}]\textsubscript{o} that half saturates pH\textsubscript{i} changes does not necessarily correspond to the one that half saturates transport of NH\textsubscript{4}\textsuperscript{+}. To deduce the flux of NH\textsubscript{4}\textsuperscript{+}, we had recourse to a mathematical model.

from 10 mM NH\textsubscript{4}\textsuperscript{+} and for which the response to subsequent control application of 0.5 or 1 mM NH\textsubscript{4}\textsuperscript{+} was closely similar to the initial response (in the record of Fig. 7 A, a final application of 20 mM NH\textsubscript{4}\textsuperscript{+} was made). To make sure that the effect of NH\textsubscript{4}\textsuperscript{+} 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\textsubscript{i} change (Fig. 7 A). Data were analyzed only for experiments in which the time for the 10--90% propionate-induced pH\textsubscript{i} change was \less than 15 s. The last part of Fig. 7 A shows that 0 K\textsuperscript{+} alone did not affect pH\textsubscript{i} on the time and pH scales of these experiments.

Fig. 7 B shows the NH\textsubscript{4}\textsuperscript{+} 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 \approx 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\textsubscript{4}\textsuperscript{+}-induced pH\textsubscript{i} change (\delta pH/\delta t) was measured before the rebound (Phase 4), between 15 and 35 s after the valves were actuated. \delta pH/\delta t[(NH\textsubscript{4}\textsuperscript{+})\textsubscript{o}] was calculated by linear regression as shown in Fig. 7 C. 

The mean value of \delta pH/\delta t for [NH\textsubscript{4}\textsuperscript{+}]\textsubscript{o} that half saturates pH\textsubscript{i} changes does not necessarily correspond to the one that half saturates transport and pH\textsubscript{i} changes is indirect, the value of [NH\textsubscript{4}\textsuperscript{+}]\textsubscript{o} that half saturates pH\textsubscript{i} changes does not necessarily correspond to the one that half saturates transport of NH\textsubscript{4}\textsuperscript{+}. To deduce the flux of NH\textsubscript{4}\textsuperscript{+}, we had recourse to a mathematical model.
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 (Fᵣₑₐ in Fig. 2 D), and we assume that the flux of NH₄⁺ (FₙH₄ in Fig. 2 D) results from simple diffusion (Fick’s law). To deduce the flux of NH₄⁺ through the cotransporter (FₙH₄ 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: \( \beta = 12 \text{ mM, } \text{pH}_i = 7.4, \tau_{\text{reg}} = 3 \text{ min, and } P_{\text{NH₃}} = 13 \mu \text{m s}^{-1} \). The surface-to-volume ratio, \( S/V \), with its attendant uncertainty, was used to calculate \( P_{\text{NH₃}} \), but cancels out in the calculations.

As a first step, a constant inward FₙH₄ (inFₙH₄) was imposed for 30 s, with [NH₄⁺]₀ (plus [NH₃]₀) set to 2 mM. The resulting \( \delta \text{pH}/\delta t \) was calculated 15 s after the onset of the imposed inFₙH₄ and plotted against inFₙH₄ for various PₙH₃ (7, 13, and 19 \mu m s⁻¹; Fig. 8 A). Increasing PₙH₃ increased \( \delta \text{pH}/\delta t \), but only slightly, showing that PₙH₃ is not a major rate-limiting factor.

A similar simulation, still using an imposed inFₙH₄, was then performed in the presence of various [NH₄⁺]₀ (plus [NH₃]₀). Increasing [NH₄⁺]₀ increased [NH₃]₀, reduced outward FₙH₄, and, as expected, reduced \( \delta \text{pH}/\delta t \) (inFₙH₄) (Fig. 8 B). It is clear that the experimental result in which \( \delta \text{pH}/\delta t \) was smaller for an application of 20 mM ammonium than for 10 mM (Fig. 7) does not necessarily imply inward FₙH₄ (20 mM NH₄⁺) < inward FₙH₄ (10 mM NH₄⁺). We also note that since the relation of \( \delta \text{pH}/\delta t \) to inFₙH₄ is curved (Fig. 8, A and B), \( \delta \text{pH}/\delta t \) vs. [NH₄⁺]₀ will saturate more rapidly than will inFₙH₄ vs [NH₄⁺]₀.

The pHᵢ peak reached after withdrawal of external ammonium must depend both on [NH₄⁺]ᵢ at the end of the ammonium application, and on the effluxes of NH₄⁺ and of NH₃ after withdrawal. To start modeling this, we considered the case of a 30-s application of 2 mM extracellular NH₃ with a constant inFₙH₄ (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 FₙH₄ = outFₙH₄ = [NH₄⁺]ᵢ. With no loss of generality, this can be written: outFₙH₄ = outFₙH₄,max × \([\text{NH}_4^+]_/\text{[NH}_4^+\text{]}\)max, where \([\text{NH}_4^+]_/\text{[NH}_4^+\text{]}\)max is the intracellular NH₄⁺ concentration reached at t = 30 s and outFₙH₄,max is an initially arbitrary constant corresponding to the maximum transient outFₙH₄. As illustrated in Fig. 8 C, the rebound acidification on removal of extracellular NH₄⁺ is maximal for zero outFₙH₄ and decreases with increasing outFₙH₄. Let \( \Delta \text{pH} \) (inFₙH₄) be the total pHᵢ change induced by a 30-s inFₙH₄ followed by an outFₙH₄ defined as above. From the experimental data, the mean ratio \( \Delta \text{pH} / (\delta \text{pH}/\delta t) \) 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 in

![Image](https://via.placeholder.com/552x726)

**Figure 8.** Use of the cell model (Fig. 2 D) to derive inward FₙH₄ ([NH₄⁺]₀) from measured pHᵢ changes induced by brief applications of NH₄⁺. (A) Constant inward FₙH₄ (inFₙH₄) was imposed on the model for 30 s \( \delta \text{pH}/\delta t \), calculated 15 s after onset, was plotted against inFₙH₄. Data are shown for simulations with [NH₄⁺]₀ set to 2 mM (to fix [NH₃]₀) and PₙH₃ = 7, 13, and 19 \mu m s⁻¹. (B) As in A, \( \delta \text{pH}/\delta t \) was plotted versus inFₙH₄ for simulations with [NH₄⁺]₀ = 0.5 mM (triangles), 1 and 2 mM (diamonds), and 5, 10, and 20 mM (circles). PₙH₃ = 13 \mu m s⁻¹. Because increasing [NH₄⁺]₀ increases [NH₃]₀, \( \delta \text{pH}/\delta t \) (inFₙH₄) is smaller for higher [NH₄⁺]₀. (C) Simulations in which an inFₙH₄ was imposed for 30 s and followed by an outward FₙH₄ (outFₙH₄). inFₙH₄ was set to 6.65 mM min⁻¹ and [NH₄⁺]₀ was fixed by setting [NH₃]₀ = 2 mM; this gave \( \delta \text{pH}/\delta t = 0.44 \text{ pH unit min}^{-1} \), equal to the mean measured pHᵢ change induced by 2 mM NH₄⁺ in 0 K⁺ for cells with baseline pHᵢ = 7.4. At t = 30 s, FₙH₄ switched instantaneously from inFₙH₄ to maximum outFₙH₄, outFₙH₄,max, and decreased to zero as [NH₄⁺]ᵢ decreased to zero (see text). Simulations for outFₙH₄,max equal to 0, -6.65, and -20 mM min⁻¹ show that the rebound acidification after 30 s decreased when outFₙH₄,max increased. (D) Plot of \( \Delta \text{pH} \) (inFₙH₄) measured as baseline pHᵢ (7.4) minus the minimal pHᵢ reached during the rebound acidification after 30 s of influx inFₙH₄. Simulations for 0.5 mM (triangles) or 20 mM (circles) NH₃ o (−NH₃) show that [NH₃]₀ has little effect on \( \Delta \text{pH} \) (inFₙH₄), outFₙH₄,max = 0 (E) \( \text{outFₙH₄,max} \) (NH₄⁺) calculated from \( \delta \text{pH}/\delta t ([\text{NH}_4^+]_0) \) (○) and \( \Delta \text{pH} ([\text{NH}_4^+]_0) \) (○) of Fig. 7 D. The points were fitted by Michaelis-Menten curves (R = 0.963 and 0.994, respectively) with apparent constants K_in equal to 5.9 ± 1.3 and 7.8 ± 0.7 mM.
Fig. 8 C is 0.56 min for outF\textsubscript{NH4\textsuperscript{max}} = 0, which we accept as an approximation. \(\Delta p\text{H} \text{/(inF}_{\text{NH4}}\)) is very little affected by \([\text{NH}_4\textsuperscript{+}])\text{_o} \) (still for an imposed inF\textsubscript{NH4}; Fig. 8 D), much less so than is \(\Delta p\text{H}/\Delta t\) (Fig. 8 B). Thus, the inverse operation of estimating inward F\textsubscript{NH4}(\text{[NH}_4\textsuperscript{+}])\text{_o} \) is better done from \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}])\text{_o} \) than from \(\Delta p\text{H}/\Delta t\text{([NH}_4\textsuperscript{+}])_o \). By comparing experimental \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}])\text{_o} \) (Fig. 7 D) and simulated \(\Delta p\text{H}(\text{inF}_{\text{NH4}}) \) (Fig. 8 D), we calculated inF\textsubscript{NH4}(\text{[NH}_4\textsuperscript{+}])\text{_o} \) (Fig. 8 E). The points were well fitted by a Michaelis-Menten equation of the form (Eq. 7):

\[
\text{inF}_{\text{NH4}}(\text{[NH}_4\textsuperscript{+}])\text{_o} = \frac{\text{inF}_{\text{NH4\textsuperscript{max}}} \times \text{[NH}_4\textsuperscript{+}])\text{_o}}{K'_{m} + \text{[NH}_4\textsuperscript{+}])\text{_o}}. \tag{7}
\]

The constant, \(K'_{m}\), corresponding to half saturation of inward F\textsubscript{NH4}, was 7.8 \( \pm \) 0.7 mM. Variant analyses from \(\Delta p\text{H}/\Delta t\text{([NH}_4\textsuperscript{+}])_o \) or using Lineweaver-Burke plots gave lower values, down to 4.9 mM (Marcaggi, 1999). We conclude that \(K'_{m} = 7.8 \text{ mM}\) is a conservative estimate of the affinity (an upper limit for \(K'_{m}\)) of the transporter for \([\text{NH}_4\textsuperscript{+}]) \) in these experimental conditions.

\[\text{Functional Selectivity for NH}_4\textsuperscript{+} \text{ over K\textsuperscript{+}}\]

Having established the dependence of inF\textsubscript{NH4} on \([\text{NH}_4\textsuperscript{+}])\text{_o} \) (for 30 s applications of ammonium), we then extended the approach to analyze the inhibitory effect of K\textsuperscript{+}. Fig. 9 A shows an experiment in which cells were superfused for 30 s with \([\text{NH}_4\textsuperscript{+}]) \) in 0 or 10 mM K\textsuperscript{+}. \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}]) \) from six such experiments is shown plotted with double inverse scales as a function of \([\text{NH}_4\textsuperscript{+}])\text{_o} \) in Fig. 9 B. Using the model, as described above, a value of inF\textsubscript{NH4}(\text{[NH}_4\textsuperscript{+}]) \) was deduced for each measurement of \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}]) \) 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 inF\textsubscript{NH4\textsuperscript{max}}).

Fig. 9 D illustrates how K\textsuperscript{+} 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\textsuperscript{+}] in the extracellular clefts (Coles and Orkand, 1983). But in isolated cell bundles, the depolarization might be greater and in some way affect \([\text{NH}_4\textsuperscript{+}]) \) uptake. We therefore used Ba\textsuperscript{2+}, which blocks the depolarization for at least 45 s after the application of K\textsuperscript{+} (Fig. 9 D), to study the effect of K\textsuperscript{+} on \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}]) \) in the absence of changes in membrane potential. In confirmation of Marcaggi et al. (1999), Ba\textsuperscript{2+} (at 5 mM) had in itself no effect on \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}]) \) (n = 5; not shown). Nor did it have a significant effect on the inhibition of \(\Delta p\text{H}(\text{[NH}_4\textsuperscript{+}]) \) produced by raising K\textsuperscript{+} to 20 mM (n =

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**Figure 9.** Inhibition of inward \([\text{NH}_4\textsuperscript{+}]) \) flux by external K\textsuperscript{+}. (A) Comparison of responses to 2 and 5 mM \([\text{NH}_4\textsuperscript{+}]) \) in 0 and 10 mM K\textsuperscript{+}. For each response, \([\text{NH}_4\textsuperscript{+}]) \) was applied for 30 s when applied in 0 K\textsuperscript{+}; K\textsuperscript{+} was removed from the superfusate 15 s earlier. (B) Double inverse plot of mean \(\Delta p\text{H} \text{/(inF}_{\text{NH4}}\) vs. \([\text{NH}_4\textsuperscript{+}])\text{_o} \) (n = 6). Straight lines passing through mean data points intersect the abscissa at \([\text{NH}_4\textsuperscript{+}])\text{_o} = 5.02 \text{ mM} \) (0 K\textsuperscript{+}, ○) and 6.90 \text{ mM} \) (10 mM K\textsuperscript{+}, ●). (C) Double inverse plot of \(\text{[NH}_4\textsuperscript{+}]) \text{_o} \) vs. \(\text{[NH}_4\textsuperscript{+}]) \text{_o} \) is the mean value during the \(\text{[NH}_4\textsuperscript{+}]) \) application calculated from \(\Delta p\text{H}\) of the six experiments of B using the relation of Fig. 8 E. Straight lines passing through mean data points intersect the abscissa for \([\text{NH}_4\textsuperscript{+}])\text{_o} = 5.16 \text{ mM} \) (0 K\textsuperscript{+}, ○) and 7.09 \text{ mM} \) (10 mM K\textsuperscript{+}, ●). (D) Intracellular recording from a retinal slice with a micro- electrode in the glial compartment. Increasing [K\textsuperscript{+}] from 10 to 20 mM in the superfusate induced a depolarization of the cell membranes. When the same increase of [K\textsuperscript{+}] 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.

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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:

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

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 dependence 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$^+$. The kinetic model of the transport process (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$_4^+$ transport of increasing [K$^+$]$_o$ during the plateau phase induced 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 pH, 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, PNH₃...) that link the transmembrane flux of NH₄⁺ to changes in pH.

Parameters for the Cell Model: pHi, β, PNH₃

The null method of pH measurement used on the isolated bundles of glial cells showed that many bundles had pHs 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). β₁[12.2 mequiv (pH unit · liter)⁻¹] is close to the value of 10.4 measured in snail neurons (Szatkowski and Thomas, 1989). Our estimate of PNH₃ (13 ± 2 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: PNH₃ 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). PNH₃ 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 compound (-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, \( \text{Cl}^- \) 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 \(-4 \text{ mV}\). 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 (\(-10 \text{ to } -75 \text{ mV}\)) 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-\( \text{Cl}^- \) 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 \( \text{NH}_4^+ \)-transport. However, since \( \text{Cl}^- \) 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 \( \text{NH}_4^+ \) would be more effective than in the isolated bundles.

\( \text{NH}_4^+/K^+ \) Selectivity of the Transporter

Until now, the few studies of competition between \( K^+ \) and \( \text{NH}_4^+ \) 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 \( \text{NH}_4^+ \)-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 \( \text{Cl}^- \)-dependent transport in bee retinal glial cells is functionally selective for \( \text{NH}_4^+ \) over \( K^+ \). Further, a minimal numerical model of the transport process in which \( \text{NH}_4^+ \) 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 \( \text{NH}_4^+ \) (Robinson and Stokes, 1959) and whose permeation through channels is most similar to that of \( \text{NH}_4^+ \) (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 \( \text{NH}_4^+ \).

Reported values for \( K_m \) (\( K^+ \))-dependent transport into erythrocytes are 55 mM (sheep; Delpire and Lauf, 1991) and 140 mM (human; Kaji, 1989). These values are higher than the \( K^* \) 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 \( K^* \) 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 \( \text{NH}_4^+ \), 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 \( \text{NH}_4^+ \) Form

We have shown that ammonium enters bee retinal glial cells overwhelmingly in the \( \text{NH}_4^+ \) 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 \( \text{NH}_4^+ \)-entry into cultured astrocytes appears to occur mainly through \( \text{Ba}^{2+} \)-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 \( \text{NH}_4^+ \) instead of as \( \text{NH}_3 \). First, at physiological \( pHS \), the majority of the ammonium is in the \( \text{NH}_4^+ \) form. Second, the entry can be coupled to a gradient. In the case of bee retinal cells in vivo, this is the \( \text{Cl}^- \) 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, \( \text{NH}_4^+ \) will contribute to the regulation of the reactions (Tsacopoulos et al. 1997a,b). In addition, \( \text{NH}_4^+ \)-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: $\text{CH}_3\text{CO-COO}^- + \text{NH}_4^+ + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{CCHN}_2\text{H}_3^- - \text{COO}^- + \text{H}_2\text{O} + \text{NAD}^+$.

Since this reaction consumes $\text{H}^+$, $\text{pH}$ is better conserved if ammonium is supplied in the $\text{NH}_4^+$ 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 $\text{NH}_3$ or $\text{NH}_4^+$ will affect $\text{pH}$ homeostasis in the brain.

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