

Intracellular pH and Chemoresponse to NH_4^+ in *Paramecium*

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Paramecium are attracted to ammonium chloride solutions relative to sodium chloride control solutions, but little is known about the mechanisms by which attraction is evoked. A known effect of ammonium solutions in other cell types is an alteration of intracellular pH. We show here that intracellular pH is elevated upon initial exposure to 5 mM NH_4Cl , but appears to decline within 10 minutes, both in wild type cells and in two mutants which do not show sustained attraction to NH_4Cl using the standard behavioral assay, the T-maze. We also present quantitative values of swimming parameters that underlie the response to NH_4Cl . Cell. Motil. Cytoskeleton 40:107–118, 1998. © 1998 Wiley-Liss, Inc.

Key words: *Paramecium*; intracellular pH; chemoresponse; ammonium; cilia

INTRODUCTION

Paramecium tetraurelia is a freshwater ciliate that lives in a chemically complex environment. As with other organisms it must sense and respond appropriately to various environmental cues. Some of these environmental cues include chemical signals that affect the behavior of the cells, causing them to disperse from, or accumulate in, the vicinity of these compounds. Attractants of *Paramecium* include folate, lactate, acetate, cyclic AMP, NH_4^+ , biotin, and glutamate among others [Van Houten and Preston, 1988; Yang, 1994] (Bell and Van Houten, unpublished results). As products of bacteria, *Paramecium*'s natural food source, these compounds may serve as food cues.

Accumulation, or attraction, of *Paramecium* is brought about by increased mean free paths of the cells due to changes in the angle and frequency of ciliary beating, resulting in increased swimming speed and decreased frequency of turning [Van Houten, 1978]. Both swimming speed and turning frequency are governed by the membrane potential of the cell. Slight hyperpolarization of the cell increases the frequency and alters the plane of ciliary beating toward the posterior of the cell, resulting in increased swimming speed, and also decreases the likelihood of a Ca^{2+} action potential-induced turn [Machemer, 1974]. Increased swimming speed along with decreased turning frequency bring about accumula-

tion of the cells in the vicinity of the attractant [Van Houten, 1978].

We have directly shown that attractants hyperpolarize the membrane [Van Houten, 1979; Preston and Van Houten, 1987]. Hyperpolarization, in turn, causes slightly increased ciliary beating frequency and fewer power stroke reversals caused by calcium action potentials. The result is a slightly smoother, faster swimming pattern and accumulation of the population of cells [Van Houten, 1990].

Several lines of evidence strongly suggest that the response to *organic* attractants is mediated by specific receptors at the cell membrane. Saturable, specific binding has been found for folate, biotin, and cyclic AMP [Schulz et al., 1984; Sasner and Van Houten, 1989; Smith et al., 1987] (Bell and Van Houten, unpublished results) and a cyclic AMP receptor protein has been purified to near homogeneity [Van Houten et al., 1991]. Addition-

Abbreviations: BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein, acetoxymethyl ester; $[\text{K}^+]_i$, intracellular potassium concentration; $[\text{K}^+]_o$, extracellular potassium concentration; pH_i , intracellular pH.

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ally, single site mutants that lack the ability to show attraction to folate or cAMP also lack specific, saturable binding of these respective compounds [DiNallo et al., 1982; Schulz et al., 1984; Smith et al., 1987].

The receptors for organic attractants appear to transduce signals to effector proteins to bring about a subsequent hyperpolarization of the cell [Van Houten and Preston, 1988]. Hyperpolarization in response to at least some organic attractants cannot be accounted for by simple K^+ or Na^+ efflux or Cl^- influx [Preston and Van Houten, 1987], and indirect evidence exists to support the role of a plasma membrane Ca^{2+} pump as the hyperpolarizing entity in chemoresponse to these attractants [Wright and Van Houten, 1990; Wright et al., 1992, 1993; Yano et al., 1996].

Attraction to NH_4^+ appears to work through a different signal transduction mechanism. First there appears to be no cell surface receptor involved. Binding studies to examine potential receptor characteristics are not possible using isotopic NH_3/NH_4^+ ligands, but behavioral interference studies could be performed using various amino compounds as competitive blockers of NH_4Cl -induced attraction. We found no interference of attraction to NH_4^+ by amino acids (including lysine, histidine, arginine, tryptophan, and glycine), nucleotides (CMP and GMP), or vitamins (including folate and thiamine) (Gagnon et al., unpublished results). Second, there appears to be no involvement of the Ca^{2+} pump [Van Houten, 1994] (see Discussion). Thus there is no evidence for a receptor for NH_4^+ .

In the absence of receptors for NH_4^+ , alteration of pH_i is a likely mechanism by which NH_4Cl causes attraction. In solution NH_4Cl is in equilibrium with the membrane permeant molecule NH_3 . When NH_3 passes into the cell it becomes protonated, alkalinizing the cell [Roos and Boron, 1981]. It is possible that perturbation of pH_i may bring about hyperpolarization of the membrane by modulating an ion conductance [Wanke et al., 1979; Bear et al., 1988] or by affecting a crucial signal transduction mechanism. In order to test the hypothesis that attraction to NH_4^+ is due to changes in pH_i , we used the pH-sensitive fluorescent dye BCECF to examine pH_i in *Paramecium* after stimulation with NH_4Cl , both in cell suspensions and in individual cells. To our knowledge this is the first study using BCECF to measure pH_i in ciliates. Intracellular pH measurements were made in wild type cells and in two mutants, which are slightly attracted to NH_4Cl in T-maze assays but cannot sustain the population accumulation [Van Houten et al., 1975, 1982]. We report here that pH_i is altered upon exposure to 5 mM NH_4Cl in both the wild type and mutants. We also analyzed swimming speed and a measure of turning frequency in order to better understand the correlation

between the alkalinization by NH_4^+ and attraction behavior of normal and mutant cells.

MATERIALS AND METHODS

Culturing of Cells

Wild type *Paramecium tetraurelia*, 51-s (sensitive to killer) and mutants derived from this stock were grown at 28°C in culture fluid consisting of wheat grass extract supplemented with $Na_2HPO_4 \cdot 7H_2O$ (1 g/l) and stigmasterol (1 mg/l). Culture fluid was inoculated with *Aerobacter aerogenes* 24 to 48 h prior to addition of *Paramecium*. Cells were harvested in late log-early stationary phase by centrifugation at 350 *g* for 2 min in oil-testing centrifuge tubes. One mutant strain was previously designated as d4-538 and is referred to here as 10-3-3 [Van Houten et al., 1982]; mutant strain 14-3-3 has no corresponding d4 designation. These two strains were isolated for their failure to be attracted to NH_4Cl [Van Houten et al., 1982].

Solutions

All buffer solutions contain 1 mM $Ca(OH)_2$, 1 mM citric acid, and ~1.3 mM Tris base plus the indicated salts at pH 7.02. BCECF-AM (Molecular Probes, Inc., Eugene, OR) was dissolved (1 mg/ml) in dimethylsulfoxide. Nigericin (Sigma, St. Louis, MO) was dissolved (5 mg/ml) in methanol.

T-Maze Assays

The T-maze assay employs a three-way stopcock with a two-way bore [Van Houten et al., 1975, 1982]. One arm of the stopcock contained the control solution while the opposite arm contained the test solution. The test and control solutions differed by only one ion pair, but were otherwise identical in pH and ionic strength. Harvested cells were washed twice through 13 ml of the control buffer at 100*g* for 1 min and resuspended in fresh control buffer. Cells were loaded into the stopcock via the central arm and the plug was turned to allow cells to swim back and forth between either arm. After the desired time (2 or 30 min) the plug was closed, the arms were emptied, and a sample of cells was counted. The index of chemokinesis (I_{Che}) was calculated as the number of cells counted from the test arm/number of cells counted from both the test and control arms. $I_{Che} > 0.5$ indicates attraction to the test solution; $I_{Che} < 0.5$ indicates repulsion.

Video Recording and Computer Analysis of Behavior

Harvested cells were washed twice in 5 mM NaCl or 5 mM NH_4Cl buffer solution by centrifugation and incubated in 5 mM NaCl or 5 mM NH_4Cl buffer, respectively, to adapt at room temperature for at least 30 min. Immediately before recording, 100 μ l of stimulus

solution was spread thinly over a clean microscope slide, allowing cell motility in only two dimensions. Two microliters of the cell suspension were added to the microscope slide, just out of the video camera's field of view. Taping was started immediately and cells swam into the field of view (cells generally were recorded swimming into the field of view within 2 to 5 sec of being added to the slide). Recording proceeded for at least 15 sec. Cell density was adjusted so that there was usually less than 15 to 20 cells on screen at any time. Cells were transferred from 5 mM NaCl buffer to 5 mM NaCl (control) or 5 mM NH₄Cl (test) buffer, and from 5 mM NH₄Cl buffer to 5 mM NH₄Cl (control) or 5 mM NaCl buffer (test).

Swimming behavior of cells was observed on a monitor (RCA TC1214) connected through a video cassette recorder (Sony, Park Ridge, NJ, SLV-R5UC) to a video camera (Cohu 6410, San Diego, CA) mounted on a StereoZoom 7 dissecting microscope (Bausch & Lomb, Rochester, NY). Video data was processed using a video processor (VP 110, Motion Analysis Corp., Santa Rosa, CA) and analyzed using ExpertVision software (version 3.14, Motion Analysis) and a modified user program graciously provided by Kevin Clark and David Nelson [1991]. Data were analyzed in a procedure similar to that previously described by Clark and Nelson [1991], adapted from Sager et al. [1988]. Briefly, the first 15 sec of each taping was processed at a capture rate of 15 frames/sec and the video outlines of each cell were used to calculate the geometric center (centroid) of each cell. The centroids were used to calculate each cell's swimming path. Because the ExpertVision software often could not correctly reconstruct paths when two or more cells came in contact, efforts were made to reduce chance collisions by keeping the total number of cells on screen at about 15 to 20. Additionally, the maximum number of pixels allowed to define an individual cell outline was delimited and a linear prediction extrapolation was incorporated into the path calculations. Files containing path data were edited to delete any artifactual paths that may have been included. Paths were smoothed twice to reduce video noise.

These paths were then used to calculate two values that are useful in describing *Paramecium* behavior. These parameters, previously defined by Clark and Nelson [1991], describe the average swimming speed of the cells while not undergoing any turns (straight swimming speed) and the percentage of time that the cells spend deviating from straight swimming (percent directional changes, or PDC). Directional changes were defined by determining a threshold angular value which a straight swimming cell would not exceed in the time interval between two frames (1/15 sec). This angular threshold had previously been determined for *Paramecium* to be

17° [Clark and Nelson, 1991]. However, we found some straight swimming cells to slightly exceed 19° in this same time interval (data not shown). We also found that setting a threshold above 19° could also exclude some actual turns from being detected as such. We, therefore, selected 19° as our threshold for an angular change to be considered a turn. PDC was calculated as follows:

$$\text{PDC} = \frac{\text{Amount of time spent swimming} \geq 19^\circ}{\text{Total Path Time}}$$

After videotaping cells in the desired conditions, measurement of swimming parameters was facilitated by calculation and use of the geometric centers of each video image (centroids). To ensure that spuriously low swimming speeds were not determined as a result of cell turning, the average swimming speed was measured as the mean speed for each cell-path taken while not undergoing any turns. Swimming speed was calculated as the linear distance between each consecutive centroid in the same swimming path, divided by the time separating those points (1/15 sec) [Clark and Nelson, 1991]. In short, angular changes were determined from each set of three consecutive centroids, and directional changes were noted each time a cell exceeded an empirically determined threshold angle (19°). The total number of times that all cells exceeded 19° from one path step to the next was totaled, the sum multiplied by the time of the path step (1/15 sec), and that product was expressed as a percentage of the total path time.

Swimming speed data were compared between test and control trials using the Mann-Whitney U test.

pH_i Measurement of Cell Suspensions

Fluorescence measurements were performed using an Hitachi (Danbury, CT) F-2000 fluorescence spectrometer equipped with a magnetic stirrer. The fluorometer was interfaced with a personal computer (AT&T 6386SX/EL) and data were obtained and analyzed using an intracellular cation measurement software package (Hitachi).

Harvested cells were centrifuged through approximately 13 ml of 5 mM NaCl buffer twice and then transferred to a conical polystyrene centrifuge tube containing 5 µM BCECF-AM in 5 mM NaCl buffer for a total volume of 5 ml. Cells were incubated in this suspension for 30 min at room temperature in the dark, then centrifuged at 100g for 1 min and transferred to approximately 13 ml 5 mM NaCl buffer. Cells were centrifuged through this buffer and this wash step was repeated twice. The cells were then resuspended in approximately 2 ml of 5 mM NaCl buffer until used for pH_i measurement.

To measure pH_i at various time points after introduction to a stimulus, 0.2 ml of the cell suspension was transferred to a centrifuge tube containing the stimulus

solution, incubated for an appropriate amount of time, and then centrifuged at 100g for 1 min. The cells were transferred to a quartz or UV-quality optical glass cuvette containing approximately 1.1 ml of the stimulus solution. The concentration of cells in the cuvette generally ranged between 4,000 and 15,000 cells/ml for the wild type, or between 10,000 and 40,000 cells/ml for the mutants. With the cells stirring, the cuvette was excited at 506 and 440 nm, and emission intensity recorded at 527 nm. pH_i was determined using a calibration curve of the ratio of fluorescence intensities at 506 and 440 nm (I_{506}/I_{440}) vs. a variety of pH_i values. The pH_i values were altered using the K^+/H^+ ionophore nigericin [Thomas et al., 1979] and by setting $[K^+]_o$ equal to $[K^+]_i$ (approximately 18 mM) [Hansma, 1974] (see below). pH in the cuvette was then altered using HCl and NaOH and was monitored using a pH microelectrode (MI-410, Microelectrodes, Inc., Londonderry, NH) and millivolt meter (MV-1, Microelectrodes, Inc.). Calibrations were performed for each population of cells used.

For pH calibration using nigericin, it was necessary to have the value of K_i . The value of $[K^+]_i$ used in our studies was 18 mM, a value obtained from flame photometric studies in *Paramecium tetraurelia* [Hansma, 1974]. This $[K^+]_i$ agrees with concentrations derived from electrophysiological studies in *P. tetraurelia* (18 mM) [Oertel et al., 1978] and ^{86}Rb efflux studies in *P. tetraurelia* (12 to 24 mM) [Hansma, 1981]. Electrophysiological studies using the K^+ reversal potential in *P. caudatum*, however, suggest an $[K^+]_i$ of 34 mM [Ogura and Machemer, 1980]. Ogura and Machemer's results disagree, however, with earlier electrophysiological studies on *P. caudatum*, which demonstrated $[K^+]_i$ of about 17.5 mM [Naitoh and Eckert, 1973]. Additionally, the cellular conditions between the two methods of measuring pH_i were quite different. In suspension, cells were constantly agitated by stirring, and the effect of this upon pH_i is not known. Measurement of pH_i in single cells required their immobilization. The deciliation process may affect pH_i homeostasis in the cell if, as with Ca^{2+} channels, mechanisms essential for pH regulation are partitioned into the ciliary membrane [Dunlap, 1977]. Although not identical, the pH_i s are similar in both methods (see results below).

pH_i Measurement Using Individual Cells

Harvested cells were loaded with BCECF/AM as described above for cell suspension experiments. After incubating in BCECF/AM for 30 min the cells were centrifuged out of dye at 100g for 1 min and washed twice through 13 ml fresh 5 mM NaCl buffer. The loaded cells were then deciliated after the method of Ogura and Machemer [1978]. Cells were resuspended in a total volume of 2 ml of 5 mM NaCl, 5% ethanol (v/v) and triturated with a Pasteur pipette for 2 min. Deciliated cells

were transferred to fresh 5 mM NaCl buffer and allowed to rest at least 10 min prior to pH_i recording. Cells were layered onto a coverslip coated with 2.5% gelatin (w/v in distilled H_2O) and allowed to sediment to the substrate prior to measuring pH_i . Cells were superfused alternately with either 5 mM NaCl buffer or 5 mM NH_4Cl buffer. To some cells 5 mM NH_4Cl buffer was applied with a puffer while being continuously superfused with 5 mM NaCl buffer. Cells were observed under a Nikon inverted microscope with attached photometer (model D104, Photon Technology International, Inc., South Brunswick, NJ). Only cells which appeared healthy (no blistering of the cell surface, regular contraction of contractile vacuoles, absence of swollen vacuoles) were used for the experiments. Cells were excited at 506 nm and 440 nm and emission read at 527 nm and data were processed using DeltaScan software, version 2.06 (Photon Technology International, Inc.). Approximately 2 h after removal from the BCECF loading solution, cells started to show evidence of compartmentalization of dye (characterized by a punctate distribution of the dye within the cell). To avoid complications from measuring BCECF sequestered within vacuoles or other compartments, cells showing evidence of compartmentalization were not used. It was observed that when cells containing compartmentalized dye were exposed to NH_4Cl , no measurable change in pH_i occurred (Davis, unpublished observations). Recordings were calibrated using a BCECF calibration curve (I_{506}/I_{440} vs pH) generated in vitro in 5 mM NaCl buffer. We considered this adequate for measurements of qualitative changes since the spectral properties of the dye, when measured in loaded single cells and in solution, were the same (data not shown).

RESULTS

Measurement of pH_i in Cell Suspensions

To determine whether NH_4Cl could alter intracellular pH in *Paramecium*, pH_i was measured before and after NH_4Cl stimulation using the pH-sensitive fluorescent dye BCECF, which was calibrated intracellularly. Cell suspensions of wild type *Paramecium* exhibit a basal pH_i 6.68 ± 0.02 (s.e.m., $n = 5$) (Fig. 1A). When transferred from 5 mM NaCl to 5 mM NH_4Cl , wild type cells show an initial elevation of pH_i to 6.80 ± 0.05 (s.e.m., $n = 5$), which appears to gradually decline below basal levels within 10 min. This acidification is not dependent upon removal of external NH_4^+ . In the two mutants that are unable to sustain attraction to NH_4Cl , the basal pH_i appears lower over the 10 min time course: pH_i for 10-3-3 is 6.40 ± 0.08 (s.e.m., $n = 5$) (Fig. 1B), for 14-3-3 pH_i is 6.54 ± 0.07 (s.e.m., $n = 5$) (Fig. 1C). These are markedly depressed relative to the wild type, and in fact the basal pH_i of strain 10-3-3 is significantly lower

than in wild type (as determined via Mann-Whitney U-test, $P < 0.05$). Basal pH_i notwithstanding, stimulation with NH_4Cl in these strains causes elevations in pH_i (6.62 ± 0.05 and 6.71 ± 0.08 s.e.m., $n = 5$, respectively) comparable in magnitude to the wild type response, although the initial alkalinizations appear to be slower (compare the peak alkalinization in Fig. 1).

Measurement of pH_i in Individual Cells

Measurement of pH_i in cell suspensions may accurately represent population responses to a chemical stimulus but can also introduce artifacts not attributable to stimulus. For example, mechanical stimulation at the anterior of the cell induces a depolarization due to an inward Ca^{2+} conductance, while similar stimulation at the posterior of the cell causes a hyperpolarization of the cell due to an outward K^+ conductance [Machemer, 1988]. The effects of membrane potential or specific ion fluxes on pH_i have not been determined in *Paramecium*. To eliminate possible problems occurring from mechanical stimulation of the cells (for which we perhaps could not control with cells in suspension kept in NaCl) and also to examine the time course of the response of single *Paramecium* to NH_4Cl , pH_i measurements were also performed on single cells that were deciliated to keep them immobilized during observation.

Intracellular pH was measured in single cells superfused with 5 mM NaCl buffer. After a baseline pH_i was established, the superfusate was switched to 5 mM NH_4Cl buffer. Once the maximal change in pH_i was achieved, the superfusate was switched back to NaCl. The readings were performed over a total of 500 sec for each experiment, and data were analyzed from those cells that (1) had a steady baseline pH_i , (2) demonstrated a pH_i response to

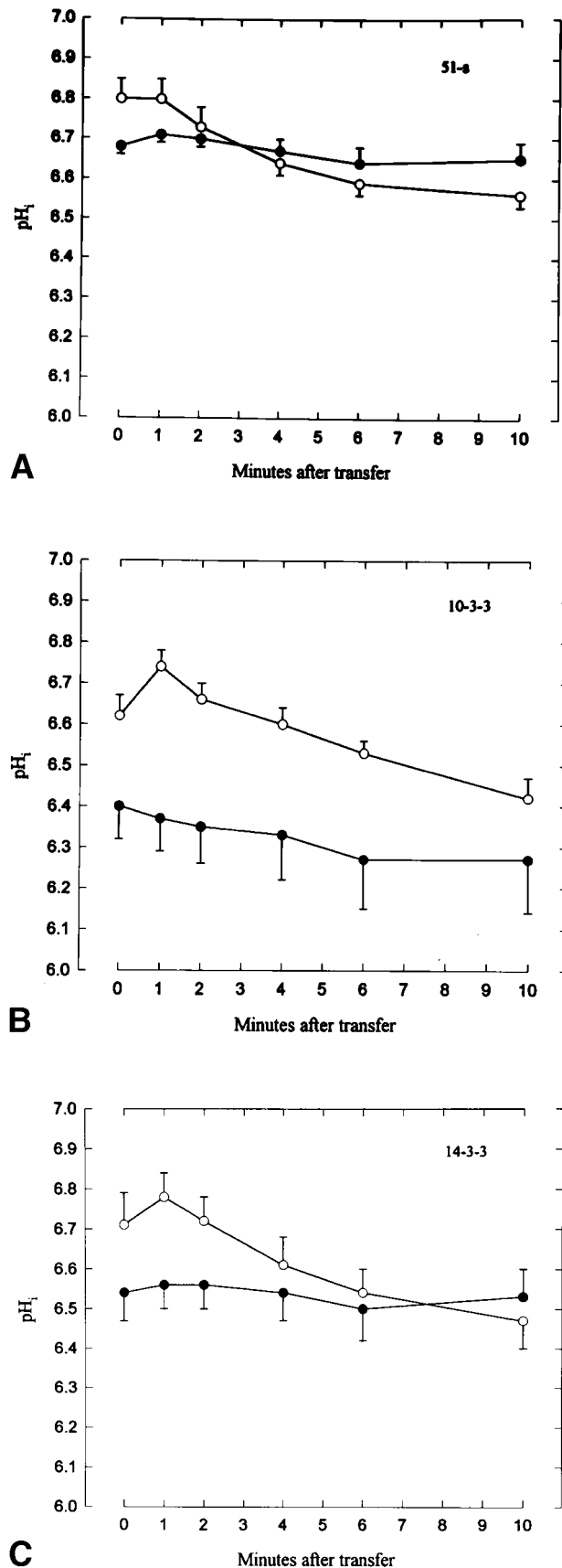


Fig. 1. **a:** Intracellular pH response of wild type (51-s) to NH_4Cl in suspension. Cells were loaded with BCECF in 5 mM NaCl buffer for 30 min and transferred to fresh 5 mM NaCl (control, solid circle) or to 5 mM NH_4Cl (test, open circle) in quartz cuvettes. Cells were excited at 506 and 440 nm while emission was monitored at 527 nm. Readings were performed at the indicated time after transfer. Data shown are the average of 5 experiments. Error bars represent s.e.m. **b:** Intracellular pH response of 10-3-3 to NH_4Cl in suspension. Cells were loaded with BCECF in 5 mM NaCl buffer for 30 min and transferred to fresh 5 mM NaCl (control, solid circle) or to 5 mM NH_4Cl (test, open circle) in quartz cuvettes. Cells were excited at 506 and 440 nm while emission was monitored at 527 nm. Readings were performed at the indicated time after transfer. Data shown are the average of 5 experiments. Error bars represent s.e.m. **c:** Intracellular pH response of 14-3-3 to NH_4Cl . Cells were loaded with BCECF in 5 mM NaCl buffer for 30 min and transferred to fresh 5 mM NaCl (control, solid circle) or to 5 mM NH_4Cl (test, open circle) in quartz cuvettes. Cells were excited at 506 and 440 nm while emission was monitored at 527 nm. Readings were performed at the indicated time after transfer. Data shown are the average of 5 experiments. Error bars represent s.e.m.

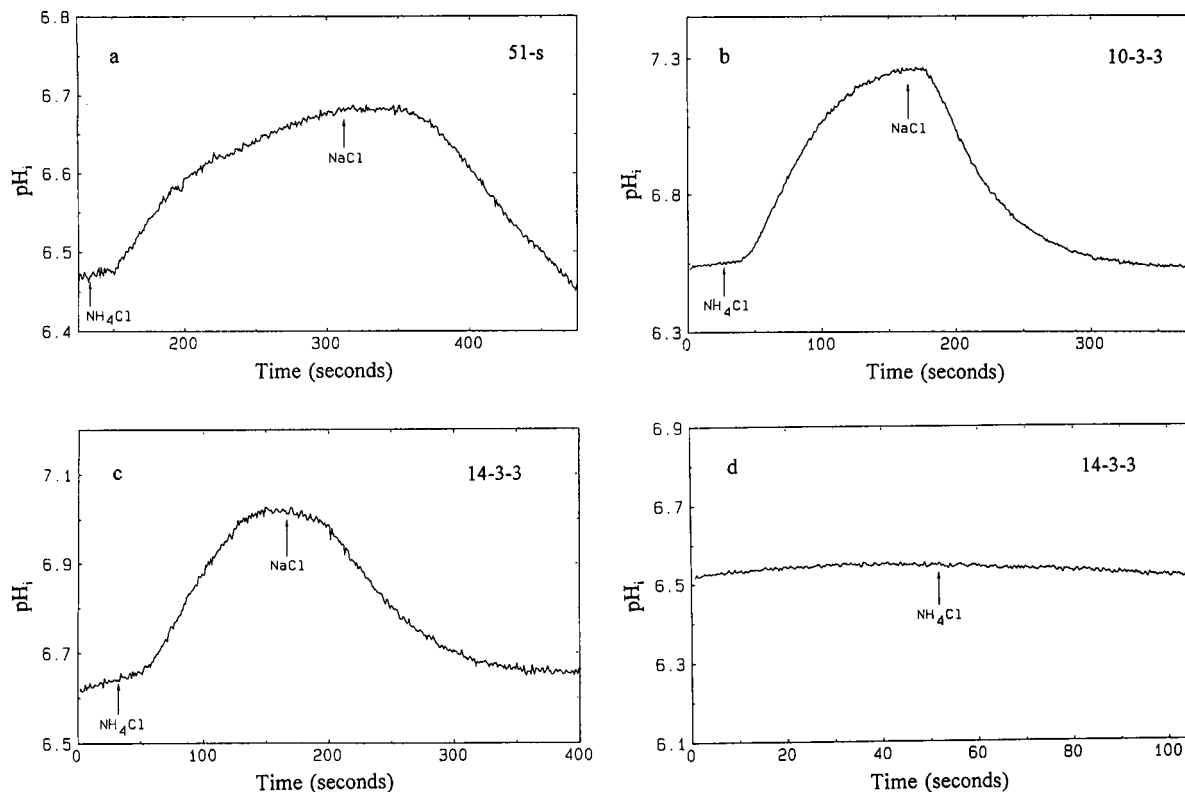


Fig. 2. Sample trace of pH_i measurements in single wild type and mutant cells. Deciliated cells loaded with BCECF were perfused with 5 mM NaCl buffer and excited at 506 and 440 nm, while emission intensity was monitored at 527 nm. The perfusate was switched to 5 mM NH_4Cl buffer and then back to 5 mM NaCl at the corresponding

arrows. In every case, cells of wild type (a) and 10-3-3 (b) showed responses to NH_4Cl . Though not shown in trace (a), pH_i did not drop below 6.45. Cells from 14-3-3 however showed responses to NH_4Cl in about 50% of the trials (c), while the remainder of the cells show no observable response (d).

NH_4Cl , and (3) returned to approximately basal pH_i level following return to NaCl. These criteria were met for most of the cells observed (see Fig. 4a-c).

From these single cell measurements, the basal pH_i of wild type cells (≈ 6.6) appears slightly lower than that determined in suspension measurements (see Table II). Wild type cells alkalinize upon exposure to NH_4Cl , and this alkalinization is reversible, although the extent of recovery is often inconsistent between individual cells, with some cells acidifying to levels below basal pH_i , while others do not recover entirely to the baseline over the course of the trace (data not shown).

Basal pH_i measurements for 10-3-3 and 14-3-3 appear to be comparable to that of the wild type (see Table II). In contrast, in the suspension measurements, the mutants had a lower basal pH_i (Figs. 1-3). These mutants, like wild type, show alkalinization upon exposure to NH_4Cl and recovery when the superfusate is switched back to NaCl, and the extent of recovery also is inconsistent between individual cells. It should be noted that only approximately 50% of strain 14-3-3 cells respond to NH_4Cl stimulation (Fig. 4d). Average values for 14-3-3, however, were only taken from those cells

from which pH_i responses were elicited. However, both populations of cells would contribute to the population suspension measurements.

For NH_4Cl to affect chemosensory signal transduction through intracellular alkalinization, pH_i would have to be altered rapidly. Rapid application of 5 mM NH_4Cl to wild type cells is achieved with a puffer at increasing puffer durations (Fig. 3) and the resulting measured intracellular responses to NH_4Cl exposure are rapid and dependent on the duration of exposure, up to a maximum of 10 sec (not shown). Upon removal of the stimulus, these transient alkalinizations are followed by brief acidifications to levels below the basal pH_i . As the duration of exposure to NH_4Cl increases, the magnitude of post-alkalinization acidification also increases (Fig. 3). Intracellular pH after these acidifications recovers to approximately basal levels ($pH_i \approx 6.85$ seen at the beginning of the experiment in Fig. 3 vs. $pH_i \approx 6.81$ at the end, not shown in Fig. 3). Application of NaCl control solution with the puffer method did not elicit changes in intracellular pH, ruling out mechanical stimulation involvement in the intracellular response to NH_4Cl (data not shown).

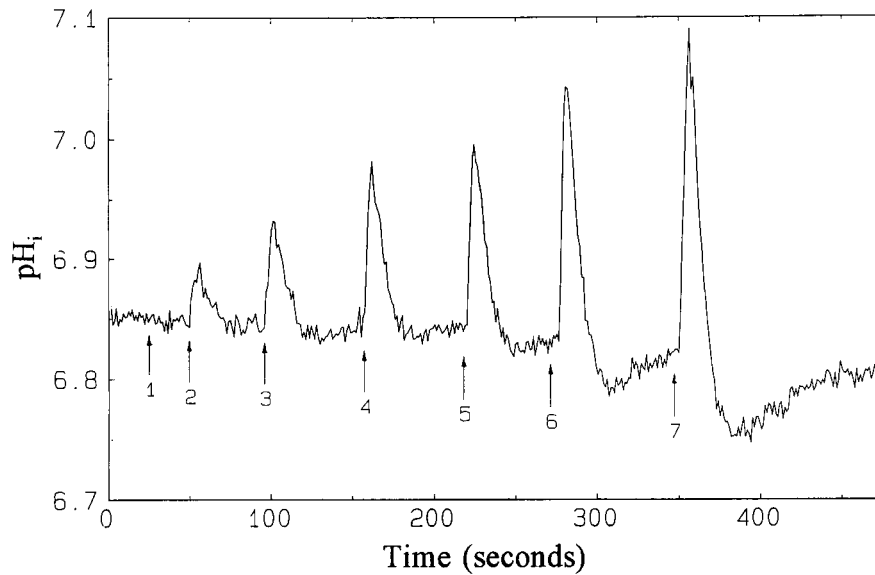


Fig. 3. Effect of rapid exposure to NH_4Cl on pH_i . Deciliated wild type cells loaded with BCECF were continuously perfused with 5 mM NaCl buffer and excited at 506 and 440 nm, while emission intensity was

monitored at 527 nm. At points indicated by the arrows 5 mM NH_4Cl was puffed onto the cell with the following durations: (1) 100 msec, (2) 300 msec, (3) 500 msec, (4) 750 msec, (5) 1 sec, (6) 2 sec, (7) 4 sec.

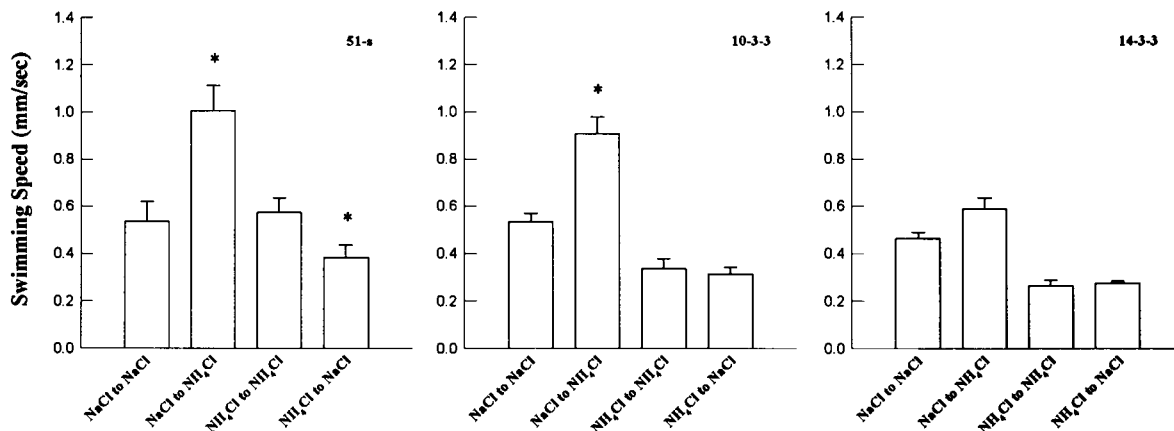


Fig. 4. Swimming speed (mm/sec) for strains 51-s (wild type), 10-3-3, and 14-3-3. Cells from each population were incubated for 30 min in 5 mM NaCl or 5 mM NH_4Cl buffer and then transferred to 5 mM NaCl or 5 mM NH_4Cl buffer, as indicated. Behavior was examined over the

first 15 sec after transfer. Errors bars represent standard error of the mean (s.e.m.). $n = 6$ experiments for 51-s and 10-3-3, $n = 5$ experiments for 14-3-3. Asterisks indicate significant difference from the control using the Mann-Whitney U test ($P < 0.05$).

Analysis of Swimming Behavior

Two mutant strains were previously described as showing an initial attraction to NH_4Cl , but not sustaining attraction after 30 min in the T-maze assay although they can respond normally to other stimuli [Van Houten et al., 1982]. We confirmed that the mutant populations of 10-3-3 and 14-3-3 show a slight attraction after 2 min in T-maze assays, but this attraction is not sustained and the responses are significantly different from the wild type after 30 min (Table I). In contrast, wild type *Paramecium* (strain 51-s) show a slight attractant response to NH_4Cl vs. NaCl after 2 min using the T-maze assay (Table I). The

TABLE I. T-Maze Analysis of Behavioral Response to NH_4Cl †

Strain	I_{Che} (2 min)	n	I_{Che} (30 min)	n
Wild Type	0.64 ± 0.08	9	0.83 ± 0.09	35
10-3-3	0.61 ± 0.08	9	0.54 ± 0.10	9*
14-3-3	0.61 ± 0.07	9	0.55 ± 0.13	12*

†Cells were assayed for their response to 5 mM NH_4Cl vs. 5 mM NaCl using T-mazes (see Materials and Methods). T-mazes were run for 2 or 30 min. $I_{\text{Che}} > 0.5$ indicates attraction to test solution; $I_{\text{Che}} < 0.5$ indicates repulsion. Data shown are the average and S.D. of several experiments, n shown is the number of T-mazes for 2 or 30 min, respectively.

*Significantly different from wild type ($P < 0.05$) using the Mann-Whitney U-test.

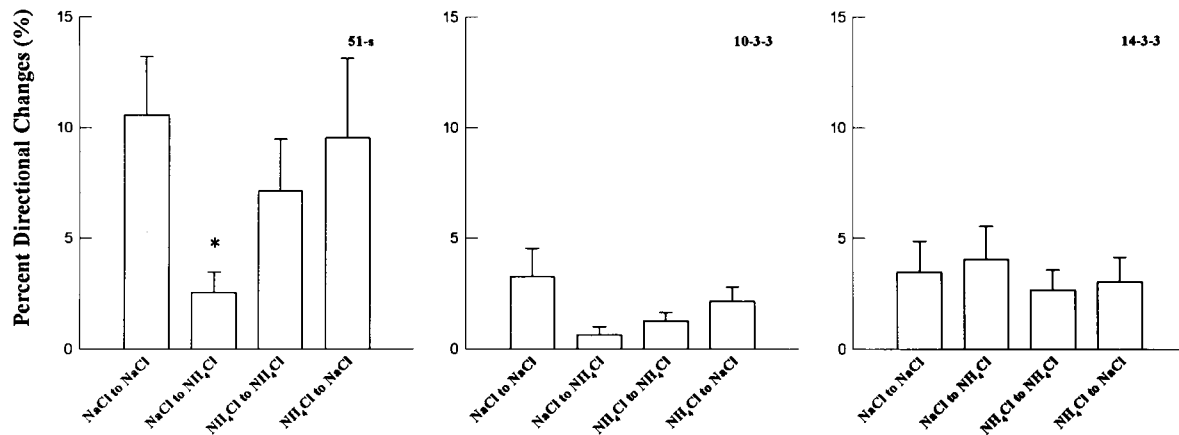


Fig. 5. Percent directional changes (PDC) for strains 51-s (wild type), 10-3-3, and 14-3-3. Cells from each population were incubated for 30 min in 5 mM NaCl or 5 mM NH₄Cl buffer and then transferred to 5 mM NaCl or 5 mM NH₄Cl buffer, as indicated. Behavior was examined

over the first 15 sec after transfer. Errors bars represent s.e.m. $n = 6$ experiments for 51-s and 10-3-3, $n = 5$ experiments for 14-3-3. Asterisks indicate significant difference from the control using the Mann-Whitney U test ($P < 0.05$).

attraction is sustained and increases over a 30 min time course.

While the T-maze assay is useful in determining a population behavioral response, it cannot be used to analyze the swimming behavior parameters of individual cells that contribute to the population response. To better characterize the swimming parameters of *Paramecium* that are affected by NH₄Cl, computerized motion analysis is used.

The swimming parameters that are relevant to chemoattraction are swimming speed and frequency of turning [Van Houten, 1978]. We have shown previously that speed and frequency of turning (with adaptation) are sufficient parameters to simulate chemoresponse in T-mazes [Van Houten and Van Houten, 1982; Van Houten, 1990]. Here we measure swimming speed and percent directional change (PDC), which is a function of turning frequency (see Materials and Methods).

Swimming speed and PDC are measured for two situations that a cell in a T-maze assay might encounter: after crossing the boundary from NaCl into NH₄Cl, or after swimming from NH₄Cl into NaCl. Speed and PDC are also measured for the appropriate control transfers: from NaCl to NaCl or from NH₄Cl to NH₄Cl, respectively. Wild type cells significantly increase swimming speed upon introduction to 5 mM NH₄Cl from 5 mM NaCl, compared with their speed in the control transfer from 5 mM NaCl to 5 mM NaCl (Fig. 4). Likewise, swimming speed significantly decreases after cells, adapted to 5 mM NH₄Cl, are transferred to 5 mM NaCl, compared with speed in the control transfer from 5 mM NH₄Cl to 5 mM NH₄Cl. PDC values decrease after introduction to NH₄Cl, and increase in cells as they are transferred from NH₄Cl to NaCl, but this latter change is not statistically significant (Fig. 5). We also observed that

PDC, in control or stimulus conditions, show a high degree of variability from one population to the next (data not shown).

The behavioral mutant 10-3-3 also exhibits a significant increase in swimming speed when encountering NH₄Cl from NaCl (Fig. 4), but no significant differences are noted for PDC (Fig. 5). Neither PDC nor swimming speed are altered by transfer from NH₄Cl to NaCl. Mutant strain 14-3-3 does not show significant changes in swimming speed (Fig. 4) or PDC (Fig. 5) after either of the experimental transfers ($P > 0.05$).

DISCUSSION

Intracellular pH

Paramecium tetraurelia is a freshwater ciliate that has the ability to sense and respond to various chemical signals in its environment. Certain chemical compounds, such as lactate, acetate, folate, cyclic AMP, biotin, glutamate, and NH₄Cl, are attractants of *Paramecium*. Here we examine *Paramecium* to see whether NH₄Cl alters pH_i and whether these changes correlate with modulation of swimming behavior necessary for attraction. The motivation for examining pH_i comes from the differences between the signal transduction pathways for other attractants and NH₄Cl. First, the calcium pump is not involved in the NH₄Cl chemoresponse. We have three lines of indirect evidence for this. (1) Lithium, which reduces Ca²⁺ homeostasis efflux from cells and perturbs chemoresponse to acetate, folate, and lactate, has no effect on chemoresponse to NH₄Cl, suggesting that Ca²⁺ efflux may not be linked with attraction to NH₃ as it is with other attractants [Wright et al., 1992]. (2) The Ca²⁺ homeostasis mutant, K-shy [Evans et al., 1987], does not respond to acetate or folate, but responds normally to

NH₄Cl [Van Houten, 1990]. (3) Antisense down regulation of calmodulin levels to indirectly affect the pump has no effect on increased motility in NH₄⁺Cl, which is characteristic of behavior in attractants; however, the same antisense treatment inhibits increased motility in response to the attractant acetate [Yano et al., 1996]. Second, intracellular cyclic AMP levels rapidly rise after stimulation by glutamate, but not after stimulation with other organic attractants or NH₄Cl [Yang and Van Houten, 1993; Yang et al., 1997]. This further points to at least three different signal transduction pathways for the attractants acetate, glutamate and NH₄Cl. Third, no analog appears to compete for NH₄Cl attraction, reinforcing the possibility that there is no cell surface receptor for NH₄Cl. In light of these observations, we wanted to explore the possibility that NH₄⁺ acted by alkalinizing the cell, possibly by entering as NH₃ without interacting with a cell surface receptor.

Our first approach was to measure p*H*_i in suspensions of cells. Basal p*H*_i was found to be ≈ 6.7 in the wild type cells, and this increased by about 0.1 pH unit after exposure to NH₄Cl. Interestingly, after maximal alkalinization, p*H*_i appears to decline within 10 min to a level around or below basal p*H*_i. This is surprising since intracellular acidification to values close to or lower than basal levels is often seen after removal of external NH₄⁺, due to the dissociation of intracellular NH₄⁺ that was taken up slowly during incubation in ammonium solution [Roos and Boron, 1981]. While this slow uptake of NH₄⁺ can also account for a slow acidification as we see in *Paramecium* following an initial rapid NH₄Cl-induced rise [Boron and DeWeer, 1976], this acidification would not be expected to reach the magnitude and relatively rapid time course as shown in Figure 1 [Madshus, 1988].

For example, p*H*_i in squid giant axons acidifies after NH₄Cl-induced alkalinization from approximately 7.7 pH units to almost 7.6, but this acidification requires almost 2 h, and the basal p*H*_i is never approached [Boron and DeWeer, 1976].

Alkalinizations in response to NH₄Cl were also observed in the mutant strains. In suspension, the mutants exhibited basal levels of p*H*_i much lower than the wild type (p*H*_i ≈ 6.7 for wild type cells [Fig. 1a], <6.40 for 10–3–3 [Fig. 1b], ≈ 6.55 for 14–3–3 [Fig. 1c]). These data suggest that (1) the mutants might possess some faulty p*H*_i regulatory mechanism and (2) this regulatory mechanism (or perhaps simply the low basal p*H*_i) might somehow be involved in the lack of sustained attraction to NH₄Cl.

We also measured p*H*_i in single wild type cells, which clearly demonstrated an intracellular alkalinization as a result of NH₄Cl (Figs. 2, 3). While the p*H*_i values obtained by single cell measurements appear to differ slightly from those obtained in suspension, especially for

basal p*H*_i of wild type cells (p*H*_i 6.68 ± 0.02 in cell suspension measurements, vs. 6.56 ± 0.08, s.e.m., n = 3, in single cell measurements), the absolute values should be regarded cautiously since the single cell data are taken from a much smaller sample size and from deciliated cells. Additionally, the BCECF calibration curve used for these single cell experiments was generated with dye in buffer, whereas dye loaded within the cells was used for calibration for the cells in suspension. Since it was possible that the spectral properties of BCECF in buffer (5 mM NaCl buffer) might differ from those exhibited in the cell, we determined that the spectra of the dye in buffer and in individual cells were the same before proceeding with the single cell measurements. Another potential source of disparity between the cell suspension and the microfluorometric measurements is the possibility of error in the assumed intracellular [K⁺] necessary for the in vivo calibration of suspension data. In vivo calibrations using nigericin are performed for the cell suspension measurements in the presence of external K⁺ [Thomas et al., 1979]. The external [K⁺] is generally set near the intracellular [K⁺] to allow the intracellular [H⁺] to equilibrate to the external [H⁺]. If this chosen external [K⁺] is different from the actual intracellular [K⁺], the calibration curve may be shifted, giving spurious results (see Materials and Methods for choice of K_i value).

The qualitative agreement in p*H*_i measurements in suspension and single cells confirms the alkalinizing effect of NH₄Cl on wild type *Paramecium*. p*H*_i values for the mutants also are in fair agreement between suspension and single cell measurements. The absolute differences between these methods of measuring *Paramecium* intracellular pH could be due to differences in calibration of the dye (see Materials and Methods) or the deciliation of the cells used in single cell measurements. Unfortunately, it is not possible to keep deciliated cells in adequate suspension by stirring alone without damaging them to provide a deciliated suspension control. While the two methods are in qualitative agreement in measurements of wild type cells, the single cell measurements do not bear out the low basal p*H*_i measurements made in suspension cultures of mutants 10–3–3 and 14–3–3. Of the two mutants, 14–3–3 had the lower basal pH value in suspension measurements, and in the single cell measurements, we used values only from those cells that showed p*H*_i alterations in response to NH₄Cl. The cells that did not respond to NH₄Cl were not included in the data in single cell measurements (Table II), but these unresponsive cells, about half of those tested, would be included in the suspension measurements. The difference among the 14–3–3 cells is not due to mixtures of genotypes because the cells have been subcloned and the population should be genetically identical. (Very small changes in speed and PDC are adequate to explain accumulation of populations

TABLE II. Intracellular pH From Single-Cell Measurements*

Strain	Basal pH_i (before NH_4Cl)	Peak pH_i (after NH_4Cl)
Wild type (51-s)	6.56 ± 0.14	6.88 ± 0.18
10-3-3	6.51 ± 0.16	7.09 ± 0.18
14-3-3 ^a	6.55 ± 0.07	6.90 ± 0.18

*Intracellular pH measured in individual cells before (basal) and after stimulation with NH_4Cl . Deciliated cells loaded with BCECF were bathed in 5 mM NaCl buffer and the perfusate was switched to 5 mM NH_4Cl buffer. Data shown are the averages and SD for $n = 3$ cells (wild type and 10-3-3) or $n = 5$ cells (14-3-3).

^aData representing only those cells that responded to NH_4Cl (see text).

of cells [Van Houten and Van Houten, 1982]. Therefore, behavioral data do not assist in determining more about the heterogeneity of cell pH responses in 14-3-3 because of the inherent large variability in the behavioral measurements, which would obscure the small differences in speed and PDC between responding and non-responding populations of 14-3-3.) Additionally, it is not likely that the subset of cells that are unresponsive (approximately 50% of those examined) are impermeable to NH_3 , but the reason for their failure to alkalinize is not known. These cells, like 10-3-3 and wild type, are attracted to acetate solutions, which we know acidify the wild type cells [Davis, 1994], but this does not shed more light on possible mechanisms for their defects in response to NH_4Cl .

Perhaps the most persuasive demonstration of alkalization of single cells in response to NH_4Cl are the data from puffing the stimulus onto the cells (Fig. 3). Also demonstrated with puffer application is a tendency for pH_i to acidify after removal of external ammonium. The magnitude of this acidification appears to correlate with the duration of NH_4Cl -exposure, as has been seen in other systems such as squid giant axon [Boron and DeWeer, 1976]. This is presumably due to prolonged influx of NH_4^+ into the cell, which dissociates to NH_3 and H^+ and is maintained in the cell when NH_4Cl is removed. The exposures of single cells to NH_4Cl are much shorter than the exposures of cells in suspension (e.g., 200 sec vs. 20 min), which could explain why we do not see recovery of the pH in single cells in NH_4Cl . The acidification induced by puffer application of NH_4Cl is reminiscent of that seen in cell suspension in the continued presence of NH_4Cl , although it is uncertain whether these phenomena are related. Measurements of pH_i in individual cells that were superfused first with NaCl then NH_4Cl and then NaCl again did not consistently show a post-alkalinization acidification. It is possible that acidification might occur but might not be seen simply because replacement of NH_4Cl by NaCl in the bath was very gradual compared to the rapid return to NaCl with the puffer technique.

Comparison With Previous Work on Paramecium

Microelectrode measurements in a different species, *P. caudatum*, indicate a pH_i of 6.80 [Umbach, 1982]. These are similar to (and support) our cell suspension results for wild type cells. Umbach found that treatment of cells with NH_4Cl resulted in an elevation of pH_i to >7.1 , more than 0.2 pH units higher than our observations. However, the concentration of NH_4Cl used by Umbach was 10 mM, twice our concentration, which may account for the magnitude difference between the two sets of results. Additionally, Umbach did not report a post-alkalinization decrease of pH_i after exposure to NH_4Cl even after 10 min. This, however, may be attributable to a slow rate of bath change, since peak alkalization did not occur until about 10 min after switching to NH_4Cl , at which point the superfusate was returned to NH_4^+ -free solution [Umbach, 1982].

Correlation Between pH_i and Motility

In order to determine whether the observed changes in pH_i responses are related to motility responses to stimulus, we analyzed both population and individual cell behavior. We confirmed with T-maze assays that wild type cells show attraction to NH_4Cl relative to NaCl over a short time (within 2 min) and that this attraction is sustained and increases over 30 min, while the T-maze analysis of the mutants showed they have the ability to detect and respond to NH_4Cl , but only initially. These strains showed slight attraction to NH_4Cl after 2 min, but this attraction was not sustained after 30 min (Table I).

We proceeded to examine in wild type and mutants two swimming parameters that are the most important contributors to the attractant response: swimming speed and frequency of turning [Van Houten, 1990; Van Houten and Van Houten, 1982]. Computerized motion analysis was used to determine cell swimming speed and percent directional changes, or PDC, which is a representative parameter of turning frequency [Clark and Nelson, 1991]. Swimming responses to most attractants include a moderate increase in speed and a slight decrease in turning frequency relative to behavior in control solutions [Van Houten, 1978]. Conversely, removal of cells from a relative attractant to control results in a decrease in swimming speed and an increase in turning frequency [Van Houten, 1978]. Therefore, the response of cells transferred both to and from NH_4Cl was also observed.

Computerized analysis showed that when wild type cells were transferred from NaCl to NH_4Cl , the cells' swimming speed increased, and PDC decreased, consistent with an attractant response. Conversely, swimming speed decreased and PDC increased upon transfer from NH_4Cl to NaCl, observations that are consistent with a relative repellent response. The swimming speed of wild type cells after the two control transfers (from NaCl to

NaCl or from NH_4Cl to NH_4Cl) was approximately the same and was consistent with behavioral adaptation back to a basal level given sufficient time after transfer from a relative repellent (NaCl) to an attractant (NH_4Cl). However, PDC was highly variable and the increase was not statistically significant.

Basal swimming speed of the mutants in NaCl was similar to that of the wild type and, as in the wild type, strain 10-3-3 showed significant increases in swimming speed, but here the similarity ended. Mutant 10-3-3 did not show significant changes in speed when transferred from NH_4Cl to NaCl and showed no significant change in PDC under either transfer condition. There were no statistically significant changes in swimming speed or PDC when strain 14-3-3 was transferred either to or from NH_4Cl . It is possible, however, that any statistical differences may be masked by the variance of behavior noted between one mutant population and the next.

Mechanism

There is a general temporal correlation between the rapid change in pH_i in normal cells, the attraction of these cells to NH_4Cl , and the expected change in speed and PDC that are known to be necessary for population accumulation in attractant stimuli [Van Houten, 1990]. However, the site of action of the pH effect is not known at this time. It is possible that pH has an effect on dynein function, changing ciliary beating frequency and power stroke (hence turns). Sea-water induced initiation of motility in sea urchin sperm is dependent upon intracellular alkalinization, which activates flagellar dyneins [Christen et al., 1983]. However, this would not address the mechanism by which cells hyperpolarize in NH_4Cl , and hyperpolarization has effects on axonemal function. Alternatively, there could be a direct effect on membrane potential, ion channels, or pump function by pH_i . At the moment, we have no evidence to distinguish between these alternative mechanisms.

It is difficult to explain the defects in the mutants that are unable to sustain a response to NH_4Cl . First, one would expect to see an initial change in speed and in mutant PDC upon transfer from NaCl to NH_4Cl because the cells can, over 2 min, show attraction to NH_4Cl in T-mazes. The cells do show alkalinization, although of different magnitudes and their basal pH_i levels are below those of wild type cells. Notably in suspension measurements, the alkalinization appears to be slower than in the wild type cells (compare peak alkalinizations in Figs. 1-3). The slower time course is not born out in the single cell measurements for 10-3-3, but half of the population of 14-3-3 shows no change in pH_i at all in NH_4Cl (Fig. 2).

We know from experiments with Pawn mutants that cannot make abrupt swimming path turns, that the modulation of turning frequency is essential for chemore-

sponse, including in T-mazes [Van Houten, 1990]. Therefore, we expected to see modulation of PDC even in the mutants because they are initially attracted in T-mazes, and intracellular alkalinization does occur, albeit more slowly, in NH_4Cl . However, the expected changes in speed and PDC did not occur. Therefore, either pH_i does not govern the initial change in speed and PDC or the mutants may just be slow to translate the change in pH_i into a change in speed and PDC in the short time after transfer. This latter explanation is a distinct possibility because the time course of the alkalinization differs among wild type and mutants in suspension measurements, although we did not attempt a kinetic analysis.

pH Regulation in *Paramecium*

We have little insight into pH_i -regulatory mechanisms in *Paramecium* at present. Amiloride, a blocker of Na^+/H^+ exchange has been used in perturbing the attractant effects of acetate on *Paramecium* [Van Houten and Preston, 1985]. Including 0.5 mM amiloride in both arms of the T-maze effectively blocked attraction of cells to Na-acetate from NaCl. However, this is a secondary effect since amiloride itself is a strong repellent in Na^+ , but not K^+ , solutions, and amiloride does not inhibit attraction to K-acetate relative to KCl. Thus, some Na^+ -dependent carrier may be at work in the cell, which, if inhibited, could result in depolarization of the cell.

Many candidate transporters and mechanisms could affect *Paramecium* pH homeostasis, such as an electrogenic ATP-dependent $\text{Ca}^{2+}/\text{H}^+$ antiport [Yu et al., 1993] and H^+ ATPase pump. There is evidence for a H^+ pump from cloning open reading frames in *Paramecium* (Van Houten and Elwess, unpublished results). However, a great deal remains to be explained in *Paramecium* pH control. Until other possible contributors to pH homeostasis are explored, we can only speculate about the site of defects in the mutants and mechanism(s) of NH_4 acidification.

Summary

In summary, we have shown that NH_4Cl , at a concentration that is attractant in behavioral assays, does alkalinize pH_i . We have also demonstrated a surprising drop in pH_i within 10 min after continuous exposure to NH_4Cl , both in the wild type and in behavioral mutants that show initial but not sustained attraction to NH_4Cl in the standard behavioral assay, the T-maze. Interestingly, in these mutants there is an initial NH_4^+ alkalinization similar in magnitude to that displayed by the wild type, but the basal pH_i of these mutants appears to be lower. *Paramecia* swim faster and turn less frequently upon stimulation with the attractant NH_4Cl . Mutants unable to sustain attraction to NH_4Cl show diminished behavioral responses and these behavioral defects likely contribute

to abnormal population responses in T-maze testing for attraction to NH_4Cl relative to NaCl . However, it is not possible at this time to assign a cause of their population behavior defect to these swimming parameters.

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