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Chemosensory Transduction in *Paramecium*

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Summary

***Paramecium tetraurelia* is attracted and repelled by a variety of chemical stimuli. The attractants probably signify the presence of nutrients. For attractants, there are at least three signal transduction pathways that all lead to a hyperpolarization of the cell that results in relatively fast and smooth swimming. The three pathways differ in stimuli, receptor mechanisms and second messengers. Receptors for one and possibly two of the pathways couple surface events to the activation of a plasma membrane calcium pump. One pathway is not receptor-mediated, but affects intracellular pH by diffusion of the stimulus across the membrane. A variety of techniques are described in an effort to probe the steps of each of these pathways, which remain only partially defined.**

Key words: *Paramecium*; Cycle AMP; Glutamate; Chemoresponse; Signal transduction; Cilia; Intracellular pH.

Introduction

Paramecium tetraurelia detects environmental stimuli that include chemical cues. The cells respond to these cues by changing swimming patterns that ultimately lead to attraction or repulsion of populations of cells. This paper will focus primarily on three signal transduction pathways for attraction of these ciliates to chemical cues that most likely signal the presence of bacteria, that is, food [22]. First, however, it will be useful to explain the physiology underlying the behavioral response.

Ciliary beating controls the swimming patterns of paramecia and ciliary beat (the beat frequency, angle of cilia to the body, and "reversal" of the power stroke) has been shown to be controlled in turn by membrane potential [reviewed ref. 10]. Generally speaking, with a relative hyperpolarization, the beat frequency increases and the cilia beat more directly toward the posterior, making the cell swim more quickly. A relative depolarization does the converse. A sufficiently large depolarization will initiate a calcium action potential, which will transiently increase calcium in the cilia and change the power stroke of the cilia so that the cell backs up for a body length or two. Once calcium is reduced by calcium pumping or sequestration, the cilia will beat with the normal power stroke. The whole maneuver takes less than one second and the result is a turn of the cell, which sets off in an almost randomly established new direction.

We have observed that, generally, attractant stimuli cause smooth, fast swimming, characteristic of a hyperpolarization; and that repellents cause depolarization and the converse behavioral response [19, 21]. Electrophysiological measurements confirm that attractant and repellent stimuli do, indeed, hyperpolarize and depolarize respectively [13, 20]. Figure 1 shows more recent confirmation of the smooth swimming paths stimulated by the attractant glutamate and short, jerky paths stimulated by the repellent inosine monophosphate (IMP).

Manipulation of speed of swimming and frequency of turning with adaptation is sufficient to cause a population of paramecia to accumulate near, or disperse from, the source of chemical cues [20, 23, 24]. Perhaps the more important parameter for a chemoresponse is the ability to execute a turn because Pawn mutants [15], which are unable to turn because they are defective in generating the calcium action potential, are not generally attracted or repelled [19].

Materials and Methods

Cell cultures: *Paramecium tetraurelia* stock 51-S (sensitive to killer) were used throughout, with the exception of two mutants derived from 51-S and described [24]. Culturing is described elsewhere [16, 24].

Electrophysiology: Standard methods for measuring membrane potential and voltage clamping are described elsewhere [13, 20].

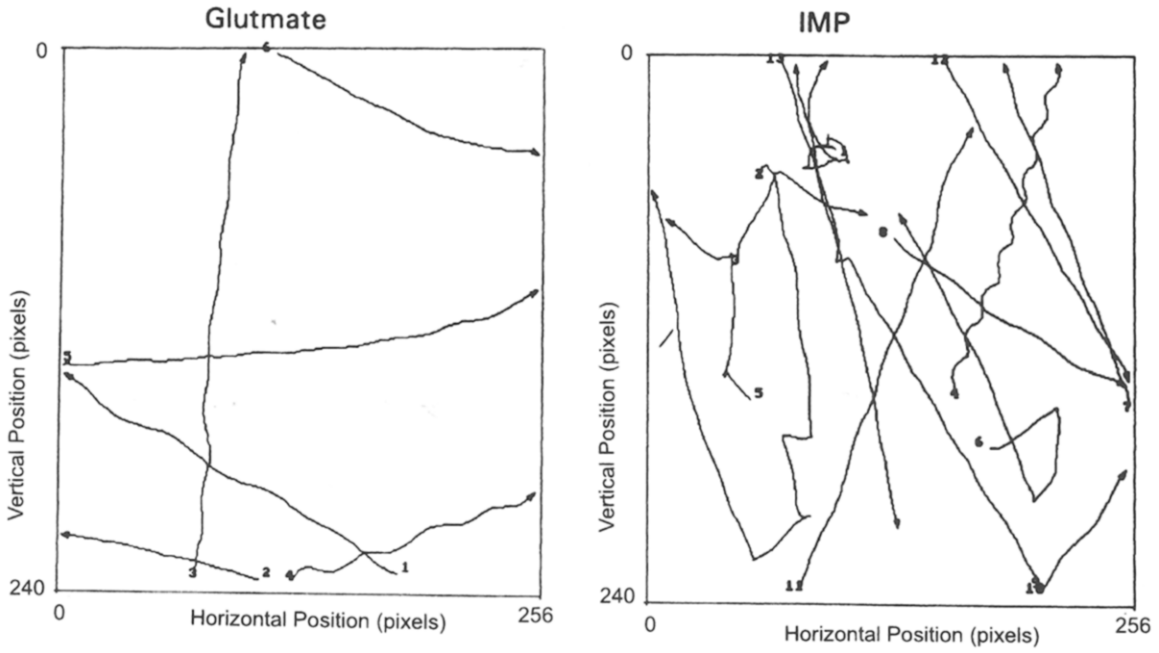

Fig. 1 Tracks of swimming cells in 5 mM K-L-glutamate in chemokinesis buffer and 1 mM K_2IMP in chemokinesis buffer. Tracks were generated by analysis of video tapes of the swimming cells using Expert Vision software and Motion Analysis system hardware. Cells swim fast and smoothly in glutamate, and swim more slowly and turn frequently in IMP.

Membrane biochemistry: See [26] for descriptions of affinity chromatography and membrane preparation. The pellicle preparation, calcium ATPase assays and calmodulin overlays were done as described elsewhere [29–31].

Fluorescent dye for pH measurement: Use of BCECF-AM for intracellular pH measurement was done as described elsewhere [5]. Cells were incubated with 5 µM BCECF-AM in the chemokinesis buffer for 30 min, removed from the dye by centrifugation, resuspended in buffer (e.g. chemokinesis buffer used for behavioral assays, 5 mM KCl, 1 mM $Ca(OH)_2$, 1 mM citric acid, 1.3 mM Tris base, pH 7), and then washed again immediately before use. A Hitachi 2000 fluorometer was used for cation analysis of populations. A Delta scan system was used (in collaboration with J. Fiekers, Univ. of Vermont) for individual cell analysis. Calibration of pH was done using nigericin, 18 mM KCl extracellular solution, and known pH adjusted with NaOH.

Cloning and molecular biology: The cloning of the calcium pump gene is described elsewhere [6]. The subcloning, mutagenesis and expression of the calmodulin binding domain is described in [35] and Yano, personal communication. The use of antisense oligonucleotides is described [35].

Behavioral assays: T-mazes were used as behavioral assays of chemoresponse as described [24].

Immunocytochemistry: *P. tetraurelia* were fixed in 2% paraformaldehyde, permeabilized with 0.05% Triton X-100, incubated with a 1:100 dilution of antiserum: phosphate buffered saline (PBS), incubated with a 1:1000 dilution of secondary antibody (10 µg/ml) (with fluorescein or Texas red conjugate). The cells were observed with a BioRad 200 confocal microscope.

Results and Discussion

Pathway 1: The stimuli cAMP, folate, acetate, lactate and biotin, among others, attract paramecia probably through the same signal transduction pathway. There are cell surface binding sites for cAMP, folate, acetate and biotin that can be analyzed for their number, affinity and other properties [1, 2, 16–18]; one receptor protein for cAMP has been purified [26], and other binding proteins that could be receptors are partially purified [2, 11, 12; W. Bell and C. Paquette, personal communication]. These biochemical, binding kinetic studies, and interference studies using behavioral assays of chemoresponse [reviewed in 2, 21, M. Gagnon and W. Bell, personal communication] suggest that the responses to these stimuli begin at separate receptor sites. After these stimuli bind to receptor, the cell hyperpolarizes 8–10 mV and this change in membrane potential affects ciliary beating making the cells swim more quickly and smoothly. Between the receptor and the hyperpolarization, there are many undefined steps in signal transduction, but we do know that the hyperpolarizations for folate and acetate are not due solely to Na or K channel activity since no reversal potential or dependence on extracellular K or Na could be demonstrated [13]. However, there is a measurable conductance for acetate stimulation (0.2 nA, Preston and Van Houten, unpub-

lished). We believe that this conductance is generated by the activation of a calcium pump of the plasma membrane.

There is a demonstrable calcium ATPase activity of the cell pellicle membranes [29], which are the surface membranes after removal of cilia and with cytoskeleton attached [3]. The activity has all the hallmarks of a plasma membrane calcium pump: it has ATP, Mg, and Ca dependence; a requirement for Na or K for full activity; is inhibited by vanadate, calmodulin inhibitors, but not oligomycin or azide; affinity of ~90 nM for calcium. The pellicle membranes, but not the ciliary membranes, show a phosphoenzyme intermediate of about 133 kD, the expected mass of a calcium pump of the plasma membrane as opposed to intracellular pumps [29, 31] (intracellular calcium pumps would not be calmodulin regulated, and therefore, would not have the additional C terminal amino acids that work as an autoinhibitory peptide and calmodulin binding domain for activation by calmodulin).

Indirect, correlative evidence links this calcium pump of the pellicle to chemoresponse: 1) lithium inhibits the pump ATPase activity, the calcium homeostasis efflux from paramecia, and the chemoresponse to acetate, folate, cAMP but not the chemoresponse to ammonium, a control attractant for a different pathway; 2) K-shy A/B double mutant (courtesy of Dave Nelson) is not attracted to these same stimuli but is attracted to ammonium [30]. This mutant has an undefined defect in calcium homeostasis [8].

The gene for one isoform of a plasma membrane calcium pump has been cloned [6] and is 43% identical at the amino acid level to the human sequence. The C terminus autoinhibitory end is shorter than in most plasma membrane calcium pumps, except for that in *Entamaoeba histolytica* (acquisition number U20321) and therefore, we expressed the C terminus to verify that it did bind to calmodulin. The C terminus sequence was mutated to remove UAA codons for glutamine, and expressed in bacteria as a glutathione S-transferase fusion protein. Both the cleaved protein (approx. 10 kD) and fusion protein bind to calmodulin in calmodulin overlays of electroblots and both are recognized by an antibody against the last 17 amino acids of the pump (Fig. 2), as is a 130 kD pellicle protein.

In order to take a different approach to testing for the role of the pump in chemoresponse, we adopted the methods of Hinrichsen to study *Paramecium* calmodulin and electroporated antisense or sense oligonucleotides (ODNs) against calmodulin mRNA into cells [9]. Our rationale was that down regulation of calmodulin should affect pump activity, and we found that the antisense ODNs inhibited the chemoresponse to acetate but not to ammonium [35]. The effect of antisense oligonucleotides on response to glutamate could not be assessed because the electroporation itself disrupted the cells' ability to respond to glutamate. We did, however, demonstrate that the antisense oligonucleotides reduced calmodulin mRNA levels and protein levels using a semi-quantitative RT-PCR method and microscale ELISA assay [data not shown, 27].

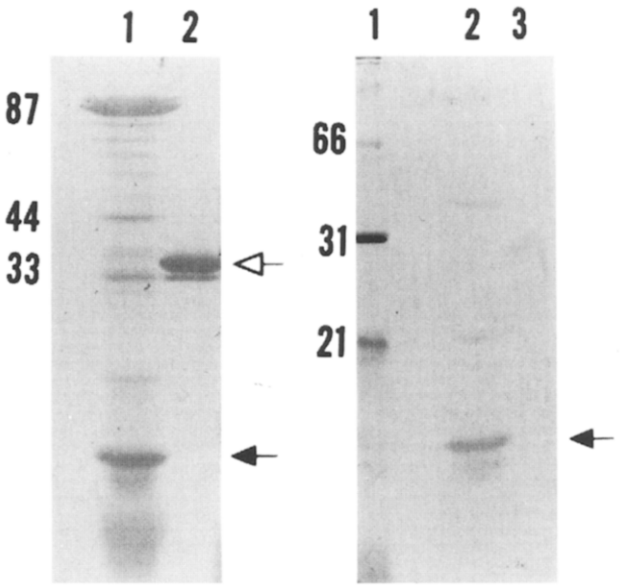

Fig. 2. Calmodulin binding domain. The calmodulin binding domain of the *Paramecium* plasma membrane calcium pump was expressed as a GST fusion protein and cleaved from GST by thrombin in the presence of BSA. **A.** Lane 1: calmodulin binding domain peptide (filled arrow) and BSA, lane 2: expressed GST alone (open arrow) separated on SDS PAGE and stained with amido black. **B.** SDS PAGE separation of same proteins as in A, but incubated with biotinylated calmodulin and developed with avidin-alkaline-phosphatase [see 29, 31]. Note calmodulin binding to the calmodulin binding domain peptide (closed arrow).

The autoinhibitory C terminus of the pump is being over-expressed in paramecia in order to explore the role of the pump further. The C terminus not only binds to calmodulin [36], but also has sites for activation by phosphorylation by PKA and PKC, which makes it a very interesting site for multiple regulation mechanisms [7, 28].

We have developed antibodies for both the cAMP receptor and calcium pump C terminus, and we have used these antibodies in confocal microscopy for the visualization of the location of these signal transduction components [36]. The receptor is strictly surface-associated (Fig. 3A) and immunoreactivity is in a pattern on the surface that corresponds to areas of the cortical units between the ridges (Fig. 3B). The pump antibody shows some immunoreactivity with trichocysts but the surface labeling corresponds, like that of the receptor

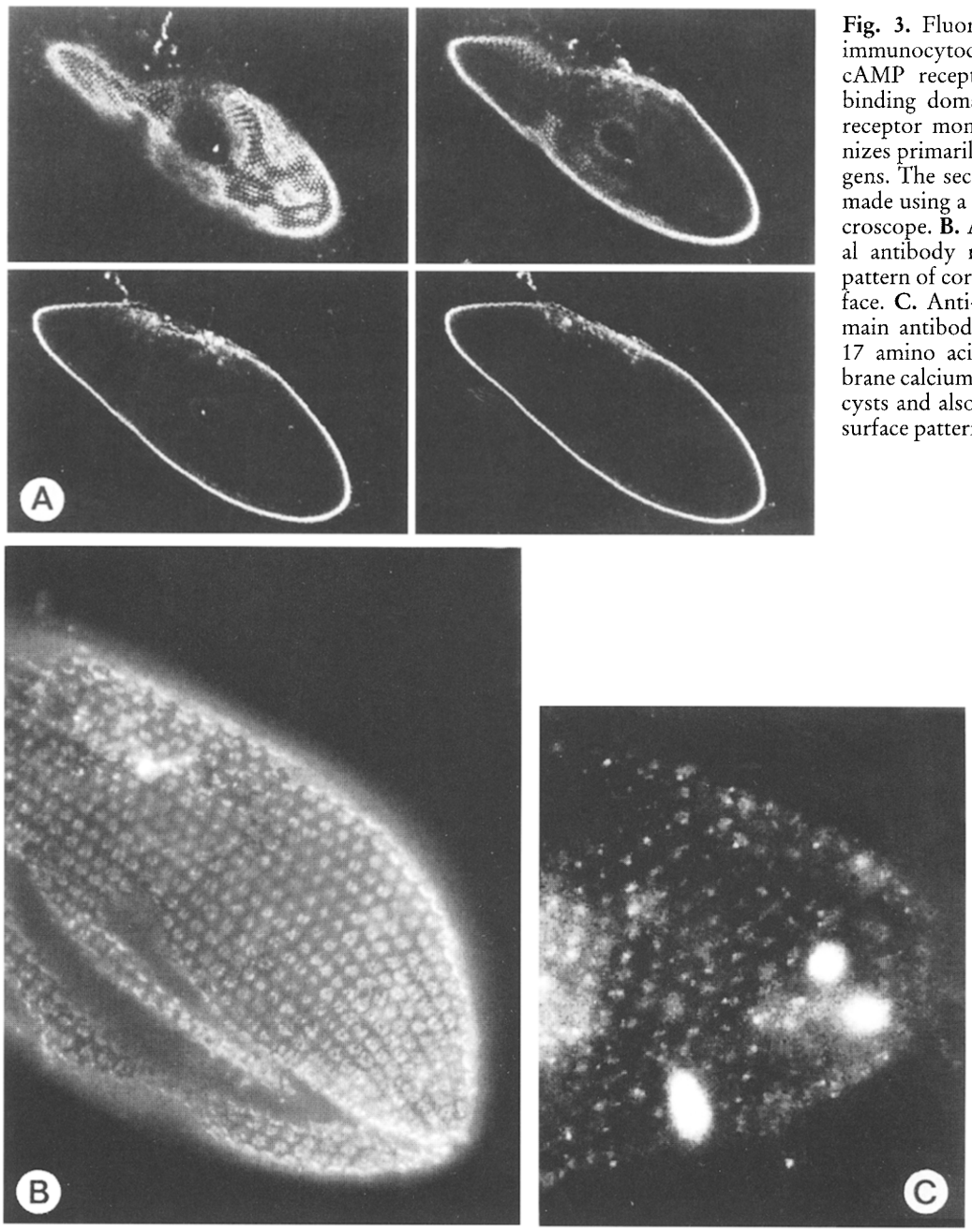

Fig. 3. Fluorescence micrographs of immunocytochemistry using anti-cAMP receptor and anti-calmodulin binding domain antibodies. **A.** Anti-receptor monoclonal antibody recognizes primarily surface associated antigens. The sections are optical sections made using a BioRad 200 confocal microscope. **B.** Anti-receptor monoclonal antibody recognizes antigens in a pattern of cortical units on the cell surface. **C.** Anti-calmodulin binding domain antibody (made against the last 17 amino acids of the plasma membrane calcium pump recognizes trichocysts and also an antigen that shows a surface pattern.

immunoreactive, to areas of the cortical units. There are important differences between the surface immunoreactivity patterns. The areas of immunoreactivity to the pump are restricted to one or two central areas of each unit (Fig. 3C), probably to the base of the one to two cilia per unit, while the receptor immunoreactivity is more broadly spread and generally not found at the center where the cilia arise. However, double labeling does show a mingling of the immunoreactivities (data not shown), which is consistent with the physical or indirect interaction of the receptor and pump in the transduction pathway. We would expect some overlap if the two signal transduction components were coupled and this area of study is being pursued.

Pathway 2: The second pathway for chemoresponse is for glutamate and IMP. There are specific binding sites for glutamate on the *Paramecium* surface, some of which are specific for glutamate and some of which can also bind IMP [33, 34]. One interpretation of the binding data is that there are receptors that mediate the attractant response to glutamate and others that mediate the repellent response to IMP and at which glutamate acts as an antagonist of the repellent response. Other interpretations cannot be ruled out at this point.

Glutamate induces a very rapid increase in intracellular cAMP in the cells. In relatively slow measurements beginning at 1 second, we see intracellular cAMP increase by 3-fold in glutamate stimulated cells, but we are actually measuring its declining phase (Fig. 4A). In collaboration with Helmut Plattner, we find that intracellular cAMP actually increases by 3-fold in 30 msec and 7 fold in 200 msec (Fig. 4B) [32]. These measurements require a rapid mixing apparatus that allows for rapidly stimulation of intact cells. The results are consistent with a role for cAMP in the signal transduction pathway as opposed to a slower process such as adaptation.

The coupling of the receptor to the adenylyl cyclase is not clear at this time. A G-protein could fill this role, but there is only preliminary evidence for trimeric G proteins in *Paramecium tetraurelia* (de Ondarza, personal communication), and no evidence yet for a role in chemoresponse. At this meeting there are reports of *Tetrahymena* (Renaud, personal communication) and *Euplotes* (Luporini, personal communication) G_α proteins being involved in signal transduction, which makes it all the more feasible that such G proteins in *Paramecium* have a role in chemoresponse.

Glutamate has been shown by Preston [14] and ourselves to hyperpolarize cells, albeit at different concentrations and conditions. The hyperpolarization could be due to channel activity [14] but there also is evidence for activation of the calcium pump: 1) Lithium inhibits glutamate chemoresponse, 2) K-shy A/B is not attracted to glutamate, 3) calmodulin antisense oligonucleotides in stably transformed cells inhibit glutamate chemoresponse [Yano, personal communication, W. Q. Yang, Ph. D. thesis, 1995; and 33, 34]. The coupling between receptor and adenylyl cyclase again is not clear, but heterologous PKA can activate the pump activity by 85% *in vitro* [33, 34], opening the possibility for the cAMP increase in response to glutamate to modulate the pump and generate a hyperpolarization by pump activation.

Pathway 3: The third pathway has only one stimulus to date, NH_4Cl. No other amine-containing compound that we have tried will compete or interfere with the response to NH_4Cl (Gagnon and Van Houten, personal communication). At pH 7, NH_4Cl will be in equilibrium with NH_3, which can cross the cell membrane and alkalinize the cells. We have shown with the fluorescent dye, BCECF, that cells in suspension alkalinize rapidly in NH_4Cl and then slow-

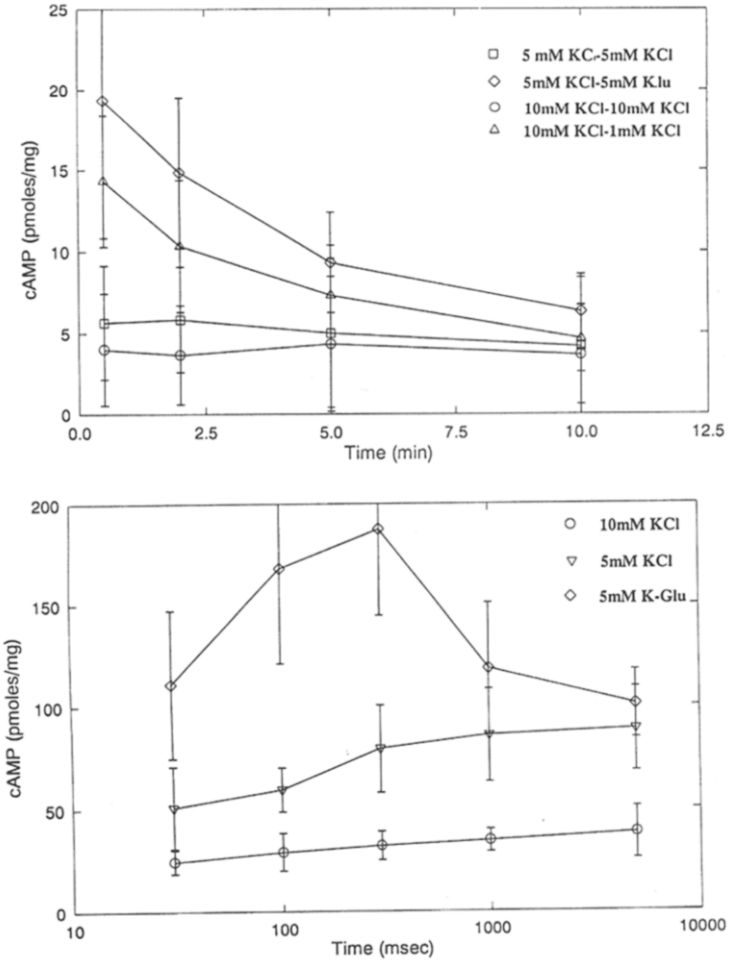

Fig. 4. Intracellular cyclic AMP increases with glutamate stimulation. **A.** Slow time course of cAMP increase with glutamate stimulation. **B.** Rapid time course using quench flow apparatus in collaboration with H. Plattner [32] (with permission).

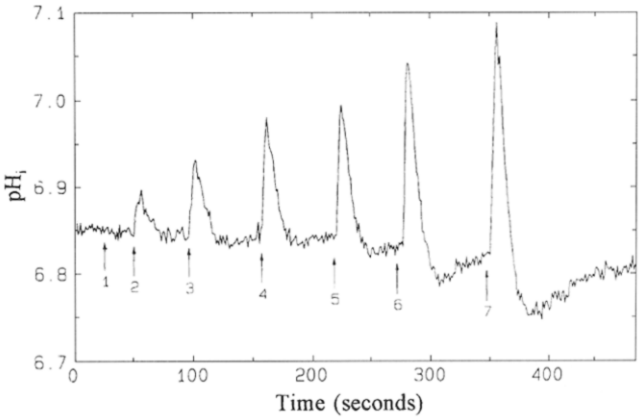

Fig. 5. Fluorescence from a single cell loaded with BCECF-AM pH sensitive fluorescent dye. NH_4Cl is puffed onto a single cell and fluorescence intensity increases, indicating an increase in pH inside the cell. As duration of the puff increases from 100 msec, 300 msec, 500 msec, 750 msec, 1 sec, 2 sec to 4 sec, the intracellular alkalinization increases (with permission).

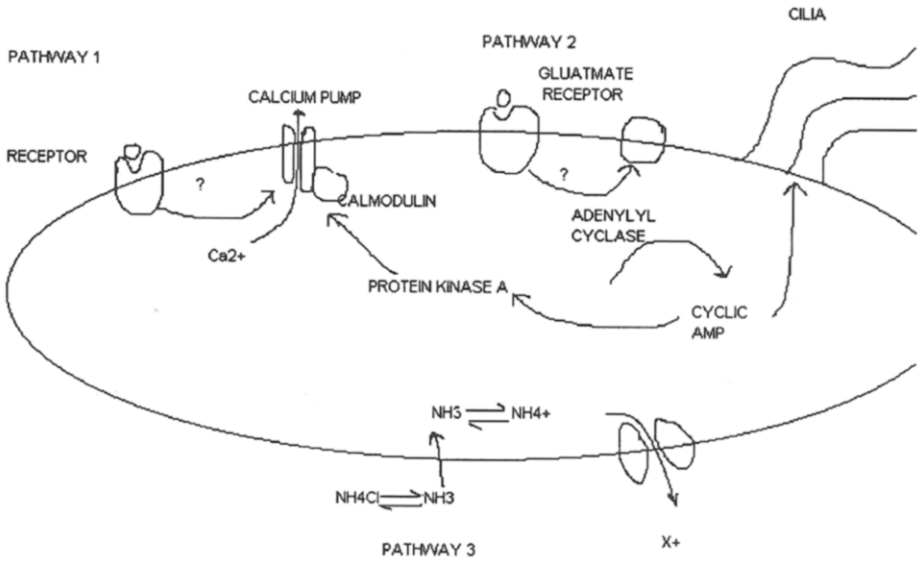

Fig. 6. Schematic of three pathways for *Paramecium* chemosensory transduction for attractants.

ly acidify [5]. Likewise, individual cells show rapid, reversible alkalinizations and slow acidifications (Fig. 5). There are two mutants that are attracted to NH_4Cl but cannot sustain this attraction [24]. Both show rapid alkalinization, but also show unusually acidic basal pH [4, 5].

We do not yet know very much about intracellular pH control in *Paramecium,* but we expect that there is an amiloride-sensitive Na/H exchanger that is not involved in chemoresponse [25] and that there could be a pH sensitive channel that is responsible for the NH_4Cl induced hyperpolarization.

Summary: There are at least three attractant signal transduction pathways in *Paramecium tetraurelia* (Fig. 6), and each ultimately induces a hyperpolarization and consequent swimming patterns that lead to relative accumulation or dispersal of cells. The coupling between receptors and enzymes such as adenylyl cyclase or the calcium ATPase is not yet known. The pump gene has been cloned and its sequence presents strategies for us to probe its role in chemoresponse.

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