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Genetic dissection of attractant-induced conductances in *Paramecium*

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Summary

***Paramecium tetraurelia* is attracted to acetate and biotin by swimming smoothly and fast up gradients of these attractants, and turning immediately and slowing down when leaving these stimuli. We use a group of mutants, each with a different defect in an identified ion conductance, to show that these two stimuli open different ion channels, and the behaviors that occur upon application of stimulus (on-response) and removal of stimulus (off-response) have different roles in attraction to these two stimuli. The most important parameters for successful attraction to acetate are the on-response behaviors of fast swimming with few turns, and the mutants' behavior suggests that $I_{K(Ca,h)}$ is the conductance involved that initiates this behavior. $I_{K(Ca,h\ or\ d)}$ appears to be important to the on-response in biotin; the results with mutants suggest that the biotin off-response depolarization is initiated by an I_{Ca}, which can be large enough or close enough to channels to open $I_{K(Ca,d)}$, $I_{Na(Ca)}$ and $I_{Mg(Ca)}$.**

Key words: *Paramecium*, mutant, conductance, chemoattraction, channel, biotin, acetate.

Introduction

Paramecium tetraurelia employ multiple chemosensory pathways in order to move to areas populated by their bacterial prey (Van Houten, 1994). A number of bacterial metabolites are attractant stimuli, including glutamate, folate, biotin, cAMP, acetate and ammonium (Bell et al., 1998; Yang et al., 1997; Van Houten, 1994) and, except for ammonium, these stimuli bind to cell surface receptors to initiate the responses. In contrast, the attractant NH_4Cl diffuses as ammonia across the cell membrane, which results in alkalinization, hyperpolarization and altered swimming behavior (Davis et al., 1998). The response of the cells to the application of any of the other stimuli is an immediate decrease in frequency of turning and an increase in swimming speed. The removal of the stimuli results in an increase in turning and decreased speed. The abrupt turns in the *Paramecium* swimming path are attributed to calcium action potentials from activation of ciliary voltage-gated calcium channels, and consequent change in power stroke of the cilia due to the transiently increased ciliary calcium (Eckert, 1972).

The changes in swimming behavior associated with the application of attractant (on-responses) and removal of attractant (off-responses) are characteristic of relative hyperpolarization and depolarization of the cells (Bonini et al., 1986; Eckert, 1972; Machemer, 1989). Electrophysiological recording from cells confirms that the cells hyperpolarize upon application of attractant stimuli, using concentrations and conditions that would not cause artifacts of junction potentials (Van Houten, 1979; Preston and Van Houten, 1987). The most likely mechanism of on-response hyperpolarization is activation of a K^+ conductance, and, indeed, a transient K^+ conductance was identified upon stimulation with glutamate (Preston and Usherwood, 1988).

The on-response hyperpolarization in attractants is sustained as long as the stimulus is present, and we attribute this to a calcium plasma membrane pump (PMCA) conductance. We have several indirect lines of evidence to support this attribution (reviewed in Van Houten, 1998). Together, the evidence points to a PMCA electrogenic conductance during the on-response to attractants.

The removal of attractant elicits an immediate turn of the cell and slower swimming, both consistent with depolarization in the off-response. A turn is caused by the transient increase in ciliary calcium from voltage-gated calcium channels, and a depolarization brings the membrane potential closer to threshold for action potential, increasing the frequency of turns (Nakaoka and Machemer, 1990). Depolarization is also associated with a decrease in ciliary beat frequency, and slower swimming (Nakaoka and Machemer, 1990).

We use the T-maze for an assay of chemoresponse behavior (Van Houten et al., 1982). This simple apparatus allows cells to swim between two arms containing relative attractant and

repellent solutions. Cells repeatedly leave and re-enter the attractant, spending the majority of time in the attractant solution. Using computer simulations of the T-maze, we found that the immediate responses of the cells upon crossing the step gradient boundary between attractant and control were important parameters for attraction. As simulated cells enter and distribute between the two arms of the T-maze, decreased turning and smooth fast swimming as the cells leave the control solution and move into attractant, and an immediate turn response as the cells leave attractant and move into the control solution are crucial to successful attraction.

While we know a great deal from others' work on the biophysics of the ciliary beat, we do not know the identities of the ion channels that participate in the chemoresponse to stimuli like acetate and biotin, with the exception of the voltage-gated calcium channels of the cilia. Using Pawn mutants that have no functional voltage-gated calcium channels, we showed that without the ability to generate an abrupt turn the mutants are not normally attracted or repelled (Van Houten, 1978). Here we present observations of a group of mutants in *P. tetraurelia* that have defects in specific conductances in order to dissect the conductances that are responsible for on- and off-responses to two stimuli, acetate and biotin.

Materials and methods

Cell culture

Paramecium tetraurelia (Wenrich and Sonneborn) strains *51S* (sensitive to killer), *Cam 1-1*, *Cam 11* (Kink et al., 1990) *Cam 4* and *eccentric* (*XntA*) (Ling et al., 1994) (R.R.P., unpublished data) were grown at 28°C in a wheat-grass medium inoculated with *Klebsiella pneumoniae* (Sasner and Van Houten, 1989).

Solutions

Solutions used in behavioral assays contained 1 mmol l^{-1} $Ca(OH)_2$, 1 mmol l^{-1} citric acid and approximately 1.3 mmol l^{-1} Tris base in addition to the attractant and control compounds noted. Experiments were conducted at either pH 6.7 or 7.0 with the appropriate cation (K^+, Na^+, Ca^{2+} or Mg^{2+}) for that particular experiment matched with either acetate, biotin or Cl^- as a control (all chemicals from Sigma Chemical, St Louis, MO, USA, unless otherwise noted). For example, a Na-biotin solution would be tested against a NaCl solution in a behavioral assay.

Electrophysiological recordings

Membrane currents were measured under two-electrode voltage clamp as described (Preston et al., 1990). Glass electrodes were filled with either 0.5–1.0 mol l^{-1} KCl or 0.5–1.0 mol l^{-1} CsCl, depending on the current being measured. The cell membrane was voltage clamped near resting potential (–40 mV) and currents were recorded as the contents of the experimental chamber were changed with a perfusion system at a rate of 1 ml s^{-1} in a 1 ml bath. Recordings

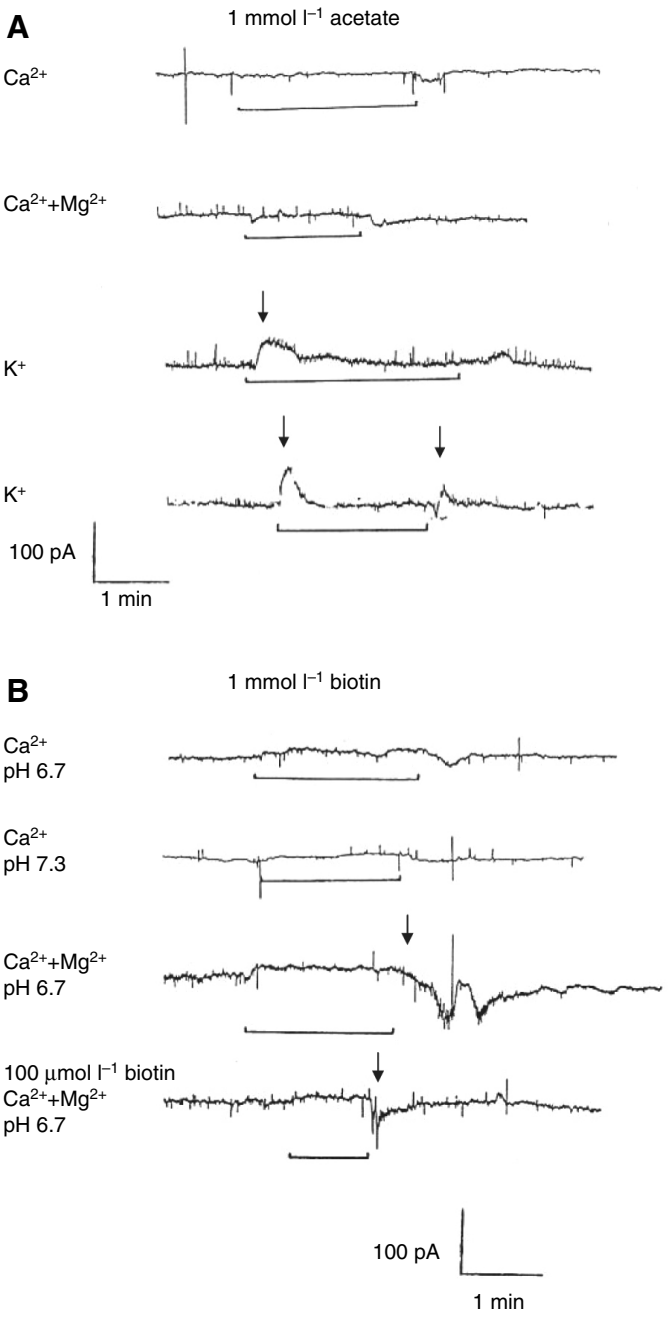

Fig. 1. Voltage-clamp recordings from cells in acetate or biotin. The cells were held at resting potential (–30 mV) using two-electrode voltage-clamp. (A) Solid bars indicate application of 1 mmol l^{-1} acetate. Lower two traces were recorded using K^+-filled electrodes with 1 mmol l^{-1} K^+ in the bath. K^+ currents were suppressed in the middle and upper traces using CsCl and tetraethylammonium (TEA), leaving Ca^{2+} (1 mmol l^{-1}) and Mg^{2+} (5 mmol l^{-1}) as the predominant charge carriers. Arrows show the outward conductances upon addition of acetate and removal of acetate. (B) Solid bars indicate application of variable concentrations of biotin at variable pH. K^+ currents were suppressed in the middle and upper traces using CsCl and TEA leaving Ca^{2+} (1 mmol l^{-1}) and Mg^{2+} (5 mmol l^{-1}) as the predominant charge carriers. Arrows show the inward conductances upon removal of biotin.

from among 20 cells that are shown in Fig. 1 were selected to illustrate the inward and outward conductances that suggested to us models that we then explored with studies of mutant behaviors.

Behavioral assays

T-maze assays were conducted as previously described (Van Houten et al., 1982). Paramecia in control solution were placed in a stopcock and allowed to swim freely between the two glass arms of the T-maze apparatus that contained the test and control solutions. After 30 min the stopcock was closed, isolating the test and control arms, and aliquots from each were counted. The number of cells in the test arm was divided by the total number of animals in both arms to yield an index of chemokinesis (I_{che}). Values greater than 0.5 indicate attraction.

Videotapes of cells entering control or test solution were analyzed with Expert Vision software (version 3.14, Motion Analysis Corp., Santa Rosa, CA, USA) and a modified user program provided by K. Clark and D. L. Nelson. Analysis of swimming speed and turning frequency (% directional change) were conducted as described by Clark and Nelson (Clark and Nelson, 1991).

Cells were incubated in the appropriate buffer for at least 30 min before transfer to the buffer in which they were analyzed. Paramecia were pipetted in 2–3 μl into a 50 μl volume of test solution on a glass slide placed on a StereoZoom 7 dissecting microscope (Bausch and Lomb, Rochester, NY, USA). Cells were just off screen when put on the slide and videotaped using a video camera (Cohu 6410, San Diego, CA, USA) attached to a Sony SLV-R5UC video cassette recorder as they swam into view (~1 s) (Davis et al., 1998). Cell images were processed using a VP 110 video processor (Motion Analysis Corp.).

Results

Voltage clamp studies of cells stimulated by acetate or biotin

We measured conductances under two general voltage clamp conditions: in the absence of K^+ and Na^+ and with CsCl electrodes to block K^+ conductances, and in the presence of K^+ with KCl electrodes. Cells were superfused with the stimulus acetate or biotin in buffer and both the on- and off-responses were recorded.

Acetate

Cells show a very small and transient inward conductance of about 1–5 pA upon both addition and removal of acetate (see arrows in representative recordings in Fig. 1A). If the small inward conductances in acetate were carried by Ca^{2+}, and if they were sufficiently large, they should activate the Na^+ or Mg^{2+} currents, hence magnifying any inward conductance elicited by acetate because, in *P. tetraurelia*, inward Mg^{2+} and Na^+ conductances are activated by a rise in intracellular Ca^{2+} levels (Preston, 1990). However, in the presence of Mg^{2+}, the small inward conductances initiated by acetate are not changed. When extracellular K^+ is present, upon addition of acetate and at times upon its removal, there are small outward conductances of 10–30 pA, which are likely to be K^+ conductances activated by intracellular Ca^{2+} from I_{Ca}.

Biotin

Cells in biotin show a very different pattern of conductances (Fig. 1B). There is a sustained outward conductance upon addition of biotin (about 5–10 pA) and a larger, transient inward conductance of approximately 10 pA upon removal of biotin. This off-response conductance can be enhanced to about 40 pA with the addition of Mg^{2+} to the bath. In Mg^{2+} solutions, even 100 μmol l^{-1} biotin, a tenfold lower concentration of biotin than we generally use for behavior tests, can elicit a measurable off-response conductance. K-biotin elicits a transient outward conductance followed by a sustained outward conductance during the on-response. The replacement of K-biotin with KCl (stimulus off) elicits an inward conductance, usually followed by an outward conductance. For K-biotin, two different pH conditions were used because of our observation that K-biotin is a better attractant at low pH (6.7) and, unless otherwise stated, the recording and behavior are measured using biotin solutions of pH 6.7 (see below). Consistent with this observation about behavior and pH, there is little conductance change at relatively high pH (7.3). Since the calcium-activated Na channel carries H^+ as well as Na^+, we attribute the inward conductances at low pH in K^+ to Ca^{2+} activation of the Na^+ channel.

Use of conductance mutants in T-maze assays of chemoresponse

We tested chemoresponse behavior using T-maze assays of mutants that have specific defects. Each mutant has a single site mutation that leads to characterized conductance defects (Table 1). Mutant *Cam 11* has a mutation in the gene for calmodulin, and consequently has lost the calcium activated Na^+ conductance ($I_{Na(Ca)}$) (Saimi, 1986; Kink et al., 1990). Mutant *Cam 4* likewise has a mutation in the gene for calmodulin, and has lost a calcium activated K^+ conductance $I_{K(Ca,d)}$. The mutation in *Cam 1-1*'s calmodulin gene produces defects in both of the major Ca-dependent K^+ conductances ($I_{K(Ca,d)}$ and $I_{K(Ca,h)}$) (Preston et al., 1990). Mutant *XntA* has lost the calcium activated Mg^{2+} conductance ($I_{Mg(Ca)}$) and also has a reduced calcium activated Na^+ current ($I_{Na(Ca)}$) (Preston and Kung, 1994). In all cases below, the Mann–Whitney *U*-test was used to determine statistically significant differences from wild type at the $P<0.05$ level (Tables 1 and 2).

Acetate T-mazes

Cam 4 with defect in $I_{K(Ca,d)}$

Wild-type cells (*51-S*) are about equally well attracted to K^+ and Na^+ salts of acetate and to K-acetate with Mg^{2+} in the bath (Table 1). Mutant *Cam 4*, likewise is attracted to K^+ and Na^+ salts of acetate, indicating that $I_{K(Ca,d)}$ plays little or no role in the acetate on- or off-responses.

Table 1. *T-maze assays of chemoresponse to acetate solutions*

Strain	Defect	K-acetate	Na-acetate	Mg+K-acetate
51-S, wild type		0.83±0.04[g,h]	0.84±0.03[e,g]	0.90±0.03[h]
Cam 4	$I_{K(Ca,d)}$ Δ	0.88±0.02[e,g,h]	0.74±0.04[e,g]	nd
Cam 1-1	$I_{K(Ca,d,h)}$ Δ	0.62±0.04[d,f]	0.60±0.02[d,f]	nd
Cam 11	$I_{Na(Ca)}$ Δ	0.70±0.02[b,f]	0.52±0.02[a,d,e,g]	nd
XntA	$I_{Mg(Ca)}$ Δ $I_{Na(Ca)}$ ↓	0.71±0.02[d,f]	0.70±0.03[e]	0.63±0.02[d]

Values are the means ± 1 s.e.m. of nine or more T-mazes. The concentration of acetate tested was 5 mmol l^{-1} *vs* 5 mmol l^{-1} of the appropriate cation balanced with Cl^-. Values are calculated as the number of cells in the test arm of the T/total number of cells in both test and control arms.

Values >0.5 indicate attraction; <0.5 indicate repulsion. Statistical significance was tested with the Mann–Whitney *U*-test. Comparisons were done within both columns and rows. Differences were determined to be significant at $P<0.01$. Symbols represent treatments statistically different from that respective experiment; Δ=lost; ↓=diminished.

[a]Different from K-acetate; [b]different from Na-acetate; [c]different from Mg^{2+}+K-acetate; [d]different from wild type; [e]different from *Cam 11*; [f]different from *Cam 4*; [g]different from *Cam 1-1*; [h]different from *XntA*; nd, not done.

Cam 1-1 with defect in $I_{K(Ca,h,d)}$

Cam 1-1, which shares with *Cam 4* the loss of $I_{K(Ca,d)}$ but has also lost $I_{K(Ca,h)}$, shows significantly reduced chemoresonse to K- or Na-acetate (Table 1). Considering that *Cam 4* is not defective in its chemoresponse to these stimuli, we attribute the decrease in chemoresponse of *Cam 1-1* cells to the loss of $I_{K(Ca,h)}$. $I_{K(Ca,h)}$ may be the conductance implicated in the initial hyperpolarization of the on-response.

Cam 11 with defect in $I_{Na(Ca)}$

Cam 11, with its loss of $I_{Na(Ca)}$, shows chemoresponse to K-acetate within the normal range, but has a complete loss of chemoresponse to Na-acetate (Table 1). Despite the loss of attraction in Na-acetate, we believe that $I_{Na(Ca)}$ plays no significant role in chemoresponse to acetate because the loss of chemoresponse in K^+-free (Na) acetate solutions is a secondary effect of the mutation. *Cam 11* in K^+-free media has an extremely low resting membrane potential [near the equilibrium potential for K^+ (E_K) (Satow and Kung, 1976)], which prevents the cell from responding with an adequate hyperpolarization for the on-response or depolarization of the off-response.

To test further the potential role of $I_{Na(Ca)}$ in acetate chemoresponse, we tested mutant *XntA*, which has lost $I_{Mg(Ca)}$ and has a greatly reduced $I_{Na(Ca)}$, and found that *XntA* is normally attracted in K- and Na-acetate solutions (Table 1).

Table 2. *T-maze assays of wild-type chemoresponse to biotin solutions*

K-biotin		Na-biotin		Mg-biotin
pH 6.7	pH 7.0	pH 6.7	pH 7.0	pH 7.0
0.72±0.01	0.61±0.04*	0.79±0.02	0.72±0.02	0.89±0.01*

Values are the means ± 1 s.e.m. of nine or more T-mazes. The concentration of biotin tested was 1 mmol l^{-1} *vs* 1 mmol l^{-1} of the appropriate cation balanced with Cl^-.

Statistical significance was tested with Mann–Whitney *U*-test. *Statistically significantly different from K-biotin pH 6.7 at the $P<0.05$ level.

Biotin T-mazes

Cam 4 and Cam 1-1 with defects in $I_{K(Ca,d)}$ and $I_{K(Ca,h,d)}$

Cam 4 mutants that have lost the $I_{K(Ca,d)}$ conductance show significantly stronger attraction than wild type in Na-biotin (Tables 2 and 3). *Cam 1-1*, which has lost both the depolarization- and hyperpolarization-activated $I_{K(Ca)}$, shows significantly stronger attraction in K-biotin (pH 6.7) and Na-biotin pH 7 than wild-type cells (Tables 2 and 3). Thus, when the $I_{K(Ca,d)}$ is lost in *Cam 4* or when both $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ are lost in *Cam 1-1*, there is an enhancement of chemoresponse.

Cam 11 and XntA with defects in $I_{Na(Ca)}$ and $I_{Mg(Ca)}$

To test the hypothesis that the depolarizing off-response is initiated by I_{Ca} and, therefore, can potentially be enhanced with activation of $I_{Na(Ca)}$ or $I_{Mg(Ca)}$ in Na, low pH or Mg solutions, we tested wild-type cells in Na-biotin, Mg-biotin and K-biotin at pH 7 and 6.7 (Table 2). Wild-type cells show significantly stronger attraction to biotin at pH 6.7 than at pH 7. Wild-type cells are also significantly better attracted in Mg-biotin pH 7 than in K-biotin at pH 7, or even Na-biotin at pH 6.7 or 7.

Cam 11, which has lost the $I_{Na(Ca)}$, and *XntA*, which has defects in $I_{Na(Ca)}$ in addition to its loss of $I_{Mg(Ca)}$, both show in K-biotin at pH 6.7 slightly depressed chemoresponses that nonetheless are within the wild-type range (Table 3). *Cam 11* shows significant loss of chemoresponse in Na-biotin at pH 6.7, but this result cannot be used to implicate its lost $I_{Na(Ca)}$ in an important function like the off-response depolarization because, as described above, *Cam 11* in K^+-free Na^+ solutions has a severely reduced resting potential. However, resting membrane potential of *Cam 11* appears to be normal in Mg^{2+} solutions, judging from the swimming speed and behavior in K^+, Na^+ and Mg^{2+} solutions (W.E.B. and J.L.V.H., unpublished observation) and direct measurements (R.R.P., unpublished observations). *Cam 11* shows strong attraction to Mg-biotin.

Table 3. *T-maze assays of chemoresponse to biotin solutions*

Strain	Defect	K-biotin, pH 6.7	Na-biotin, pH 7	Mg-biotin, pH 7
Wild type		0.72±0.01[c,g]	0.79±0.02[c,e,f,g]	0.89±0.01[a,b,e,h]
Cam 4	$I_{K(Ca,d)}$ Δ	0.73±0.03[b,g]	0.91±0.01[a,d,e]	–
Cam 1-1	$I_{K(Ca,d,h)}$ Δ	0.85±0.02[d,e,f,g]	0.89±0.01[d,e]	–
Cam 11	$I_{Na(Ca)}$ Δ	0.66±0.03[c,g]	0.53±0.03[c,d,f,g]	0.79±0.03[a,b,d,g]
XntA	$I_{Mg(Ca)}$ Δ $I_{Na(Ca)}$ ↓	0.67±0.04[c,g]	–	0.50±0.02[a,d,e]

Values are the means ± 1 s.e.m. of nine or more T-mazes. The concentration of biotin tested was 1 mmol l^{-1} *vs* 1 mmol l^{-1} of the appropriate cation balanced with Cl^-. Statistical significance was tested with Mann–Whitney *U*-test. Comparisons were done with both columns and rows. Differences were determined to be significant at $P<0.01$. Symbols represent treatments statistically different from that respective experiment; Δ=lost; ↓=diminished.

[a]Different from K-biotin pH 6.7; [b]different from Na-biotin pH 7; [c]different from Mg-biotin pH 7; [d]different from wild type; [e]different from *Cam 11*; [f]different from *Cam 4*; [g]different from *Cam 1-1*; [h]different from *XntA*; nd, not done.

Swimming behavior assays of individual cells

The T-maze results for the conductance mutants lead us to ask about the swimming and turning behavior of individual cells, which we measure as percent directional change (turning) and speed. In these experiments that examine the on-response, control cells are videotaped as they swim across a boundary from control solution to more control solution and we refer to test cells as those that swim from control to attractant solution. For the off-response, control cells swim from attractant into more attractant while test cells swim out of attractant into the control solution. We are not attempting to use the same solutions for wild type and all the mutants even though strength of attraction can vary with counter-ion. Here we are using a selection of strains and conditions to correlate turning or speed with strength of attraction. Also, *Cam 11* must be tested in K^+ to avoid artifacts of abnormal resting potential of this mutant in the absence of K^+. Therefore, the statistical analysis that we used in this part of the study was regression analysis.

Cells swimming into and out of acetate

We assayed behavior of wild type, *Cam 11*, *XntA* and *Cam 1-1* in acetate solutions. The data in Fig. 2 are arranged by descending order of T-maze attraction to acetate: wild type in Na-acetate 0.84±0.03; *Cam 11* in K-acetate 0.70±0.02; *XntA* in Na-acetate 0.70±0.03; *Cam 1-1* in K-acetate 0.62±0.03). Fig. 2A shows the average numbers of turns (expressed as % directional change or PDC) of cells entering acetate compared to control cells. Fig. 2B shows the turns upon leaving acetate, and Fig. 2C shows the speed of control cells and those entering acetate. The wild type, *Cam 11* and *XntA* all turn less frequently and speed up as they enter acetate. *Cam 1-1* shows no significant change in turning or speed upon entering acetate. Upon leaving acetate, wild-type cells turn more, as do *Cam 11* cells. However, *XntA* and *Cam1-1* cells do the opposite and depress the frequency of turning compared with controls.

When the data are treated not as absolute frequencies of turning, but rather as net changes in turning upon entering acetate (Fig. 3A), the differences among the strains of cells become more apparent. Wild type shows larger changes in turning and speed than *Cam 11*. *XntA* shows a very large

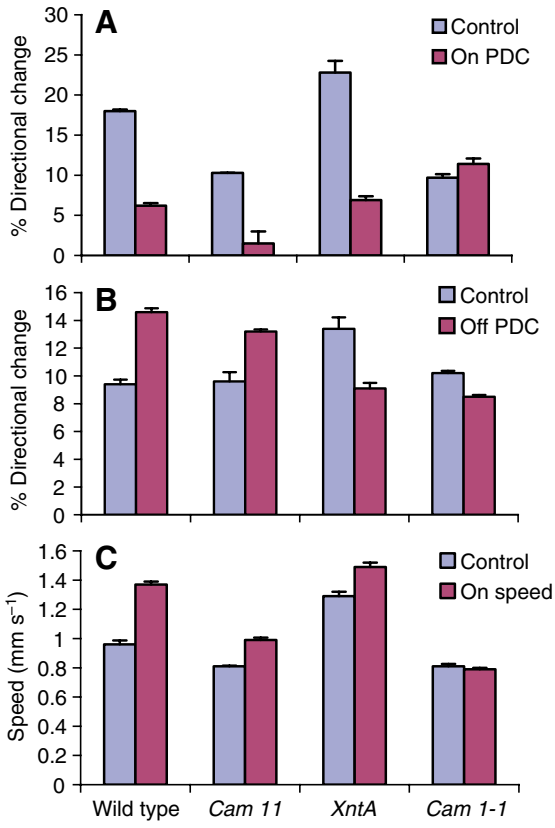

Fig. 2. Behavior of cells entering and leaving acetate. Cells were analyzed using Motion Analysis as they entered 5 mmol l^{-1} acetate in buffer from a control buffer or *vice versa*. Cells were monitored for turns (% change in direction, PDC) and speed. (A) Cell turning upon entering acetate (On PDC); (B) cell turning upon leaving acetate (Off PDC); (C) speed in mm s^{-1} upon entering acetate (On speed). (A) Wild-type, *Cam 11* and *XntA* On PDCs were significantly different from Controls. (B) Wild type and *Cam 11* Off PDCs were significantly different from Controls. (C) Wild-type and *Cam 11* speeds were significantly different from control (Mann–Whitney *U*-test, $P<0.05$). The wild-type and *XntA* cells were analyzed in Na-acetate; *Cam 1-1* and *Cam 11* were analyzed in K-acetate. (Values are means ± 1 s.e.m. of 45 or more measurements.)

decrease in turning upon entering acetate, but an unexpected decrease in turning upon leaving acetate as well. *Cam 1-1* shows small changes in turning and speed, which are opposite to those shown by wild-type cells. A comparison of Fig. 3B,C shows that there is a significant correlation of the speed change upon entering acetate with magnitude of T-maze responses (R^2=0.945, Pearce r=0.972), but other parameters do not show statistically significant correlations.

Cells swimming into and out of biotin

For turning and swimming speed, we observed wild type moving into and out of solutions in Na-, Mg- and K-biotin solutions; *Cam 1-1* and *XntA* in Na-biotin; and *Cam 11* in K-biotin. Results for turning are shown in (Fig. 4) and the strains are arranged by descending T-maze values (wild type in Mg-biotin 0.89±0.01; *Cam 1-1* in K-biotin 0.85±0.02; wild type in Na-biotin 0.79±0.02; wild type in K-biotin 0.72±0.01; *Cam 11* in K-biotin 0.66±0.03; *XntA* in Na-biotin 0.63±0.04; and *XntA* in Mg-biotin 0.50±0.02). Comparison of Fig. 4A and B shows that the absolute values for turning frequency moving into biotin correlate significantly with the magnitude of T-maze results (Fig. 4A, R^2=0.844, Pearce r=0.7126). Turning frequency in the off-response (Fig. 4C) and speed (not shown) in the on-response do not correlate significantly with the T-maze data.

The transformation of the data into net differences from control in turning frequency of the on- and off-responses highlight the differences among the strains (Fig. 5). *Cam 11* and *XntA* that have both lost $I_{Na(Ca)}$ show very small changes

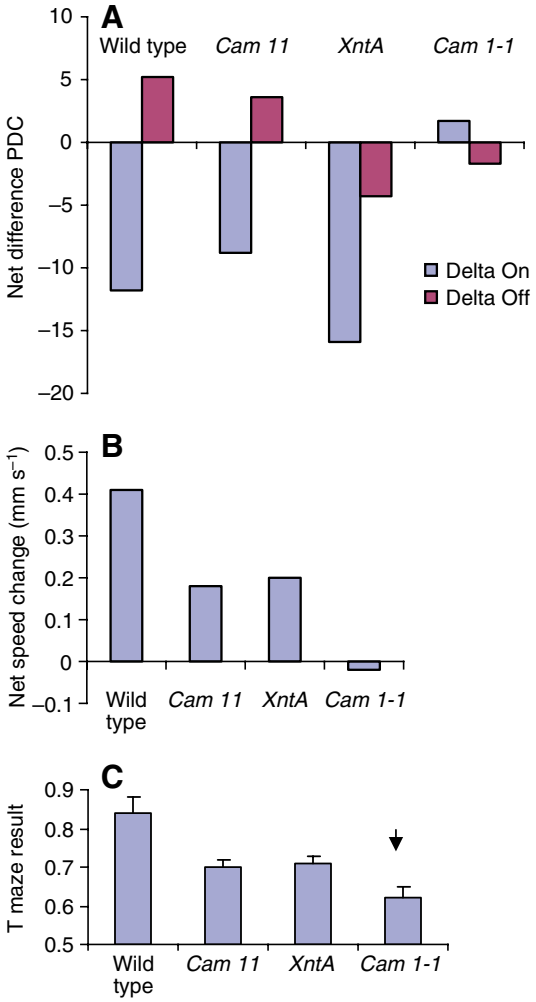

Fig. 3. Cell behavior upon entering or leaving acetate. The data from Fig. 2 were transformed into differences between the control value and experimental value for on- and off-response PDCs (A) and on-response speed in mm s^{-1} (B). (C) The T-maze values for wild type and mutants in A and B. (Values >0.5 indicate attraction, <0.5 indicate repulsion.) Arrow indicates significant difference with wild type at P<0.01, Mann–Whitney U-test.

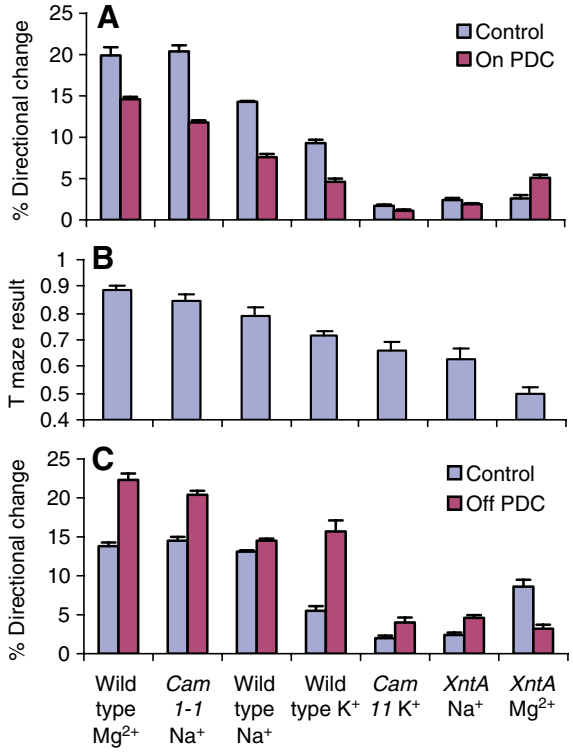

Fig. 4. Behavior of cells entering and leaving biotin solutions. Cells were analyzed using Motion Analysis as they entered 1 mmol l^{-1} biotin in buffer from a control buffer or *vice versa*. Cells were monitored for turns (% change in direction, PDC) and speed. (A) Cell turning upon entering biotin (On PDC); (B) the T-maze values for the wild type and mutants in biotin for comparison with A because the T-maze values correlate well with On-PDC responses; (C) Cell turning upon leaving biotin (Off PDC). (A) *Cam 1-1* and wild type in Na-biotin On PDCs were significantly different from Controls (P<0.05). (B) All Values are means ± 1 s.e.m. of 45 or more measurements. Mann–Whitney U-test was used for statistical analysis of T-maze data; all values except for *Cam 11* were different from wild type in Na^+ and Mg^{2+} at P<0.05 level. (C) Wild type in Mg-biotin and *Cam1-1* Off PDCs were significantly different from Controls. The wild type cells were analyzed in K-biotin pH 6.7, Na-biotin pH 7, Mg-biotin pH 7; *Cam 1-1* in Na-biotin pH 7; *Cam 11* K-biotin pH 6.7; *XntA* in Na-biotin pH 7 and Mg-biotin pH 7.

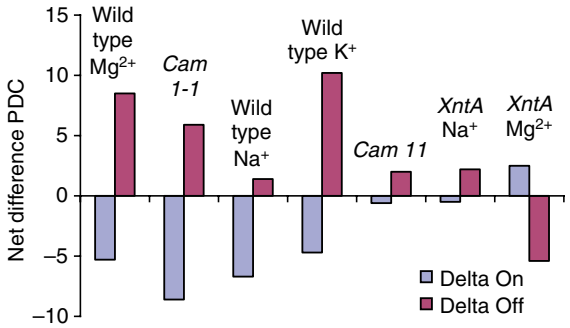

Fig. 5. Cell behavior upon entering or leaving biotin solutions. The data from Fig. 4 were transformed into differences between the control value and experimental value for on- and off-response PDCs.

in both the on- and off-responses in K- and Na-biotin, respectively. *XntA* that is not attracted at all to Mg-biotin shows statistically significantly different and opposite responses to biotin than wild type in Mg-biotin.

Discussion

Conductance measurements

The limited number of conductances that we measured in acetate and biotin did not constitute an exhaustive study, but we present them here because they suggested to us two different models for chemoresponses in these stimuli.

The application of acetate activates a Ca^{2+}-dependent K^+ conductance ($I_{K(Ca)}$) that initiates the hyperpolarization in the stimulus. There is a small, sustained outward conductance, which we attribute to the plasma membrane calcium pump (PMCA). Upon removal of acetate, there is an influx of Ca^{2+} (I_{Ca}), large enough at times to activate a small outward K^+ conductance, but not large enough to activate an inward Na^+ or Mg^{2+} conductance when these ions are in the bath. Alternatively, the Ca^{2+} influx does not occur in close enough proximity to the Ca^{2+}-activated Na^+ and Mg^{2+} channels to activate them.

In the case of biotin, it is difficult to observe an initial K^+ conductance that probably initiates the hyperpolarization, but there is a sustained outward conductance, which we attribute to the PMCAs. There appears to be an inward Ca^{2+} (I_{Ca}) conductance upon removal of biotin, and this off-response inward conductance is amplified in low pH, Na^+ (not shown), or Mg^{2+}. This is consistent with the activation of $I_{Na(Ca,d)}$, which also carries H^+, or activating $I_{Mg(Ca)}$ when there is Na^+ or Mg^{2+} in the bath or the bath is of acidic pH (R.P.P., unpublished observations).

T-maze analysis

Our studies of mutants in T-maze assays implicate $I_{K(Ca,h)}$ and rule out a role for $I_{K(Ca,d)}$ in attraction to acetate because mutant *Cam 4* that has lost $I_{K(Ca,d)}$ shows normal chemoresponse while *Cam 1-1*, which has lost both $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$, shows greatly reduced chemoresponse. Since the *Cam 1-1* mutant shows reduced attraction but not neutral responses to acetate, the $I_{K(Ca,h)}$ must be necessary but not sufficient for optimal chemoresponse behavior. Other mutants with altered $I_{Na(Ca)}$ or $I_{Mg(Ca)}$ show no significant differences from wild type in their attraction to acetate.

Mutant strains as well as wild type in various salts of biotin demonstrate a role for $I_{K(Ca,h)}$ and/or $I_{K(Ca,d)}$ in chemoresponse to biotin, but a very different role from that in acetate. When $I_{K(Ca,h)}$ is eliminated by mutation, the attraction response is not reduced as in acetate, but rather is enhanced in biotin. Mutant *Cam 1-1* shows significantly higher T-maze results than wild type in K- and Na-biotin, and *Cam 4* shows significantly higher attraction in Na-biotin (Table 2). Therefore, a K^+ conductance appears to be activated in biotin chemoresponse, perhaps normally short circuiting and reducing the effectiveness of the depolarizing off-response. We discuss below that when the K^+ conductance is eliminated by mutation, the off-response is increased (compare *Cam 1-1* in Na-biotin to wild type in Na-biotin off-response in Fig. 4C).

Other conductances that can contribute to but are not necessary for chemoresponse in biotin are $I_{Na(Ca)}$ and $I_{Mg(Ca)}$. Wild-type cells can be attracted in K-biotin at pH 7 (Table 2), that is, under conditions when $I_{Na(Ca)}$ and $I_{Mg(Ca)}$ are not activated. However, cells are more effectively attracted to K-biotin at pH 6.7 or to Na-biotin at pH 6.7 or 7, and show the strongest attraction to Mg-biotin even at pH 7. When $I_{Na(Ca)}$ is eliminated as in *Cam 11*, the attraction is greatly diminished for K-biotin at pH 6.7, presumably because $I_{Na(Ca)}$ is not functional to carry protons or Na^+. However, when *Cam 11* is in Mg-biotin attraction is strong and normal, presumably because *Cam 11* has a functional Mg^{2+} channel allowing for a strong off-response. When $I_{Mg(Ca)}$ is eliminated as in *XntA*, there is little attraction to Na-biotin and no attraction to Mg-biotin at all, which emphasizes the importance of the depolarizing off-response conductances.

We interpret these results to support the model that in Na-biotin or biotin solutions of pH 6.7, the off-response depolarization can be augmented with the I_{Ca} activation of $I_{Na(Ca)}$, which conducts protons as well as Na^+. When Mg^{2+} is available, the off-response depolarizing I_{Ca} activates $I_{Mg(Ca)}$, which even more effectively depolarizes the cell and causes the largest increase in turning frequency as cells leave biotin.

Individual cell turning and speed measurements

In previous computer simulation studies (Van Houten and Van Houten, 1982), we found that some aspects of swimming behavior are critical to accumulation or repulsion in the T-mazes. Most relevant to these studies is the observation that boundary responses are necessary but not sufficient for accumulation. That is, an immediate response when crossing a step gradient is essential as the cells move into attractant (hyperpolarize and swim smoothly and straight) and out of attractant (depolarize and immediately turn). It appears that the behaviors of wild type and mutants in acetate solutions (Figs 2 and 3) implicate the immediate on-response, particularly the speed increase, in successful attraction to acetate.

Behavior of wild type and mutants in biotin solutions likewise implicates the on-response depression of turning as critical in successful attraction to biotin, but speed increases do not correlate well with attraction in T-mazes (Figs 4 and 5). The off-response increase in turning and changes in speed, likewise, do not correlate with successful T-maze attraction for either stimulus. However, we note that the wild-type cells increase their speed significantly (0.93±0.03 in control *vs* 1.14±0.02 mm s^{-1}) when cells leave biotin, which is unexpected given the large off-response depolarization and inward conductances.

Models

Our data on behavior of wild type and mutant cells in acetate solutions lead us to a model for acetate attraction that begins with a hyperpolarizing K^+ conductance to initiate the on-response behavior of fast swimming with few turns. We believe that this is the most important aspect of successful attraction to acetate and the $I_{K(Ca,h)}$ is the conductance involved. The significant correlation of the on-response increase in swimming speed with T-maze values fits with the initiation of the on-response by $I_{K(Ca,h)}$. The loss of this conductance in mutant *Cam 1-1* correlates with diminished attraction and with speed and turning changes that are the opposite of the wild type. The small residual attraction of the mutant *Cam 1-1* to acetate can be attributed to the activation of the plasma membrane calcium pump, which we believe normally sustains the hyperpolarization in wild-type cells. Our observations of cells that have 60% reduced calmodulin levels (by transformation with anti-sense calmodulin expression vectors) show that these cells have no sustained hyperpolarizing conductance and also no significant attraction to acetate (Yano et al., 1996) (R.R.P., W.E.B., J. Yano and J.L.V.H., unpublished observations).

A cell in biotin appears to experience very different chemosensory conductances. The tests of mutants in T-mazes point to $I_{K(Ca,d\ or\ h)}$ short-circuiting a conductance that is critical for attraction because elimination of $I_{K(Ca,h\ and\ d)}$ in mutant *Cam 1-1* leads to increased attraction, just the opposite as for acetate. The conductances that might be short-circuited by $I_{K(Ca)}$ appear to be $I_{Na(Ca)}$ and $I_{Mg(Ca)}$. When $I_{Na(Ca)}$ or $I_{Mg(Ca)}$ is lost by gene mutations in *Cam 11* and *XntA*, which we previously thought to be associated with the depolarizing off-response, to our surprise, *Cam 11* and *XntA* attraction to biotin is compromised and on-response speed and turning changes are small or even opposite to the wild type. We had not anticipated a role for conductances lost in these mutants ($I_{Na(Ca)}$ and $I_{Mg(Ca)}$) in the on-response. We associate $I_{Na(Ca)}$ with the depolarization off-response and turning upon leaving biotin, as supported by the electrophysiological recordings (Fig. 1). However, we find a significant correlation of decreased turning in the biotin on-response with attraction strength, and we do not see a role for $I_{K(Ca)}$ in determining the on-response behavior (see Fig. 4A wild type *vs Cam 1-1*). Additionally, the unexpected transient increase in speed as wild-type cells leave biotin could be explained with an activated $I_{K(Ca)}$. Therefore, we propose that the hyperpolarization on-response in biotin is initiated by a conductance other than $I_{K(Ca,h\ or\ d)}$ and that on-response behavior is important to the attraction outcome. The off-response depolarization is initiated by an I_{Ca}, which can be large enough or close enough to channels to open $I_{K(Ca,d)}$, $I_{Na(Ca)}$ and $I_{Mg(Ca)}$. The Na and Mg conductances can enhance the off-response depolarization, but $I_{K(Ca,d)}$ dampens it. When $I_{K(Ca,d)}$ is eliminated by mutation, attraction is stronger. Therefore, we attribute importance to both the on- and off-responses for optimal attraction to biotin. Strength of attraction correlates to decrease in on-response turning and enhancement or loss of the off-response conductances greatly affect attraction outcomes. With this model we can account for the loss of attraction of *XntA* and *Cam 11*, but we cannot account for the loss of on-responses of PDC and speed when these mutants are introduced to biotin.

In summary, attraction signal transduction pathways for two stimuli, acetate and biotin, involve conductances for on- and off-responses differently. We presumed that the off-response would be more important to the outcome of attraction to biotin than the on-response, but we did not foresee the role of the $I_{K(Ca)}$ in wild-type attraction to biotin on- or off-responses. Likewise, without the mutants, we would not have uncovered a dampening role for $I_{K(Ca)}$ or the potential roles for $I_{Na(Ca)}$ and $I_{Mg(Ca)}$ in the on-response. These studies point to the usefulness of the *P. tetraurelia* behavioral mutants in dissecting complex pathways.

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