Chemoreception in *Paramecium tetraurelia*:
acetate and folate-induced membrane hyperpolarization

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Summary. Acetic and folic acids hyperpolarize the membrane potential of *Paramecium tetraurelia* in a concentration-dependent manner. The membrane responses are accompanied by small changes in cell resistance, and are significantly reduced by increasing extracellular cation concentrations, suggesting that the attractants bring about the membrane potential change by increasing cell permeability to cations. The inability to show a reversal potential for the hyperpolarization to attractants suggests that the effects of cations on the response are non-specific, however. The possible roles of Ca^{++}, K^+, and Na^+ in the attractant-induced responses were further investigated by applying acetate and folate to cells with genetic defects in specific ion conductances, by collapsing the driving forces for these ions, and by testing the effects of ion channel blockers on the responses. These studies suggest that the membrane responses to attractants are not due to the direct effects of increased or decreased membrane permeability to cations.

Attempts to block the acetate and folate-induced hyperpolarization by collapsing surface potential or using a mutant with reduced surface charge were inconclusive, as were studies on the possible role of attractant transport in the membrane responses.

We hypothesize that the membrane hyperpolarization may be due to either the indirect effects of increased calcium permeability, to extrusion of calcium through activation of a calcium pump, or to a proton efflux.

Introduction

The ability to sense and recognize chemicals is fundamental to life. Virtually every living organism, from bacteria to man, can 'smell' or 'taste' chemical components of the air that it breathes or of the water in which it swims. However, despite the ubiquity of chemosensation, it has not yet proved possible to describe fully the means by which any one organism perceives chemicals and then transduces this information to produce a response. Even in the case of bacteria, where a genetic approach has allowed an almost complete dissection of the molecular components of the chemosensory-motor response pathway, the means by which the different components interact is still uncertain (see Ordal 1985). In vertebrates, very little is known of the mechanisms of chemosensory transduction, in part due to the fact that the specialized cells responsible for olfaction and gustation are inaccessible and difficult to isolate in the quantities required for biochemical analysis.

We have chosen the ciliated protozoan *Paramecium* as a 'model' system to study chemoreception. Like bacteria, this organism is easy to manipulate genetically and biochemically, but it also possesses an excitable membrane that is in many respects similar to that of a neurone or of a specialized olfactory or gustatory receptor cell. An additional advantage is that the activity of the cell's locomotory organelles, the cilia, are under the direct control of the membrane potential, so that a behavioural response to a stimulus can readily be explained in terms of the membrane potential change that it produces.

Paramecium exhibits two well defined components of swimming behaviour. The first was originally described by Jennings (1906), who noted that a cell backs up and turns upon colliding with a solid object. This classic 'avoidance reaction' reflects regenerative membrane depolarization, resulting from increased membrane permeability to calcium. Calcium then flows into the cell and acti-

vates a ciliary reversal mechanism, causing the cell to turn. Conversely, membrane hyperpolarization causes an increase in posteriorly directed ciliary beat frequency, so that the cell swims faster forward (see review by Kung and Saimi 1982). The antagonistic effects of these two membrane responses allows the cell a fine control over its swimming behaviour, so that an external stimulus need produce only a small membrane potential change to effect a large response.

The ways in which the two components of swimming behaviour interact to produce accumulation in or repulsion from chemical stimuli has been detailed by Van Houten (1978, 1979). Briefly, chemicals which elicit small depolarizations repel paramecia by increasing the frequency of cell turning, thus increasing the chances of the cell leaving the area of stimulation. Hyperpolarizing stimuli, in addition to causing the cell to swim faster forward, also lessen the chance of regenerative depolarization and hence cell turning; these stimuli are attractant. The present study was undertaken to determine how two such attractant stimuli, acetic and folic acids, are able to bring about membrane hyperpolarization in *Paramecium tetraurelia*.

Materials and methods

Cells and culture conditions. Paramecium tetraurelia stock 51 s, and mutants derived from this wild-type stock, were grown on either Cerophyl rye medium, or a rye grass seed infusion. Cultures were inoculated with *Klebsiella pneumoniae* 1–24 h prior to use and were maintained at 22 °C.

Solutions. Unless otherwise stated, all solutions contained 1 mM Ca(OH)$_2$, 1 mM citric acid, and about 1.3 mM Tris (hydroxymethyl)amino methane (Tris) base, pH 7.0. This buffer solution maintains a stable pH indefinitely and is routinely used in both electrophysiological and behavioural studies. The ionic composition of the test and control solutions were matched at all times. '5 mM KCl buffer' comprised 1 mM Ca(OH)$_2$, 1 mM citrate, 5 mM KCl, 1.3 mM Tris, pH 7.0. All chemicals were of analytical grade and were obtained from Sigma Chemical Co., Fisher Scientific Products, or J.T. Baker Chemicals.

Deciliation. Cells were collected following negative geotactic migration through 5 mM KCl buffer, then absolute ethanol was added to a final concentration of 5% (v/v). Deciliation was effected by agitating the cell suspension for 2 min (Ogura and Machemer 1978), after which the cells were washed in fresh buffer and left 10 min prior to intracellular recording.

Intracellular recording. Standard electrophysiological techniques were used to monitor the membrane potential of *P. tetraurelia* (Naitoh and Eckert 1972). Specimens from logarithmic growth phase cultures were placed in a Plexiglas recording bath (capacity 1 ml) mounted on the stage of an Olympus CK inverted microscope. The bath was perfused continually with test or control solutions, driven by a Buchler 'polystaltic' pump at a flow rate of 1 ml/min. The bath was held at ground by
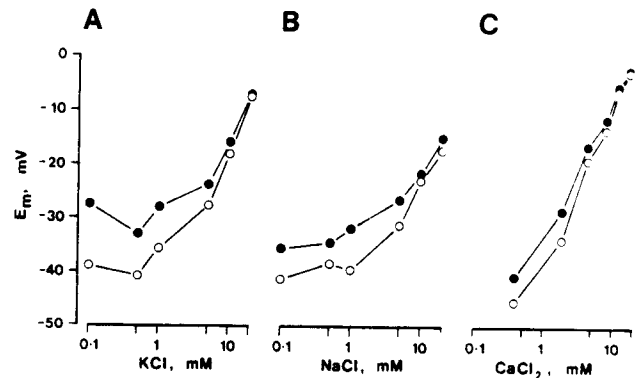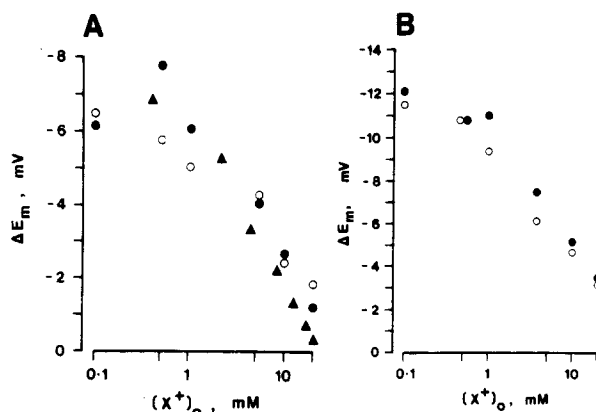

Fig. 1 A–C. Effects of cations on membrane potential responses to acetate. Closed circles represent membrane potentials (E_m) in control solutions, open circles are membrane potentials in 5 mM acetate. A Effects of increasing [K$^+$]$_o$ on membrane potential in 5 mM NaCl or 5 mM Na-acetate. B and C Effects of increased [Na$^+$]$_o$ (B), or [Ca^{++}]$_o$ (C) on membrane potential in 5 mM KCl or 5 mM K-acetate. Data points are the means of 3–8 determinations
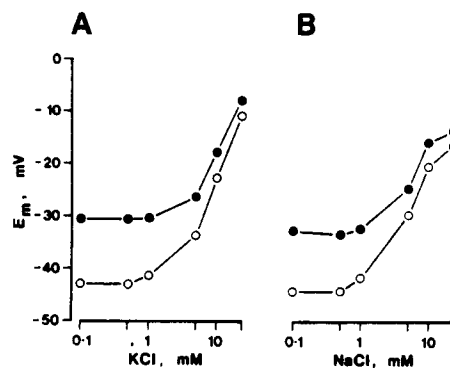
Fig. 2 A, B. Effects of increased extracellular cation concentration on membrane potential responses to folate. Closed circles represent membrane potentials (E_m) in control solutions, open circles are membrane potentials in folate. A effects of KCl on membrane potentials in 5 mM NaCl or 2.5 mM Na$_2$ folate. B effects of NaCl on membrane potentials in 5 mM KCl and 2.5 mM K$_2$ folate. Data points are the mean membrane potentials of 5–7 cells

means of a 2% agar bridge, into which was inserted an Ag/AgCl wire. Intracellular recording and current injection glass capillary microelectrodes were, unless otherwise stated, filled with 0.5 M KCl, tip resistance 60–80 $M\Omega$. Potential signals from the recording microelectrode were amplified using a WPI M701 amplifier, and were displayed on a Tektronix 5112 dual beam oscilloscope. Permanent records were made using a Gould 220 chart recorder. A Grass SD 9 stimulator provided a source of current for intracellular injection, via the recording electrode (facilitated by a bridge circuit included in the amplifier), or via a second, stimulating glass capillary microelectrode. The amount of current injected into the cell was measured using a WPI IVA current to voltage converter placed between the agar bridge and ground.

Membrane potential (E_m) values were determined 6 min after applying a test solution (Van Houten 1979). Membrane potential changes (ΔE_m) are the steady-state differences be-

Fig. 3A, B. Effects of increased extracellular cation concentration on hyperpolarization to acetate and folate. **A** mean membrane potential changes (ΔE_m) induced by 5 mM K- or Na-acetate relative to 5 mM KCl or NaCl. Closed circles represent the effects of KCl on the hyperpolarization, open circles show the effects of NaCl, triangles, effects of $CaCl_2$. **B** effects of increased KCl (closed circles) and NaCl (open circles) concentration on the membrane potential changes (ΔE_m) to Na_2 folate or K_2 folate

Table 1. Effects of mutation-induced perturbation of ion channel function on membrane potential responses of *P. tetraurelia* to acetate and folate

	ΔE_m, mV	
	Acetate	Folate
Wild type	-7.4 ± 0.7 (8)	-11.4 ± 1.6 (22)
Deciliated	-7.9 ± 0.3 (10)	-11.4 ± 0.7 (9)
Pawn	-7.3 ± 0.4 (5)	-11.6 ± 1.1 (6)
Dancer	-7.5 ± 0.4 (4)	-11.2 ± 1.3 (14)
tea A	-6.7 ± 0.4 (7)	-11.4 ± 1.0 (10)
Pantophobiac	-6.9 ± 1.0 (5)	-11.1 ± 0.9 (9)
tea B	-5.4 ± 0.8 (8)	-11.8 ± 1.2 (12)

Data are the mean $\pm$ SD membrane potential responses of wild-type cells, deciliated cells (lacking voltage-dependent Ca^{++} currents), 'Pawn' mutants (with greatly reduced voltage-dependent Ca^{++} channel activity), 'Dancer' mutants (with enhanced voltage-dependent Ca^{++} conductance), *tea A* (enhanced K^+-rectifying current), 'Pantophobiac' (a mutant with reduced Ca^{++}-dependent K^+ channel activity), and *tea B* (reduced surface charge) to 5 mM K-acetate and 2.5 mM K_2 folate, relative to 5 mM KCl. Figures in brackets indicate number of determinations

tween the membrane potential in the test solution and in control solution.

Results

The characteristics and concentration dependence of the hyperpolarizations of the membrane potential of *P. tetraurelia* to acetic and folic acids are decribed in some detail elsewhere (Preston, Schulz and Van Houten, in preparation). When bathed in 5 mM KCl buffer, *P. tetraurelia* has a membrane potential of -32.3 ± 2.5 mV (mean $\pm$ SD, $n = 79$). When this solution is replaced by 5 mM K-acetate, the ciliate typically hyperpolarizes by about 7.5 mV; an approximate -11.5 mV membrane potential change is observed in response to 2.5 mM K_2 folate relative to 5 mM KCl (Table 1). Membrane responses to both attractants are fully reversible.

Mechanism of hyperpolarization in attractants

In order to determine the nature of possible membrane permeability increases during hyperpolarization to attractants, the effects of increasing $[Ca^{++}]_o$, $[K^+]_o$, or $[Na^+]_o$ on the membrane potential of *P. tetraurelia* in acetate and folate were examined. Contrary to expectations, increased concentrations of any one of the three cations decreased the magnitude of the induced membrane potential changes (Figs. 1, 2). In the case of acetate, the membrane response was reduced to less

than a millivolt by 20 mM $[X^+]_o$ (Fig. 3). Further increases in cation concentration are lethal, so it was not possible to determine if the responses reversed polarity as $[X^+]_o$ was raised above 20 mM. Since it is unlikely that acetate or folate cause simultaneous increases in membrane permeability to all three cations, the individual roles of Ca^{++}, K^+, and Na^+ in attractant-induced hyperpolarization were examined in more detail.

Ca^{++} permeability

a) *Paramecium* possesses a major voltage-dependent calcium conductance that mediates depolarizing action potentials in this unicell. The channel can be inactivated genetically (the 'Pawn' mutant) or, since the channel is resident in the ciliary membrane (Ogura and Takahashi 1976; Dunlap 1977), can be eliminated by deciliation. Both Pawn mutant d4-95 and cells deciliated in 5% (v/v) ethanol show normal membrane potential changes in response to 2.5 mM K_2 folate and 5 mM K-acetate (Table 1). Conversely, the activity of the voltage-dependent Ca^{++} channel can be enhanced through mutation; mutant d4-623 ('Dancer', Hinrichsen and Saimi 1984) also shows normal membrane potential responses to 2.5 mM K_2 folate and 5 mM K-acetate (Table 1).

In addition to the voltage-dependent channel of the ciliary membrane, *Paramecium* contains other Ca^{++} conductances that have proved inaccessible to genetic modification. Therefore, at-

Table 2. Effects of drug-induced suppression of ion currents on membrane potential responses to acetate and folate

		ΔE_m, mV
a)	W7	-11.1 ± 1.4 (6)
	EGTA	-10.8 ± 0.8 (6)
b)	K_2 folate	-11.6 ± 1.6 (10)
	Na_2 folate	-12.4 ± 1.8 (11)
c)	KCl control	
	acetate	$- 3.7 \pm 1.4$ (5)
	folate	$- 4.3 \pm 1.1$ (10)
	TEA^+/Cs^+	
	acetate	$- 2.9 \pm 1.5$ (9)
	folate	$- 4.9 \pm 1.6$ (13)
d)	Control	$- 5.6 \pm 0.9$ (11)
	Ruthenium red	$- 4.5 \pm 1.7$ (10)
	Pre-soaked	$- 5.8 \pm 0.9$ (6)

a) Effects of W7, a voltage-dependent Ca^{++} channel inhibitor, on the membrane potential change (ΔE_m) to folate were tested at a concentration of 90 µg/ml. W7 was present in both the control and test solutions (5 mM KCl versus 2.5 mM K_2 folate). Effects of decreased $[Ca^{++}]_i$ on membrane potential responses to 2.5 mM K_2 folate were examined by filling recording microelectrode with 0.25 M K_2EGTA, buffered to pH 7.0 with Tris base. Membrane responses were measured only after the effects of diffusion of EGTA from the electrode tip on membrane potential were apparent.

b) Folate was applied to cells either in the absence of external sodium, or in the absence of external potassium. Data are mean membrane potential changes induced by 2.5 mM K_2 folate relative to 5 mM KCl ($[Na^+]_o = 0$), or 2.5 mM Na_2 folate relative to 5 mM NaCl ($[K^+]_o = 0$)

c) Specimens were impaled with glass capillary recording microelectrodes containing 2 M CsCl. As an ionic strength control, cells were bathed in 19 mM KCl and their responses to 5 mM K-acetate and 2.5 mM K_2 folate ($[K^+]_o = 19$ mM) examined. Recording bath was then filled with a solution containing 10 mM TEA^+, 4 mM Cs^+, and 5 mM KCl ($[X^+]_o = 19$ mM) and the responses to acetate and folate re-examined

d) Effects of ruthenium red on acetate-induced membrane potential changes. 5 mM acetate was applied to three groups of cells: Control – no prior exposure to ruthenium red, Ruthenium red – 5 mM acetate was applied in the presence of 0.1 mM ruthenium red, 6–10 min after first exposure to the dye, Pre-soaked – 5 mM acetate was applied to cells that had been soaking for 3 h in ruthenium red. Data are means $\pm$SD of (*n*) determinations

tempts were made to block any possible attractant-induced increases in $[Ca^{++}]_i$ pharmacologically.

b) W7 [N-(6 aminohexyl)-5-chloro-1-naphthalene-sulphonamide] is a calmodulin antagonist that inhibits the voltage-dependent Ca^{++} current of *P. caudatum* (Hennessey and Kung 1984). Concentrations of W7 that block Ca^{++}-conductance in *P. caudatum* (150 µg/ml) cause the immediate death of *P. tetraurelia*. At 70 µg/ml the drug is no longer toxic, but at this concentration it fails to inhibit Ca^{++}-spiking. Thus as a compromise, cells were

bathed in 90 µg/ml W7 and the membrane potential response to 2.5 mM K_2 folate examined in the brief (10 min) period before cell death. In the absence of a voltage clamp, it is uncertain that W7 completely inhibits the Ca^{++} current, but W7-treated cells fail to show regenerative depolarizations to 400 ms, 1 nA outward current pulses. W7 had no effect on the magnitude of the membrane potential change induced by folate (Table 2a).

c) In order to block increases in $[Ca^{++}]_i$ resulting from increased membrane permeability to Ca^{++}, or from possible mobilization of intracellular Ca^{++} stores, specimens were injected with EGTA. This was effected by using a recording electrode filled with 0.25 M K_2EGTA buffered to pH 7.0 with Tris. The electrode tip breaks during cell penetration, allowing its contents to diffuse into the cell body. The conversion of the normally graded action potential to an all-or-none spike (by suppression of Ca^{++}-dependent K^+ channel activation [Satow 1978]), and the eventual death of the cell, was taken as evidence of increased intracellular EGTA concentration. Data in Table 2a show that EGTA had no effect on the magnitude of the hyperpolarization induced by 2.5 mM K_2 folate however.

K^+ and Na^+ permeability

Figure 3 suggests both a K^+- and an Na^+-dependence of the membrane potential responses to acetate and folate, but neither ion is essential for membrane hyperpolarization: 2.5 mM K_2 folate and 5 mM K-acetate hyperpolarize paramecia relative to 5 mM KCl in the absence of Na^+, and both 2.5 mM Na_2 folate and 5 mM Na-acetate produce equivalent membrane potential changes relative to 5 mM NaCl in the absence of K^+ (Table 2b). These observations argue against specific roles for K^+ and Na^+ in the responses to attractants, but it is possible that there is a cation efflux that does not discriminate between K^+ and Na^+. The concentration of K^+ and Na^+ within *P. tetraurelia* is of the order of 18 mM and 3–4 mM, respectively (Hansma 1974, 1979, 1981). If the hyperpolarizations are due to the efflux of one or either cation, it should be possible to block (or at least reduce) the responses to attractants by raising both $[K^+]_o$ and $[Na^+]_o$ to approximate their intracellular concentration, thereby reducing the driving force for both ions. As a control, cells were bathed in 20 mM KCl. Addition of 5 mM folate ($[K^+]_o = 20$ mM) produced a mean ($\pm$SD) membrane potential change of -5.7 ± 1.0 mV (*n*=6). The con-
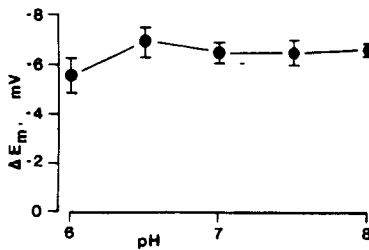
Fig. 4. Effects of pH on membrane hyperpolarization to acetate. Tris buffer was used to vary the pH of the control and test solutions. Data points represent mean ($\pm$SD, $n=3$–7) membrane potential changes (ΔE_m) observed when 5 mM KCl is replaced with 5 mM K-acetate
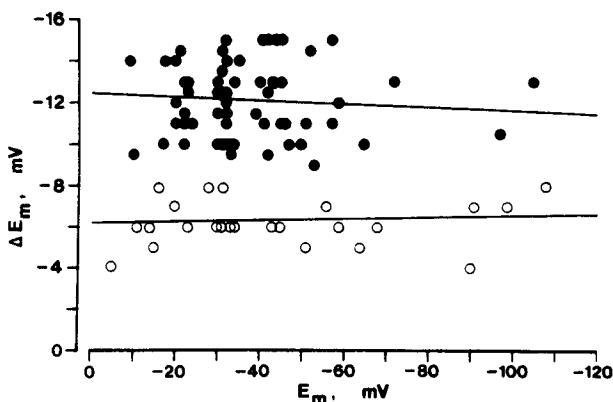

Fig. 5. Voltage independence of the membrane potential responses to acetate and folate. Current injection was used to vary the membrane potential (E_m), and the resultant effects on the magnitude of the hyperpolarization (ΔE_m) to 2.5 mM K$_2$ folate (closed circles) or 5 mM K-acetate (open circles) examined. Lines fitted by regression analyses

trol solution was then replaced with a mixture of 16 mM KCl and 4 mM NaCl ($[X^+]_o = 20$ mM) and the responses to 5 mM folate re-examined. The 6.9 $\pm$ 0.7 mV (mean $\pm$ SD, $n=7$) hyperpolarization was not significantly different from the control response. We therefore conclude that the hyperpolarization to attractants cannot result from either a K$^+$ or an Na$^+$ efflux. The following experiments were carried out to independently confirm this conclusion.

a) A number of mutants are available that have reduced or enhanced K$^+$ conductances. Of these, *tea A* (d4-152) exhibits a stronger rectifying K$^+$ current (Satow and Kung 1976), while 'pantophobiac' (d4-620) lacks most of the slow Ca^{++}-dependent K$^+$ current (Saimi et al. 1983). Both mutants show wild-type responses to acetate and folate (Table 1).

b) Extracellularly-applied tetraethylammonium ion (TEA$^+$) and Cs$^+$ blocks greater than 90% of

the voltage-dependent K$^+$ current in *P. tetraurelia* (Hinrichsen and Saimi 1984). The effect of eliminating this current on the responses to attractants was tested by using 2 M CsCl microelectrodes to impale deciliated cells. The cells were bathed in 5 mM KCl buffer supplemented with 10 mM TEA$^+$ and 4 mM CsCl. The hyperpolarizations induced by replacing 5 mM KCl with either 5 mM K-acetate or 2.5 mM K$_2$ folate were not significantly different from those in control solutions lacking TEA$^+$ and Cs$^+$ (Table 2c).

c) When *P. tetraurelia* is bathed in 5 mM NH$_4$Cl and then exposed to 2.5 mM (NH$_4$)$_2$ folate, the ciliate hyperpolarizes by 11.6 $\pm$ 1.7 mV (mean $\pm$ SD, $n=5$). In the absence of external K$^+$ or Na$^+$ the outward driving force for both ions is greatly enhanced, but the hyperpolarization does not show the corresponding increase in magnitude that would be expected if membrane permeability to either cation were increased by the stimulus.

There remain several possible mechanisms by which the membrane responses to attractants in *P. tetraurelia* might be affected:

a) influx of Tris buffer or citric acid,
b) transport of folate or acetate,
c) non-specific effects of attractants on membrane surface potential, or
d) stimulus-induced extrusion of Ca^{++} or H$^+$.

a) Citrate can be removed from the buffer solution and MOPS can be used in the place of Tris without affecting behavioural or electrophysiological responses of *Paramecium* to attractants (data not shown): it is therefore unlikely that the hyperpolarizations result from increased membrane permeability to either compound.

b) (i) To test the possibility of the attractant-induced membrane responses being caused by the stimulus entering the cell, the effect of varying external pH, and hence the degree of acid dissociation, on hyperpolarization to acetate (pK$_a$ 4.7) were examined. The membrane responses are not significantly affected by a change in pH from 6 to 8 (Fig. 4).

(ii) Paramecia sequester radiolabelled acetic and folic acids (Van Houten 1976; Schulz et al. 1984). When incubated overnight in growth medium supplemented with 2.5 mM folate, the intracellular folate concentration increases from 2 μM to 130 μM (as determined by radioimmunoassay, S. Schulz, personal communication) yet the cells hyperpolarize normally in 2.5 mM K$_2$ folate (-12.3 ± 1.7 mV, mean $\pm$ SD, $n=8$).
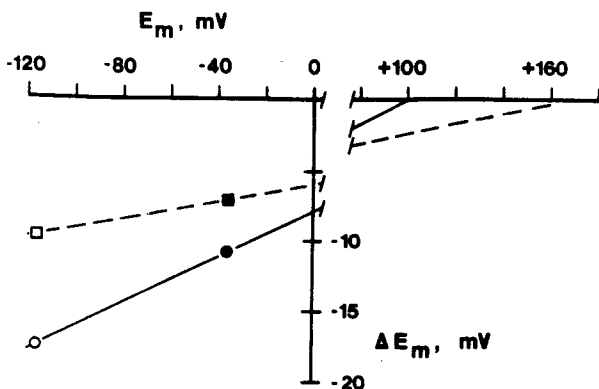
Fig. 6. Membrane potential responses of the restless mutant to acetate and folate in the presence or absence of external potassium. Closed symbols represent membrane potential changes (ΔE_m) induced by 2.5 mM K_2 folate (closed circle) or 5 mM K-acetate (closed square) relative to 5 mM KCl. Cells were then bathed in 5 mM NaCl, causing an approximate -80 mV drop in membrane potential, and the responses to 2.5 mM Na_2 folate (open circle) and 5 mM Na-acetate (open square) examined. Lines connecting the two sets of data can be extrapolated back to the abscissa to give an estimated 'reversal potential'. Data points are mean responses of 8–13 cells

c) The non-specific effects of cations on the attractant-induced hyperpolarizations suggest that acetate or folate might exert their effects by interaction with the surface potential of *P. tetraurelia*. This hypothesis was tested by examining the voltage-dependence of the responses, by measuring changes in membrane resistance during hyperpolarization, and by testing the effects of impermeable cations on the membrane responses.

(i) The voltage dependence of the membrane responses to folate and acetate was tested by injecting sustained inward or outward current pulses to shift the membrane potential from rest. The membrane potential of *P. tetraurelia* becomes unstable under such conditions which hampers attempts to measure steady-state voltage changes to acetate and folate application, but the responses are not obviously voltage dependent over a range of 0 mV to -110 mV (Fig. 5).

Contrasting with these data is the apparent membrane potential-dependence of the responses of mutant d4-647 ('restless') to folate and acetate. Restless possesses a defect that causes a rapid loss of $[K^+]_i$ when transferred to K^+-free media (Richard et al. 1985). This K^+ efflux is accompanied by increased membrane potential (from -36.6 ± 4.2 mV in 5 mM KCl to -117.0 ± 12.7 mV in 5 mM NaCl, means $\pm$ SD, $n = 12$ and 8 respectively), thus providing a means of manipulating membrane potential without injecting current. Mutant cells in 5 mM KCl buffer show wild-type

membrane potential responses to 2.5 mM K_2 folate and 5 mM K-acetate, but significantly larger hyperpolarizations to sodium salts of the two attractants in K^+-free solutions (Fig. 6). When these membrane potential changes are plotted against resting potentials in K^+ and Na^+, the resulting lines that connect the two sets of data can be extrapolated back to $+170$ mV in the case of acetate, and $+101$ mV for folate (Fig. 6): membrane potentials that are close to the estimated calcium equilibrium potential for *P. tetraurelia* ($+105$ mV in Ca-citrate buffer: $[Ca^{++}]_i$ is estimated to be 0.1 μM [Naitoh and Kaneko 1972]). At present, it is not possible to say whether the enhanced responses in K^+-free solutions result from the effects of increasing membrane potential, or from the secondary effects of ion substitution on a mutant membrane. The inability to show that the hyperpolarizations reverse direction when current is used to shift membrane potential (Fig. 5) favours the latter hypothesis. Theoretically, it should be possible to place restless in a K^+-free environment and then inject current to bring the mutant's membrane back toward -40 mV. If the resultant response to folate equalled that in K^+ solutions, it is likely that restless does provide an accurate means of determining a reversal potential for the responses. Unfortunately, the membrane potential of restless becomes unstable when depolarized by greater than 50 mV, and the cell generates repeated and prolonged all-or-nothing spikes ($+60$ mV, 2–14 s). Measurements of responses to folate are unreliable under such conditions.

(ii) The resistance of the membrane of *P. tetraurelia* during hyperpolarization in K_2 folate or K-acetate was monitored by injecting repeated (0.8 Hz) 0.3 nA, 500 ms inward current pulses. The observed voltage responses to current injection are small and variable, therefore data were normalized by taking the mean response to ten current pulses immediately following a change of solution in the recording bath (5 mM KCl to test solution, and vice versa) and expressing this figure as a percentage of the mean of ten current pulse responses immediately prior to solution changeover. The results are presented in Fig. 7.

Folate concentrations of less than 1.0 mM produce very little change in membrane resistance. At concentrations in excess of 1.0 mM, a net membrane resistance increase is observed as folate replaces the control solution, but even at 2.5 mM, the evoked change is less than 10%. In contrast, concentrations of acetate that are sufficient to hyperpolarize the cell also produce decreased membrane resistance with a corresponding resistance
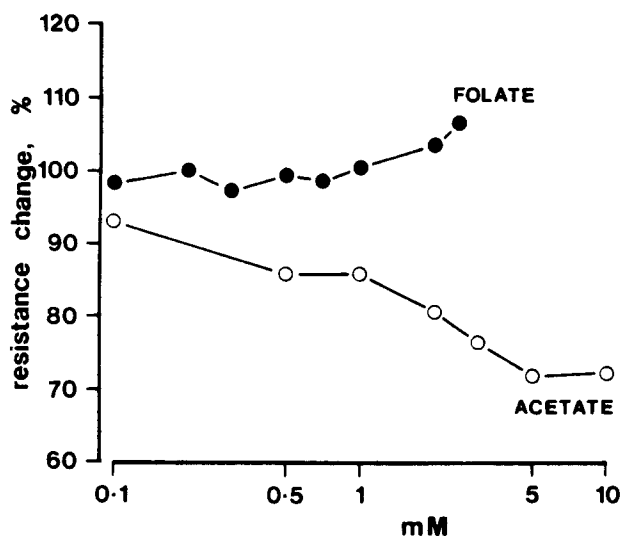
Fig. 7. Changes in membrane resistance during acetate and folate induced hyperpolarization. Data points are the mean ($n = 4$–14) percent changes in membrane resistance observed following hyperpolarization to increasing concentrations of K-acetate (open circles) or K_2 folate (closed circles) relative to equivalent concentrations of KCl

increase as control solution replaces acetate in the recording bath.

(iii) Application of 0.1 mM ruthenium red, an impermeable, polycationic dye, to *P. tetraurelia* produced a comparatively slow depolarization of the membrane potential (of the order of 12 mV over 6–10 min). Subsequent application of 5 mM K-acetate in the continued presence of ruthenium red caused a hyperpolarization that was not significantly different from the cells' response to acetate in the absence of ruthenium red (Table 2d). Folate is precipitated by millimolar concentrations of polycations, so it was not possible to test the effects of ruthenium red on the membrane responses to this attractant.

If paramecia are incubated in 0.1 mM ruthenium red for 2.5–3 h, the ciliates gradually lose their spontaneous turning behaviour and fail to show avoidance responses to mechanical stimulation. These cells still hyperpolarize normally when introduced to 5 mM K-acetate, however (Table 2d).

(iv) The 'TEA-insensitive' mutant, *tea B*, shows an approximate +10 mV shift in surface potential compared with wild type (Satow and Kung 1981). While application of 2.5 mM K_2 folate to *tea B* elicits a hyperpolarization that is indistinguishable from a wild type response, the membrane potential change induced by 5 mM K-acetate is significantly reduced (Table 1).

Discussion

To summarize: acetate and folate hyperpolarize the membrane potential of *P. tetraurelia* in a concentration dependent manner. These responses are accompanied by only small changes in membrane resistance and their magnitude is significantly reduced by increasing concentrations of cations in the extracellular medium. This decrease in response size probably results from ionic strength effects rather than specific alterations of driving forces on membrane permeable ions that might carry a hyperpolarizing current.

The data presented above suggest that neither Cl^-, K^+, nor Na^+ are involved in the membrane responses of *P. tetraurelia* to acetate and folate. Naitoh et al. (1972) previously established that Cl^- does not significantly contribute to the resting potential of *Paramecium* and that there is no increase in permeability to Cl^- during current-induced excitation, so it is unlikely that a Cl^- flux mediates the attractant-induced hyperpolarizations. In addition, the effects of acetate and folate on membrane potential are examined relative to Cl^-: cells hyperpolarize as, for example, 2.5 mM K_2 folate or 5 mM K-acetate replaces 5 mM KCl in the recording bath. In both examples, the induced potential change is sustained in the absence of external chloride.

The membrane responses to attractants might be expected to be due to an outward K^+ flux, since this cation mediates hyperpolarization to posterior mechanostimulation in *Paramecium* (Naitoh and Eckert 1973). However, in addition to the results of applying acetate and folate to cells with genetic or pharmacologically-induced defects in K^+ or Na^+ channel function, there are three lines of evidence that rule out the possibility of the attractant responses involving monovalent cations. Firstly, substituting NH_4^+ for K^+ and Na^+ has no effect on the magnitude of the hyperpolarization, despite the enhanced outward driving force for these ions. Secondly, collapsing this driving force by raising external K^+ and Na^+ levels to approximate internal concentrations similarly had no effect on the attractant responses. Lastly, if a K^+ or an Na^+ flux were the mechanism, or at least part of the mechanism of hyperpolarization in acetate and folate, injecting current to modify the membrane potential of *P. tetraurelia* should have shown a reduction in the size of the responses with increasing cell negativity. Contrasting with these results is the strong dependence of the membrane potential responses to attractants on external cation concentration (Fig. 3). Since this depen-

dence cannot be due to decreasing the driving force for cations, it must be assumed that increasing the ionic strength of the medium adversely affects an intramembranous component that mediates hyperpolarization to attractants. The non-specific effects of cations on the excitability of *Paramecium* have been recognized for some time. Naitoh and Eckert (1968) initially took the decrease in the membrane potential of *P. caudatum* with increasing $[X^+]_o$ to result from an indiscriminate increase in membrane permeability to cations. It has since been suggested that the membrane of *Paramecium* is more selective in its permeability, and that cations depolarize the ciliate by screening negative charges on the cell surface (Eckert and Brehm 1979). These charges are inherent in membrane proteins and polar lipid head groups when the cell is bathed in low ionic strength media, and collectively form a surface potential that extends some distance into the external medium. When cations bind to these charges, there is a drop in the potential difference across the inner and outer surfaces of the membrane. This drop is not seen by an intracellular recording microelectrode, but it acts on intramembranous components, including ion channels. A resultant selective cation influx or anion efflux restores the potential drop across the inner and outer membrane surfaces and is recorded as cell depolarization. Although still untested and open to interpretation, this hypothesis could explain many aspects of *Paramecium* behaviour.

Changes in surface potential are known to alter membrane conductance behaviour (Frankenhäuser and Hodgkin 1957) and have been suggested as a mechanism of chemosensory transduction in a number of sytems (Gingell 1971). Kashiwayangi and Kurihara (1984) and Kumazawa et al. (1985) used a mouse neuroblastoma cell line as a model system in an attempt to discern the mechanism by which odorants and tastants trigger receptor potentials in Metazoa. These cells do not originate from a specialized chemosensory system, but they respond to odors such as camphor, and to bitter substances such as quinine with membrane depolarization at concentrations equivalent to those necessary to produce a response from olfactory or gustatory receptor cells. The authors conclude that since there is little change in membrane resistance during depolarization, and that the response is voltage-independent, odorants and tastants effect membrane potential changes by interaction with the cell surface potential. The ciliated protozoan *Tetrahymena pyriformis* similarly responds to salts, odorants and bitter substances with membrane depolarization and negative che-

motaxis (Ueda and Kobatake 1977; Ataka et al. 1978; Aiuchi et al. 1980; Tanabe et al. 1984). These responses are sensitive to the amount of polar lipids in the cell membrane, so it is likely that stimulus transduction is affected by charge screening or by the charge redistribution that occurs when electrically neutral compounds dissolve in the lipid portion of the membrane (Tanabe et al. 1979).

There are some indications that acetate and folate may similarly act on the surface potential of *P. tetraurelia* to account for at least part of the attractant-induced hyperpolarization. Neither acetate nor folate produce the large changes in membrane resistance that might be expected if they were opening or closing ion channels in the membrane. Acetate elicits a greater change in membrane resistance than folate but the two attractants have opposite effects: folate application leads to a small resistance increase, while acetate decreases membrane resistance. It is unlikely that acetate and folate should hyperpolarize by two different mechanisms that are similarly sensitive to external cations and insensitive to perturbation of ion channel function. Secondly, the hyperpolarizations to folate and acetate are similarly membrane potential independent; there is no indication of a reversal potential for either response (Fig. 5). While these observations suggest some form of surface charge involvement in the responses of *P. tetraurelia* to attractants, there is evidence that argues against surface charge being the sole mechanism of transducing chemostimulation. Firstly, although mutant *tea B* shows a significantly smaller hyperpolarization to 5 mM K-acetate compared with the wild type (Table 1), it responds normally to 2.5 mM K_2 folate. Satow and Kung (1981) estimated that the surface potential of this mutant to have been reduced by about 10 mV, an effect which could be mimicked in wild type paramecia by increasing the concentration of cations in the external medium. The effects of a cation-induced 10 mV depolarization on the magnitude of the responses to acetate and folate can be seen in Fig. 3: the two attractants elicit significantly smaller membrane potential changes when $[X^+]_o$ is raised. Thus, if surface charge were the sole means by which chemicals hyperpolarize *Paramecium*, mutant *tea B* would be expected to show a similarly reduced capacity to respond to both acetate and folate. Lastly, if acetate and folate were acting solely through a surface potential change, the polycationic dye ruthenium red should compete with attractants for anionic sites on the cell surface and thereby block the hyperpolarization. Its lack of effect on the membrane response to acetate (Ta-

ble 2d) again suggests that changes in surface potential are not the mechanism by which chemosensation is transduced in *P. tetraurelia*.

The effects of attractants on the membrane potential of the restless mutant in the presence or absence of external potassium are intriguing (Fig. 6), since they hint at Ca^{++} involvement in the hyperpolarization. The nature of this involvement is uncertain however. *P. tetraurelia* requires at least $10^{-5} M$ $[Ca^{++}]_o$ for survival, so that it has not been possible to show an absolute dependence of the membrane responses on Ca^{++} by removing the ion from the external medium. It is unlikely that the voltage-dependent Ca^{++} channels are involved in the hyperpolarizations since a) deciliated cells and Pawn mutants, both of which lack functional Ca^{++} channels, show wild type responses to acetate and folate, and b) an increase in intraciliary Ca^{++} concentration would initially cause depolarization and reversed ciliary beating. A possible explanation for the observed membrane responses is that attractants cause a decrease in voltage-dependent Ca^{++} conductance to hyperpolarize the cell, but in the absence of a significant increase in membrane resistance during attractant application, this seems unlikely. A simultaneous increase in K^+ or Na^+ permeability could explain the small change in membrane resistance; this possibility is being further investigated under voltage clamp.

There has recently been a growing appreciation of the role of calcium as a second messenger of chemostimulation in a variety of systems (reviewed by Rasmussen and Barrett 1984), a role that does not necessarily involve activation or deactivation of ion channels. It is possible to envision a similar role for calcium in the responses of *P. tetraurelia* to folate and acetate, whereby binding of attractant to the cell surface causes mobilization or displacement of a plasma membrane-bound pool of Ca^{++}. The released cation then activates a Ca^{++}-ATPase, causing Ca^{++} to be pumped out of the cell with resultant membrane hyperpolarization. The cause of the initial Ca^{++} release may be twofold. Firstly, specific or non-specific attractant binding may disturb the negative charge density on the outer cell surface and, as a result, cause positive charges to be displaced from the cytoplasmic side of the membrane. Secondly, it is possible that binding of attractants to specific receptors causes lipid turnover within the membrane, promoting the release of Ca^{++} from highly charged phospholipid head groups. A similar mechanism has been proposed to account for the release of a plasma membrane-bound pool of 'trigger' calci-

um immediately after binding of the attractant f-methionyl-leucyl-phenylalanine to leukocytes (Smolen and Weissmann 1982; Stickle et al. 1984). As in the present study (Table 2), the response is insensitive to calmodulin inhibitors and EGTA (Smolen and Weissmann 1982).

Reports of Ca^{++}-ATPase activities in *Paramecium* are numerous (reviewed by Riddle et al. 1982): Andrivon et al. (1983) electrophoretically resolved 15 proteins with such activity in extracts of whole cells. At least two of these are associated with cilia and, while it has not proved possible to assign specific functions to any of these enzymes, one is presumed to be involved in the extrusion of Ca^{++} from the intraciliary space following activation of voltage-dependent Ca^{++} channels in the ciliary membrane (Browning and Nelson 1976; Martinac and Hildebrand 1981). However, it is unlikely that a ciliary calcium pump is responsible for the membrane hyperpolarizations to acetate and folate since these responses remain following artificial deciliation of *P. tetraurelia*. In addition, Riddle et al. (1982) report the likely candidate for a ciliary Ca^{++}-pump to be inhibited by ruthenium red: data in Table 2 clearly show the responses of *Paramecium* to acetate to be insensitive to ruthenium red, despite its inhibitory effects on responses to depolarizing stimuli. Noguchi et al. (1979) and Bilinski et al. (1981) have characterized Ca^{++}-ATPases associated with pellicle (cell body) membranes of *Paramecium*, but the kinetic parameters and ion sensitivities of the enzymes are very similar to that of the ciliary Ca^{++}-ATPase, leading Riddle et al. (1982) to suggest that the three may be identical. However, comparatively little is known of the function or possible localization of any of the 15 Ca^{++}-ATPases described by Andrivon et al. (1983), so it is conceivable that the cell body contains an electrogenic Ca^{++} pump of the type observed in erythrocytes (Gimble et al. 1982; Smallwood et al. 1983) that hyperpolarizes the cell by Ca^{++} extrusion following attractant binding. Ideally, it should be possible to mimic attractant-induced membrane potential changes by artificially increasing $[Ca^{++}]_i$. Attempts to raise internal calcium by intracellular injection have produced hyperpolarizations of variable magnitude, such as reported by Satow (1978). Presumably the depolarizing effects of injecting a divalent cation are offset to various degrees by activation of hyperpolarizing Ca^{++}-dependent K^+ or Na^+ conductances in the cell membrane.

Finally, there is the possibility of the involvement of a cation/proton extrusion mechanism in the responses of *P. tetraurelia* to attractants. Acti-

vation of a proton pump has also been implicated in the mechanism of gustation, since amiloride, a Na^+/H^+ exchange inhibitor (Benos 1982), reduces salt taste (Schiffman et al. 1983; Desimone et al. 1984). This pump is electroneutral, but the effects of amiloride on attraction of *Paramecium* to acetate have recently been tested (Van Houten and Preston 1985). Amiloride has no effect on attraction to acetate in potassium solutions, but blocks the response to sodium salts. Electrophysiological data suggest that amiloride has no effect on the size of the hyperpolarization to either the potassium or sodium salt of acetate, so the diuretic may interfere with regulation of intracellular sodium, and hence resting membrane potential and turning frequency. The mechanism of amiloride action on the behavioural response is being further investigated.

References

Aiuchi T, Tanabe H, Kurihara K, Kobatake Y (1980) Fluorescence changes of rhodamine 6G associated with chemotactic responses in *Tetrahymena pyriformis*. Biochim Biophys Acta 628:355–364

Andrivon C, Brugerolle G, Delachambre D (1983) A specific Ca^{2+}-ATPase in the ciliary membrane of *Paramecium tetraurelia*. Biol Cell 47:351–364

Ataka M, Tsuchii A, Ueda T, Kurihara K, Kobatake Y (1978) Comparative studies on the reception of bitter stimulus in the frog, *Tetrahymena*, slime mold and *Nitella*. Comp Biochem Physiol 61 A:109–115

Benos DJ (1982) Amiloride: a molecular probe of sodium transport in tissues and cells. Am J Physiol 242:C131–C145

Bilinski M, Plattner H, Tiggemann R (1981) Isolation of surface membranes from normal and exocytotic mutant strains of *Paramecium tetraurelia*. Ultrastructural and biochemical characterization. Eur J Cell Biol 24:108–115

Browning JL, Nelson DL (1976) Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. I. $^{45}Ca^{2+}$ fluxes across the resting and excited membrane. Biochim Biophys Acta 448:338–351

Desimone JA, Heck GL, Mierson S, Desimone SK (1984) The active ion transport properties of canine lingual epithelia in vitro. J Gen Physiol 83:633–656

Dunlap K (1977) Localization of calcium channels in *Paramecium caudatum*. J Physiol (Lond) 271:119–133

Eckert R, Brehm P (1979) Ionic mechanisms of excitation in *Paramecium*. Annu Rev Biophys Bioeng 8:353–383

Frankenhäuser B, Hodgkin AL (1957) The action of calcium on the electrical properties of the squid axon. J Physiol (Lond) 137:218–244

Gimble JH, Waisman DM, Gustin M, Goodman DBP, Rasmussen H (1982) Studies of the Ca^{2+} transport mechanism of human erythrocyte inside-out membrane vesicles. Evidence for the development of a positive interior membrane potential. J Biol Chem 257:10781–10788

Gingell D (1971) Cell membrane surface potential as a trans-

ducer. In: Bittar EE (ed) Membranes and ion transport, vol 3. Wiley & Sons, Bath, pp 317–357

Hansma HG (1974) Biochemical studies on the behavioral mutants of *Paramecium aurelia*: ion fluxes and ciliary membrane proteins. PhD dissertation. University of California, Santa Barbara

Hansma HG (1979) Sodium uptake and membrane excitation in *Paramecium*. J Cell Biol 81:374–381

Hansma HG (1981) Turnover of monovalent cations in *Paramecium*. J Exp Biol 93:81–92

Hennessey TM, Kung C (1984) An anticalmodulin drug, W-7, inhibits the voltage-dependent calcium current in *Paramecium caudatum*. J Exp Biol 110:169–181

Hinrichsen RD, Saimi Y (1984) A mutation that alters properties of the calcium channel in *Paramecium tetraurelia*. J Physiol (Lond) 351:397–410

Jennings HS (1906) Behavior of the lower organisms. Columbia University Press, New York

Kashiwayanagi M, Kurihara K (1984) Neuroblastoma cell as a model for olfactory cell: mechanism of depolarization in response to various odorants. Brain Res 293:251–258

Kumazawa T, Kashiwayanagi M, Kurihara K (1985) Neuroblatoma cell as a model for a taste cell: mechanism of depolarization in response to various bitter substances. Brain Res 333:27–33

Kung C, Saimi Y (1982) The physiological basis of taxes in *Paramecium*. Annu Rev Physiol 44:519–534

Martinac B, Hildebrand E (1981) Electrically-induced Ca^{2+} transport across the membrane of *Paramecium caudatum* measured by means of flow-through technique. Biochim Biophys Acta 649:244–252

Naitoh Y, Eckert R (1968) Electrical properties of *Paramecium caudatum*: modification by bound and free cations. Z Vergl Physiol 61:427–452

Naitoh Y, Eckert R (1972) Electrophysiology of the ciliate Protozoa. In: Kerkut GA (ed) Experiments in physiology and biochemistry, vol 5. Academic Press, New York, pp 17–31

Naitoh Y, Eckert R (1973) Sensory mechanisms in *Paramecium*. II. Ionic basis of the hyperpolarizing mechanoreceptor potential. J Exp Biol 59:53–65

Naitoh Y, Kaneko H (1972) ATP-Mg^{2+} reactivated triton-extracted models of *Paramecium*: modification of ciliary movement by calcium ions. Science 176:523–524

Naitoh Y, Eckert R, Friedman K (1972) A regenerative calcium response in *Paramecium*. J Exp Biol 56:667–681

Noguchi M, Inoue H, Kubo K (1979) Calcium-activated adenosine triphosphatase activity of pellicles from *Paramecium caudatum*. J Biochem 85:367–373

Ogura A, Machemer H (1978) Electrophysiological consequence of deciliation in *Paramecium*. Acta Protozool 18:183

Ogura A, Takahashi K (1976) Artificial deciliation causes loss of calcium dependent responses in *Paramecium*. Nature 264:170–172

Ordal GW (1985) Bacterial chemotaxis: biochemistry of behavior in a single cell. CRC Crit Rev Microbiol 12:95–130

Rasmussen H, Barrett PQ (1984) Calcium messenger system: an integrated view. Physiol Rev 64:938–983

Richard EA, Hinrichsen RD, Saimi Y (1985) A single gene mutation that affects a potassium conductance and resting membrane potential in *Paramecium*. J Neurogenet 2:239–252

Riddle LM, Rauh JJ, Nelson DL (1982) A Ca^{2+}-activated ATPase specifically released by Ca^{2+} shock from *Paramecium tetraurelia*. Biochim Biophys Acta 688:525–540

Saimi Y, Hinrichsen RD, Forte M, Kung C (1983) Mutant analysis shows that the Ca^{2+}-induced K^+ current shuts off

one type of excitation in *Paramecium*. Proc Natl Acad Sci USA 80:5112–5116

Satow Y (1978) Internal calcium concentration and potassium permeability in *Paramecium*. J Neurobiol 9:81–91

Satow Y, Kung C (1976) A 'TEA$^+$-insensitive' mutant with increased potassium conductance in *Paramecium aurelia*. J Exp Biol 65:51–63

Satow Y, Kung C (1981) Possible reduction of surface charge by a mutation in *Paramecium tetraurelia*. J Membr Biol 59:179–190

Schiffman SS, Lockhead E, Maes FW (1983) Amiloride reduces the taste intensity of Na$^+$ and Li$^+$ salts and sweeteners. Proc Natl Acad Sci USA 80:6136–6140

Schulz S, Denaro M, Xypolyta-Bulloch A, Van Houten J (1984) Relationship of folate binding to chemoreception in *Paramecium*. J Comp Physiol A 155:113–119

Smallwood JI, Waisman DM, Lafreniere D, Rasmussen H (1983) Evidence that the erythrocyte calcium pump catalyzes a Ca^{2+}:nH$^+$ exchange. J Biol Chem 258:11092–11097

Smolen JE, Weissmann G (1982) The effect of various stimuli and calcium antagonists on the fluorescence response of chlorotetracycline-loaded human neutrophils. Biochim Biophys Acta 720:171–180

Stickle DF, Daniele RP, Holian A (1984) Cytosolic calcium, calcium fluxes, and regulation of alveolar macrophage superoxide anion production. J Cell Physiol 121:458–466

Tanabe H, Kurihara K, Kobatake Y (1979) Sensitization of chemotactic response by changing the lipid composition of surface membrane in *Tetrahymena pyriformis*. Biochim Biophys Acta 553:396–403

Tanabe H, Kamo N, Kobatake Y (1984) Fluorometric estimation of surface potential change associated with chemotactic stimulation in *Tetrahymena pyriformis*. Biochim Biophys Acta 805:345–353

Ueda T, Kobatake Y (1977) Hydrophobicity of biosurfaces as shown by chemoreceptive thresholds in *Tetrahymena*, *Physarum* and *Nitella*. J Membr Biol 34:351–368

Van Houten JL (1976) Chemokinesis in *Paramecium*: A genetic approach. PhD Dissertation, University of California, Santa Barbara

Van Houten J (1978) Two mechanisms of chemotaxis in *Paramecium*. J Comp Physiol 127:167–174

Van Houten J (1979) Membrane potential changes during chemotaxis in *Paramecium*. Science 204:1100–1103

Van Houten J, Preston RR (1985) Effect of amiloride on *Paramecium* chemoresponse. Chem Senses 10:466