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SEXUAL BEHAVIOUR IN *EUPLOTES RAIKOVI* IS ACCOMPANIED BY PHEROMONE-INDUCED MODIFICATIONS OF IONIC CURRENTS

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Summary

In the marine ciliate *Euplotes raikovi*, pheromone released by a complementary mating type (nonself pheromone) induces typical sexual behaviour, whereas self pheromone released by the same mating type generally has no effect. Nonself pheromone evokes a reduction of the mean walking speed by 66 %, a threefold increase in the frequency and duration of long-lasting rest phases and a doubling in the number of side-stepping reactions. Consequently, translocation is strongly reduced and the cells remain in a small area. This could increase the probability of finding a sexual partner for pair formation (conjugation). The usual pattern of rhythmic, spontaneous depolarizations controlling the walking rhythm is absent in nonself-pheromone-stimulated cells. The remaining depolarizations arise from a 4 mV hyperpolarized membrane potential and do not reach the usual amplitudes of 15–20 mV but only of 6–10 mV. In addition, the amplitudes of K^+ currents are increased at depolarizations of more than 20 mV by at least 30 %. Hyperpolarization- and depolarization-activated Na^+ current amplitudes are increased, whereas the Ca^{2+} current amplitude remains nearly unaffected.

Key words: ciliary reversal, K^+ current, membrane potential, voltage-dependent Ca^{2+} current, side-stepping reaction, ciliate, *Euplotes raikovi*.

Introduction

Some species of ciliates can be divided into a variety of mating types distinguished by chemical substances termed gamones or pheromones (Dini and Nyberg, 1993; Mulisch, 1996; Raffioni et al., 1992). These species-specific molecules are either anchored to the cell membrane, as in *Euplotes crassus* and *Paramecium* spp. (Mulisch, 1996; Kitamura and Hiwatashi, 1980; Kitamura, 1988), or released into the surrounding medium, as in *Euplotes raikovi* (Ortenzi and Luporini, 1995; Luporini et al., 1983, 1995). In *Euplotes raikovi*, they have been identified as polypeptide pheromones and are closely related to each other by their molecular architecture (Mronga et al., 1994; Luporini et al., 1995). Each mating type produces a specific protein that is either incorporated into the plasma membrane as a receptor protein or released as a pheromone protein. Both proteins are produced as isoforms by alternative mRNA splicing (Luporini et al., 1995). The pheromone protein binds with similar, but not identical, affinities to the receptor protein of its own mating type and to the receptor protein of other mating types. Pheromone of its own mating type (self pheromone) and pheromone of complementary mating types (nonself pheromone) bind in a competitive fashion to the same receptor protein, and the quantitative relationship between bound pheromone types determines whether the vegetative or the mating stage should follow (Ortenzi and Luporini, 1995). A preponderance of bound nonself pheromone induces mating and generates specific mating behaviour that leads to the formation of cell pairs (conjugation).

In ciliates, the membrane potential and the intraciliary Ca^{2+} concentration control the powerstroke direction, the frequency of ciliary beating (Eckert, 1972; Naitoh and Kaneko, 1972) and, thereby, the translocation of cells (Machemer and Sugino, 1989; Lueken et al., 1996). In *Paramecium* spp., depolarizing receptor potentials activate Ca^{2+} conductances within the ciliary membrane, and the resultant rise in the intraciliary Ca^{2+} concentration evokes ciliary reversal and thereby backward swimming. Hyperpolarizing receptor potentials, induced by augmentation of a K^+ conductance, cause faster forward swimming by increasing the frequency and amplitude of ciliary beating (Machemer, 1989; Machemer and Teunis, 1996).

In *Euplotes raikovi*, as described for *Euplotes vannus* (Lueken et al., 1996), a pattern of rhythmic, spontaneous depolarizations controls a rhythm of speed decrease and increase in forward-walking cells. Prolonged depolarizations induce decelerations to zero velocity followed by fast backward jerks as in *Bursaridium difficile* (Berg and Sand, 1994). The present study describes nonself-pheromone-induced modifications of this depolarization pattern as well as of walking behaviour.

Materials and methods

Cells and culture

Complementary mating types II and XI of *Euplotes raikovi* (termed *Euplotopsis raikovi* by Borror and Hill, 1995) were used. They belong to the clones $1bF_113$ and $1aF_13N$, respectively (Luporini et al., 1983), and were obtained from Dr P. Luporini (Department of Cell Biology, University of Camerino, Italy). Cells were cultivated in artificial sea water and fed with bacteria as described previously for *Euplotes vannus* (Lueken et al., 1987). The voltage-clamp-induced individual current components from the total current pattern of *Euplotes raikovi* were compared with the ion currents of *Euplotes vannus* (clone D35; termed *Moneuplotes vannus* by Borror and Hill, 1995) formerly identified by our group (see Fig. 4). All experiments were performed at room temperature (20–22 °C) on cells that had been starved for 13 days.

Solutions

Artificial sea water (ASW) was prepared from analysis-grade salts (in mmol l^{-1}): NaCl, 465; $NaHCO_3$, 2.5; KCl, 10; $CaCl_2$, 10.4; $MgCl_2$, 24.8; $MgSO_4$, 28.1. The pH was adjusted with HCl to 8.0–8.1. Behavioural as well as electrophysiological experiments were performed in ASW.

Pheromone solutions were obtained by sterile filtration of the culture medium from either mating type. They were applied to the complementary mating type or, as a control, to the pheromone-producing mating type. The effectiveness of nonself-pheromone solution was assessed by the induction of intraclonal conjugation.

Behavioural experiments

The observation chamber was a plastic Petri dish (Nunc, diameter 35 mm), designed for cell culture purposes, filled with 200 µl of medium containing cells and 1800 µl of experimental solution. Cells moving on the bottom of the chamber were observed using an inverted microscope (Olympus CK2) and video-recorded at 50 frames s^{-1} under darkfield illumination. Randomly selected walking tracks were digitized in real time using a frame grabber board (PIP Video digitizer board 1024B, Matrox, Canada) and BIOTRACK software (Fa. Vogel Bildverarbeitungssysteme, D-91054 Erlangen, Germany). These tracks were resolved into time segments of 80 ms using WINTRACK software, version 1.6 (Fa. Vogel). Distances were calibrated using an object micrometer, and direct measurements were made on the video monitor. Recordings were made using Panasonic cameras (WV-BL 600 and WV-CL 502) and Panasonic recorders (AG-6720-E and NV-FS200-EG). The latter has a slow-motion and single-image mode that produces half-image steps of 20 ms.

Electrophysiological recordings

Only cells of mating type II were investigated using electrophysiological methods. By analogy to behavioural experiments, 900 µl of either pheromone solution was added to 100 µl of cell suspension. No further change of solution was made because such changes induce a loss of mating competence in microelectrode-penetrated cells. Therefore, cells were investigated either in self or in nonself pheromone. The numbers of cells tested in each solution are given in the figure legends.

Shortly (5 min) after cell transfer, most cells had settled at the bottom of the chamber. One of these cells was captured using a suction micropipette, penetrated with a single microelectrode and finally released from the pipette. Cells could rotate around the tip of the inserted microelectrode. A single-electrode voltage-clamp system (npi SEC 1L, H.-R. Polder, D-71732 Tamm, Germany), an analog-to-digital interface board (Tecmar, Scientific Solutions Division, Solon, OH, USA) connected to a personal computer and pCLAMP software version 6.0.2. (Axon, Foster City, CA, USA) were used. Switching frequency was 10 kHz with a duty cycle of 50 %. Recordings were filtered on-line at 1 kHz with an eight-pole Bessel filter (48 dB per octave). Microelectrodes, filled either with 0.5 mol l^{-1} KCl or 0.5 mol l^{-1} CsCl to block K^+ channels, had resistances of 30–40 MΩ. Microelectrodes filled with 0.33 mol l^{-1} KCl were used to reduce the electromotive force for outwardly directed K^+ currents. Direct changes of the pheromone-containing extracellular K^+ concentrations were not possible because the pheromone solutions were obtained from the culture medium with a standard K^+ concentration and because of problems associated with mechanical agitation during solution changes. The free-running membrane potential was recorded in the current-clamp mode at zero-current injection at a sampling rate of 1000 Hz for 1 min periods. During voltage-clamp experiments, the holding potential was adjusted with respect to the cell-specific resting potential at which the transverse cirri did not move. This resting potential is not stable in *Euplotes raikovi* owing to spontaneously generated depolarizations. The lowest level of these membrane potential fluctuations, i.e. the resting potential, is usually preferred to the depolarized levels and will be termed the preference potential. The current–voltage diagrams show currents activated by voltage steps (ΔV_m) in relation to the holding potential, which was set to equal the preference potential for each cell. Differences in the preference potentials of different cells of up to 6 mV are evened out by this method of data collection and presentation. This method also allows the calculation of a mean voltage threshold for the activation of ciliary reversal that was determined with incremental, depolarizing voltage steps of 2 mV. Passive membrane properties were determined by applying a set of ten 90 ms current injections of −0.1 nA. The input resistance was derived from the mean ohmic voltage drop at 80 ms and was used to correct the current recorded by subtracting the linear leakage current.

The significance of all electrophysiological data was tested using the Mann–Whitney *U*-test ($P<0.05$). Values are presented as means ± S.E.M.

Results

Behaviour of pheromone-stimulated cells

In self-pheromone solution, cells covered long distances by walking forward (Fig. 1) at a mean speed of 162.3±13.2 µm s^{-1} (N=12). In nonself-pheromone solution, the mean speed was

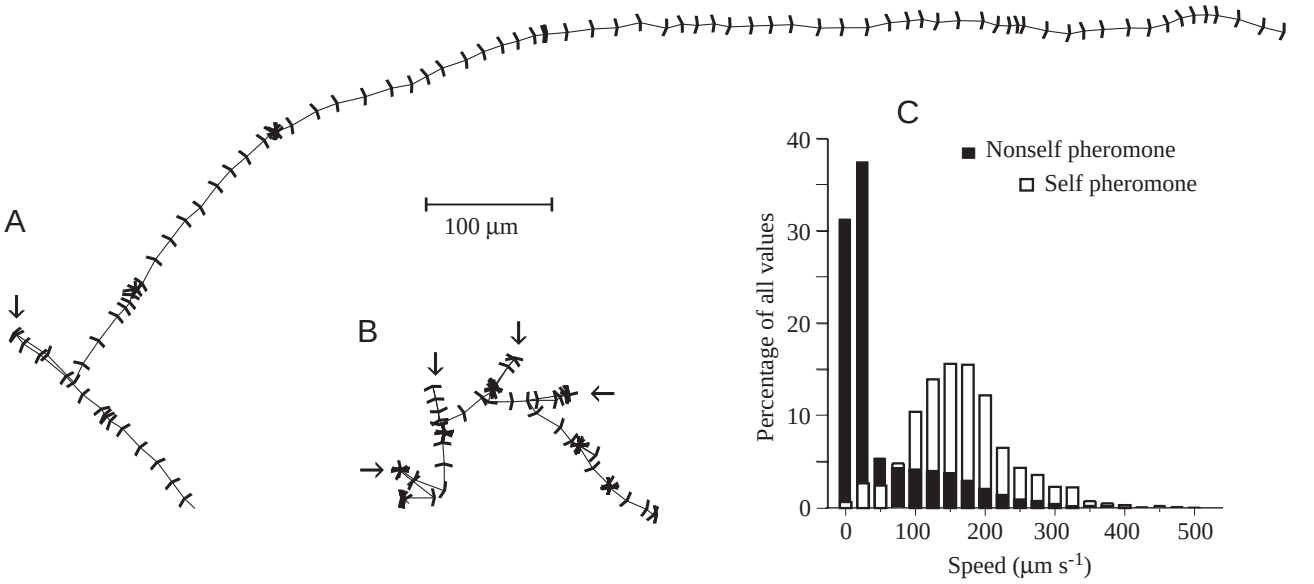


Fig. 1. Representative 8 s tracks and speed distribution of walking cells in self and in nonself pheromone. (A) The track produced by a self-pheromone-stimulated cell moving forward in a slow-and-fast rhythm as indicated by the density of arrowheads. The forward movement is interrupted by only one side-stepping reaction (arrow). The specimen covers a distance of approximately 800 µm within 8 s. (B) The track produced by a nonself-pheromone-stimulated cell. This cell performs side-stepping reactions (arrows) as well as long-lasting stops much more frequently than the cell in A, leading to a significantly reduced translocation distance of only 170 µm in 8 s. (C) Histogram showing the relative speed distribution of walking cells in self and in nonself pheromone (N=12 cells in each group). Slowly moving cells or those that stopped provided more measurement opportunities than did quickly moving cells that left the visual field rapidly. Therefore, the distribution is expressed as a percentage of all the values obtained in each medium.

reduced to 55.6±7.9 µm s^{-1} (N=12; P<0.05). Translocation of nonself-pheromone-stimulated cells was low, because cells in self pheromone performed 11±4.2 stops min^{-1} (N=12) and 9±2.2 ciliary reversals min^{-1} (N=12), whereas cells in nonself pheromone performed 34±5 stops min^{-1} (N=12) and 18±2.6 ciliary reversals min^{-1} (N=12); the number of stops does not include the stop during a ciliary reversal. Consequently, the speed distribution of nonself-pheromone-stimulated cells clearly shifted to smaller values (Fig. 1C). In solution without any pheromone, cells showed no statistically significant differences from cells in self pheromone. They walked at a mean speed of 186.6±23.1 µm s^{-1} (N=9) and performed 9.3±3.1 ciliary reversals min^{-1} (N=9) and 13.6±8.3 stops min^{-1} (N=9).

Effects of pheromone on membrane potential

In *Euplotes raikovi*, as described previously for *Euplotes vannus* (Lueken et al., 1996), a pattern of rhythmic, spontaneous depolarizations controls a rhythmical reduction in speed of forward-walking cells. In nonself-pheromone-stimulated cells and with 0.5 mol l^{-1} KCl in the microelectrode, the frequency and duration of depolarizations were reduced (Fig. 2). The amplitude of the depolarizations was 15–20 mV in controls and 6–10 mV in nonself-pheromone-stimulated cells. This range was reduced in nonself-pheromone-stimulated cells because of the reduced amplitude of depolarizations. In addition, the preference potential shifted by approximately –4.3 mV in nonself-pheromone-stimulated cells (Table 1, asterisks). Rare hyperpolarizations in the latter cells occurred spontaneously, but repeatedly. Fluctuations of membrane potential in cells measured in self-pheromone

Table 1. *Characteristics of the freely running membrane potential dependent on pheromone type and KCl concentration inside the microelectrode*

	Self pheromone	Nonself pheromone	KCl concentration inside the microelectrode (mol l^{-1})
Potential range (mV)	34±3.4	21±2.8	0.5
	19.8±1.6	23.3±2.5	0.33
Highest potential value (mV)	–7.9±0.8	–21.7±1.7	0.5
	–0.2±1.2	0.8±0.5	0.33
Lowest potential value (mV)	–39.9±3.5	–42.7±2.1	0.5
	–20.0±1.6	–22.5±2	0.33
Preference potential (mV)	–31.4±2.7*	–35.7±2*	0.5
	–16.4±1‡	–17.3±0.9‡	0.33
N (cells)	9	11	0.5
	5	5	0.33

Values are means ± S.E.M.

The significance of the change in preference potential was determined using the Mann–Whitney U-test (*P<0.05).

The difference for the 0.33 mol l^{-1} KCl microelectrode was not significant (‡P>0.05).

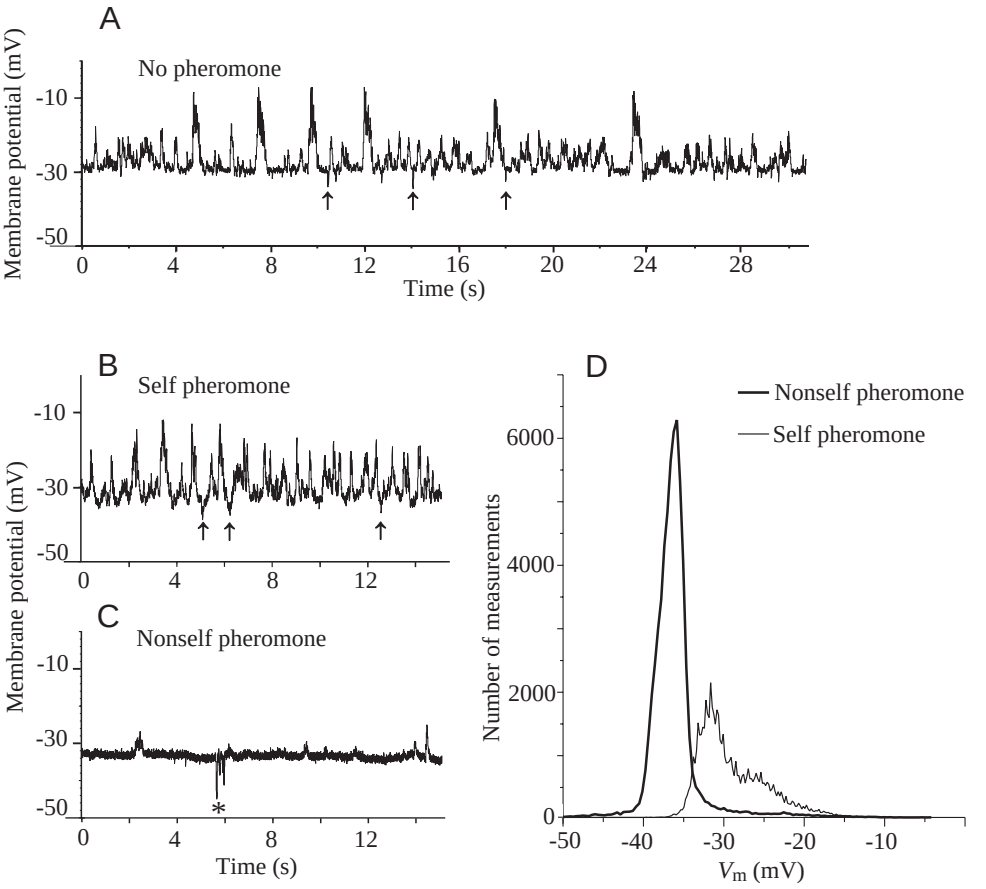


Fig. 2. Representative recordings of the freely fluctuating membrane potential of *Euplotes raikovi*. (A) In pheromone-free solution (fresh artificial sea water), spontaneous depolarizations occur rhythmically, and the amplitudes of brief hyperpolarizations (arrows) are low. (B) In self-pheromone solution, spontaneous depolarizations also occur rhythmically, and hyperpolarizations (arrows) are brief and of low amplitude. (C) In nonself-pheromone solution, the frequency and amplitude of depolarizations are reduced. Rare hyperpolarizations with increased amplitude occur spontaneously (asterisk). (D) Distribution of membrane potential values from 1 min recordings from a single cell. Nonself pheromone induces a decrease in the membrane potential (V_m) range and a shift of the preference potential to a more negative value. For statistical data see Table 1.

solution were similar to those in cells that were exposed to a pheromone-free solution (Fig. 2A,B). This demonstrates that the exposure to nonself pheromone itself, rather than the absence of self pheromone, inhibits the generation of spontaneous potential fluctuations.

The effects of nonself pheromone failed to occur when the microelectrode was filled with 0.33 mol l^{-1} KCl instead of 0.5 mol l^{-1} KCl (Fig. 3; Table 1). A reduction in the K^+ concentration in the microelectrode might result in a decrease in the intracellular K^+ concentration, i.e. a decreased K^+ equilibrium potential. A lower K^+ equilibrium potential reduces the electromotive force for outwardly directed K^+ currents. Consequently, the preference potential would shift to more depolarized values, and the probability of generating potential fluctuations would be increased. Indeed, in nonself-pheromone-stimulated cells with 0.33 mol l^{-1} KCl in the microelectrode, the same fluctuation pattern occurred as was found in cells exposed to self pheromone and using 0.5 mol l^{-1} KCl microelectrodes (compare Fig. 3B with Fig. 2B).

Effects of pheromone on input resistances

The input resistances of cells in nonself pheromone (147±19 MΩ, N=15) were higher than those in cells in self pheromone (95±8 MΩ, N=8) when electrodes had been filled with 0.5 mol l^{-1} KCl. When electrodes had been filled with 0.33 mol l^{-1} KCl, no significant difference was found between cells in self-pheromone solution (71±4 MΩ, N=4) and in nonself-pheromone solution (80±5 MΩ, N=4).

Effects of pheromone on ion currents

The pattern of membrane currents found in *E. raikovi* is similar to that in *E. vannus* (Krüppel and Lueken, 1988). Because of the smaller surface area of *E. raikovi*, the current amplitudes in this species were considerably lower than in *E. vannus* (Fig. 4). In *E. vannus*, the hyperpolarization-activated inward current is carried by Na^+ (Krüppel, 1993), and the depolarization-activated inward current is composed of an early, voltage-dependent, transient Ca^{2+} current (Krüppel and Wissing, 1996) and a late Ca^{2+}-dependent Na^+ current (Krüppel and Lueken, 1990). The fast and slow outward K^+ currents ($I_{K,fast}$ and $I_{K,slow}$; Krüppel et al., 1991; Westermann et al., 1990) (Figs 4, 5) and the inward Ca^{2+} and Na^+ currents (see Fig. 6) could also be distinguished in *E. raikovi*, but the plateau of $I_{K,slow}$ was influenced by a small inward Na^+ current (I_{Na}).

At depolarizations of more than 20 mV, $I_{K,fast}$ and $I_{K,slow}$ were increased by at least 30 % in nonself-pheromone-stimulated cells (Fig. 5A,B). In addition, the reversal potential of total currents (predominantly carried by $I_{K,slow}$ and to a small amount by I_{Na}, see above), measured after 140 ms of 160 ms voltage pulses, was shifted by approximately –4.2 mV (Fig. 5C). This suggests either that the Na^+ current (I_{Na}) was decreased or that $I_{K,slow}$ was increased (or a combination of

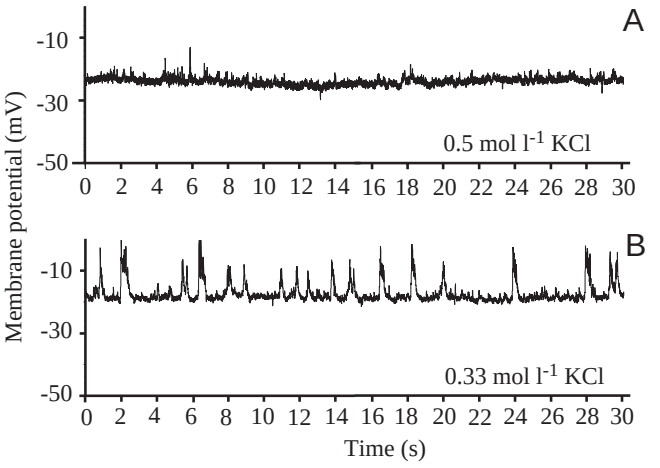


Fig. 3. Dependence of the effects of nonself pheromone on the membrane potential of *Euplotes raikovi* on the KCl concentration inside the microelectrode. (A) 0.5 mol l^{-1} KCl leads to inhibition of the membrane potential fluctuations. (B) 0.33 mol l^{-1} KCl leads to an increase in the number, duration and amplitude of depolarizations.

these two effects) in the presence of nonself pheromone. At low levels of depolarization, however, the late, depolarization-activating inward current component was slightly increased in nonself-pheromone-stimulated cells.

The voltage-dependent transient inward Ca^{2+} current remained nearly unaffected by nonself pheromone (Fig. 6). Therefore, the increase in $I_{K,fast}$ is not associated with any reduction in the Ca^{2+} current. Summation of the Ca^{2+} current with the late K^+ current can be excluded owing to the fast inactivation of the Ca^{2+} current (Krüppel and Wissing, 1996).

The late hyperpolarization-dependent inward Na^+ current is activated by nonself pheromone at hyperpolarizations of −10 mV, whereas in the presence of self pheromone this current occurred at hyperpolarizations of approximately −40 mV (Fig. 7).

Effects of pheromone on the threshold for ciliary reversal

Ciliates reverse the beat direction of their cilia (e. g. *Paramecium* spp.) or their cirri (e.g. *Stylonychia*/*Euplotes* spp.) when they are depolarized to a voltage threshold that activates inward Ca^{2+} currents (Machemer and Deitmer, 1987; Lueken et al., 1996). In *E. raikovi*, this threshold was lowered by nonself pheromone from 8.6±0.7 mV (N=8) to 6.3±0.5 mV (N=8; P<0.05) when 0.33 mol l^{-1} KCl electrodes were used.

Discussion

In ciliates, successful sexual stimulation by pheromones is indicated by a change of locomotory behaviour resulting in the formation of pairs (Ricci, 1996). In both *E. raikovi* and in *E. vannus* (Lueken et al., 1996), rhythmical, spontaneous fluctuations in membrane potential correspond with a rhythmical reduction in the speed of forward-walking cells. Inhibition of these rhythms allows pair formation. In the present study, we have analysed pheromone-induced changes in behaviour and in the electrophysiological properties of *E. raikovi*.

Behaviour of pheromone-stimulated cells

After stimulation with nonself pheromone, walking cells remain in a small area (small radius of action) because they make frequent long-lasting stops, more frequent side-stepping reactions and their walking speed is reduced. A slower speed reduces the intensity of cell/cell collisions and thereby the intensity of the typical mechano-responses (the ‘avoiding reaction’ and ‘escape response’) that would normally separate cells. The general reduction of translocation could increase the probability of finding a conjugation partner.

Effects of pheromone on membrane potential

In the ciliates *Stylonychia mytilus*, *Euplotes vannus* and *Bursaridium difficile*, spontaneous membrane potential depolarizations have been recorded in standard solutions (Machemer and Deitmer, 1987; Krüppel and Lueken, 1988; Berg and Sand, 1994). In *Paramecium aurelia*, however, such depolarizations are induced only in Na^+-containing solutions (Satow and Kung, 1974). Presumably, the ratio of Ca^{2+}, K^+ and Na^+ conductances at the resting potential determines whether spontaneous activity is generated (Hinrichsen and Schultz, 1988). In *Euplotes raikovi*, the spontaneously generated depolarizations are inhibited in nonself-pheromone-stimulated cells as a result of a slight hyperpolarization of the preference potential (see Fig. 2). Such a hyperpolarizing effect might be

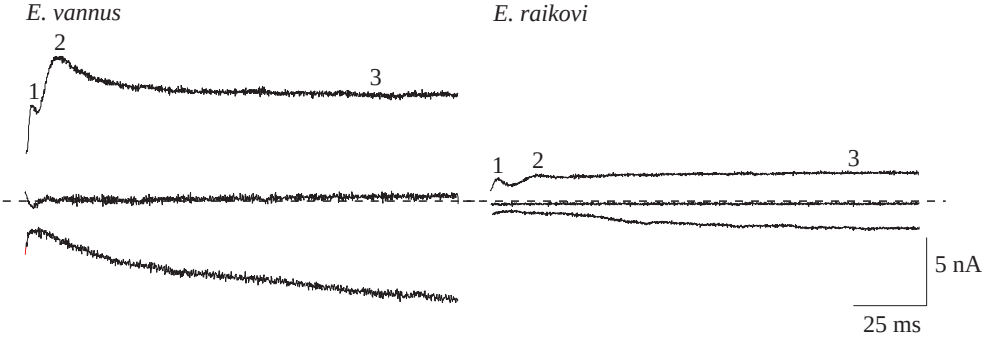
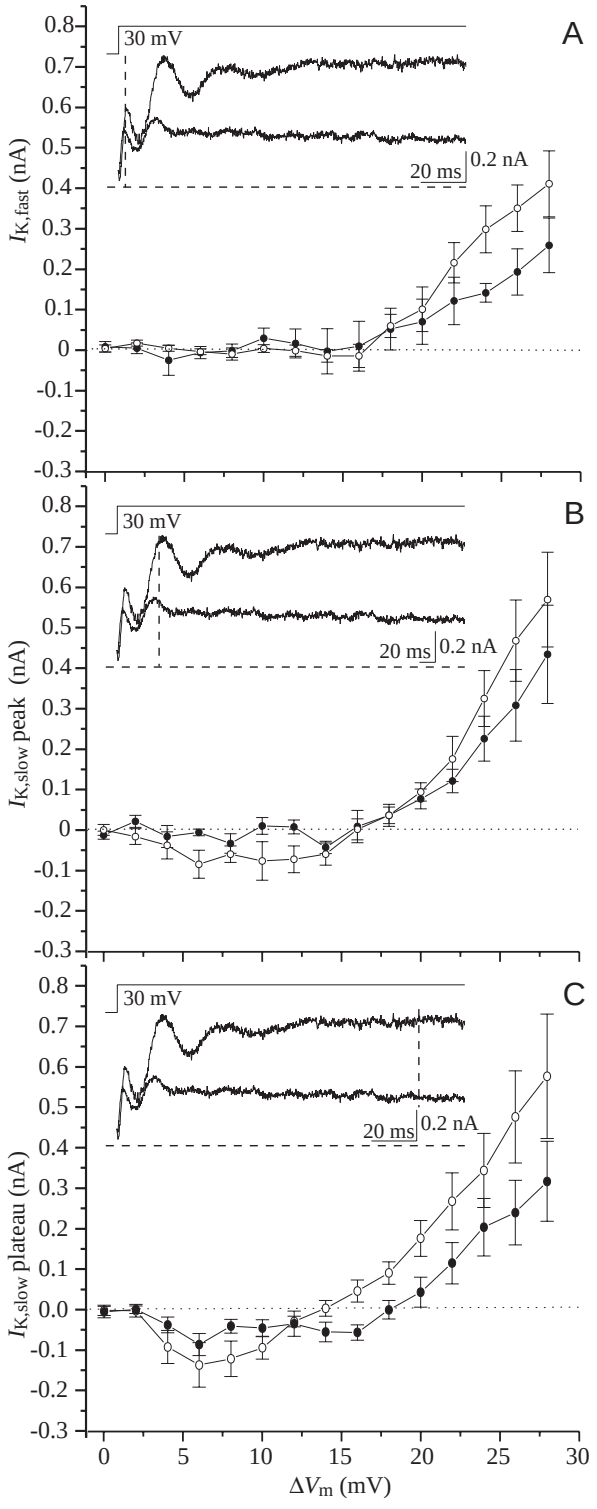


Fig. 4. Comparison of representative total current patterns from a *Euplotes vannus* and a *Euplotes raikovi* cell. The fast outward K^+ current ($I_{K,fast}$) (1) as well as the peak (2) and the plateau (3) of the slow outward K^+ current ($I_{K,slow}$) are clearly visible in both species. Because of the smaller cell surface area of *E. raikovi*, its current amplitudes were only one-quarter of those measured in *E. vannus*. Step potentials were −65 mV, 0 mV and +55 mV, respectively. Holding potentials were −35 mV in *E. vannus* and −30 mV in *E. raikovi*.

caused by an increased resting conductance for K^+ or a reduction in the resting conductance for Ca^{2+} and/or Na^+ (gK^+, gCa^{2+} and gNa^+, respectively). The input resistance, which is inversely proportional to the total membrane conductance, does not change in nonself-pheromone-stimulated cells when 0.33 mol l^{-1} KCl electrodes are used and is increased when 0.5 mol l^{-1} KCl electrodes are used, indicating that a reduction in $gCa^{2+}$and/or gNa^+ is more likely than an increase in gK^+.



How the different intracellular K^+ concentrations that presumably result from the use of the two types of K^+ electrode produce these different effects on the input resistance remains obscure. The spontaneous membrane potential depolarizations are not inhibited by nonself pheromone when 0.33 mol l^{-1} KCl electrodes are used. If the intracellular K^+ concentration changes from 0.5 to 0.3 mol l^{-1}, then the K^+ equilibrium potential should become 10 mV more positive (extracellular K^+ concentration was 10 mmol l^{-1}). Consequently, the total membrane potential should be depolarized to some extent, and any pheromone-induced hyperpolarizing effect might be compensated. The same result should be obtained, without the uncertainty of the real intracellular [K^+], by increasing the extracellular K^+ concentration. This method, however, was rejected in the present study to avoid mechanical disturbance of cells during the solution change (see Materials and methods). In fact, for this change in the electrode [K^+], the preference potential shifts by approximately 15–18 mV (Table 1). This unexpectedly large shift could result from secondary effects such as an amplification of depolarization through an increase in potential-dependent Na^+ and/or Ca^{2+} conductances.

Effects of pheromone on ion currents

In nonself-pheromone-stimulated *E. raikovi*, the depolarization-activated total outward current is increased. The early outward currents are predominantly carried by K^+, and the late outward current is carried by K^+ and to lesser extent by Na^+ (see Fig. 4). A late-activating inward current is increased at low depolarizations, and a large outward current is increased at depolarizations above 20 mV (see Fig. 5). The latter data are best explained by an augmentation of a Na^+/K^+ conductance which passes ion currents dependent on the driving force for either ion. At low levels of depolarization, i.e. at a high driving force for Na^+, an inward Na^+ current would result, and at high levels of depolarization, i.e. at high driving force for K^+, an outward K^+ current would result. In *E. vannus*, two depolarization-activated, late ion currents have been identified which have been named $I_{K,slow}$ and $I_{Na,Ca}$ (Westermann et al., 1990; Krüppel and Lueken, 1990). It is not yet known whether these ionic currents use the same or two different channel populations. The slight increase of inward

Fig. 5. Depolarization-activated outward K^+ currents in self- and in nonself-pheromone-stimulated cells. The time at which measurements were taken is indicated by the dashed vertical lines in the insets. The outward currents of nonself-pheromone-stimulated cells (dotted lines in inset, open circles in figure) are of increased amplitude relative to controls (solid lines in inset, filled circles in figure). (A,B) The fast and slow outward K^+ currents ($I_{K,fast}$ and $I_{K,slow}$) rise proportionally with the amplitude of the applied voltage pulse (ΔV_m, N=8 cells in each case). (C) The reversal potential of the total outward current shifts by –4.2 mV (N=9 cells in each case). The holding potential of all cells corresponded to the preference potential determined previously. Electrodes were filled with 0.33 mol l^{-1} KCl. Values are means ± S.E.M.

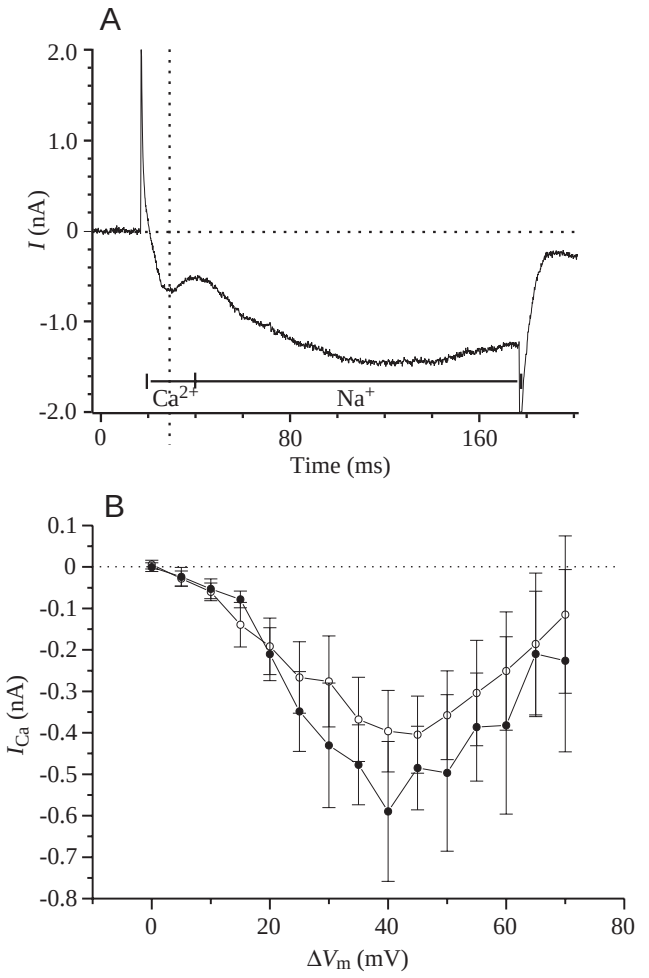


Fig. 6. Inward Ca^{2+} and Na^+ currents recorded after blocking the outward K^+ currents using 0.5 mol l^{-1} CsCl inside the microelectrode. (A) An example of depolarization-activated inward currents (*I*) showing the fast-activating inward Ca^{2+} current and the slow-activating inward Na^+ current. The voltage pulse (ΔV_m) was +30 mV. The cell was exposed to self-pheromone solution. (B) Current–voltage diagram of inward Ca^{2+} currents (I_{Ca}). The differences between cells in nonself pheromone (open circles) and in self pheromone (filled circles) are not significant. Values are means ± S.E.M. (*N*=9 cells in each case).

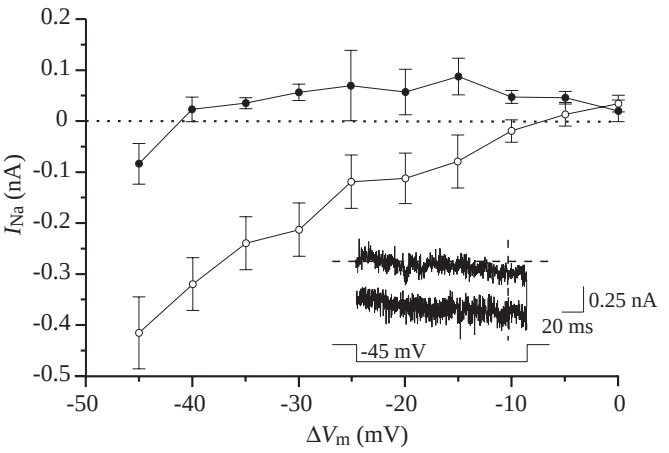


Fig. 7. Current–voltage diagram of the hyperpolarization-activated inward Na^+ current (I_{Na}) of cells in self pheromone (filled circles) and in nonself pheromone (open circles) after inhibition of outward K^+ currents by Cs^+. In nonself-pheromone-stimulated cells, the inward Na^+ current activates at low hyperpolarizations, shifting the threshold for activation from −40 mV to −10 mV. Values are means ± S.E.M. (*N*=7 cells in each case). The inset shows representative traces from one individual in self-pheromone solution (solid line) and another individual in nonself-pheromone solution (dotted line). The electrode was filled with 0.33 mol l^{-1} CsCl.

current at small depolarizations and the general increase in the outward currents at larger depolarizations would also help to explain the occurrence of the small number of low-amplitude depolarizations observed in nonself pheromone (see Fig. 2C). Any spontaneous depolarization would be repolarized quickly before the usual amplitude is reached by an increase of these conductances.

In addition to the pheromone-induced increase in outward currents, a hyperpolarization-activated inward current is also induced in these cells. By analogy to the inward rectification in *E. vannus* (Krüppel, 1993), this inward current in *E. raikovi* may be carried by Na^+. In *Paramecium tetraurelia*, one inward Na^+ current activates upon hyperpolarization (Saimi, 1986) and another upon depolarization (Saimi and Kung, 1980), giving rise to speculations that both ionic currents flow through the same population of channels. If the hyperpolarization-induced inward current and the depolarization-induced currents in *E. raikovi* use the same population of channels, the nonself pheromone would act by increasing one common conductance.

Ciliary reversal is based on the free Ca^{2+} concentration inside the cilia (Mogami et al., 1990, 1991). The necessary increase in Ca^{2+} concentration is achieved by a voltage-gated inward Ca^{2+} current (Pernberg and Machemer, 1989, 1995). In nonself-pheromone-stimulated *E. raikovi*, the number of stops and ciliary reversals is augmented and the threshold potential for ciliary reversals is lowered, although the depolarization-activated inward Ca^{2+} current is unaltered (see Fig. 6). Consequently, the augmentation of the intraciliary Ca^{2+} concentration is probably not caused by an increased influx of extracellular Ca^{2+} through the membrane of the cilia.

A hyperpolarization-activated inward Ca^{2+} current has been described in *Paramecium tetraurelia* (Preston et al., 1992). We can speculate on the existence of such an inward current in the present study, because nonself pheromone induces hyperpolarization in *E. raikovi*. Hennessey et al. (1995) and Evans and Keller (1997) describe different kinds of receptor-mediated, voltage-independent inward Ca^{2+} currents in *Paramecium tetraurelia* and *Chlamydomonas reinhardtii*. A Ca^{2+} conductance could exist in *E. raikovi* that is affected by nonself pheromone and either is superimposed upon other conductances or is unmeasurable for reasons not yet known. The release of Ca^{2+} from intracellular stores (Erxleben and Plattner, 1994; Länge et al., 1995; Plattner et al., 1991) could

also lead to an increase in the intraciliary Ca^{2+} concentration that is not measurable using standard electrophysiological methods. In *Paramecium caudatum*, the injection of Ca^{2+} results in a reversal of ciliary beating and in an increased beat frequency without a change in resting potential level (Nakaoka and Machemer, 1990). We suggest that, in *E. raikovi*, nonself pheromone induces the augmentation of intraciliary [Ca^{2+}] which additionally could be involved as a second messenger in preparing the cells for conjugation. In *E. vannus*, both the inward Na^+ current and the outward K^+ currents are Ca^{2+}-dependent (Krüppel and Lueken, 1990; Krüppel et al., 1991). Accordingly, a nonself-pheromone-mediated increase in the intracellular Ca^{2+} concentration could have enhanced the Na^+ and K^+ conductances in *E. raikovi*. Inositol 4,5-trisphosphate (InsP_3) could act as a second messenger releasing Ca^{2+} from alveolar sacs or from the endoplasmatic reticulum. Miwa and Wada (1995) unravelled the role of InsP_3 in conjugation in *Paramecium bursaria* by demonstrating that cells began conjugation 30 min after the injection of 10 pl of a 120 μmol l^{-1} InsP_3 solution. An alternative explanation for the effects of nonself pheromone is an enhancement of the sensitivity of the motility apparatus to the intracellular Ca^{2+} concentration, thus inducing responses that are normally associated with elevated Ca^{2+} concentrations.

In conclusion, nonself pheromone changes ion currents and locomotory behaviour, resulting in conjugation. The intraciliary and/or intracellular Ca^{2+} concentrations seem to play an important role in this process. Since the effects of nonself pheromone are multi-layered, there is a need for further investigations to elucidate the signal transduction pathway involved in the sexual behaviour of ciliates.

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References

Berg, T. O. and Sand, O. (1994). Spontaneous all-or-nothing action potentials in the ciliate *Bursaridium difficile*. *J. Euk. Microbiol.* **41**, 13–17.

Borror, A. C. and Hill, B. F. (1995). The order Euplotida (Cilophora): taxonomy, with division of *Euplotes* into several genera. *J. Euk. Microbiol.* **42**, 457–466.

Dini, F. and Nyberg, D. (1993). Sex in ciliates. In *Advances in Microbial Ecology* (ed. J. G. Jones), pp. 85–153. New York: Plenum Press.

Eckert, R. (1972). Bioelectric control of ciliary activity. *Science* **176**, 473–481.

Erxleben, C. and Plattner, H. (1994). Ca^{2+} release from subplasmalemmal stores as a primary event during exocytosis in *Paramecium* cells. *J. Cell Biol.* **127**, 935–945.

Evans, J. H. and Keller, L. R. (1997). Receptor-mediated calcium-influx in *Chlamydomonas reinhardtii*. *J. Euk. Microbiol.* **44**, 237–245.

Hennessey, T. M., Kim, M. Y. and Satir, B. H. (1995). Lysozyme act as a chemorepellent and secretagogue in *Paramecium* by activating a novel receptor-operated Ca^{2+} conductance. *J. Membr. Biol.* **148**, 13–25.

Hinrichsen, R. D. and Schultz, J. E. (1988). *Paramecium*: a model system for excitable cells. *Trends Neurosci.* **11**, 27–32.

Kitamura, A. (1988). Mating-type substances. *Paramecium* (ed. H. D. Görtz), pp. 85–94. Berlin: Springer Verlag.

Kitamura, A. and Hiwatashi, K. (1980). Reconstitution of mating active membrane vesicles in *Paramecium*. *Exp. Cell Res.* **125**, 486–489.

Krüppel, T. (1993). Inward rectification by hyperpolarization-activated Na current in the marine ciliate *Euplotes vannus*. *J. Membr. Biol.* **133**, 263–270.

Krüppel, T. and Lueken, W. (1988). Membrane excitability and membrane currents in the marine ciliate *Euplotes vannus*. *Eur. J. Protistol.* **24**, 11–21.

Krüppel, T. and Lueken, W. (1990). Calcium-dependent sodium current in the marine ciliate *Euplotes vannus*. *J. Membr. Biol.* **116**, 79–86.

Krüppel, T., Westermann, R. and Lueken, W. (1991). Calcium-dependent transient potassium outward current in the marine ciliate *Euplotes vannus*. *Biochim. Biophys. Acta* **1062**, 193–198.

Krüppel, T. and Wissing, F. (1996). Characterisation of the voltage-activated calcium current in the marine ciliate *Euplotes vannus*. *Cell Calcium* **19**, 229–241.

Länge, S., Klauke, N. and Plattner, H. (1995). Subplasmalemmal Ca^{2+} stores of probable relevance for exocytosis in *Paramecium*. Alveolar sacs share some but not all characteristics with sarcoplasmic reticulum. *Cell Calcium* **17**, 335–344.

Lueken, W., Krüppel, T. and Gaertner, M. (1987). Effects of raised $[K^+]_o$ and $[Ca^{2+}]_o$ on conjugation and on electrophysiological parameters in the marine ciliate *Euplotes vannus*. *J. Exp. Biol.* **130**, 193–202.

Lueken, W., Ricci, N. and Krüppel, T. (1996). Rhythmic spontaneous depolarizations determine a slow-and-fast rhythm in walking of the marine hypotrich *Euplotes vannus*. *Eur. J. Protistol.* **32**, 47–54.

Luporini, P., Miceli, C. and Ortenzi, C. (1983). Evidence that the ciliate *Euplotes raikovi* releases mating inducing factors (gamones). *J. Exp. Zool.* **226**, 1–9.

Luporini, P., Vallesi, A., Miceli, C. and Bradshaw, R. A. (1995). Chemical signaling in ciliates. *J. Euk. Microbiol.* **42**, 208–212.

Machemer, H. (1989). Cellular behaviour modulated by ions: electrophysiological implications. *J. Protozool.* **36**, 463–487.

Machemer, H. and Deitmer, J. W. (1987). From structure to behaviour: *Stylonychia* as a model system for cellular physiology. In *Progress in Protistology*, vol. 2 (ed. J. O. Corliss and D. J. Patterson), pp. 213–330. Bristol: Biopress Ltd.

Machemer, H. and Sugino, K. (1989). Electrophysiological control of ciliary beating: a basis of motile behaviour in ciliated protozoa. *Comp. Biochem. Physiol.* **94**A, 365–374.

Machemer, H. and Teunis, F. M. (1996). Sensory-motor coupling and motor responses. In *Ciliates, Cells as Organisms* (ed. K. Hausmann and P. C. Bradbury), pp. 379–402. Stuttgart, Jena, New York: Gustav Fischer Verlag.

Miwa, I. and Wada, T. (1995). Light pulses and injection of IP_3 induce mating ability in *Paramecium bursaria*. *J. Exp. Zool.* **272**, 338–344.

Mogami, Y., Fujima, K. and Baba, S. A. (1991). Five different states of ciliary activity in the epaulette of echinoplutei. *J. Exp. Biol.* **155**, 65–75.

Mogami, Y., Pernberg, J. and Machemer, H. (1990). Messenger role of calcium in ciliary electromotor coupling: a reassessment. *Cell Calcium* **11**, 665–673.

Mronga, S., Luginbühl, P., Brown, L. R., Ortenzi, C., Luporini, P., Bradshaw, R. A. and Wüthrich, K. (1994). The NMR solution structure of the pheromone Er-1 from the ciliated protozoan *Euplotes raikovi*. *Protein Science* **3**, 1527–1536.

Mulisch, M. (1996). Nuclei and sexual reproduction. In *Protozoology*, 2nd edition (ed. K. Hausmann and N. Hülsmann), pp. 216–230. Stuttgart: Georg Thieme Verlag.

Naitoh, Y. and Kaneko, H. (1972). Reactivated Triton-extracted models of *Paramecium*: Modification of ciliary movement by calcium ions. *Science* **176**, 523–524.

Nakaoka, Y. and Machemer, H. (1990). Effects of cyclic nucleotides and intracellular Ca on voltage-activated ciliary beating in *Paramecium*. *J. Comp. Physiol.* A **166**, 401–406.

Ortenzi, C. and Luporini, P. (1995). Competition among homologous polypeptide pheromones of the ciliate *Euplotes raikovi* for binding to each other's cell receptors. *J. Euk. Microbiol*. **42**, 242–248.

Pernberg, J. and Machemer, H. (1989). Depolarization-induced membrane current components in *Didinium*. *J. Comp. Physiol*. **164**, 551–562.

Pernberg, J. and Machemer, H. (1995). Voltage-dependence of ciliary activity in the ciliate *Didinium nasutum*. *J. Exp. Biol*. **198**, 2537–2545.

Plattner, H., Lumpert, C. J. and Knoll, G. (1991). Stimulus–secretion coupling in *Paramecium* cells. *Eur. J. Cell Biol.* **55**, 3–16.

Preston, R., Saimi, Y. and Kung, C. (1992). Calcium current activated upon hyperpolarization of *Paramecium tetraurelia*. *J. Gen. Physiol.* **100**, 233–251.

Raffioni, S., Miceli, C., Vallesi, A., Chowdhury, S. K., Chait, B. T., Luporini, P. and Bradshaw, R. A. (1992). Primary structure of *Euplotes vannus* pheromones: comparison of five sequences of pheromones with variable mating interactions. *Proc. Natl. Acad. Sci. USA* **89**, 2071–2075.

Ricci, N. (1996). Ethology of ciliates. In *Ciliates, Cells as Organisms* (ed. K. Hausmann and P. C. Bradbury), pp. 403–416. Stuttgart, Jena, New York: Gustav Fischer Verlag.

Saimi, Y. (1986). Calcium-dependent sodium currents in *Paramecium*: mutational manipulations and effects of hyper- and depolarization. *J. Membr. Biol.* **92**, 227–236.

Saimi, Y. and Kung, C. (1980). A Ca-induced Na-current in *Paramecium*. *J. Exp. Biol.* **88**, 305–325.

Satow, Y. and Kung, C. (1974). Genetic dissection of active electrogenesis in *Paramecium aurelia*. *Nature* **247**, 69–71.

Westermann, R., Krüppel, T. and Lueken, W. (1990). Contributions of fast and slow potassium outward currents to repolarization of the action potential in *Euplotes vannus*. *Acta Protozool*. **29**, 305–313.