Effects of Calmodulin Antisense Oligonucleotides on Chemoresponse in *Paramecium*

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Abstract

The calcium/calmodulin-regulated Ca-ATPase of the plasma membrane is implicated in *Paramecium* chemosensory transduction. Calmodulin antisense oligonucleotides electroporated into *Paramecium* disrupt chemosensory responses to sodium acetate but not to ammonium chloride. **Chem. Senses 21: 55–58, 1996.**

Introduction

Paramecium tetraurelia shows attraction to small molecules that probably indicate the presence of bacteria. Glutamate, folate, acetate and ammonium are among the stimuli that we have identified (Van Houten, 1978, 1994; Van Houten and Preston, 1988). While all of these stimuli hyperpolarize the cells causing relatively smooth and fast swimming that eventually, with adaptation, results in accumulation of cells in the attractant stimuli (Van Houten, 1990), there also appear to be differences among the transduction mechanisms for these stimuli.

At least three chemosensory transduction pathways exist in *Paramecium* (Van Houten, 1994), and these are epitomized by attraction to acetate, ammonium and glutamate. Briefly, stimuli can be divided into three groups by apparent dependence upon the calcium plasma membrane pump activity and by the intracellular messenger cyclic AMP. Acetate and possibly glutamate are thought to hyperpolarize the cells by activation of the calcium pump, which could easily generate a sustained conductance sufficiently large to hyperpolarize the cells (Wright and Van Houten, 1990). If the hyperpolarization were due to a pump current, there could be a reversal potential that is not characteristic of a calcium channel (Läuger, 1991), and there would be no effects from changing extracellular K^+ or Na^+ or both. This matches our observations for acetate chemoresponse hyperolarization (Preston and Van Houten, 1987). Attraction to ammonium chloride (NH_4Cl), in contrast, does not appear to be by a receptor-mediated process but, rather, acts by diffusion of ammonia across the membrane and alkalinization of the cell (Davis and Van Houten, 1994). This division into two mechanisms is further supported by the correlative evidence that LiCl inhibits attraction to acetate and glutamate but not ammonium, and LiCl also inhibits calcium homeostasis efflux by 50% (Wright *et al.*, 1992). Moreover, K-shy, a mutant with calcium homeostasis problems (Evans *et al.*, 1987), is not attracted to acetate or glutamate but is normally attracted to ammonium (Wright *et al.*, 1992; Yang and Van Houten, 1993). Acetate and glutamate do not completely share the same transduction pathway because glutamate is the only stimulus among those tested that induces a rapid and robust increase in cyclic AMP (Yang, 1995; Yang *et al.*, 1994). This increase is sufficiently rapid to be part of the stimulus transduction mechanism in addition to slower processes such as adaptation.

While it is important to test the role of the plasma membrane Ca-ATPase in chemoresponse because of its integral role in homeostasis, it is not possible to completely eliminate this pump activity by drug or mutation and still test for chemoreponse behavior. Therefore, we have turned to antisense oligonucleotide technology to partially down-regulate calmodulin and examine the effects on chemoresponse behavior.

Methods, results and discussion

Paramecium tetraurelia are electroporated with antisense oligodeoxynucleotides (ODNs) that are complementary to the 5′ end of calmodulin mRNA, including the translation start site (Hinrichsen *et al.*, 1992). Electroporation was achieved under the following conditions to prepare cells for electrophysiology: 400 V (field strength 2000 V/cm), 300 μF and low resistance using a Cell Porator Electroporation system (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA). The cells were harvested from the early stationary phase and washed twice with 10 mM HEPES (pH 7.2). The cell pellet was suspended in HEPES buffer at 6×10^5 cells/ml. The cell suspension (250 μl) was mixed with 30 μg ODN with 3′ cholesterol modification in 3–10 μl water (the final concentration of ODN was ~15 μM) in the electroporation cell with 0.4 cm between the electrodes. The mixtures were kept on ice for 10 min before and after electroporation. The viability of electroporated cells was <1%. The living cells were isolated and washed three times in the resting solution [1 mM $Ca(OH)_2$, 1 mM citric acid, 1 mM Tris base, 1 mM KCl, pH 7.2], then kept in resting medium at 22°C.

There are calcium-dependent Na and K conductances in *Paramecium* (Preston *et al.*, 1991). The Na conductance has been shown directly by patch clamp to be calcium/calmodulin-regulated while the K conductance has not, although it is defective in certain calmodulin mutants (Saimi and Martinac, 1989; Preston *et al.*, 1990; Saimi and Ling, 1990). These conductances alter the kinetics of the action potential, which is a graded calcium action potential. During the action potential, calcium enters the cilia through voltage-gated channels, causing a change in the ciliary beat pattern. While calcium remains high, the ciliary beating pattern causes the cells to swim backward. Normally this backward swimming is very short in duration, but can be prolonged with the opening of the calcium/calmodulin-dependent Na channel, which sustains the depolarization of the cell, or inhibition of the calcium/calmodulin-dependent K conductance, which normally functions to repolarize the cells. The Na conductance appears to be more sensitive to calmodulin levels, as shown in experiments in which antisense ODN-treated cells can be 'cured' of the loss of Na conductance with injection of calmodulin (Hinrichsen *et al.*, 1992). More calmodulin is required to effect a 'cure' of the defects in the Na conductance than in the K conductance. Consequently, with antisense down-regulation of calmodulin, the behavior associated with the Na conductance disappears before that associated with the K conductance. That is, cells show short duration of backward swimming as the first behavioral effect of ODN electroporation with anti-calmodulin sequences (Hinrichsen *et al.*, 1992).

Cells were electroporated with calmodulin antisense ODNs and later selected for their short backward swimming behavior in a depolarizing solution that would activate the voltage-dependent calcium and calcium/calmodulin-dependent channels (20 mM TEA^+, 10 mM NaCl, 1 mM $CaCl_2$, 1 mM HEPES, pH 7.2). Normal cells show prolonged backward swimming in this solution. However, at 24 h cells that showed very abbreviated backward swimming (0 to <10 s backward swimming) were collected and tested for chemoresponse behavior by motion analysis. Sense ODN-electroporated cells, like untreated normal cells, almost all swam backward in the test solution for >10 s, and therefore were taken at random. Results of a sample experiment are shown in Table 1.

Cells selected after antisense or sense ODN electroporation were transferred to control chemokinesis solution [1 mM $Ca(OH)_2$, 1 mM citric acid, 1 mM Tris base, plus salt indicated, at pH 7.0]. After 30 min or more, they were

Table 1 Duration of backward swimming of cells electroporated with sense and antisense ODNs

Duration of backward swimming (s)	Sense ODN	Antisense ODN
0–5		84.9
5–10		3.0
10–15	10.5	6.1
15–20	36.8	3.0
20–25	36.8	3.0
25–30	15.8	
30–35		

Data are percent cells showing duration of backward swimming. Measurements were taken 23–24 h after electroporation of calmodulin antisense ODN. Sense and antisense distributions are significantly different by the Mann–Whitney *U*-test ($P < 0.0001$). $n = 19, 33$. The results are from one experiment, which was repeated with similar results >10 times.

transferred by micropipet to a shallow pool of the same buffer or buffer with stimulus. The cells were first placed just out of the field of view of the video camera (Cohu 6410 video camera mounted on a Stereo Zoom 7 Bausch & Lomb dissecting microscope) to avoid monitoring mechanical stimulation and motion artifacts from the addition of the cells. As the cells swam into view (2–5 s later), they were videotaped for 1 min. The tapes were later analyzed for speed of swimming using a 15 s segment (at 15 samples/s) as close as possible to the beginning of the 1 min of taped swimming. Ciliary beating frequency and angle change with hyperpolarization (Machemer, 1974), causing an increase in swimming speed and decrease in the frequency of action potentials which cause turns. All the attractant stimuli being tested elicited a hyperpolarization of normal cells, making swimming speed a sensitive measure of effects of the stimuli on the cells. Other tests of chemoresponse behavior that require a larger population of cells, such as T-maze assays, await more stably transformed and larger populations of cells.

It should be noted that the duration of backward swimming in highly depolarizing stimuli was used only to select cells that were affected by the electroporation of the calmodulin antisense ODNs, and that the behavior in chemical attractant stimuli is dependent upon small hyperpolarizations and not upon the calmodulin-dependent Na or K conductances.

Table 2 Motion analysis measurement of speed of cells in control and attractant solutions

Oligonucleotide	Speed when transferred to	
	Control solution	Stimulus solution
	NaCl	NaOAc
Sense	0.87 ± 0.27 (n = 127)	1.02 ± 0.33 (n = 131)[a]
Antisense	0.94 ± 0.33 (n = 123)	0.93 ± 0.35 (n = 132)
None	1.22 ± 0.10 (n = 128)	1.47 ± 0.10 (n = 112)[a]
	NaCl	NH_4Cl
Sense	0.74 ± 0.21 (n = 103)	1.35 ± 0.43 (n = 77)[a]
Antisense	0.91 ± 0.23 (n = 95)	1.30 ± 0.28 (n = 121)[a]
None	1.22 ± 0.10 (n = 128)	1.52 ± 0.09 (n = 97)[a]
	NaCl	Na-glutamate
Sense	0.81 ± 0.30 (n = 96)	0.74 ± 0.25 (n = 85)
Antisense	0.83 ± 0.21 (n = 91)	0.69 ± 0.18 (n = 79)[a]
None	1.08 ± 0.33 (n = 42)	1.21 ± 0.30 (n = 53)[a]

Concentrations of salts indicated are 5 mM in chemokinesis buffer [1 mM $Ca(OH)_2$, 1 mM citric acid, 1 mM Tris base, pH 7 0] All cells were collected from late log phase cultures. However, untreated cells were not kept for 24 h in buffer, in which the cells stop growing and show slower basal speed of swimming. Therefore, relative changes in swimming speed and not the absolute values of swimming speed should be compared.
[a]Significantly different from speed in control solution by Mann–Whitney U-test ($P < 0.003$).

Swimming speed was analyzed using a Motion Analysis System, with Expert Vision and modifications made by K. Clark (Clark and Nelson, 1991). Tapes were edited to avoid analysis of artifacts and crossed paths. The speeds of individual cells in control and test solutions were compared using the Mann–Whitney U-test (Table 2). Cells electroporated with calmodulin sense or antisense ODNs increased their swimming speed in NH_4Cl relative to NaCl. However, those electroporated with calmodulin antisense ODNs failed to speed up in sodium acetate (Na-OAc) relative to NaCl. Sense ODNs did not interfere with this speed increase in control groups. While untreated cells showed expected increases in speed in Na-L-glutamate relative to NaCl control, any electroporation treatment, regardless of the ODN, interfered with the effect of L-glutamte on speed. Therefore, it is not possible to interpret the results from the L-glutamate tests. Tests of antisense transcription on glutamate response await the development of stably transformed cells that have longer to recover.

Changes in speed of untreated cells have been measured previously (Van Houten, 1978; Wright *et al.*, 1992; Yang, 1995), and the differences in speed between cells reacting to the control stimulus were expected to be small, but statistically significant. The speeds presented for controls in Table 2 are consistent with these previous studies. Moreover, Table 2 shows that calmodulin antisense ODNs interfere with the characteristic increase in speed in stimulus Na-OAc, which we believe is dependent upon the plasma membrane Ca-ATPase. The interference is specific and not merely due to untoward effects of electroporation or ODNs on motility because the sense ODNs did not interfere with the swimming speed increase in Na-OAc and there was no effect of either ODN on the speed increases in the stimulus NH_4Cl.

It is important to note that for Table 2 all cells were collected from late log phase cultures. However, untreated cells were not kept for 24 h in buffer, in which the cells stop growing and show slower basal speed of swimming. Therefore, relative changes in swimming speed and not the absolute values of swimming speed should be compared.

In summary, swimming speed increases are part of the response of paramecia to attractant stimuli. After electroporation of calmodulin antisense ODNs, there is a loss of the Na-OAc-induced increase in swimming speed while there is no effect on the NH_4Cl-induced increase. The signal transduction pathway initiated by Na-OAc is thought to

involve plasma membrane calmodulin-regulated Ca-ATPase, whereas the pathway initiated by NH_4Cl does not. While the results of Table 2 are not proof of involvement of the plasma membrane Ca-ATPase in chemosensory signal transduction, they are completely consistent with it.

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