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Lysozyme Acts as a Chemorepellent and Secretagogue in *Paramecium* by Activating a Novel Receptor-operated Ca^{++} Conductance

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Abstract. Using combined intracellular recordings and behavioral bioassays, it was found that lysozyme has two different effects in *Paramecium,* depending upon the concentrations used. At low concentrations (0.5 nM to 1.0 µM) it acts as an effective chemorepellent that causes reliable electrophysiological changes. Lysozyme-induced somatic depolarizations, isolated by blocking K^+ channels with Cs-TEA, showed concentration dependencies that were well correlated with chemorepulsion. Ion dependency experiments showed that these were Ca^{++} based depolarizations. Addition of either Na^+ or Mg^{++} improves chemorepulsion by providing additional depolarizations. Both the depolarizations and chemorepulsion were blocked by 10 µM neomycin, suggesting that the depolarization is necessary for this chemosensory transduction event. At higher concentrations (100 µM), lysozyme is a secretagogue. A transient inward current, recorded in Ca^{++} alone solutions with Cs-TEA present, was seen in response to high lysozyme concentrations. The amplitude of this inward current was well correlated with exocytosis. Addition of neomycin (1.0 mM) eliminated both the inward current and exocytosis, suggesting a causal relationship. Neither amiloride or W-7, compounds previously suggested to affect the electrophysiological responses to secretagogues, had any significant effects. The mucopolysaccharide hydrolysis activity of lysozyme was not required for any of these responses. We propose that *Paramecium* have a high affinity receptor on the body plasma membrane that responds to either lysozyme or a related compound to cause an increase in a novel body Ca^{++} conductance. This receptor-operated Ca^{++} conductance causes membrane depolarization and chemorepulsion at low concentrations and triggers a sufficient Ca^{++} influx at high concentrations to cause exocytosis.

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Key words: Chemosensory transduction — Ion channels — Exocytosis — Calcium — Paramecium — Receptors — Lysozyme

Introduction

The unicellular eukaryotic cell *Paramecium* responds to sensory stimuli (touch, heat or chemicals) with changes in the body (somatic) membrane potential (Saimi & Kung, 1987; Machemer, 1988*a,b;* Hennessey, 1989; Van Houten, 1994). The cilia are not involved in any of these stimuli-induced membrane potential changes because they are still seen in deciliated cells. In general, hyperpolarizing stimuli cause faster forward swimming (Machemer, 1988*a,b;* Nakaoka & Iwatsuki, 1992) and strong depolarizing stimuli can elicit a Ca^{++} based action potential, Ca^{++} influx and consequent backward swimming (Eckert, 1972). Small depolarizations also have reliable effects on slowing swim speed, making swim speed a sensitive indicator of membrane potential changes (Machemer, 1988*a,b*). Therefore, sensory information is integrated in the form of somatic membrane potential changes and the result can be estimated by swimming behavior bioassays. Intracellular electrophysiology is used to confirm any membrane potential changes suggested by such bioassays.

The general model in *Paramecium* is that chemoattractants cause hyperpolarization of the body plasma membrane potential (Van Houten, 1979) and that chemorepellents cause somatic depolarizations (Van Houten, 1979; Clark et al., 1993; Hennessey et al., 1994). Although exceptions to this generalization have been described (Van Houten, 1979) this general model is similar to the integration of excitatory (depolarizing) and inhibitory (hyperpolarizing) synaptic inputs in neuronal decision making. In both cases, sufficient receptor-regulated somatic depolarizations trigger action potentials. In *Paramecium,* they are graded, Ca^{++}-based, ciliary action po-

tentials that not only affect membrane potential but intraciliary Ca^{++} concentrations as well.

Depolarizing chemorepellents, such as quinidine, have been described in *Paramecium* (Van Houten, 1979) but the high concentrations (mM) involved make it difficult to study receptor-regulated events. L-glutamate has also been shown to be a depolarizing chemorepellent in *Paramecium* at a specific concentration but at different concentrations it is also a hyperpolarizing chemoattractant (Preston & Usherwood, 1988). Chemorepellents have also been described in *Tetrahymena* (Kohidai et al., 1994) and bacteria (Tso & Adler, 1974) but accompanying intracellular electrophysiological analyses have not been reported.

Three different categories of chemorepellents have recently been described in *Paramecium,* all of which are effective at micromolar concentrations. They are external GTP (Clark et al., 1993), oxidants (Hennessey et al., 1994) and secretagogues (Francis & Hennessey, 1995). The somatic membrane receptors involved in these responses have not yet been purified and the membrane conductance changes responsible for these depolarizations have not been fully elucidated.

While chemoreceptors have been described in *Paramecium* that mediate cAMP-induced hyperpolarizing responses and chemoattraction (Van Houten et al., 1991), the conductance changes involved have also not been fully described. It has been suggested the cAMP-induced hyperpolarization of *Paramecium* (Wright et al., 1993) and *Dictyostelium* (Van Duijn et al., 1990) may not involve membrane ion channels but may be mediated by an electrogenic plasma membrane ion pump, such as a Ca^{++}-ATPase.

Of all of the chemorepellents recently described for *Paramecium,* the secretagogues are least understood. Secretagogues are compounds that trigger the fusion of cytoplasmic secretory granules (called trichocysts) with the plasma membrane, resulting in release (exocytosis) of the granule contents in the form of long protein spines. Trichocyst release may play a significant role in escape behavior from predators (Harumoto, 1994). There have been many secretagogues described in the literature, many of which are polycations (Plattner, Sturzl & Matt, 1985; Satir & Bleyman, 1993). Secretagogues such as aminoethyldextran (AED) and veratridine both are assumed to work by way of an increase in intracellular Ca^{++} (Matt, Bilinski & Plattner, 1978; Satir et al., 1988; Kerboeuf & Cohen, 1990; Knoll et al., 1993; Plattner et al., 1994) and both activate Ca^{++}-dependent ion currents (Erxleben & Plattner, 1994) but the Ca^{++} source is controversial. A secretagogue-induced inward current was first documented for the response of *Paramecium* to alcian blue by Vuoso (1990) but the Ca^{++} dependency was not established. A somatic Ca^{++} channel was proposed for the action of the secretagogues AED (Kerboeuf & Cohen, 1990) and veratridine (Plattner et al., 1994) but they presented no electrophysiological analysis to support this. Conversely, it has been suggested that the secretagogue-induced elevation of intracellular Ca^{++} does not involve Ca^{++} influx (Knoll et al., 1993; Erxleben & Plattner, 1994). Since an AED-induced $^{45}Ca^{++}$ influx has been described in *Paramecium* (Kerboeuf & Cohen, 1990), the question of whether there is a secretagogue-receptor activated Ca^{++} conductance is still unresolved.

Of the many secretagogues available for study, we have chosen to use lysozyme because it is nontoxic, it can be obtained in a very pure form and it is effective as a chemorepellent at very low concentrations (1.0 nM to 0.1 μM) (Hennessey & Becker, 1994).

Materials and Methods

Cell Cultures

Wild type (type 51s) and mutant (tam 8, trichocyst nondischarge) *Paramecium tetraurelia* were grown to late log phase (4 day) in the axenic medium of Soldo and Van Wagtendonk (1969). The other behavioral mutants cam[11] (formerly fast-2, d4-91), pawn B (d4-95) and cam[1] (formerly pantophobiac A, d4-622) were gifts from Dr. C. Kung and the MAG 160 (eccentric or xntA) was a gift from Dr. R. Preston. The tam 8 mutant was a gift from Dr. J. Beisson. These mutants, along with wild type controls, were grown in bacterized medium (Sonneborn, 1970). All cultures were grown at 25°C.

Chemicals and Solutions

The general wash solution consisted of 1.0 mM MOPS, 4.0 mM KCl, 50.0 μM $CaCl_2$ and pH = 7.2 with Tris-base. In most behavioral experiments, the test solution was 1.0 mM MOPS, 1.0 mM $CaCl_2$, pH = 7.2 with Tris-base with all ions added as chloride salts. When higher concentrations of lysozyme or neomycin (added as the sulfate salt) were added to any solutions, the pH was adjusted to pH = 7.2 with further addition of Tris-base. The W-7 was made as a stock solution in DMSO and added by dilution so that the final DMSO concentration never exceeded 0.018%. Other calcium channel blockers were obtained from Alomone Labs, Israel.

The standard electrophysiological recording solutions were either 1.0 mM MOPS, 4.0 mM KCl, 1.0 mM Ca Cl_2, pH = 7.2 with Tris-base (with 500 mM KCl electrodes) or the "Cs-TEA" condition with 1.0 mM MOPS, 4.0 mM CsCl, 10.0 mM TEA-Cl, 1.0 mM $CaCl_2$, pH = 7.2 with Tris-base (with 2.0 M CsCl electrodes). When other ions were added to the recording solutions, the Ca^{++} concentrations was always kept at 50 μM to keep cells healthy on the electrodes. The electrode resistances varied between 20 to 200 megaohms.

All of the compounds used were supplied by Sigma Chemical Co., St. Louis, MO.

Electrophysiology

Standard one electrode intracellular membrane potential recordings, current injection and two electrode voltage clamp analysis were similar to the procedures reported previously (Satow & Kung, 1979; Hennessey & Kung, 1987). Membrane potentials and ion currents were displayed on a digital oscilloscope and retained on a chart recorder with the paper moving at a speed of 2.5 cm/min. For voltage-clamp experiments, the holding potential was equal to the membrane potential under those conditions. In the Cs-TEA experiments, the holding potential was −25 m V. The cells were always recorded under continuous bath perfusion at a rate of about 20.0 ml/min. The recording bath had

a volume of about 1.0 ml. Solutions were changed by switching valves connected to different solutions without changing the flow rate of the perfusion system. In all of the electrophysiological analyses, cells were preincubated in the wash solution for at least 4.0 hours. Cells were deciliated by vortexing for 1.0 min after addition of 5.0% EtOH (Ogura & Machemer, 1980).

Behavioral Assays

The three-way stopcock chemokinesis assay of Van Houten et al. (1975) was used to verify that lysozyme is a chemorepellent. The wild-type cells used were grown in bacterized medium so that the results can be compared to previously reported chemokinesis works, the majority of which were done with cells grown in bacterized medium. Washed cells were placed in the entry arm, lysozyme solutions in the test arm and control solution in the control arm. The stopcock was left open for 15 min. The I_{che} (index of chemotaxis) statistic was determined differently than as originally described by Van Houten et al. (1975). The number of cells in the control arm was divided by the number of cells in both control and test arms to get an index of chemorepulsion. This I_{che} varied between 0.5 and 1.0, with 1.0 being complete repulsion and 0.5 being no preference for either arm.

Cells were also scored for the percent of cells showing avoiding reactions (%AR) by simply transferring single cells to test solutions and observing them under a standard binocular microscope. A cell that showed any backward swimming response (avoiding reaction) was scored as a positive responder. Ten cells were done for each trial and the mean of the ten responses was calculated. Each trial was repeated three times and the %AR was expressed as the mean ± SD. In most behavioral assays, the cells were preincubated in the control solution used for at least 4.0 hours.

Lysozyme Hydrolysis Activity Assay

Lysozyme hydrolyzes mucopolysaccharides in the cell walls of bacteria to lyse the bacteria. This activity is assayed by monitoring the decrease in OD_{450} (optical density at 450 nm) of a suspension of *Micrococcus lysodeikticus*. As the cells are lysed by the action of lysozyme, the solution clears. The hydrolysis activity was determined by adding 0.1 ml of a test sample to 2.9 ml of a *Micrococcus* suspension (10 mg/80 ml) and measuring the change on OD_{450} as a function of time in a Shimadzu UV1600 spectrophotometer. One unit of activity is defined as a change in OD_{450} of 0.001/min.

Trichocyst Release

In response to secretagogues, such as 100 μM lysozyme, *Paramecium* release trichocysts as long protein spines that can be seen as a puff of white material when viewed with bright background lighting through a binocular microscope. Verification that the material is trichocysts is easily seen by viewing these "puffs" under phase contrast microscopy. To quantitate release, cells were transferred to a test solution and individually scored as either firing or not. The extent of release from individual cells was not assayed because, unlike the response to picric acid (Satir & Bleyman, 1993), the released trichocysts did not remain associated with the cells after lysozyme-induced release. The mean response of ten cells was used to determine the percent cells releasing trichocysts and this was repeated three times for a total of 30 cells.

Results

Responses to Lysozyme in Behavioral Assays

Lysozyme is a chemorepellent in the three-way stopcock chemokinesis assay of Van Houten et al. (1975), showing maximal avoidance above 5.0 μM lysozyme (Fig. 1). Cells actively avoided entering the test arm at these concentrations and accumulated in the control arm. The control solution used contained 1.0 mM MOPS, 1.0 mM $CaCl_2$ and pH = 7.2.

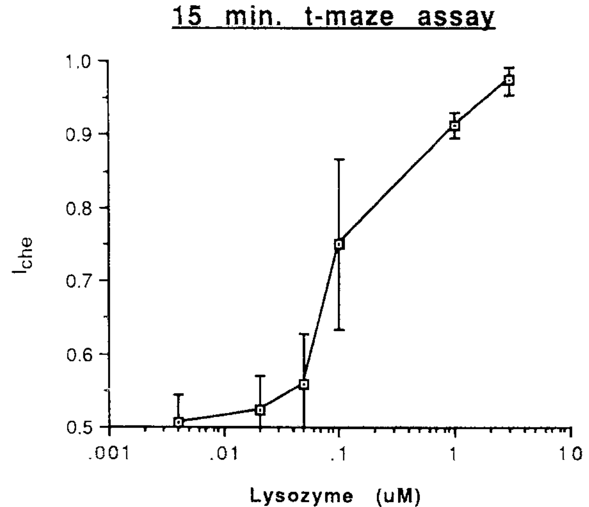

Fig. 1. Lysozyme is a chemorepellent in the classical three-way stopcock assay. Bacterized cells were rinsed in the wash solution (*see* Materials and Methods) and put in the entry arm of the three-way stopcock. Control solution was put in the control arm and lysozyme solutions (in the same solution, pH adjusted to 7.2) were put in the test arm. The stopcock was left open for 15 min and then the cells were removed and counted. The I_{che} was determined as a ratio of the number of cells in the control arm divided by the number in the test and control arms. Therefore, I_{che} = 1.0 is complete avoidance while I_{che} = .50 is no preference. Each point represents the mean ± SD (n = 3).

Scoring the %AR of individual cells proved to be a simpler and more sensitive method than the three-way stopcock for quantitating the responses of cells to lysozyme. Cells grown in axenic medium required only 0.1 μM lysozyme to show 100% AR (in the same buffer as cells in Fig. 1) and their responses were improved even more when 1.0 mM $MgCl_2$ and 10.0 mM NaCl were added. As shown in Fig. 2, cells showed significant avoiding reactions in lysozyme concentrations as low as 5.0 picomolar when Mg^{++} and Na^+ were added. Cells grown in bacterized medium were generally less sensitive to lysozyme, 100% AR required at least 1.0 μM lysozyme in the $CaCl_2$ alone solution (Fig. 2).

The effects of Mg^{++} and Na^+ on the lysozyme response is due to the activation of Ca^{++}-dependent channels during this response. Wild-type *Paramecium* have well characterized Ca^{++}-activated Na^+ channels (Saimi & Kung, 1980; Saimi, 1986) and Ca^{++}-activated Mg^{++} channels (Preston, 1990) that can contribute to membrane potential changes when internal Ca^{++} is elevated. As shown in Fig. 3*A*, addition of external Na^+ increases the responses of wild type and the Ca^{++}-activated Mg^{++} channel mutant, MAG160, to a low concentration of

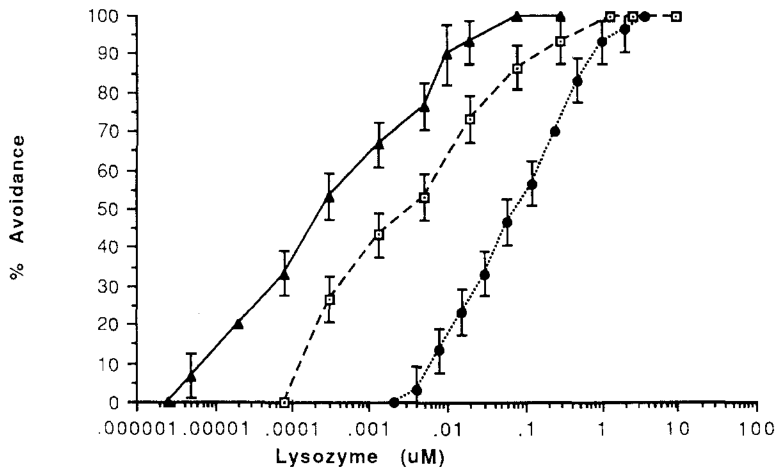

Fig. 2. Both cells grown in axenic and bacterized medium showed increases in avoiding reactions (AR) with increasing lysozyme. Avoiding reactions are a representative response to depolarizing repellents. The %AR was determined by scoring responses of individual cells (*see* Materials and Methods) in the same conditions as used in Fig. 1. The %AR of bacterized cells (closed circles) increases with lysozyme concentrations but the responses of axenic cells (open squares) occur at much lower lysozyme concentrations. Adding 1.0 mM $MgCl_2$ and 10 mM NaCl to the test solution (closed triangles) increased the sensitivity of axenic cells to lysozyme. Each data point represents the mean ± SD of at least 3 experiments each.

lysozyme (0.2 nM) but not those of the Ca^{++}-activated Na^+ channel mutant, cam[11]. The cam[11] mutant does not depolarize in Na^+ solutions while the wild type and MAG160 cells do. Similarly, increasing external Mg^{++} concentrations improves the responses of both wild type and cam[11] but not those of the MAG160 mutant (Fig. 3*B*). The MAG160 mutant does not properly depolarize in Mg^{++} solutions. Therefore, the improvement of lysozyme-induced responses in Na^+ and Mg^{++} solutions requires their appropriate ion channels, both of which are activated by elevated internal Ca^{++}. Neither channel is strictly required for lysozyme-induced AR, however. This suggests that internal Ca^{++} levels are elevated by even nanomolar concentrations of lysozyme. Unlike Mg^{++} and Na^+, addition of up to 16.0 mM K^+ or 4.0 mM Ca^{++} did not increase the %AR in the same lysozyme concentrations as above.

Lysozyme's hydrolyzing activity is not involved in its effects on swimming behavior or exocytosis. When a 1.0 μM solution of lysozyme was boiled for 5 min (and cooled to room temperature), its hydrolytic activity was eliminated but it still caused 100% of the cells placed in it to show strong avoiding reactions. Addition of triacetylchitotriose (0.1 mM), a competitive inhibitor of lysozyme hydrolysis activity, did not prevent lysozyme from causing 100% of the cells to show avoiding reactions either. Lysozyme is also a secretagogue (Harumoto, 1994; Hennessey & Becker, 1994) and its ability to cause exocytosis was not altered by either boiling or addition of triacetylchitotriose (*personal observations*).

Electrophysiological Responses to Lysozyme

Paramecium show transient membrane potential changes in response to secretagogues such as lysozyme. Intracellular electrophysiological recordings show a transient hyperpolarization and a depolarization in response to 100 μM lysozyme when KCl electrodes were used with the 4.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM MOPS solution (Fig. 4*A*). The trichocyst nondischarge mutant, tam 8, was used in all of the following experiments to eliminate any possible exocytosis artifacts. These lysozyme-induced responses varied, sometimes the depolarization preceded the hyperpolarization and sometimes no depolarization was seen. The variability of these responses made them difficult to quantitate because of their often biphasic nature. Under voltage clamp conditions (and the same K^+-Ca^{++} solution), a transient outward current was seen (Fig. 4*D*), sometimes accompanied by an inward current.

The lysozyme-induced hyperpolarization is due to the activation of a K^+ conductance because it could be effectively blocked by Cs-TEA. A problem with the Cs-TEA condition is that it depolarizes the cell, causing trains of action potentials. Lysozyme addition stops the action potentials and produces a transient depolarization (Fig. 4*B*). Since action potentials are eliminated under voltage clamp, the lysozyme response is now seen as a transient inward current with Cs-TEA, sometimes followed by a slower inward current (Fig. 4*E*).

The somatic receptor potential for the lysozyme response was further isolated for more detailed studies. A reproducible, transient, lysozyme-induced depolarization was unveiled when the action potentials were eliminated by deciliation (Ogura & Machemer, 1982) and the K^+ channels blocked by Cs-TEA (Hennessey & Kung, 1987) (Fig. 4*C*). A similar response was seen with boiled lysozyme (100 μM). When current was injected through the recording electrode to estimate membrane resistance (as represented by a change in voltage by Ohm's law), lysozyme (0.1 mM) caused up to a twofold decrease in membrane resistance at the peak of the depolarization. Membrane resistance returned following repolarization, even in the continued presence of lysozyme (*data not shown*). The transient decreased membrane resistance is consistent with an increased membrane ion conductance during the depolarization. Under voltage clamp, the same conditions produced a transient inward current, often (but not always) followed

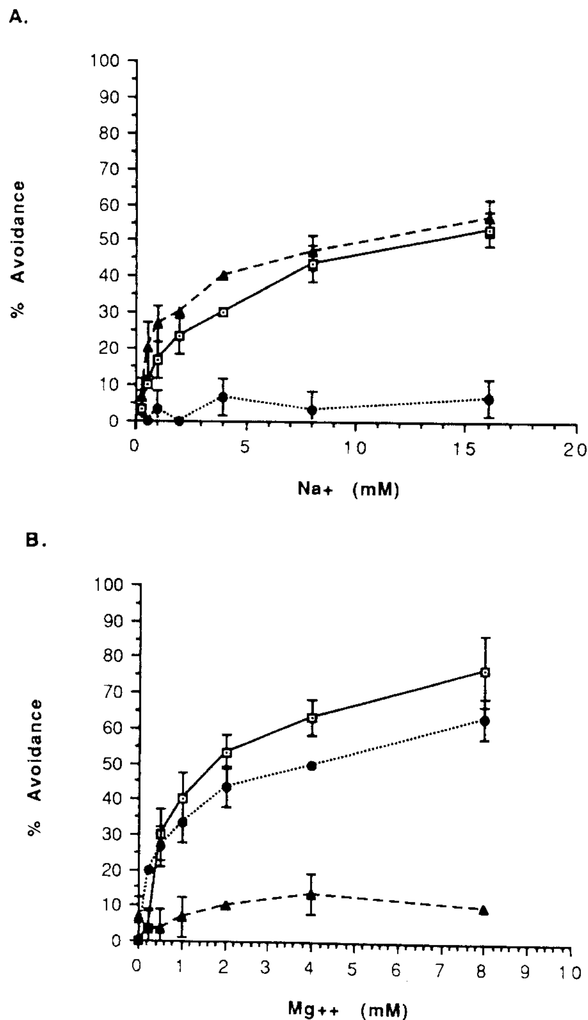

Fig. 3. The lysozyme responses of wild type and behavioral mutants were Mg^{++} and Na^{+} dependent. (*A*) Addition of Na^{+} to the test solution increased the responses of wild type (open squares) and the Mg^{++} channel mutant MAG160 (closed triangles) to 0.2 nM lysozyme but not those of the Na^{+} channel mutant, cam^{11} (closed circles). (*B*) Addition of Mg^{++} to the test buffer increased the responses of the wild type (open squares) and the cam^{11} mutants (closed circles) but not those of the Mg^{++} channel mutants, MAG160 (closed triangles). Each point represents the mean ± SD of at least 3 experiments.

by a slower inward current (Fig. 4*F*). The first transient is what we refer to as the somatic, lysozyme-induced receptor current.

The transient lysozyme-induced depolarization could be repeatedly elicited in deciliated tam 8 (with Cs-TEA), provided sufficient time was allowed to wash out the lysozyme between presentations (Fig. 5). The repeatability of this depolarization response was also often seen in wild type (*data not shown*) where trichocyst discharge occurred during the first presentation of lysozyme but not in any subsequent presentations.

Cells had to be very healthy on the electrode to show repeatable responses. Cell health was judged by lack of cell swelling (or "blistering") and obvious contractile vacuole activity. In intact cells, responsiveness was assured by observing a strong ciliary reversal in response to injected current (1.0 nA) through the recording electrode.

Similar lysozyme-induced depolarizations could be seen at low concentrations of lysozyme (0.1 μM) and these depolarizations could be blocked by 10 μM neomycin (Fig. 6). While cells show 100% avoiding reactions at 0.1 μM lysozyme, addition of 10 μM neomycin reduces the %AR to 38.0 ± 4.0% (n = 3). The %AR at 40 μM neomycin is zero. This is well below the concentration necessary to fully inhibit I_{Ca} (Gustin & Hennessey, 1988). However, 10.0 μM neomycin is sufficient to eliminate the 0.1 μM lysozyme-induced depolarization (Fig. 6*C*). At these concentrations, neomycin may be a competitive inhibitor for this lysozyme effect because the inhibition can be overcome by a ten-fold excess of lysozyme (Fig. 6*D*).

No other Ca^{++} channel blockers had any inhibitory effects on the avoiding reactions seen in 0.1 μM lysozyme. The toxins tested at 2.0 μM concentrations were calcicludine, calciseptine and w-conotoxins (GVIA, MVIIA, MVIIC and SVIB) while taicatoxin was at 0.04 μM. Other Ca^{++} channel blockers tested were nifedipine, nimodipine, nitrendipine and PN200-110, all at 10 μM concentrations. None of these compounds inhibited lysozyme-induced avoiding reactions. Amiloride, a compound that has been previously described as a blocker of secretagogue-induced currents (Erxleben & Plattner, 1994), did not affect the lysozyme-induced depolarization (Fig. 6*B*). Neither amiloride or W-7 (another compound previously suggested as a blocker of secretagogue-induced inward currents) (Erxleben & Plattner, 1994) caused dramatic reductions in the lysozyme-induced (0.1 mM) inward current. The inward currents were 0.71 ± .11 nA (n = 5), 0.51 ± .12 nA (n = 5) and 0.61 ± .16 nA (n = 8) for controls, 1.0 mM amiloride and 10 μM W-7 respectively for deciliated cells with Cs-TEA. However, the inward current seen in response to 100 μM lysozyme was completely eliminated by 1.0 mM neomycin.

The isolated lysozyme-induced depolarization is Ca^{++} dependent. When Ca^{++} is the only divalent ion present, the membrane potential reached during the lysozyme-induced depolarization increased 20 m V/10-fold change in Ca^{++} added (Fig. 7). The Ca^{++} dependence is not due to any contribution of ciliary voltage-dependent Ca^{++} channels because it is seen in both deciliated cells (Fig. 7) and in pawn B, a mutant with a defective ciliary Ca^{++} channel activity (*data not shown*). This is similar to the slope of Ca^{++} dependence seen for the anterior mechanosensory Ca^{++} current (Ogura & Machemer, 1980). A similar Ca^{++} dependence was seen for both the tam 8 (trichocyst nondischarge mutant) and the MAG160 (Ca^{++}-activated Mg^{++} channel mutant, Preston & Kung, 1994) (Fig. 7), showing that their lysozyme-induced depolarizations were due to changes in Ca^{++}

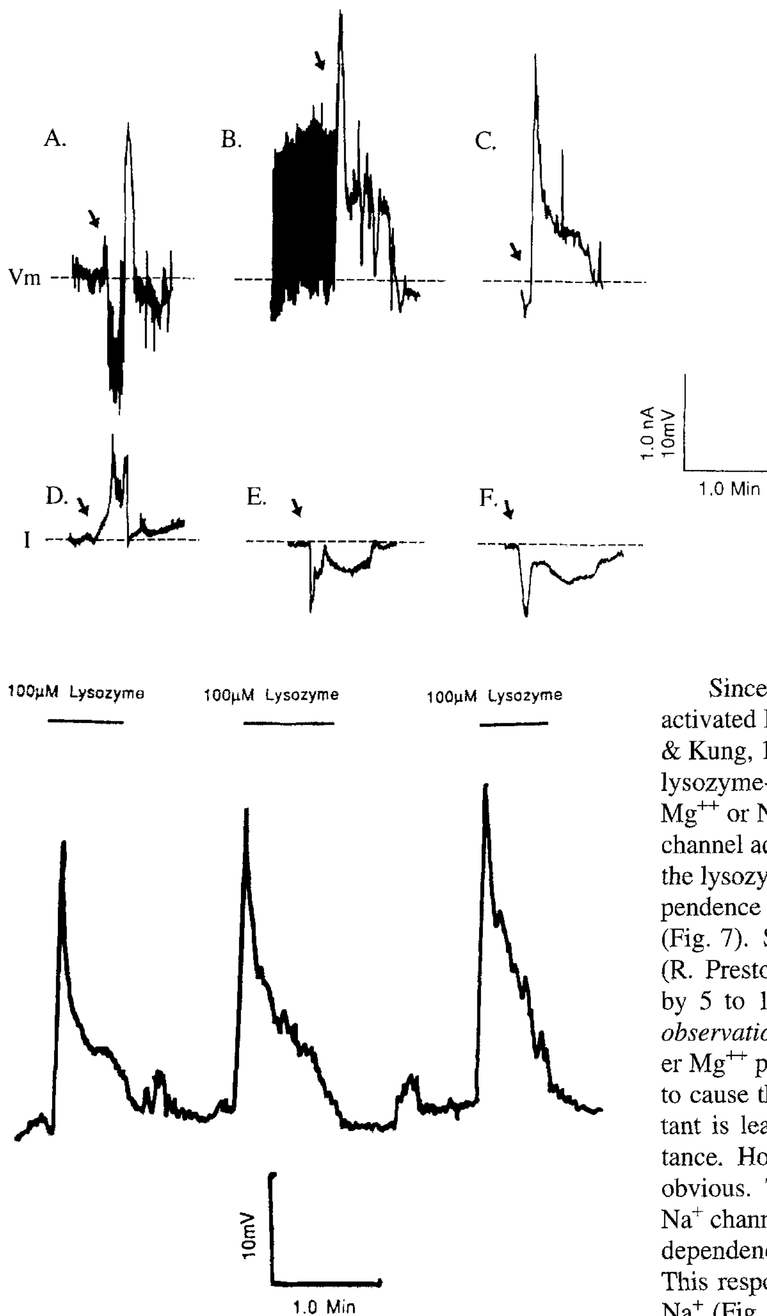

Fig. 4. The electrophysiological responses of the trichocyst nondischarge mutant, tam 8, could be seen under various conditions. In each trace, 100 µM lysozyme was added after a 20 to 40 sec delay (at the arrow) and was present during the rest of the trace. Traces *A* through *C* are membrane potential measurements and traces *D* through *F* are from voltage clamped cells. (*A*) Intact cells in solutions containing 1.0 mM $CaCl_2$ and 4.0 mM K^+ (and 500 mM KCl electrodes) show biphasic response in response to lysozyme (100 µM). (*B*) When Cs-TEA is added (with 2.0 M CsCl electrodes) to block K^+ channels, intact cells produce action potentials due to the depolarization. Addition of 100 µM lysozyme interrupts these action potentials, producing a single transient depolarization in the continued presence of lysozyme. (*C*) Deciliation removes the action potentials, unveiling the underlying transient, lysozyme-induced depolarization. (*D*) Intact cells show a transient outward current under voltage clamp (V_h = −25 m V) in the same conditions as 4*A*. (*E*) Intact cells show a transient inward current under voltage clamp in the same conditions as 4*B*. Action potentials are not seen because the cell is voltage clamped. (*F*) Deciliated cells show the same responses as intact cells in Cs-TEA.

Fig. 5. Lysozyme-induced depolarizations can be repetitively elicited. This representative deciliated tam 8 cell was recorded with Cs-TEA and 100 µM lysozyme was added as shown by the bars above each transient depolarization. Between each addition, the cell was washed with the control solution.

conductances that were unaffected by their mutations. All of these ion dependency experiments were performed with 100 µM lysozyme but the 0.1 µM lysozyme response (Fig. 6*A*) also showed a Ca^{++} dependency with a slope of 20.0 m V/10-fold increase in Ca^{++}.

Since wild-type *Paramecium* have separate Ca^{++}-activated Mg^{++} (Preston, 1990) and Na^+ channels (Saimi & Kung, 1980; Saimi, 1986), the question of whether the lysozyme-induced depolarization could be carried by Mg^{++} or Na^+ was addressed in mutants that lacked these channel activities. When the MAG160 mutant was used, the lysozyme-induced depolarization showed a Mg^{++} dependence of about 13.0 m V/10-fold change in Mg^{++} (Fig. 7). Since the MAG160 mutant is somewhat leaky (R. Preston, *personal communication*) and depolarized by 5 to 10 m V in 5.0 mM Mg^{++} solutions (*personal observation*), it is difficult to distinguish between whether Mg^{++} permeates the same conductance that Ca^{++} does to cause the transient depolarization or whether the mutant is leaky enough to provide another Mg^{++} conductance. However, the Na^+ permeability question is more obvious. The cam[11] mutant, which lacks Ca^{++}-activated Na^+ channels (Saimi, 1986), also shows virtually no Na^+ dependence for the lysozyme-induced depolarization. This response only changes 4.0 m V/10-fold change in Na^+ (Fig. 7). Therefore, the lysozyme-induced depolarization can be carried by Ca^{++} (and possibly by Mg^{++}) but not solely by Na^+.

Lysozyme causes two different electrophysiological and behavioral responses, dependent upon the concentration used. At low lysozyme concentrations (less than 1.0 µM), the concentration dependence for the amplitude of the lysozyme-induced somatic depolarization was well correlated with the concentration dependence for producing avoiding reactions (Fig. 8). At higher concentrations (above 1.0 µM), the size of the lysozyme-induced inward current is well correlated with trichocyst release

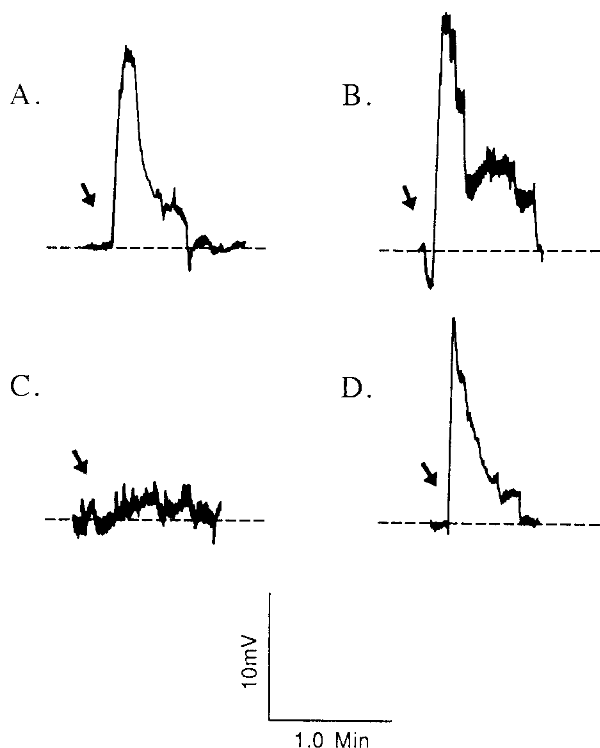

Fig. 6. The transient, lysozyme-induced depolarization has different sensitivities to different pharmacological agents. Deciliated tam 8 (with Cs-TEA) was used in all of these traces. Lysozyme addition began at the arrow. (*A*) The response to 0.1 μM lysozyme is very similar to the responses to 100 μM lysozyme (*see* Fig. 4*C* and 5). (*B*) Addition of 1.0 mM amiloride does not dramatically alter the response to 0.1 μM lysozyme. Perfusion with amiloride began 30 sec before addition of lysozyme and amiloride together (at arrow). (*C*) Addition of 10 μM neomycin (in the presence of 0.1 μM lysozyme) blocks the depolarization. The sequence of additions is similar to that used with amiloride (*above*). (*D*) Raising the lysozyme concentration to 100 μM overcomes the inhibition by 10 μM neomycin.

(Fig. 9) while the amplitude of the lysozyme-induced depolarization is not (compare Fig. 8 and Fig. 9). The lysozyme-induced depolarizations peak well before the concentrations necessary for trichocyst release. However, the inward current increases with lysozyme concentrations above 1.0 μM until they plateau, as does trichocyst release. Therefore, depolarization and chemorepulsion are associated with low concentrations of lysozyme while the inward current and exocytosis require high (100 μM) concentrations.

Abbreviations

AED:	aminoethyldextran
AR:	avoiding reactions
DMSO:	dimethyl sulfoxide
I_{che}:	index of chemotaxis
MOPS:	morpholinopropanesulphonic acid
TEA:	tetraethylammonium
Tris:	tris(hydroxymethyl)aminomethane

Discussion

Lysozyme is a Chemorepellent at nM to μM Concentrations

In the classical three-way stopcock assay of Van Houten et al. (1975), cells actively avoided entering lysozyme-containing solutions at concentrations greater than 0.1 μM (Fig. 1). This confirms that lysozyme is a chemorepellent. The chemorepulsion was due to lysozyme-induced avoiding reactions (AR) because the %AR was increased by lysozyme in a concentration dependent manner (Fig. 2). Therefore, lysozyme acts as a "Type I" chemorepellent (Van Houten, 1979) because it increases the frequency of avoiding reactions. The mucopolysaccharide hydrolysis activity of lysozyme is not necessary for these behavioral responses.

The %AR bioassay was quicker, easier and more sensitive than the 15-min three-way stopcock assay for quantitating these chemorepellent responses. As shown in Fig. 2, lysozyme-induced responses of axenic cells could be detected in this convenient bioassay at concentrations as low as 0.3 nM in Ca^{++} alone solutions and the responsiveness was increased by addition of Mg^{++} and Na^{+} so that concentrations as low as 5.0 pM could be detected. This extremely low concentration response suggests a very high affinity receptor for lysozyme. Cells grown in bacterized medium were generally less responsive to lysozyme and also improved with addition of Mg^{++} and Na^{+}, but not to the level of axenic cells.

The speed of this bioassay is important because cells adapt to 0.1 μM lysozyme within 10 min (the %AR becomes zero) and can deadapt after an additional 10 min in the wash solution (cells return to 100% AR). To test whether this adaptation is due to a general loss of responsiveness, lysozyme-adapted cells were assayed for their duration of backward swimming in 30 mM K^{+} (Schusterman et al., 1978). Neither the duration of 30 mM K^{+} backward swimming or the %AR in 1.0 μM GTP (Clark et al., 1991) were decreased in lysozyme adapted cells (*personal observations*). Therefore, this will serve as an excellent model system for studying the regulatory mechanisms involved in chemosensory adaptation (T.M. Hennessey and M.Y. Kim, *in preparation*).

Since this is a simple and rapid bioassay, it can be easily used to screen for drugs and mutations that inhibit either the ionic conductances involved in the responses to lysozyme or some other aspect of the chemosensory transduction pathway. These screens would provide preliminary information and would have to be followed by electrophysiological analyses to assure that the drugs are not simply inhibiting the ability of the cell to respond to depolarizing stimuli in general.

Mg^{++} and Na^{+} improved the chemorepellent nature of lysozyme by way of additional depolarizing contributions from Ca^{++}-activated Mg^{++} and Na^{+} channels. The

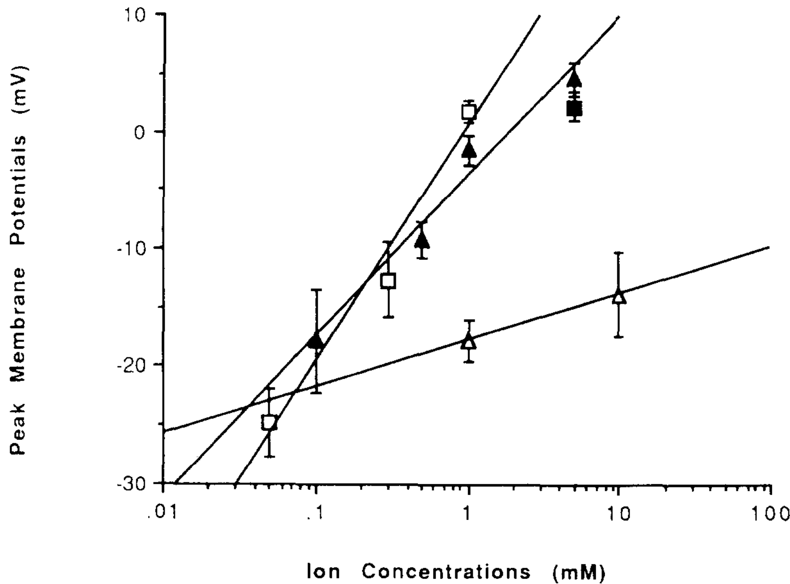

Fig. 7. The lysozyme-induced depolarizations are calcium-dependent. Deciliated cells were used in all of the following experiments (with Cs-TEA and 50 μM Ca^{++}). In the tam 8 mutants, the peak membrane potentials reached by the transient depolarizations (in 100 μM lysozyme) show a Ca^{++} dependency of about 20 m V/10-fold change in extracellular Ca^{++} (open squares). This depolarization can be carried by Mg^{++} in both tam 8 (closed squares) and MAG160 (closed triangles) with about 14.0 m V/10-fold change in Mg^{++}. The cam[11] mutant, which lacks the Ca^{++}-activated Na^+ channel (open triangles), shows a very poor Na^+ dependence of about 4.0 m V/10-fold change in Na^+. Each point represents the mean ± SD of at least 3 cells. ■ tam Mg^{++}; ▲ MAG Mg^{++}; △ CAM Na^+; □ tam Ca^{++}.

ion channel mutant MAG160, which is defective in its Ca^{++}-activated Mg^{++} channel (Preston & Kung, 1994), did not improve its %AR in response to a low concentration of lysozyme (0.2 nM) in Mg^{++} solutions while wild type and cam[11] did (Fig. 3*B*). Similarly, increasing extracellular Na^+ had no effect on the responsiveness of the cam[11] mutant to 0.2 nM lysozyme because it lacks the Ca^{++}-activated Na^+ channel (Fig. 3*A*). Therefore, lysozyme causes the opening of Ca^{++}-activated Mg^{++} and Na^+ channels at very low concentrations to increase the cellular responsiveness to this stimulus when these ions are present. This suggests that either internal Ca^{++} levels are elevated by even nanomolar concentrations of lysozyme or lysozyme directly activates these channels. The reason that cells did not increase their responsiveness in higher Ca^{++} (up to 4.0 mM) may be related to the fact that responsiveness to many stimuli is reduced at high Ca^{++} (Hook & Hildebrand, 1980; Clark & Nelson, 1991) because of a positive shift in the voltage dependence for the ciliary Ca^{++} channel (Satow & Kung, 1979). We did not analyze the effects of lower Ca^{++} on lysozyme-induced AR because this response itself requires external Ca^{++} (Hildebrand & Dryl, 1983).

Lysozyme Generates Somatic Depolarizing Receptor Potentials

The biphasic response to lysozyme, seen in Fig. 4*A*, is due to the sum of a transient depolarization and hyperpolarization. Biphasic responses associated with exocytosis have been described in the related ciliate *Didinium* (Hara & Asai, 1980; Hara & Naitoh, 1985) and in the *Paramecium* responses to the secretagogues alcian blue (Vuoso & Satir, 1994) and AED (Cohen & Kerboeuf, 1993; *personal observations*). It is likely that the hyperpolarization seen in Fig. 4*A* is due to the opening of Ca^{++}-activated K^+ channels because Ca^{++}-activated K^+ currents have been documented in response to other secretagogues in *Paramecium* (Erxleben & Plattner, 1994). We propose that the initial Ca^{++} source to activate these channels is the same lysozyme-activated body Ca^{++} conductance that is responsible for the lysozyme-induced-depolarization but we cannot rule out additional possible contributions from internal calcium stores.

We have further isolated the depolarization aspect of the secretagogue response by blocking the K^+ channels with Cs-TEA. This isolated secretagogue-induced depolarization was not seen in the work of Erxleben and Plattner (1994) because their solutions always contained either Na^+, K^+ or both. Therefore, the majority of the responses seen in their work were due to Ca^{++}-activated channels.

Contrary to the results of Erxleben and Plattner (1994), we saw no inhibition of secretagogue-induced electrophysiological responses by either amiloride or W-7. Even the lysozyme-induced hyperpolarization was seen in the presence of amiloride (*personal observation*). Since these responses are fragile (unhealthy cells do not show them) and both amiloride and W-7 are toxic, it is likely that these compounds were not specific inhibitors. We also observed that healthy cells show multiple responses to secretagogues when the lysozyme was washed out and re-added (Fig. 5). Unhealthy cells did not show this repeatability.

Both the lysozyme-induced hyperpolarization and depolarization are transient. We propose that the hyperpolarization (Fig. 4*A*) is transient because the Ca^{++} source is transient (Fig. 4*C*). Since the depolarization is still transient in Cs-TEA, K^+ conductances are not necessarily involved in the repolarization phase of the lysozyme response. However, they may serve to speed up the repolarization (compare Fig. 4*A* and 4*C*). Ciliary

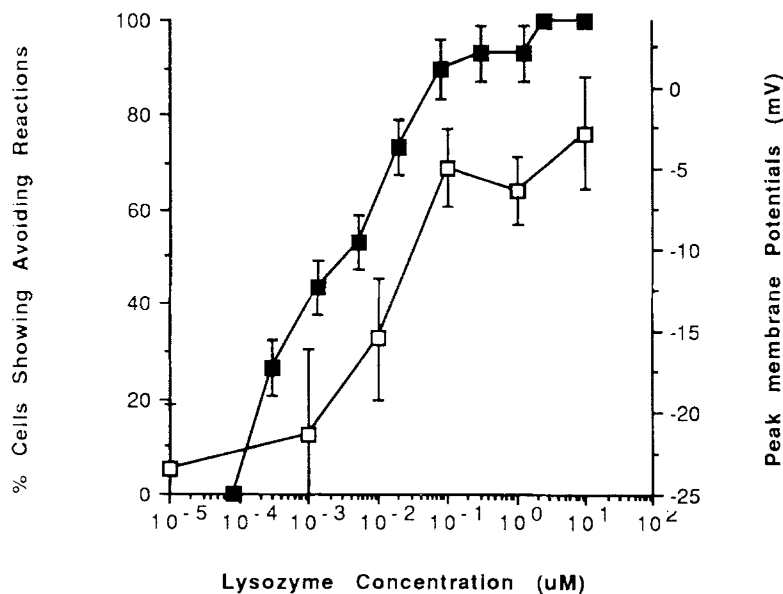

Fig. 8. There is a close correlation between the concentration dependencies of the behavioral responses to lysozyme and the lysozyme-induced depolarizations. The percent of cells showing avoiding reactions (%AR, closed boxes) showed a lysozyme dependency that closely paralleled the peak membrane potentials reached by the lysozyme-induced depolarizations (open boxes). The behavioral responses were measured with tam 8 cells. The depolarizations were also measured in deciliated tam 8 cells with Cs-TEA. Each point represents the mean ± SD of at least 3 experiments. ■ %AR; □ Vm.

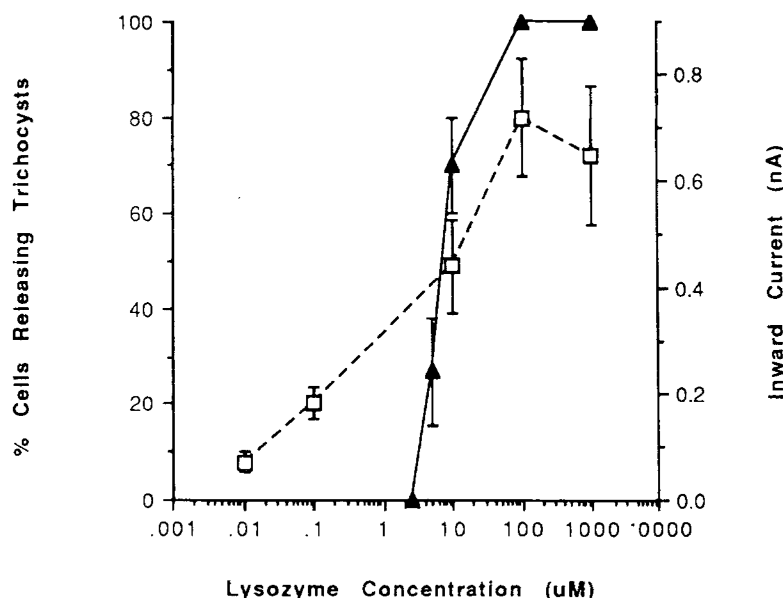

Fig. 9. There is also a close correlation between the concentration dependencies of the magnitude of the lysozyme-induced inward current and trichocyst release. The percent of wild type axenic cells releasing trichocysts (closed triangles) was scored as described in Materials and Methods. The inward Ca^{++} was measured in intact (axenic) tam 8 cells with Cs-TEA (*see* Fig. 4*E*). Each point represents the mean ± SD of at least 3 experiments. ▲ % Firing; □ Ca^{++} current.

conductances are not involved at all because both the transient depolarization (Fig. 4*C*) and the transient inward current (Fig. 4*F*) are seen in deciliated cells. We preferred to use deciliated cells instead of Pawn B because these mutants are somewhat leaky (*personal observation*). The fact that the inward currents are still transient (Fig. 4*E* and 4*F*) suggests that the deactivation (or inactivation) mechanisms is not voltage dependent.

Transient somatic depolarizations are also seen in response to GTP (Clark et al., 1993) but they differ because they oscillate. It is possible that there are separate membrane receptors for GTP and lysozyme because 10 μM neomycin acts like a competitive inhibitor for lysozyme responses but it has no effect on GTP responses (*personal observations*). Also, cells that are adapted to lysozyme still show unaltered GTP responses, consistent with the possibility that there are separate receptors for these two chemorepellents. The GTP induced depolarizations are also Ca^{++} dependent (T.M. Hennessey, *in preparation*) but it is not known if the lysozyme-induced Ca^{++} conductance is also shared by the GTP response pathway. These transient repellent responses are different from the sustained membrane potential changes seen with attractants (Van Houten, 1979) and some repellents (Van Houten, 1979; Hennessey et al., 1994).

The Lysozyme-induced Depolarizations are Carried by Calcium

When Ca^{++} was the only added divalent ion, the membrane potential reached by the lysozyme-induced depo-

larization was a function of the external Ca^{++} concentration with a dependency close to that predicted by the Nernst equation for a divalent cation (Fig. 7). It is not known why the slope was only 20 m V/10-fold change in Ca^{++} instead of the predicted 29 m V/10-fold change in Ca^{++} but this value is similar to that seen for the anterior mechanosensory Ca^{++} channel (Eckert, 1972; Ogura & Machemer, 1980). Therefore, the depolarization was due to a change in Ca^{++} conductance, consistent with either the opening of a membrane Ca^{++} channel or the activation of a Ca^{++} transporter. This conductance may also pass Mg^{++} but it is difficult to tell because the MAG160 mutant used was leaky; it showed significant depolarization in Mg^{++} solutions. There also may be other conductances to Mg^{++}-activated during this response. This conductance does not pass Na^{+} (Fig. 7) but Na^{+} addition does increase the response (Fig. 3*A*), supposedly due to the additional depolarization provided by the Ca^{++}-activated Na^{+} conductance.

Lysozyme-induced depolarizations can also be seen in 50 μM Ca^{++} solutions when either 1.0 mM Ba^{++} or 1.0 mM Mn^{++} are added. The peak membrane potentials in response to 100 μM lysozyme were -1.4 ± 1.8 m V ($n = 5$) with 1.0 mM Ba^{++} and -14.9 ± 4.0 ($n = 7$) with 1.0 mM Mn^{++}. The resting membrane potentials were -29.0 ± 2.5 for 50 μM Ca^{++}, -22.8 ± 4.0 for 1.0 mM Ba^{++} and -24.8 ± 1.8 for 1.0 mM Mn^{++}. Although this apparent nonselectivity is similar to that of the anterior mechanoreceptor Ca^{++} channel (Satow et al., 1983), it is possible that other conductances are activated during both of these depolarizing receptor potentials that can pass these divalent ions. We conclude that the lysozyme-induced depolarizations are carried by Ca^{++} under standard conditions but that Ba^{++}, Mg^{++} and Mn^{++} (to a lesser extent) can also add to these transient depolarizations. Further analysis will be necessary to determine whether these divalent ions all share a common conductance in lysozyme-induced depolarizations or, as seen with the Na^{+} effects (Figs. 3*A* and 7), may be the result of parallel activation of another conductance.

The Receptor Potential is Necessary for Chemorepulsion

The lysozyme-induced somatic depolarization (at low concentrations of less than 1.0 μM) is necessary for lysozyme chemorepulsion because neomycin inhibits both the depolarization and lysozyme-induced avoiding reactions. The depolarization is not sufficient on its own to cause ciliary reversal because no AR are seen in the Pawn B mutant in concentrations of lysozyme that cause 100% AR in wild type (less than 0.1 μM). The somatic depolarization is conducted to the cilia where it must activate voltage-dependent ciliary Ca^{++} channels to cause a Ca^{++} influx and ciliary reversal.

The Inward Current is Involved in Trichocyst Release

Higher lysozyme concentrations (100 μM) cause in inward current and exocytosis. There is a very good correlation between the amplitude of the inward current and the extent of trichocyst release (Fig. 9), suggesting that the inward current is related to trichocyst release.

Neomycin has been shown to inhibit trichocyst release in *Paramecium* (Plattner et al., 1985; Hennessey & Becker, 1994) as well as the Ca^{++} influx associated with catecholamine secretion in adrenal chromfin cells (Duarte et al., 1993). Since neomycin inhibits both the inward current and trichocyst release seen with 100 μM lysozyme (Hennessey & Becker, 1994), it is likely that the inward current is necessary to trigger exocytosis, although it may not be sufficient on its own to cause trichocyst release. Release of Ca^{++} from internal stores may also occur with secretagogues (Knoll et al., 1993; Erxleben & Plattner, 1994) but we propose that the inward current we measure is a Ca^{++} current that serves as a necessary trigger for exocytosis. This is supported by the observations that the inward current is seen in Ca^{++} only solutions and the analogous secretagogue-induced depolarization is Ca^{++} dependent (Fig. 7). Further voltage clamp analyses of reversal potentials will follow to verify the ion dependencies of this inward current.

A Novel, Secretagogue-induced, Somatic Inward Ca^{++} Current?

It is unlikely that the body Ca^{++} conductance involved in the lysozyme response is the same as any of the characterized voltage-dependent Ca^{++} conductances. The cilia are not involved so the voltage dependent ciliary Ca^{++} channel is not the lysozyme-induced Ca^{++} conductance. It is not likely to be the hyperpolarization-induced Ca^{++} channel because it is not affected by amiloride, a known blocker of this channel (Preston, Saimi & Kung, 1992).

The anterior mechanosensory Ca^{++} channel is a possibility for carrying the lysozyme-induced depolarization but since there are no mutations or blockers, it is difficult to test. One argument against this channel is that trichocyst release occurs over the whole body while the anterior mechanoreceptor channel is localized in the anterior (Ogura & Machemer, 1980). Harumoto (1994) used localized application of lysozyme to show that anterior stimulation caused backward swimming while posterior stimulation caused forward swimming. Both applications caused trichocyst release. Although this suggests that lysozyme only depolarizes on the anterior, it is also possible that Ca^{++}-activated K^{+} channels, localized on the posterior (Erxleben & Plattner, 1994) might produce an over-riding hyperpolarization in response to a lysozyme-induced posterior Ca^{++} influx. This is sup-

ported by the observation that trichocysts were released by posterior application of lysozyme (Harumoto, 1994). Unless lysozyme somehow activates the anterior mechanosensory Ca^{++} channels, it is likely that a novel, receptor-regulated Ca^{++} channel exists on the body to provide a somatic Ca^{++} influx in response to various ligands. It is also possible that the similarities seen in the apparent ion selectivities of these two conductances is due to parallel activation of the anterior mechanosensory channel during the lysozyme response. For example, since Ca^{++} stimulates contraction of the cytoskeleton (Naitoh, 1982) and cell contraction is associated with exocytosis (Cohen & Kerboeuf, 1993), it is possible that the anterior mechanosensory channels are activated during the lysozyme response as a result of the secretagogue-induced rise in intracellular Ca^{++}.

Model for the Actions of Lysozyme

These data support the model, shown in Fig. 10, that lysozyme acts either as a chemorepellent or a secretagogue, depending upon the concentration encountered by the cell. For both effects, lysozyme may be initially recognized by a high affinity body plasma membrane receptor. The high concentration effects could be due to recruitment of even more receptors.

The low concentrations necessary for lysozyme effects suggest that there may be a specific membrane receptor that recognizes lysozyme either as the proper ligand or as an agonist. This is further supported by the observation that 10 μM neomycin acts like a competitive inhibitor, suggesting a common binding site. Receptor activation would cause a change in Ca^{++} conductance on the body by an unknown mechanism. This Ca^{++} conductance change causes both a somatic depolarization and a Ca^{++} influx into the body. *Paramecium* do contain lysozyme and lysozyme hydrolysis activity can be found in the supernatant of growing cells (*unpublished observations*) but it is not known whether a receptor has evolved to recognize *Paramecium* lysozyme (as an intracellular communicator), lysozyme from another organism (such as a predator) or a polypeptide stretch that is also presented in the lysozyme molecule.

At low concentrations (less than 1.0 μM), the depolarization is sufficient to open ciliary voltage-dependent Ca^{++} channels and trigger avoiding reactions but the Ca^{++} influx, reflected by the size of the inward current in Ca^{++}-containing solutions, is not sufficient to cause trichocyst release.

At higher concentrations (greater than 1.0 μM), lysozyme causes a sufficient Ca^{++} influx to trigger trichocyst release. It is still a chemorepellent at high concentrations, but now it is also a secretagogue. At all lysozyme concentrations, the elevated Ca^{++} in the body and the cilia can also activate Ca^{++}-dependent channels

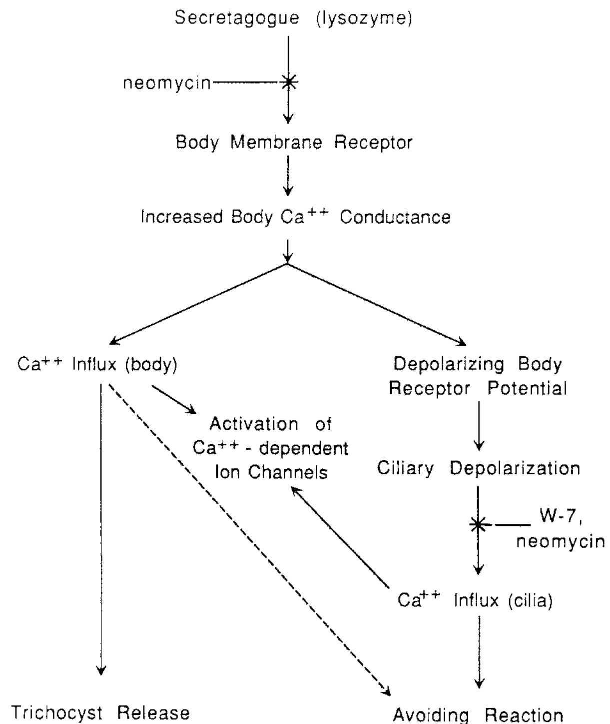

Fig. 10. Proposed model for the actions of lysozyme. Lysozyme causes an increased Ca^{++} conductance on the body plasma membrane, possibly through a novel, receptor-regulated, somatic Ca^{++} channel. This Ca^{++} conductance change has two consequences, it causes a somatic depolarization and a Ca^{++} influx into the body. The cellular responses (either trichocyst release or avoiding reactions) are dependent upon the lysozyme concentrations. At low lysozyme concentrations (less than 1.0 μM), the depolarization serves as a receptor potential to trigger ciliary depolarization and consequent ciliary reversals. At higher concentrations, the Ca^{++} influx can become sufficient to trigger trichocyst release. In both cases, elevated intracellular Ca^{++} can stimulate Ca^{++}-activated channels to open, further affecting the membrane potential changes. Sufficient lysozyme-induced Ca^{++} entry into the body can also increase intraciliary Ca^{++} concentrations to the point that it causes ciliary reversal, bypassing the voltage dependent Ca^{++} channels.

to open, providing additional depolarization in Mg^{++} and Na^{+} solutions.

The chemosensory transduction for lysozyme can be pharmacologically blocked at two different places (Fig. 10). The first is at the level of the receptor, where low concentrations of neomycin (10 to 40 μM) are sufficient to block chemorepellent responses (Fig. 6*C*). This appears to be a competitive inhibition because it can be overcome by a tenfold excess of lysozyme (Fig. 6*D*). Higher neomycin concentrations (1.0 mM) are necessary to block the inward current and exocytosis (Hennessey & Becker, 1994). The second is the ciliary Ca^{++} channel. At higher concentrations (1.0 mM) neomycin also inhibits the ciliary voltage-dependent Ca^{++} channel (Gustin & Hennessey, 1988), as does higher concentrations (100 μM) of W-7 (Hennessey & Kung, 1984). W-7 does not immediately inhibit trichocyst release (C. Erxleben & H.

Plattner, 1994, *personal observations*). Avoiding reactions can still be seen in either wild type with 100 μM W-7 or in the Pawn B mutant (Harumoto, 1994; *personal observation*) if lysozyme is high enough (100 μM), suggesting that ciliary calcium channels can be bypassed by high enough internal Ca^{++}.

Conclusions

In the general model for neuronal decision making, information can be processed by somatic integration of excitatory and inhibitory inputs, usually represented by depolarizing and hyperpolarizing receptor potentials. If a threshold depolarization is reached, an action potential is generated. The unicellular eukaryote *Paramecium* also integrates somatic sensory information in the form of the membrane potential but the response to depolarizations is a graded Ca^{++}-based action potential. Sensory responses are summed and the resultant membrane potential dictates the swimming behavior. Hyperpolarizing inputs usually come in the form of changes in ion concentrations, attractants or posterior mechanical stimulation. Depolarizing inputs come from heat, ions, mechanical stimulation or repellents. Lysozyme is a depolarizing chemorepellent. At high concentrations it is a secretagogue.

Lysozyme activates a somatic Ca^{++} conductance and this has two different consequences, a depolarization and a Ca^{++} influx. Lysozyme is a chemorepellent at low concentrations because it causes a somatic depolarization and this depolarization triggers ciliary reversal. The Ca^{++} influx is not enough to cause exocytosis but it is sufficient to open Ca^{++}-activated ion channels (Na^+, K^+ and Mg^{++}) to add further depolarizing and hyperpolarizing influences. At high lysozyme concentrations, the Ca^{++} influx is sufficient to trigger exocytosis and it becomes a secretagogue. We propose that both of these responses, chemorepulsion and exocytosis, may be triggered by the same body membrane receptor and associated Ca^{++} conductance.

The chemosensory transduction pathway for these responses could be evolutionary percursors for similar functions in "higher organisms." Considering that *Paramecium* can show modifications of sensory responses from adaptation (Schusterman, Thiede & Kung, 1978) to learning (Hennessey et al., 1979), understanding the mechanisms involved in the sensory responses in this model system will give insight into transduction strategies that may be conserved in many other eukaryotic cells.

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