Eukaryotic Unicells: How Useful in Studying Chemoreception?

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INTRODUCTION

There are compelling reasons to use unicellular organisms in the study of chemoreception, particularly when examining receptor cell function in taste and olfaction. To a student of receptor cell function, it would be useful to have a system characterized by large receptor cell size for electrophysiology, homogeneous receptor cell populations that can be grown in quantity sufficient for biochemistry, and short generation time for genetic analysis and manipulation. Unicellular organisms provide all of these characteristics and more, such as rapid, clear-cut assays of chemoresponse. Armitage,[1] Adler,[2] and Kleene[3] in recent reviews describe how bacteria can provide large amounts of material and mutants for an elegant dissection of chemosensory transduction, and these authors make the point that bacteria can clearly be of use in the study of chemoreception. There are limits to the study of sensory transduction in bacteria because, while giant bacteria can be penetrated with electrodes,[4] they are not routinely studied with conventional electrophysiology. Eukaryotic unicells, on the other hand, not only can provide a large cell for penetration by multiple electrodes, but they also have excitable plasma membranes and display neuronal properties,[5] like those of a primary neuron in chemoreception.

Eukaryotic unicellular organisms can provide useful advantages for the study of chemoreception and, while they will not replace metazoan systems in the studies of taste and smell, they can contribute information that is likely to be common to all chemoreceptor cells. Many eukaryotic unicellular chemoresponses have been described, but the most commonly studied are the chemotaxis of the slime mold *Dictyostelium*[6] in its amoeboid stage; the chemotaxis of leukocytes[7] in their response to wound and infection, and chemokineses of ciliates. Among the ciliates, *Tetrahymena* responds to peptides and amino acids,[8] which might indicate food; *Blepharisma* responds to pheromones to become mating reactive and to locate potential mates.[9] The most systematically studied ciliate chemoresponse, however, is that of *Paramecium.* The following is a description of one unicellular system, *P. tetraurelia,* to demonstrate its usefulness in studying receptor cell function.

METHODOLOGY AVAILABLE TO STUDY *PARAMECIUM*

Paramecia are grown inexpensively in mass culture axenically[10] or in bacterized rye extracts.[11] Kilogram amounts of cells can be harvested,[10] but generally there is sufficient material in one to three liters of culture to study membrane proteins.

Paramecia of several species have been studied using standard electrophysiological techniques[12] including voltage clamping.[13] Patch clamping is now being applied to the study of ion channels in the *Paramecium* membrane.[14]

The surface membrane of *Paramecium* is almost evenly divided between that covering the cilia and that covering the rest of the cell body. Cilia and their membrane can be harvested separately from the cell body membrane[15] and, likewise, the cell body membrane can be harvested free of most of the cilia.[16]

Calcium-sensitive fluorescent dyes, such as Quin-2/AM,[17] are taken into the cells and the ester bond cleaved to trap the dye in its impermeable, calcium-sensitive form inside the cell. Likewise, diacetyl-carboxyfluorescein, a pH indicating fluorescent dye,[18] is taken into the cells and deesterified.

Paramecia can be mutated using chemicals or radiation as mutagens.[19] Kung has shown that a large variety of mutants can be isolated on the basis of their altered swimming behavior and many of these mutants have altered ion channels or gates.[20] It is a remarkable feat for one laboratory to mutate and study such a large number of genes for membrane functions. This is possible in part because the components of *Paramecium* swimming behavior (frequency of turning and speed of forward swimming) are very clear and easily observed, and these components happen to be under membrane electrical control. Frequency of turning is a function of the frequency of calcium action potentials,[12] which transiently reverse the ciliary beat and cause a jerky turn, and speed of swimming is a function of frequency and angle of ciliary beating, which is controlled by the membrane potential.[21]

It has been possible to isolate mutants with specific deficits in chemoresponse behavior. For example, there are three complementation groups of mutants that specifically cannot respond to folate but that are normal in response to other chemical stimuli.[22] These mutants can be genetically characterized and show assortment typical of mutations in single genes. This is significant because the loss of single gene products that are components of the chemosensory transduction pathway makes it possible to dissect the pathway component by component. Revertants of the original mutants have also been isolated.[23] Mutants that have reverted their phenotype back to the wild type by a second-site mutation allow an analysis of a complex pathway that very often is not possible by other means. The second mutation may have no discernable phenotype by itself, but because it alters a chemoreception mutant phenotype, it is certain that the second gene product somehow participates in or affects the chemosensory pathway. (See Huang *et al.*[24] for an example of the use of revertants in *Chlamydomonas.*)

It is possible to microinject cytoplasm, RNA, or DNA into paramecia and to follow up with electrophysiological or behavioral tests in order to test the "curing" effects of the injected component on mutants or to perform quasi-complementation tests.[25] Microinjection will make it possible to transform paramecia for manipulation of cloned genes. The molecular genetics of *Paramecium* is still relatively new, and it will be necessary to meet some special problems with codon usage as its development proceeds.[26]

REVIEW OF CHEMORECEPTION IN *PARAMECIUM*

Paramecia are attracted to folate, acetate, cAMP, and other chemicals, which probably signify food. The cells respond by a kinetic mechanism that results in fast, smooth swimming up gradients of attractant.[27] Through the use of mutants, we have shown that paramecia modulate both frequency of turning and swimming speed, which add up to the net population attraction or dispersal.[27,28] From the elegant work of Jennings, Eckert, Naitoh, Kung, Machemer, and others, we know that turning and speed depend upon membrane potential.[20] An understanding of the physiology of these individual components of *Paramecium* behavior allowed us to make predictions about the effect of chemical stimuli on membrane potential during the complex swimming behavior of chemoresponse. These predictions were borne out by direct electrophysiological measurements.[29] Put simply, attractants generally hyperpolarize and repellents generally depolarize; therefore, even a complex behavior can be explained in terms of relatively simple components of *Paramecium* physiology.

How then does an external chemical stimulus affect membrane potential, which controls swimming behavior? One accepted paradigm is that receptors bind the stimulus and that this binding is transduced into internal chemical and electrical information, that is, the second and third messengers. We set about identifying receptors by first examining the number and affinities of surface binding sites, which should include the receptor. For example, binding studies with one of the available stimuli, [^{3}H]folate, indicated that there are saturable, specific binding sites on the *Paramecium* cell. Binding to these sites is reduced to a low-level, nonspecific binding in a chemoreception mutant.[30] Fluorescein-folate can also be used to study whole cell binding.[31] When dyed with fluorescein-folate, normal cells show intense fluorescence that is specific for folate, while mutant cells show little discernable fluorescence above autofluorescence.[31] Revertants that have recovered the wild-type phenotype also have recovered fluorescence in fluorescein-folate.[32] The binding of the folate conjugate is most likely to be to the outside of the cell for the following reasons: (1) the mutant has lost surface [^{3}H]folate binding capacity and (2) cells preincubated with detergent Triton X-100 to purposely permeabilize cells to the dye show an increase in fluorescence.[32] The use of fluorescein-folate has been adapted to a small scale so that individual clones in microtiter wells can be screened for binding mutants using a dissecting, epifluorescence microscope.[31]

Our next step in receptor characterization is the identification of binding proteins from the cell membrane. The *Paramecium* folate binding proteins of interest are to be found primarily on the cell body and not the ciliary membrane[30,33] and these binding sites are not likely to be evenly distributed down the cell as indicated by local perfusion during electrophysiological recording.[33] The dearth of specific binding sites on cilia does not invalidate *Paramecium* as a model for chemoreception. Cilia may still play a role in *Paramecium* chemoreception because they have an adenylate cyclase that may be part of the chemosensory pathway involved after the change in membrane potential is elicited by the stimulus (see below), and not all chemoreceptor systems, even in olfaction systems, involve cilia.[3]

We have identified folate binding proteins from the cell body membrane by affinity chromatography and determined that five of these are surface exposed, as expected for a receptor.[34] Like many other external chemoreceptor systems, the binding proteins involved in chemoreception should be of relatively low affinity; therefore, to circumvent problems of affinity chromatography of weakly binding ligands, we turned to the sensitive method of immunodetection to identify folate binding proteins.[35] The rationale was that the chemoreceptor would be among the membrane proteins to which we

could cross-link folate because cross-linking folate onto whole cells specifically inhibited attraction to folate, but not to acetate[36] (see TABLE 1).

Folate binding proteins from isolated cell body membrane that was cross-linked with folate were identified by electroblotting proteins from polyacrylamide gels onto nitrocellulose and immunodetection of the nitrocellulose with anti-folate antibodies.[35] Currently, we are cataloguing cross-linked proteins, which compare well with proteins identified by affinity chromatography, examining cross-linked proteins from chemoreception mutants, and cross-linking folate to intact cells instead of isolated membranes.

Folate is not the only stimulus that can be used in binding studies and for which there should be a receptor. Cyclic AMP is an attractant that shows saturable, specific binding to whole cells.[37] Affinity chromatography has identified only one protein that is a specific cAMP binding protein (approximately 48,000 molecular weight) from the cell body membrane (Gagnon and Van Houten, unpublished results). Nucleotides present the advantage of using [^{32}P]azido compounds that are photoreactive and will covalently cross-link cAMP to its binding site *in situ* and thereby alleviate some of the problems associated with affinity chromatography. Preliminary [^{32}P]azido-cAMP data identify one integral membrane protein of 48,000 molecular weight that is labeled

TABLE 1. Immunodetection for the Identification of Folate Binding Proteins

Stimulus	I_{che}	
	Cross-Linked Cells	Control Cells
2.5 m*M* K_2 folate	0.54 ± 0.06	0.72 ± 0.06
5 m*M* K-OAc	0.61 ± 0.06	0.67 ± 0.06

[a] Data are averages of 12 experiments for folate tests and six experiments for acetate tests ± one standard deviation. I_{che} is an index of chemoattraction. $I_{che} > 0.5$ indicates attraction; < 0.5 indicates repulsion.

on intact cells. We are pursuing the identification of the cAMP binding proteins using both normal cells and mutants that are not normally attracted to cAMP.

Binding to receptor is transduced into a change in membrane potential. The attractant-induced hyperpolarization has been studied using conventional electrophysiology, including voltage clamping. There is no obvious reversal potential and no dependence on external K or Na.[38] There are permeability studies and work with a mutant[39] (restless, courtesy of E. Richard) that point to a role for internal calcium that is not voltage dependent. Temperature affects the size of the hyperpolarization slightly, and we are inclined to consider electrogenic pumps in the production of the hyperpolarization. Studies of external pH rule out the attractant entering the cell, dissociating and activating the Na-H antiporter or other H exchange. Amiloride, a diuretic that blocks the Na-H antiporter, does block chemoresponse in Na solutions,[40] but a direct role for a Na-H exchange in the chemosensory transduction pathway needs further examination.

Receptor binding should elicit second and third messengers, which in the case of *Paramecium* must account for the change in membrane potential, change in ciliary beating, and adaptation. The first messengers to come to mind are: internal calcium, internal pH, cyclic nucleotides, and IP_3. Indirect evidence exists for a role for calcium

in chemoreception, and we are examining internal calcium movements using not only electrophysiology but also calcium-sensitive fluorescent dyes.[17] Quin-2, for example, can be loaded into the cells and gives a fluorescence signal large enough to be useful without causing extreme buffering of internal calcium with the consequent destruction of the chemoresponse. The fluorescence signal is not from dye leaking out of the cell or trapped between cilia. Quin-2-loaded cells are centrifuged to remove them from their surrounding buffer and resuspended in fresh buffer. The fluorescence signal from the supernatant is insignificant, but the cells show a large fluorescent signal. Cells incubated in Quin-2/AM for < 1 minute and not given a chance to take up dye show little fluorescence, indicating that the fluorescent signal from cells loaded with Quin-2/AM is from internalized dye and there is little trapping of dye between cilia.

Like calcium, internal pH can be examined with permeant fluorescent dyes,[18] which cells convert to a pH-sensitive form by de-esterifying the compounds, such as diacetyl-carboxy fluorescein (Van Houten and Preston, unpublished results). These dyes promise to be useful in sorting out second messenger functions particularly since changes in internal Ca are likely to be accompanied by concomitant changes in pH_i.

As pointed out by Moran,[41] the sensing of chemical stimuli must subsequently be translated into response. Cyclic nucleotides may be the second messengers that carry out this translational function for *Paramecium.* Cyclic nucleotides have been implicated in ciliary beating control.[42–44] In particular, increased levels of cAMP are associated with hyperpolarization;[44] therefore, the attractant-induced hyperpolarization may elicit a change in cyclic nucleotide levels, which, in turn, affect ciliary beating and behavior. Levels of cyclic nucleotides can be examined by RIA and HPLC,[37] among other methods.[44] It is almost certain that cyclic nucleotides will have a role somewhere in the *Paramecium* chemosensory-response pathway and the study of this role has added significance in light of the recently recognized role of adenylate cyclase in olfaction and taste.[45]

There is a recently renewed appreciation for the role of inositol phospholipids in receptor function.[46] Phosphoinositol lipids can be labeled and quantified in protozoa.[32] Lithium, which inhibits the recycling of inositol into phospholipids, profoundly affects chemoresponse;[36] however, in our hands, there is little change in phosphoinositol lipids with chemoreception stimulation, but direct measurement of IP_3 levels over time will be necessary to determine whether there is a role for IP_3 and its subsequent calcium release in *Paramecium* chemoreception. Another product of phospholipid degradation is diacylglycerol, which phorbol esters mimic in the activation of protein kinase C.[46] Phorbol esters do not affect chemoresponse, casting doubt on protein kinase C as a component of the chemosensory pathway.

There are other aspects of *Paramecium* chemoreception, such as adaptation and the effects of methylation,[47] that have not entered into this review. It is not our intent to be exhaustive but rather to give a feeling for the kinds of studies that can be accomplished with *Paramecium.* It is now time to move the *Paramecium* system into molecular genetics and cloning of genes for receptors and other pathway components as they are identified. This need to move into the molecular level is an appropriate and welcome development for chemoreception science in general.[48]

SUMMARY

The description of the chemoreception pathway in *Paramecium* is incomplete, but the technical means are available to study these pathways at the molecular level. The

hallmark of ciliates is their versatility and their most important attribute is the availability of useful mutants. It is just this versatility and amenability to genetic manipulation that will move the study of *Paramecium* chemoreception forward and provide useful information for chemoreceptor cell function in general.

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