CHEMORECEPTION: PARAMECIIUM AS A RECEPTOR CELL

Judith Van Houten and Robin R. Preston

Department of Zoology, University of Vermont
Burlington, Vermont 05405

In the sensory modalities of taste, smell and common chemical sense, there are receptor cells that make contact with the external environment and detect the presence of external chemical stimuli. The receptor cell is the site of stimulus recognition, which is thought to be mediated through binding of the stimulus to specific surface receptors and then transduction of this binding into "useful" electrical information. Information in this new form is passed on to higher order neurons and eventually is translated into a response. In order to study receptor cell function, it seems straightforward to isolate these receptor cells, identify the receptors among the membrane proteins and determine the ionic basis of receptor binding by conventional electrophysiology. However, there are limitations inherent in many of the chemosensory systems traditionally used to study chemoreception. Relatively small amounts of olfactory or taste epithelium limit the binding studies and biochemical studies necessary to identify receptor proteins; tissue is often of a mixed cell type, even when available in quantity, making it difficult to be sure of the origins of putative receptor proteins (Price, 1981; Mooser, 1981; Cagan, 1981). Hence, indirect methods (e.g. treating the tissue with n-ethylmaleimide to disrupt protein sulfhydryl bonds, and hence disrupting the chemoresponse, or demonstrating specificity and saturability of a response) are used to demonstrate that the receptor site is a protein. When a binding protein is identified, often it is not possible to draw the necessary behavioral correlations to demonstrate that the protein is the chemoreceptor. For example, a "green odorant" binding protein has been isolated from cow olfactory epithelium, but it will be difficult to demonstrate the involvement of this protein in cow chemoresponse (Bignetti et al., 1983). Small cell size and tissues comprised of more than one cell type complicate electrophysiological studies as well, although the advent of patch clamping should circumvent the size limitations (S. Kleene, personal communication; Margolis et al., 1983).

This brings us to the use of single-cell organisms, such as Paramecium, to study chemoreception. Even though unicellular organisms have no stable cell contacts that characterize metazoan systems, there are compelling reasons to use unicells in the study of chemoreception, particularly in receptor cell function. Their hallmark is versatility and their most important attribute is the availability of mutants. In particular, cells can be grown in large, homogeneous populations to provide material for biochemical analysis; the cells are large for convenient electrophysiology; and mutant cell lines provide opportunities to apply a genetic dissection to the identification of chemoreceptors and other components of the chemosensory transduction pathway.
Among the eukaryotic unicells, the most work has been done on the slime mold *Dictyostelium* and the ciliates *Tetrahymena* and *Paramecium*. *Dictyostelium* is used for its chemoresponses to folic acid and cyclic AMP (cAMP), which change over developmental time (Gerisch, 1982). However, even in this highly convenient system, no receptor mutants or electrophysiological data are available. *Tetrahymena* rivals *Paramecium* in amenability to a variety of approaches for the study of membrane functions. However, studies of *Tetrahymena* chemoresponse still focus on assay methods and on mechanisms of the behavioral response (Levandowsky et al., 1984; Leick and Leilung-Larsen, 1985). We discuss here our progress with *Paramecium* as a chemoreceptor cell, and the development of techniques that will enable a genetic dissection of the chemosensory transduction pathway.

*Paramecium* has been called a "swimming neuron" (Machener and dePeyer, 1977) and is a chemoreceptor cell. Paramecia respond to the external stimuli folic acid, acetate, lactate, cAMP and other compounds that signal the presence of their food, bacteria (Van Houten, 1978). We have developed a simple T-maze test to assay responses to these attractants and also to extremes of pH, high ionic strength and some organic membrane active compounds like quinidine, which are repellents (Van Houten, 1978). As an example, we will focus on responses to folic acid.

**Folate Chemoresponse: Binding**

It is the pterin portion of the folate molecule that is recognized by the cells in behavioral tests (Schulz et al., 1984). Attraction to folic acid is inhibited by cAMP, which shares only some structural features (Schulz et al., 1984). Folate is taken up by the cells and can be concentrated at least 50 fold from the surrounding media, as measured by radio-binding assay (Schulz and Van Houten, unpublished results). It is not possible to eliminate this uptake by low temperature or metabolic inhibitors without affecting cell integrity, therefore we measure binding of folate to cells as instantaneous binding, that is, by extrapolating the amount of \(^3\)H-folate associated with the cells in centrifugation assays to time zero (Schulz et al., 1984). \(^3\)H-folate binds specifically and saturably to whole cells with a \(K_d\) of approximately 29 \(\mu\)M (Schulz et al., 1984); binding shows the same specificity as the behavioral response and it is likely that a specific receptor exists for the binding of folate. While this may seem to be of low affinity to those who are used to dealing with neurotransmitters, it is in keeping with other external chemoreceptor systems. Estimates of half maximal response and \(K_{50}\) for vertebrate and invertebrate olfactory and taste range from 1 nM up to 700 \(\mu\)M, with \(K_{50}\) often in the \(\mu\)M range (Cagan, 1981; Hansen and Wieczorek, 1981; Price, 1981; Lancet, 1986). Similarly, paramecia respond only to relatively high levels of fermentation and other bacterial products when these are presented individually. We believe this to be a cell's means of coping with a chemically noisy environment (pond water) and to ensure a response only when bacteria are actually available. Therefore, the problems associated with biochemistry of low affinity receptors cannot necessarily be avoided but must be met head on if a variety of external chemoreceptors are to be identified and purified from *Paramecium* and other chemoreceptor cells.

Binding of folate to whole cells is primarily to the cell body and not to the cilia. Isolated cilia in filtration and centrifugation assays show <1% of the whole cell binding despite the fact that cilia are covered with approximately 50% of the surface membrane (Schulz et al., 1983; Schulz et al., 1984; Dunlap, 1977). Deciliated cells show normal folate-induced hyperpolarization (see below), which supports the notion that binding sites can be found on the cell body membrane.

A quantitative measure of folate binding is the amount of fluorescence
of cells stained with FITC-folate, that is, folate conjugated to fluorescein through reaction with fluorescein isothiocyanate (Van Houten et al., 1985). The fluorescence of stained cells is easily distinguished from autofluorescence in blind tests when the cells are examined individually under oil immersion or in microtiter wells under a dissecting microscope outfitted with cut-off filters. The FITC-folate binding is specific for folate as evidenced by inhibition of fluorescence when cells are stained with FITC-folate in the presence of excess unconjugated folate. The binding is to the exterior of the cell and not to broken cells; cells permeabilized with Triton X-100 prior to staining have an increased fluorescence, probably attributable to access to the interior of the cell. Additionally, a mutant (d4-534) that has lost both attraction to folate and surface binding (see below) due to a single site mutation (DiNallo et al., 1982) does not show FITC-folate fluorescence much above autofluorescence (Van Houten et al., 1985).

At least three genes can be mutated to decrease or eliminate attraction to folate (DiNallo et al., 1982). These mutants have single site lesions that assort in Mendelian patterns and show specific losses of folate response, while responses to other attractants and repellents remain intact. Mutants in one particular complementation group (fol1) have lost the ability to bind 3H-folate specifically and at normal levels (Schulz et al., 1984). These are the same mutants that can be distinguished from normal by FITC-folate staining above (Van Houten et al., 1985).

There are four revertants of one allele of the fol1 complementation group, mutant d4-534. At least three of these revertants have second site mutations (Van Houten and White, unpublished results). Second site revertants open up the possibility of identifying chemosensory transduction pathway components that can be identified by no other means. Mutations in genes for these components may cause no or slight phenotypic changes in the chemoreceptor of the cell. However, because they are mutations that suppress an abnormal chemoreceptor phenotype and because they are not in the originally mutated gene, they must be mutants in genes that code for products that somehow interact with the original mutant gene product. D. J. L. Luck (Huang et al., 1982) has been especially productive using revertant analysis for a fine genetic dissection of the Chlamydomonas flagellum and we expect that revertants will be useful for analysis of the Paramecium chemoreception pathway as well.

Binding Proteins

The membrane protein(s) that are part of the chemoreceptor pathway should include the receptor, which will bind folate, be exposed to external medium, and be defective in mutants missing the binding associated with chemoreceptor. We have adapted a method to isolate cell body membranes (Schulz et al., 1986) and searched through the cell body membrane proteins for folate binding proteins by affinity chromatography, cross linking and FITC-folate binding. Affinity chromatography identified about 4 proteins that specifically elute with K2-folate and not with buffers of similar ionic strength with KCl, K2-glutamate (a component of the folate molecule but is neither an attractant and nor inhibitor of 3H-folate binding), or galactose (the monomer of the Sepharose component of the affinity column). Methotrexate, a folate analog, is only a weak inhibitor of folate binding and response and selectively elutes only a subset of the folate binding proteins. Concanavalin A affinity columns were used to identify glycoproteins among the binding proteins and 125I labeling of whole cells narrowed the field of putative receptor proteins to 3 (Schulz et al., 1986).

Clear identification of the chemoreceptor among these proteins awaits comparison with protein of null mutants that are allelic to fol1 and veri-
fication of the protein's folate binding properties by crosslinking folate to receptor with immunodetection by anti-folate antibody on electropho
tests (Kerseth, Sasner and Van Houten, unpublished results). Cells crosslinked
with the N-hydroxysuccinimide ester of folate (Henderson and Zavely, 1984),
are inhibited in their response to folate, but not to another stimulus, ace
tate (Table 1). Therefore, the chemoreceptors should be among the subset of
surface proteins crosslinked and recognized on electropho
tests by antifolate antibodies (Towbin et al., 1979). We are currently producing antibodies
against folate conjugated to keyhole limpet hemocyanin (KLH) (Langone, 1982;
Hurn and Chantler, 1980). In ELISAs, the sera recognize folate conjugated
to bovine serum albumin (BSA), but not BSA alone, which indicates that these
seera will be useful in recognizing folate linked to proteins other than KLH.

Electrophysiological Correlates of Chemoreception

Binding of folate and other attractants is transduced into a hyperpolariza
tion (Van Houten, 1979). Mutant d4-534 shows only a small hyperpolariza
tion in folate, but normal hyperpolarization in other attractants. There
fore, membrane hyperpolarization is an integral part of the chemosensory
motor response pathway.

There has been a long history of fine electrophysiological work on
Paramecium's excitable membrane in order to describe the connection between
membrane electrical events and changes in ciliary movement (Eckert, 1972;
Naitoh, 1982; Kung and Saimi, 1982). A depolarization will move the mem
brane potential ($V_m$) toward threshold for an action potential, which brings
with it a transient increase in intracellular calcium. Calcium at $>10^{-6}$M
causes the cilia to transiently reverse beating direction, causing a tran
sient jerky turn in swimming path. A hyperpolarization moves $V_m$ away
from the threshold for action potentials, thereby decreasing action potential
frequency. Hyperpolarization also increases ciliary beating frequency,
which in turn increases swimming speed. Attractants generally hyperpola
rize (Van Houten, 1979) and the changes in ciliary motility caused by
hyperpolarization add up to a longer mean free path moving up the gradient
of attractant and gradual accumulation up the gradient (Van Houten, 1978;
Van Houten and Van Houten, 1982).

Chemosensitivity for folate is not uniform over the cell surface.
Deciliated cells show normal size hyperpolarizations in folate and other
attractants, confirming the binding data that indicate receptors are not
primarily on the ciliary membrane (Preston and Van Houten, 1986a; Van Hou
ten et al., 1983). Pressure perfusion of folate onto cells elicits maxi
mum hyperpolarization in the anterior portion of the cell and particularly
anterior ventral regions (Preston and Van Houten, 1986a). Gradients of
receptors are not unprecedented in Paramecium: there is a very distinct
mechanoreceptive gradient anterior to posterior (Ogura and Machemer, 1980).
Highest chemosensitivity at the anterior of the cell may facilitate its
movement into regions of attractant by eliciting fast smooth swimming as
the cell is headed in the direction of the stimulus.

The ionic basis for the hyperpolarization remains elusive. The
obvious candidates of Ca-dependent or voltage activated K and Na effluxes
are clearly eliminated by ion substitution experiments: cells show normal
hyperpolarizations to folate and other attractants in the absence of Na or
K, or when external Na and K are simultaneously fixed to eliminate the
driving force for net fluxes of both K and Na across the membrane. Mem
brane resistance in folate increases slightly and there is no obvious
reversal potential, although we have not succeeded in voltage clamping the
cells at the extreme $V_m$ of $+110$ mV necessary to clamp at $E_{Ca}$. The folate
induced hyperpolarization is not likely to be due primarily to surface
charge changes because of the specificity of the behavioral and hyperpola-
TABLE 1: Effect of Crosslinking Folate on Chemoresponse to the Stimuli Folate and Acetate

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>$I_{Che}$</th>
<th>n</th>
<th>$I_{Che}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% DMSO +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM Na₂folate</td>
<td>0.72 ± 0.06</td>
<td>12</td>
<td>0.67 ± 0.06</td>
<td>6</td>
</tr>
<tr>
<td>5 μM &quot;activated&quot; Na₂folate</td>
<td>0.54 ± 0.06</td>
<td>12</td>
<td>0.61 ± 0.06</td>
<td>6</td>
</tr>
</tbody>
</table>

Cells are treated with folate or "activated" folate in DMSO, washed in buffer, and tested in T-maze assays for response to folate (2.5 mM Na₂folate vs 5 mM NaCl) and Na-OAc (5 mM Na-OAc vs 5 mM NaCl). Index of chemokinesis ($I_{Che}$) greater than 0.5 indicates attraction; less than 0.5 indicates repulsion; 0.5 indicates no response to stimulus.

Polycations, which affect surface charge, and a surface charge mutant (Satow and Kung, 1981) do not perturb the folate-induced hyperpolarization. Studies of accumulation and hyperpolarization over a range of pH 5-8 indicate that the organic acid attractants can be fully charged and act as attractants (Schulz et al., 1985a,b). Therefore, while it is possible that the folate anion enters and directly hyperpolarizes by virtue of its net negative charge, it is not clear why pterine-6-carboxylic acid with no net charge should do likewise.

To explain the hyperpolarization, we are left with relatively few options that include: 1) receptor-mediated release of Ca from internal stores, which hyperpolarize by activating a Ca pump. This pump would have to be fast to cause the hyperpolarization as rapidly as we can perfuse the cell, but the enzymes of the rod outer segment have taught us that enzymes can account for electrical events that occur in milliseconds (Stryer, 1986). 2) Ca or folate activated $H^+$ efflux pump. We have not been able to change internal or external pH sufficiently to rule this out. It can be qualified that the putative $H^+$ efflux is not affected by amiloride in Na free solutions (Van Houten and Preston, 1985). In support of a role for Ca as Ca permeability changes of cells in acetate, another attractant. (Permeability to Ca cannot be tested in folate because of problems with precipitation.) However, the specificity of this permeability increase is difficult to sort out from the general effect of high ionic strength on hyperpolarization. A mutant, "Restless" (courtesy of E. Richard), cannot properly regulate $V_m$ in low $K$ solutions and its $V_m$ plunges toward $E_K$ in low $K$ solutions. These extremely hyperpolarized cells show an increased hyperpolarization in folate and acetate (Preston and Van Houten, 1986b). Plots of size of hyperpolarization vs $V_m$ of Restless extrapolate to near $E_{Ca}$ as reversal potential. Further clamping and study of folate mutants are necessary to solve the puzzle of the hyperpolarization.

Second Messengers

In order to investigate a possible role for Ca in the transduction pathway, we have turned to Quin-2, a calcium-sensitive fluorescent dye (Rink and Pozzan, 1985; Tsien et al., 1982). Paramecia take up and cleave the ester bond of the membrane permeable Quin-2/AM, trapping the Quin-2 inside the cell and making it available to act as a Ca indicator. Therefore, this dye and others will prove useful in examining internal free Ca levels by this very specific probe. It is difficult to manipulate external Ca for
### TABLE 2. Effects of LiCl on Chemoresponse to Folate

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Duration of Incubation</th>
<th>$I_{\text{Che}}$</th>
<th>S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM LiCl</td>
<td>5 min</td>
<td>0.66</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.60</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.34</td>
<td>0.07</td>
</tr>
<tr>
<td>2 mM NaCl</td>
<td>30 min (Control)</td>
<td>0.77</td>
<td>0.06</td>
</tr>
<tr>
<td>4 mM LiCl</td>
<td>5 min</td>
<td>0.27</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.18</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.24</td>
<td>0.04</td>
</tr>
<tr>
<td>4 mM NaCl</td>
<td>60 min (Control)</td>
<td>0.76</td>
<td>0.09</td>
</tr>
<tr>
<td>2 mM NaCl</td>
<td>30 min (Control)</td>
<td>0.67</td>
<td>0.04</td>
</tr>
<tr>
<td>1 mM Na$_2$folate</td>
<td></td>
<td>0.76</td>
<td>0.02</td>
</tr>
<tr>
<td>2 mM LiCl</td>
<td></td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>1 mM K$_2$folate</td>
<td></td>
<td>0.39</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Cells were incubated in buffer with LiCl, NaCl (control), Li$_2$folate, or Na$_2$folate (control). Cells were washed and tested for chemoresponse to 2.5 mM Na$_2$folate vs 5 mM NaCl in T-mazes. Data are averages of 3 experiments, except for the last incubation in 2 mM LiCl, which is the average of only two experiments.

conventional electrophysiological methods since external Ca cannot be completely removed. *Paramecia* will not survive with Ca < 10$^{-5}$ M, which still leaves a large driving force for Ca to enter the cell ($E_{\text{Ca}}$ +110 mV).

With the possibility of increases in internal free Ca comes the possibility of involvement of a receptor-mediated inositol phospholipid turnover cycle. Inositol triphosphate (IP$_3$) is thought to liberate Ca from the endoplasmic reticulum and thereby activate Protein Kinase C or other Ca dependent protein kinases (Nishizuka, 1984a,b). Lithium blocks this turnover cycle, inhibiting the renewal of phosphoinositol lipids for receptor-mediated degradation by phospholipase C (Berridge and Irvine, 1984; Nishizuka, 1984a,b). Lithium, but not Na, has a profound effect on Paramecium chemoresponse (Table 2), giving a tantalizing hint of a role for phospholipids in chemoreception.

**Adaptation**

Paramecia, as other chemoreceptor cells, adapt to uniform concentrations of stimulus (Van Houten et al., 1982; Van Houten and Van Houten, 1982). The molecular mechanism of this adaptation is not evident. As in bacteria, it may involve methylation of receptor or transducer proteins (Hazelbauer and Harayama, 1983). S-adenosyl-L-methionine (SAM) is a major methyl donor in eukaryotic cells and SAM has an effect on the behavioral responses of *Paramecium* to folate and acetate. This effect is not mimicked by S-adenosyl-L-homocysteine, a very similar compound except for the transferable methyl group of SAM (Van Houten et al., 1984). Therefore, it is possible that methylation by SAM or even phosphorylation (as, for example,
by protein kinase C) may mediate adaptation. There are mutants that apparently are abnormal in adaptation, that is, they are initially attracted to ammonium but fail to sustain this attraction (Van Houten et al., 1982). Such behavior is what an adaptation defect would cause in computer simulations of behavior (Van Houten and Van Houten, 1982). Mutants not affected by SAM also are available and between these sets of mutants the role of adaptation should be made more clear.

Other Chemoreception Systems in Paramacium

Folic acid is not the only organic stimulus for Paramacium. External cAMP, among other compounds, is an attractant. We study cAMP as external stimulus and have shown by HPLC methods that external cAMP is not detectably taken up into the cells and not detectably broken down when the phosphodies- terase inhibitor isobutylmethylxanthine (IBMX) is present (Smith et al., 1986). It is significant that cAMP acts externally because there have been recent reports of internal cAMP controlling ciliary beating frequency and perhaps hyperpolarization (Hennessey et al., 1985; Gustin et al., 1983; Schultz et al., 1984). These internal cAMP-induced changes look very similar to those induced by external attractants by virtue of the hyperpolarization they induce. However, we believe that internal cAMP is not involved in chemoreception because IBMX in combination with chemical stimuli does not potentiate the chemoresponses and IBMX, in both control and stimulus solutions, does not inhibit the chemoresponses (Table 3).

\[ \text{3H-cAMP} \text{ binds to cells in a saturable, specific fashion, but with low affinity (K}_d\text{ = 200 \mu M; Smith et al., 1986). However, the K}_d\text{ compares well with the half maximal concentration for behavioral responses to cAMP and for cAMP-induced hyperpolarization. We have available three mutants that are not normally attracted to cAMP (Gagnon and Van Houten, unpublished results). These mutants and the possibility of photoaffinity labeling the cAMP binding sites make the receptor approachable despite its low affinity. Cyclic AMP affinity columns consistently show one cAMP specific binding protein of approximately 48,000 dalton (Van Houten et al., 1986). This protein band specifically elutes with 5'-AMP, an inhibitor of cAMP chemoresponse behavior, but not with cGMP, which does not affect cAMP chemoresponse. In preliminary results, a protein of the same size is labeled with 32P-8-azido-}
\]

\[ \text{cAMP (Smith and Van Houten, unpublished results). However, we have not} \]

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Control Solution</th>
<th>I_{Che}</th>
<th>S. D.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX does potentiate chemoresponse when added to stimulus solution:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM Na\textsubscript{2}folate</td>
<td>2 mM NaCl</td>
<td>0.81</td>
<td>0.08</td>
<td>3</td>
</tr>
<tr>
<td>1 mM Na\textsubscript{2}folate + 1 mM IBMX</td>
<td>2 mM NaCl</td>
<td>0.42</td>
<td>0.01</td>
<td>3</td>
</tr>
<tr>
<td>IBMX does not inhibit chemoresponse when added to both stimulus and control solutions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mM Na\textsubscript{2}folate</td>
<td>5 mM NaCl</td>
<td>0.96</td>
<td>0.05</td>
<td>3</td>
</tr>
<tr>
<td>2.5 mM Na\textsubscript{2}folate + 1 mM IBMX</td>
<td>5 mM NaCl + 1 mM IBMX</td>
<td>0.98</td>
<td>0.01</td>
<td>3</td>
</tr>
</tbody>
</table>

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established the correlations necessary to establish a role for this protein in chemoreception.

CONCLUSIONS

The use of unicellular organisms circumvents problems accompanying the study of receptor cell function in some metazoan systems and, at the same time, allows the application of a variety of techniques and approaches to a single receptor cell type. *Paramecium*, in particular, presents opportunities to combine membrane protein biochemistry with electrophysiology and with the capability of generating and isolating chemoreceptor mutants to aid in the dissection of the chemosensory transduction pathway. The *Paramecium* system is still in the process of being developed as a receptor cell, but appears to have all the components necessary for a successful genetic dissection. The potential for the generation and analysis of mutants cannot be over emphasized because, in theory, each macromolecular component of the chemosensory transduction pathway can be mutated, whereas, it is not likely that there are sufficient numbers or specificities of pharmacological agents to achieve the same end. In addition, suppressor mutants often allow the only possible identification of components that interact with other pathway components, but that might not otherwise be identified. Lastly, the development of a genetic system paves the way for applying molecular techniques to the study of chemoreception, including molecular genetics (Margolis et al., 1985).

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