Chemical Senses Vol.14 no.4 pp.587–595, 1989

Evidence for a *Paramecium* folate chemoreceptor

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Abstract. *Paramecium tetraurelia* can detect and respond to folic acid in the environment. In order to understand the initial step in the sensory transduction process, we used covalent crosslinking of folate to determine if there were specific binding sites on the cell membrane. Crosslinked cells did not respond to folate but did respond to other chemoattractants. Membrane preparations were specifically covalently labeled with a chemically active form of folate. This labeling was detected with iodinated folate and a polyclonal antibody to folate. These results, as well as previous work from this laboratory, support the hypothesis that the folate chemical cue is transduced into a membrane potential change via a receptor.

Introduction

A central question of chemosensory transduction concerns the role of receptors, which are proteins that specifically bind stimuli. The receptor hypothesis is still being examined (Kashiwayanagi and Kurihara, 1985; Dionne, 1988; Lerner *et al.*, 1988). Price (1984) and Kinnamon (1988) have emphasized that olfaction and taste involve a diversity of mechanisms, not all of which necessarily use receptors. Other chemosensory systems will presumably show a similar diversity. Thus, any attempt to understand the mechanism of transduction in a chemosensory system must experimentally test the hypothesis that receptors are involved in the process.

Paramecium tetraurelia is attracted by a chemokinetic mechanism to folic acid (Van Houten, 1978), which is a necesary vitamin (van Wagtendonk, 1974) and probable food cue (Pan *et al.*, 1972). Folic acid causes membrane hyperpolarization by an as yet unknown mechanism; this in turn leads to the change in ciliary beat that causes a population of cells to accumulate (Preston and Van Houten, 1987).

Here we present evidence that the transduction process in the chemokinetic response of *Paramecium* to folate is a receptor-mediated event and propose a method to identify the receptor protein. This method includes covalent crosslinking of the ligand to the cell membrane, as performed by Henderson and Zevely (1984), followed by detection of crosslinked ligand using a radioactive label or an anti-ligand antibody. Folate can be converted to a chemically active form by incubating with carbodiimide and *N*-hydroxysuccinimide as shown in step 1 of Figure 1 (Bauminger and Wilchek, 1980). Cells or cell membrane preparations form a covalent link between the folate molecule and the adjacent protein when exposed to activated folate (Figure 1, step 2). Our aim is to identify membrane-associated folate binding proteins using the covalently attached folate as a marker and thereby to identify a subset of proteins that include the folate chemoreceptor.

Methods

Cells and culture conditions

Paramecium tetraurelia stock 51S was grown in wheat grass medium at 28°C. Medium was prepared by boiling 5 g wheat grass (Pines, Lawrence, KS) and 0.3 g proteose

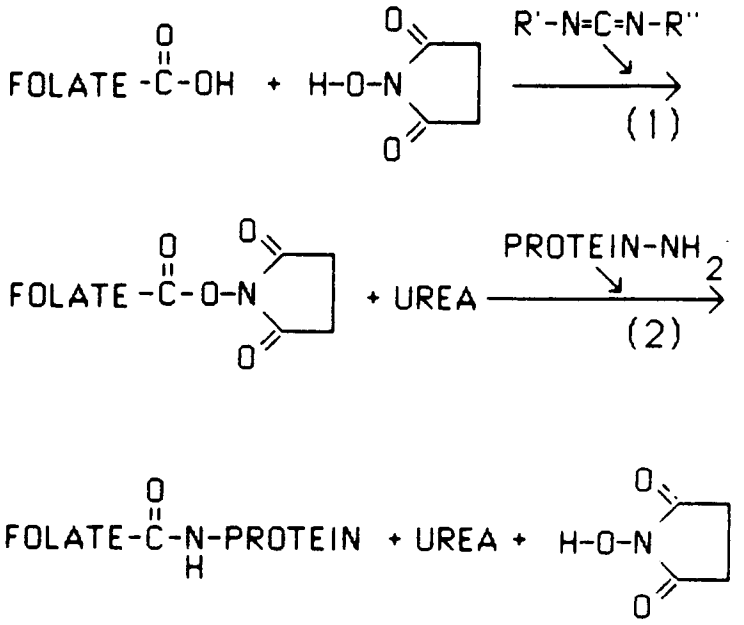

Fig. 1. Formation of activated folate by hydroxysuccinimide and carbodiimide (step 1); covalent crosslinking of folate to protein (step 2).

peptone in < 1 l distilled water for 8 min. Particulates were filtered out using Whatman no. 42 filter paper under suction, and 0.8 g Tris, 1.1 g $Na_2HPO_4.7H_2O$, 0.2 g Na_2HPO_4 and 1 mg stigmasterol added. The solution was then brought up to 1 l and autoclaved. Medium was inoculated with *Klebsiella pneumoniae* 24 h before addition of Paramecia. Cells were harvested during the stationary phase (8000 – 12 000 cells/ml).

Behavioral assays of crosslinked cells

Activated folate was prepared by an adaptation of the procedure of Henderson and Zevely (1984): 5 mM Na_2 folate, 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) in dimethylsulfoxide (DMSO) were incubated on a vibrating shaker for 1 h at room temperature. Paramecia were crosslinked by adding activated folate at 1:100 dilution to cells in 2 mM KCl, 0.1 mM $CaCl_2$, pH 7.2, and incubating with gentle shaking for 15 min. Cells were then centrifuged out of solution (200 *g*, IEC HNS-II centrifuge), washed twice by pelleting in the same buffer and immediately assayed for behavior.

Behavior was assayed as described by Van Houten *et al.* (1982). Cells were placed in a three-way stopcock and left for 30 min to disperse between the two arms, one containing the test solution (2.5 mM Na_2 folate, or 5 mM Na acetate or lactate) and the other containing the control solution (5 mM NaCl). Note that solutions also contained 1 mM $Ca(OH)_2$, 1 mM citric acid and 1.3 mM Tris, and each pH was adjusted to 7.2. After 30 min the stopcock was closed and the cells in each arm were counted. Results were expressed as an index of chemokinesis (I_{che}), defined as the number of cells in

the test arm divided by the total number of cells in both arms. All experiments were performed in triplicate. The results were analyzed with the Mann–Whitney *U* test.

Crosslinking of iodinated folate to membrane preparations

Iodinated folate was used to quantitate the amount of ligand crosslinked to membrane preparations. (Unfortunately, it was not feasible to crosslink iodinated folate to whole cells; the large volumes needed for cell viability would require an impractical amount of isotope.) Cell membrane fractions (pellicles) were prepared by modified methods of Bilinski *et al.* (1981) and A. Adoutte (personal communication). Cells were harvested by centrifugation for 2 min at ~350 *g* (IEC HNS-II centrifuge) during the early stationary phase (8000–12 000 cells/ml). The cell pellet was washed twice by centrifugation for 2 min in homogenization medium (20 mM Tris–maleate, pH 7.8, 1 mM Na_2 EDTA) and resuspended in ice-cold homogenization medium plus 1 mM phenylmethonylsulfonylfluoride (HMI). After 10 min on ice the cells were homogenized in a Potter–Elvejehm homogenizer. Cell disruption was monitored using phase-contrast microscopy and continued until >90% of the cells were ruptured. The homogenate was washed wth cold HMI (5 min at 1240 *g* in a Beckman J2-21 centrifuge), followed by vigorous mixing until the supernatant was clear (4–6 times).

Enzyme assays were performed to test the purity of the membrane preparations. Glucose-6-phosphatase and 5′-nucleotidase were assayed according to the methods of Lee and Lardy (1965). Succinate dehydrogenase activity was determined using the method of Aronson and Touster (1974). Acid phosphatase activity was determined by the method of Linhardt and Walter (1965). All enzyme assays were performed twice in duplicate at 22°C.

Iodinated folate was crosslinked to membrane preparations by incubating 5–15 μCi iodinated folate (2200 Ci/mmol, New England Nuclear, Boston, MA) with varying concentrations of EDC and NHS relative to the amount of folate (see Table II) for 3 h at room temperature with gentle shaking. To test for the specificity of the crosslinking, membrane preparations were incubated with a 100-fold excess (relative to molar amount of labeled folate) of either NaCl (to measure total binding) or Na folate (to measure nonspecific binding) for 5 min on ice and washed with a large excess of cold HMI for 5 min at 1240 *g* in a Beckman J2-21 centrifuge. These membrane preparations (1–10 mg/ml protein) were subsequently incubated with equal volumes of activated folate for 5 min on ice and the membranes washed twice as described. The washes and pellet were counted in a Picker compac 120 gamma counter and incorporation was calculated as the percentage of total counts associated with the pellet. Specific incorporation was considered to be the difference between the total and nonspecific incorporation.

Production and assay of anti-ligand antibody

Activated folate for crosslinking was prepared by incubating 60 μM Na_2 folate, 20 μM EDC and 20 μM NHS with shaking for 3 h at room temperature. Membrane preparations were subsequently crosslinked by incubating equal volumes of protein (1–10 mg/ml) and activated folate for 5 min on ice. Membranes were then washed as before (centrifuged at 1240 *g* for 5 min at 4°C in HMI). Membranes were also crosslinked

with glycine, using an identical procedure, to be used as a control antigen for ELISA.

A polyclonal antibody to folate was produced by injecting New Zealand white rabbits with folate covalently crosslinked to keyhole limpet hemocyanin (KLH). Antigen was prepared by the method of Bauminger and Wilchek (1980): 25 mg KLH. 25 mM Na_2 folate and 25 mg EDC were incubated in a total volume of 2 ml for 4 h and the product was run through a Sephadex G-50 column using phosphate-buffered saline (PBS; 0.05 M PO_4, 0.2 M NaCl, pH 7.2). The fraction that had a high absorbance at both 368 (characteristic of folate) and 280 nm (characteristic of protein) was dialyzed against distilled water, lyophilized and stored at −70°C. A bovine serum albumin (BSA) conjugate of folate was prepared in an identical manner to be used as antigen in ELISA to determine the extent of the immune response to the folate moiety.

Antibodies were produced by injecting New Zealand white rabbits with a total of 200 μg of folate−KLH at four sites (Vaitukaitis, 1981). Antigen was dissolved in 0.1 ml distilled water plus 0.1 ml Freund's complete adjuvant. Rabbits were boosted at monthly intervals with 100 μg antigen in 1:1 water:Freund's incomplete adjuvant. Blood was taken by peripheral ear vein bleeding (for titering) or cardiac puncture (for harvesting antibody) 2 weeks after boosting. Blood was allowed to coagulate at room temperature for 1 h, centrifuged at 5000 *g* for 20 min, allowed to stand at 4°C overnight and centrifuged again. The resultant sera were collected and stored at −70°C.

The sera were passed through Protein-A columns (Affi-Gel Protein-A MAPS II kit, Bio-Rad, Richmond, CA) to isolate the immunoglobulin fraction; the folate-specific antibody fraction was then purified from this fraction using a folate−Sepharose column. Antibody titers were quantitated by ELISA (Engvall and Perlmann, 1972) using folate conjugated to a different carrier (BSA) and unconjugated BSA as antigens. Briefly, ELISA procedure is as follows: 1 μg of antigen in 0.2 ml carbonate−bicarbonate buffer (pH 9.6) was incubated overnight in each well to be tested; wells were rinsed with PBS, pH 7.4, with 0.5 ml/l Tween-20 added (PBST) seven times; primary antibody diluted in PBS was incubated for 1 h at 37°C; wells were rinsed with PBST seven times; secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit, ICN ImmunoBiologicals, Lisle, IL) diluted in PBST was incubated for 1 h at 37°C; wells were rinsed with PBST seven times; plates were developed with 0.034% *O*-phenylene diamine and 0.012% H_2O_2 in 0.1 M phosphate buffer, pH 5.0; reaction was stopped by adding 0.05 ml 2 N H_2SO_4 and plates were read for optical density at 490 nm with a Bio-tek EL 307 ELISA plate reader.

Membrane preparations that had been crosslinked with folate were also used as antigen in ELISA, with sham crosslinked membrane (crosslinkers but no ligand) and glycine crosslinked membrane used as control antigens.

Results

As shown in Figure 2, covalent crosslinking of folate to whole cells specifically blocked attraction to folate. Cells treated with activated folate were essentially equally distributed between folate and control solutions (I_{che}) = 0.53 ± 0.04), and were still normally attracted to acetate and lactate. Therefore, the crosslinked cells did not respond to folate because they were specifically unable to detect it as opposed to being unable to perform in the *t*-maze test.

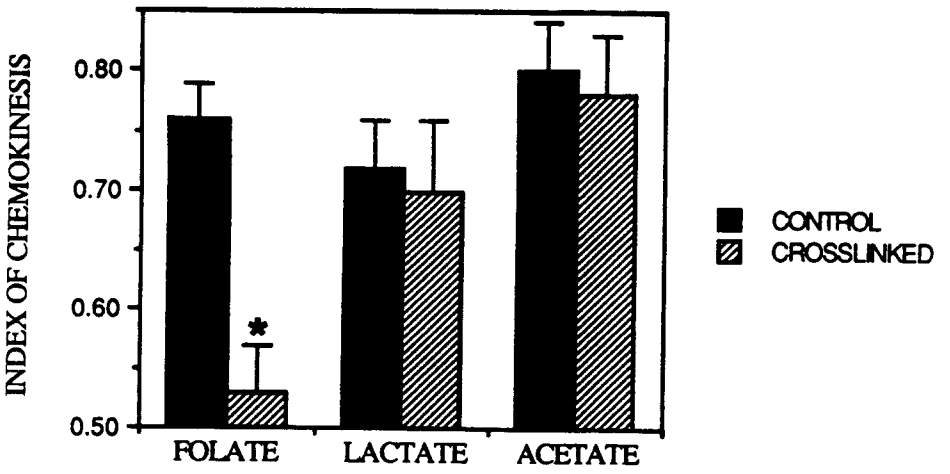

Fig. 2. Index of chemokinesis of control and folate crosslinked cells for three anionic chemoattractants. Values are averages ± SEM. Asterisk indicates significantly different from control at $P < 0.05$ level. A value of 0.50 indicates neutrality; cells are evenly dispersed between the arms of the *t*-maze. $n = 10$ for folate and 5 each for acetate and lactate, with each experiment performed in triplicate. NaCl was used in the control arm in every case; Na salts of each attractant were used.

In order to identify the membrane proteins crosslinked with folate, a preparation enriched in plasma membrane was needed. Table I shows the results of the enzyme assays comparing cell homogenates to membrane preparations for enzyme activity characteristic of surface membranes. The marker for plasma membrane, 5′-nucleotidase, was concentrated in the pellicle preparation and the other membrane-associated enzymes that serve as markers for mitochondria, microsomes and lysosomes were diminished.

As a means to determine whether the covalent incorporation of folate was specific, we compared the amount of iodinated folate incorporated into the membrane pellet in the presence or absence of 100-fold excess unlabeled folate. Activated iodinated folate was specifically incorporated into membrane fractions. Using a 14 000-fold excess of crosslinkers relative to labeled folate, 42.2% of counts were incorporated in total binding preparations, while only 11.0% of counts were incorporated in nonspecific binding preparations. With no crosslinker there was no specific incorporation of folate, and the incorporation was dependent on the ratio of crosslinker to ligand (Table II). We had hoped to improve the amount of specific incorporation by limiting the excess crosslinker but found that only with a large excess of crosslinker relative to folate was there specific incorporation.

We developed an immunodetection system for identifying folate crosslinked proteins. Polyclonal antiserum was produced against KLH−folate. The antiserum detected folate carrier protein more effectively than carrier protein alone (Table III), indicating that it could specifically detect folate. Folate-specific antibodies were isolated using a MAPS and folate−Sepharose column (Table III). These antibodies were effective in recognizing folate crosslinked to membrane preparations. Figure 3 shows that the ELISA values for sham crosslinked membrane or glycine crosslinked membrane are significantly lower than those for folate crosslinked membrane.

Table I. Enzyme activities of homogenates and membrane fractions

Enzyme	Homogenates	Pellicles
5′-Nucleotidase	29.1[a]	105.7[a]
Glucose-6-phosphatase	51.8[a]	22.6[a]
Acid phosphatase	89.4[a]	38.4[a]
Succinic dehydrogenase	0.84[b]	0.02[b]

[a]nmol P_i/mg protein × min.
[b]mU/mg protein.
Data are averages of two experiments, each performed in duplicate.

Table II. Incorporation of iodinated folate into membrane fraction with varying ratios of crosslinkers (EDC and NHS) to ligand

Crosslinkers:folate	*n*	% Specifically incorporated
0	2	−2.08 ± 2.94
10:1	2	3.18 ± 2.54
14 000:1	4	74.8 ± 16.4

Data are averages of *n* measurements ± range or SD for $n = 4$.

Table III. ELISA values for different fractions of antiserum

Fraction	Antigen	Dilution	OD
Serum	BSA−folate	1:5000	2.00
	BSA		0.07
MAPS	BSA−folate	1:10 000	0.39
	BSA		0.18
Folate	BSA−folate	1:10 000	0.64
	BSA		0.18

MAPS is the IgG fraction from a Bio-Rad Protein-A MAPS column; folate is the specific fraction eluted from the folate−Sepharose column. OD is the average of four optical density values.

Discussion

Paramecia that had been covalently crosslinked with folate were no longer able to detect folate, but retained the ability to recognize other anionic chemoattractants and respond to them in behavioral assays. A nonspecific sensory mechanism would not have been able to distinguish between chemoattractants in this experiment. If the crosslinking had interfered with swimming ability, disrupted the membrane in some way or changed a chemoreceptor common to all stimuli, the cells would not have been able to respond normally to lactate and acetate. These results also indicate that the binding site of interest is on the cell surface, since the activated folate would not have been able to cross the cell membrane by diffusion as a charged anion or by a transport carrier protein without crosslinking to the protein. Therefore, our crosslinking studies provide evidence that there is an extracellular site that is involved in folate chemoresponse and that this site is not necessary for response to other attractants, indicative of a specific ligand−receptor interaction.

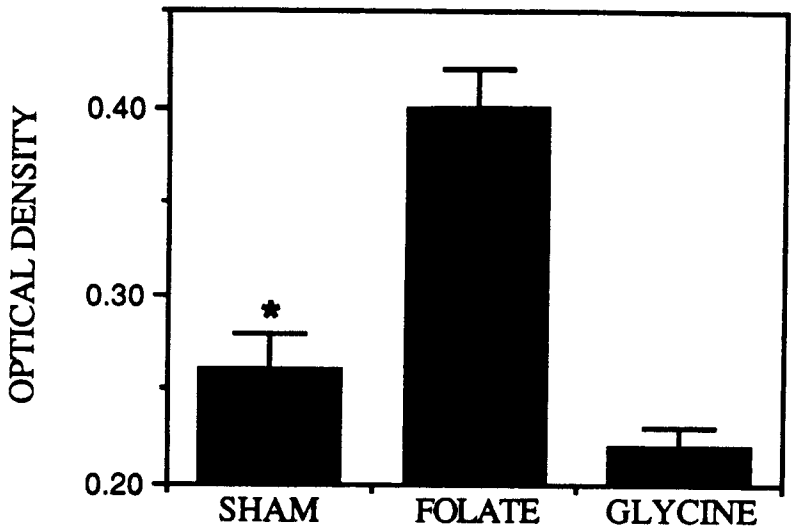

Fig. 3. ELISA values for affinity-purified antibody using sham, folate or glycine crosslinked membrane as antigen. Error bars indicate SEM. Asterisk denotes significantly different from sham using *t*-test, $P < 0.01$, $n = 4$ for each antigen.

We assume that the crosslinked cells are no longer responding to folate because the receptors are among the crosslinked proteins and their ligand binding sites are sterically blocked by the covalently attached folate. Therefore crosslinked cells cannot detect changes in folate concentration and presumably cannot respond with the changes in membrane potential that have been shown to be crucial in determining behavior (Van Houten, 1978). Henderson and Potuznik (1982), using a procedure similar to that used here, were able to block >95% of folate binding to *Lactobacillus casei*. While we are not sure of the percentage of covalently bound receptor sites in *Paramecium*, there appear to be too few functional receptors remaining after crosslinking to report changes in occupancy as the cells sample the chemical stimuli in their environment.

Iodinated folate was specifically crosslinked to membrane preparations. Excess unlabeled ligand reduced incorporation of ligand, and crosslinking was concentration-dependent. This would be the expected result if a receptor were involved (Burt, 1985). An unexpected finding was that a large excess of crosslinkers relative to ligand was necessary for specific incorporation.

Other work also supports the idea that folate chemoreception is a receptor-mediated event. There are a large number of low affinity folate binding sites ($K_D \simeq 29$ μM) on the cell body membrane. Mutants that lack normal folate binding, as assayed by tritiated folate binding, are also deficient in folate chemoresponse (Schulz *et al.*, 1984). These mutations have been shown to be due to a single site lesion (DiNallo *et al.*, 1982; Sasner *et al.*, 1989, in preparation). Taken together with the present data showing specific blocking of attraction by crosslinking ligand to the cell and specific crosslinking of ligand to membrane preparations, these observations present strong evidence for the existence of a protein on the *Paramecium* cell membrane that acts to bind folate specifically and transduce this chemical signal into the membrane hyperpolarization that leads to accumulation in folate (Van Houten, 1978).

Previous attempts to identify external folate binding protein(s) on the *Paramecium* membrane using folate–Sepharose affinity chromatography in conjunction with glycoprotein staining and cell surface iodination have resulted in identification of a subset of membrane proteins that may include the chemoreceptor (Schulz, 1987). However, judging from the tritiated folate binding studies (Schulz *et al.*, 1984), we expect the receptor to have low affinity of binding in the membrane and perhaps even lower affinity in the detergent, making identification by affinity chromatograpy tentative at best. The crosslinking method employed in this paper allowed us to circumvent the problem of low affinity binding: ligand binds to receptor *in situ* and then is covalently bound, labeling the receptor for us to follow and not requiring the protein to retain sufficient affinity for a column matrix used in *in vitro* procedures. We intend eventually to compare our results of identified folate binding proteins with the prior work using affinity chromatography.

We have show here that we have produced a polyclonal antibody that will specifically detect folate crosslinked to membrane preparations. ELISA values are significantly higher when membrane crosslinked with folate is used as antigen than when sham crosslinked membrane or glycine crosslinked membrane is used as antigen. The next logical step is to use the anti-ligand antibody to identify specific crosslinked proteins. We are currently attempting this, but problems in solubilizing the crosslinked membrane have hindered progress. Western blots and affinity columns using anti-ligand antibody to identify crosslinked receptor were unsuccessful because crosslinked membrane precipitated before entering a gel or column. Autoradiograms of iodinated folate-labeled membrane preparations revealed that all the labeled proteins remained at the top of the stacking gel. Once the obstacle of precipitation is overcome, we should be able to use our anti-ligand immunodetection system to identify the receptor.

Acknowledgements

We would like to thank Brian Cote and Jackie Isaksen for their technical help. This work has been supported by NSF and the Whitehall Foundation.

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Received on January 6, 1989; accepted on March 24, 1989