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Relationship of folate binding to chemoreception in *Paramecium*

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Summary. Paramecia respond to some soluble chemicals in their environment. Cells change their swimming pattern to accumulate in or disperse from these chemicals. Attractants of one class, which includes the folate anion, hyperpolarize the cell relative to the membrane potential in control solution (Van Houten 1979). The mechanism of transduction of the chemical cue (folate) into its characteristic electrical cue is unknown. This study investigates the role of specific binding in this transduction. While the role of uptake cannot be unequivocally established (DiNallo et al. 1980), the role of binding in chemoreception is more clear and appears to be a necessary step in the chemosensory transduction pathway. Evidence for this comes from concomitant loss of saturable folate binding with loss of chemoresponse both in mutant d4-534 and assays of binding and chemoresponse in the presence of cyclic AMP. Normal folate binding to *Paramecium tetraurelia* is characterized by a K_d of approximately 29 μM and a binding site number of approximately 6.7×10^{-11} mol/mg protein.

Introduction

Paramecium tetraurelia detect and accumulate in K_2-folate (Van Houten 1979), an essential vitamin for paramecia (Van Wagtendonk 1974). *Paramecium* cells' resting membrane potential (Vm) becomes more negative in attractant K_2-folate relative to twice the molar concentration of KCl (Van Houten 1979). Similar changes in membrane potential are associated with other attractants (Van Houten 1979, 1980) and can account for the swimming behavior changes that cause the cells to respond to attractants by accumulating in them (Van Houten 1978). In the case of folate, the negative shift in Vm of a cell can be explained by at least two possible mechanisms: folate anion may enter the cell to directly or indirectly hyperpolarize the cell, or may bind to a cell membrane component that affects membrane properties to indirectly hyperpolarize. We have attempted to distinguish between these two possibilities. We describe here our studies of the relationships between folate uptake and binding and chemoreception in *Paramecium*.

Beidler (1961) described adsorption or binding to receptor as the primary step in gustation in vertebrates. Likewise, we believed binding has a direct correlation with chemoreception in *Paramecium* and carried out a kinetic analysis of binding. In addition to normal cells and conditions, we have made use of mutant d4-534 (DiNallo et al. 1982), which is unable to normally respond to or to bind folate, and of cyclic AMP (cAMP), a competitive inhibitor of binding and inhibitor of chemoresponse. We present these studies as evidence in support of a correlation between chemoreception and binding of the attractant folate in *Paramecium*.

Abbreviations: cAMP 3′5′ adenosine monophosphate; *Vm* membrane potential

Materials and methods

Strains and cell culture. All strains used are *Paramecium tetraurelia* (formerly *P. aurelia* syngen 4), strain 51-S (sensitive) and mutants derived from 51-S. Cells are grown at 28 °C in Cerophyl medium (rye grass) with Na_2HPO_4 buffering, inoculated with *Klebsiella pneumonia*.

Solutions. The standard buffer used in behavioral and binding assays contains 1 mmol/l $Ca(OH)_2$, 1 mmol/l citric acid, and 1.3 mmol/l Trizma base (Tris(hydroxymethyl)-aminomethane), pH 7.00–7.05. Other salts vary as described in each experiment.

Behavioral assay. Chemoresponse is analyzed using a modified T-maze assay (Van Houten et al. 1982). Test and control solutions, balanced in pH and ionic strength, fill the arms of the

T-maze; cells in control solution fill the plug. Standard assays allow cells to distribute between the arms for 30 min; time course assays are stopped at specified times. Assay results are expressed as the ratio of the number of cells in the test arm to the number of cells that migrated out of the stopcock plug into either arm (I_{che}). An $I_{che} > 0.5$ indicates attraction; $I_{che} < 0.5$ indicates repulsion.

Binding assay. A centrifugation assay (modeled after Hansma and Kung 1976) was developed to measure initial rates of folic acid uptake. For each assay, 500–700 ml of *Paramecium* culture at approximately 4,000 cells/ml are harvested one day after the ciliates clear most of the bacteria from the culture fluid (early stationary phase determined by cell counts). The cells are incubated for 1 h in 2 mmol/l KCl buffer containing 2.2×10^3 units/ml of penicillin and 2 mg/ml of streptomycin. Antibiotic treatment is necessary to kill bacteria that take up and significantly affect the concentration of folate. To begin the experiment, the antibiotic treated cells are pelleted again, and the volume of the isolated pellet with residual buffer is measured for use in correcting the concentration of folate in the incubation solution after addition of the pellet. The pellet (approximately 2×10^5 cells) is then added to the incubation mixture, 8 ml of buffer containing K_2-folate at the concentration to be assayed plus 10 µl of ^{3}H-folic acid (Amersham, 1 µCi/ml). (The small amount of folic acid did not significantly affect the total carrier concentration of K_2-folate. K_2-folate was used because the behavioral assays typically balanced ionic strength and measured attraction to K_2-folate versus 2 KCl). A sample of the incubation mixture is taken before addition of the cell pellet in order to determine specific acitivity. At four times, within a short time interval (usually 30 s–5 min), the cells are resuspended by gentle shaking and duplicate 1 ml samples are withdrawn from the incubation tube. The samples are layered over 1.5 ml of wash buffer containing the same concentration of unlabeled folate and 1% sucrose in sealed 18.5 cm Pasteur pipets. Cells are centrifuged for 2 min through the folate-sucrose wash solution at 200 g in an IEC HNS-II centrifuge. Cell pellets are collected by breaking off the pipet tips. The tips are crushed in Sybron/Nalge Filmware tubes containing scintillation cocktail, and radioactivity counted in a Beckman LS7000 liquid scintillation counter. The protein content of each incubation mixture is determined using the Bio-Rad protein assay. (Protein averages 0.2–0.3 mg/ml.) Results of each experiment are expressed as moles of folate associated per mg of cell protein. Extrapolating results to time zero allows us to determine instantaneous binding.

Competition experiments follow the same procedure, with the following modification. Cyclic 3′-5′-adenosine monophosphate (cAMP) is added to the incubation and wash solutions to a concentration of 2 mmol/l. In some experiments, 2 mmol/l cAMP is also added to the antibiotic solution. Cyclic AMP and other adenine compounds were examined because adenine shares an uptake system with folate in L1210 cells (Suresh et al. 1979).

Counts were not corrected for ^{3}H-folate trapped in intercellular space because the amount trapped was over 100 fold less than the smallest amount bound and otherwise associated with the cells. In the course of a normal experiment, we substituted ^{14}C-inulin (Amersham, 50 µCi/ml) at high specific activity with no carrier inulin for ^{3}H-folate with carrier, and found 0.005% of the ^{14}C cpm in the sampled aliquots associated with the pellet. These results and the generally low ^{3}H-folate cpm associated with the pellet indicated that there is little trapping and that further study of intercellular space that varies from pellet to pellet is not necessary.

External folate concentrations were high relative to the amount bound and did not change significantly over the course of the initial rate measurements.

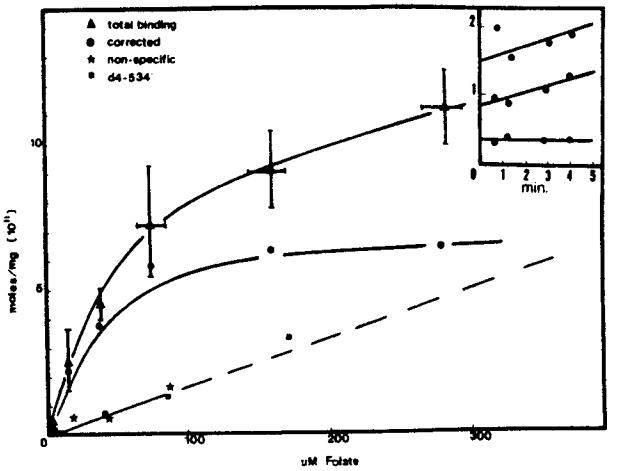

Fig. 1. Binding of ^{3}H-folate to paramecia. Instantaneous binding of ^{3}H-folate to cells is measured by centrifugation assay and plotted against folate concentration. (a) Total binding to normal paramecia (▲); binding to normal paramecia in the presence of 2 mmol/l cAMP (*); binding to normal paramecia corrected for nonsaturating binding in cAMP (●); binding to mutant d4-534 (■). Data are averages of 2–6 experiments, which are done in duplicate. Vertical bars are one standard error of the mean. Because we corrected concentration in each experiment for volume of solution that inevitably accompanied the cell pellet, it was virtually impossible to duplicate concentrations for replicate experiments. Therefore, the ranges of concentrations are given as horizontal bars. Insert shows data from typical experiment measuring amount of folate (measured as ^{3}H-folate) associated with cells as a function of time. Concentrations of folate in the incubation solutions are 100, 50 and 20 µmol/l, corresponding to lines in descending order

Results

Binding in normal cells

Binding was measured as instantaneous binding from initial rates of isotope incorporation extrapolated to time zero. Total binding, expressed as moles folate/mg cell protein, is shown in Fig. 1. Cells are generally harvested for binding studies at 4 days in culture. Total binding in 50 µmol/l K_2-folate does not change significantly with growth stage (Fig. 2, middle). Likewise, attraction measured by T-maze assays, is not affected by days in culture (Fig. 2, upper).

The amount of isotope associated with the cells decreased with increasing carrier/isotope ratio indicative of at least one binding component specific for folate (data not shown). Binding measurements would also be expected to have a non-specific component. Generally, this is measured by including a large excess of carrier folate with the isotope in the usual binding assay. However, high carrier

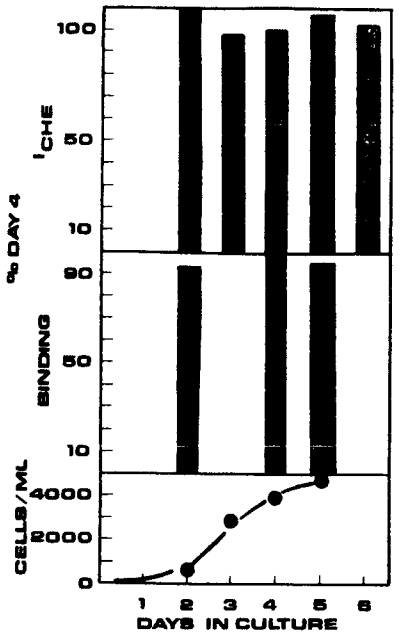

Fig. 2. Binding and attraction as a function of days in culture. Upper: Cells are usually harvested at late log/early stationary phase, typically day 3–4 in culture, for maximal cell number for T-maze assays of attraction. Attraction to 1 mmol/l K_2-folate vs 2 mmol/l KCl was measured on days 1–6 of culture and the results are expressed as % of the standard day 4 results. Data are averages from 5–6 experiments. Middle: Cells are generally harvested in late log/early stationary phase (here day 4 in culture) for binding experiments. Binding in 50 µmol/l K_2-folate was measured on Days 2, 4 and 5 of culture and expressed as % of the standard Day 4 results. Data are averages of duplicate samples. Bottom: Cell density measured by cell counts of two or more aliquots of cells on days 2–5 of culture. Results are the averages from two separate experiments. Data for T-maze assays (upper) are not from the same cell populations as in the Figures below

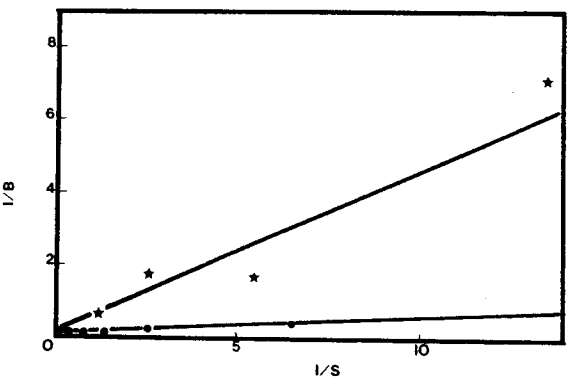

Fig. 4. Transformation of binding values. Scatchard (B/F vs B) plot of binding values. Lines are calculated from a linear program. Slope of normal binding values gives a K_d of approximately 29 µmol/l (●); binding to d4-534 (■) or in the presence of 2 mmol/l cAMP (▲) has no slope and no measurable K_d, indicative of non-saturable, nonspecific binding. Data are averages of 2 to 8 experiments

Fig. 3. Double reciprocal plot of binding values. Corrected binding data (●) and data of binding in the presence of cAMP (*) are replotted as 1/bound folate versus 1/free exogenous folate. Bound and free are in units of mol/mg cell protein. B_{max}, calculated from the y intercept, does not differ significantly between the two conditions, while the K_d, calculated from the x intercept, is increased in the presence of cAMP, characteristic of a competitive inhibitor. K_d from this plot has units that differ from K_d calculated from Fig. 4. Data are averages from 1 to 6 experiments

folate resulted in initial rates too fast for us to measure and to extrapolate to time zero with accuracy. Therefore, we used another approach to the separation of specific and non-specific binding. In our study of inhibitors of chemoresponse, we found 2 mmol/l cAMP in incubation mixtures and washes reduced binding of folate to a linear, non-saturable component (Fig. 1). In a double reciprocal plot (Fig. 3), cAMP appeared to be a competitive inhibitor of folate binding. We recognize problems with double reciprocal plots (Hollenberg and Nexø 1981). However, other data transformations, described below, are consistent with this inference. KCl and adenine do not have this inhibitory effect on binding (data not shown). Binding studies of mutant d4-534 (Fig. 1) produced a linear, non-saturable function almost identical to the cAMP binding results. (See below for more mutant analysis.) Therefore, in light of all these pieces of information, we considered binding in the presence of cAMP to be residual and non-specific and we subtracted the binding values in the presence of cAMP from total binding to produce a corrected curve (Fig. 1) that saturated at high folate concentrations.

The corrected binding data were transformed to determine the dissociation constant (K_d) for folate binding and the concentration of binding sites. In Fig. 4, a Scatchard plot of bound/free folate concentration (B/F) versus bound gives, as the inverse of the slope, a K_d of 29 µmol/l, in good agreement with the K_ds of 35 and 39 µmol/l estimated from Figs. 1 and 3, and a binding site concentra-

Table 1. T-maze assays of normal attraction to folate. Data are averages of n experiments ± one SD

Control solution	Test solution	I_{che}			
		Normal cells	n	d4-534	n
2 mmol/l KCl	1 mmol/l K_2-folate	0.76 ±0.06	6	0.57 ±0.05	8

tion of 6.7×10^{-11} mol/mg cell protein. A plot of bound folate versus log free folate (Klotz 1982) gives a B_{max} of approximately 12×10^{-11} mol/mg cell protein, in good agreement with the binding site number from the Scatchard plot (data not shown). We have checked the Scatchard plot data by the means suggested by Klotz (1982) to confirm that this is an appropriate graphical procedure. Similar Scatchard transformations of values of binding in the presence of 2 mmol/l cAMP support the assumption that this binding is nonsaturable with no measurable K_d or number of binding sites (Fig. 4).

The lowest concentration point of the Scatchard Plot is anomalously low. This point is the average of 8 experiments, but makes little difference to the calculated line through the other points. Elimination of this point gives a K_d of 31 μmol/l and binding site number of 7.1×10^{-11} mol/mg. See Discussion for further discussion of this point.

Binding is necessary for chemoresponse

Mutant d4-534 has been shown to have impaired chemoresponse to folate due to a single Mendelian mutation (Table 1) (DiNallo et al. 1982). Plots of total binding of folate (Fig. 1) show a linear, nonsaturable function similar to binding in the presence of cAMP. Transformation of these binding data for Scatchard and other analyses yield no measurable K_d (Fig. 4) or number of binding sites (Figs. 4 and 5). The concomitant loss of both functions in the same mutant indirectly support the necessity of normal binding for normal chemoresponse.

As indicated above, cyclic AMP was examined first for its inhibition of chemoattraction to folate. T-maze assays showed that normal paramecia are attracted to folic acid over a concentration range of 0.1 to 10 mmol/l, optimal attraction occurring at 1–2 mmol/l. Cyclic AMP is also an attractant; maximum response occurs at 2 mmol/l as determined from a dose response curve (Schulz, unpublished data). When present at saturating levels throughout the T-maze, cAMP significantly reduces attraction to 1 mmol/l K_2-folate, but does

Table 2. T-maze assays for inhibition of attraction by cAMP. Data are averages of n experiments ± one standard deviation. The rationale of the inhibition tests is that cells are tested for their ability to detect and respond to a gradient of attractant (folate, acetate (*OAc*), lactate, or ammonium) relative to *Cl* in the presence of a uniform, high concentration of *cAMP* throughout the T-maze. Ionic strength controls use a high uniform concentration of *KCl* throughout the T-maze in addition to the usual attractant and its counter ion in test and control solutions in the arms of the T. Motility decreases at high ionic strengths needed for these tests and variation between tests increases

Purpose	Control solution (mmol/l)	Test solution (mmol/l)	I_{che}	n
Ionic strength (Z) control	4.5 KCl	1 K_2-folate 2.5 KCl	0.71 ±0.09	6
Inhibition by cAMP	2 KCl 2.5 K-cAMP	1 K_2-folate 2.5 K-cAMP	0.55 ±0.07	5
Z control	10 KCl	5 KOAc 5 KCl	0.73 ±0.10	5
Inhibition by cAMP	5 KCl 5 K-cAMP	5 KOAc 5 K-cAMP	0.68 ±0.14	6
Z Control	10 KCl	5 K-lactate 5 KCl	0.73 ±0.14	3
Inhibition by cAMP	5 KCl 5 K-cAMP	5 K-lactate 5 K-cAMP	0.83 ±0.08	3
Z Control	5 NaCl 5 KCl	5 NH_4Cl 5 KCl	0.69	2
Inhibition by cAMP	5 NaCl 5 K-cAMP	5 NH_4Cl 5 K-cAMP	0.76 ±0.10	3

Scatchard plot could be indicating a positive cooperativity at low concentration. The resolution of low concentration binding numbers awaits a more sensitive binding assay.

It should be emphasized that the binding values are from data corrected for nonspecific binding by subtraction of binding values in presence of cAMP. Folate binding in 2 mmol/l cAMP and folate binding to d4-534 are almost identical, low level, and nonsaturating. Since we cannot measure nonspecific binding by adding a large excess of carrier folate, we have used these non-traditional measures of nonspecific binding to correct our total binding for specific binding. We are presently measuring binding to isolated cilia by a filter binding assay, which should be amenable to correction for nonspecific binding by excess carrier folate. The measurement of binding to cilia will also help to resolve the question of even distribution or localization of binding sites on cilia or cell bodies.

There is an apparent anomaly between the K_d of the binding (29 μmol/l) and concentration for half optimal attraction in T-mazes (0.5 mmol/l). We attribute the difference to the geometry of the T-maze. A diffusion gradient forms in the T-maze plug and the cells are most likely responding to this gradient when they distribute into arms of the T rather than responding to the high uniform concentrations of folate and chloride in the arms. The T-maze also measures a population response that is the end result of many 'choices' by the individual cells, which can leave and reenter arms of the T-maze many times over the course of 30 min (Van Houten et al. 1982). Therefore, we cannot determine the effective threshold or half maximal concentrations for response by using the T-mazes. We can use electrophysiology to more directly determine the minimum concentrations of folate, which elicit a hyperpolarization of the cells, a step (or steps) in the chemosensory transduction pathway much closer to the primary step of ligand binding than the population distribution in the T-maze. At present, we know cells respond to K_2-folate relative to 2 KCl below the 0.5 mmol/l range. We have not yet established a threshold or half maximal response.

Catfish tastant L-alanine binds to barbel membranes with a K_d of 4.8 μmol/l (Krueger and Cagan 1976), and mammalian tastant K_ds range from 10 μmol/l to 100 μmol/l (Cagan 1981). Therefore, the K_d of 29 μmol/l for the *Paramecium* 'tastant' folate is not unusually high. However, the binding of folate to *Dictyostelium*, which is not affected by cAMP, is significantly tighter K_d of 0.3 μmol/l (Van Driel 1981). The significance of this difference is not clear to us. It may reflect the concentration differences in folate that these cell types typically encounter in rotting logs or ponds or have evolved to detect.

Folate binding is necessary for chemoresponse

We have found evidence for a correlation between folate binding and folate chemoresponse. In two cases, mutant d4-534 and normal cells in the presence of cAMP, there are concomitant losses of binding and chemoresponse. In both cases the losses are specific for folate binding and attraction. Failure to find significant binding in d4-534 is not due to differences from normal cells in growth rate, hence in time in culture or growth condition at harvesting, because normal cells show no growth stage dependence of binding or chemoresponse (Fig. 2).

We have not yet demonstrated through genetic analysis that one mutation in d4-534 is responsible for both defective chemoresponse and binding phenotypes. We are presently addressing this problem by development of a fast binding assay to analyze F2 generations of genetic crosses of d4-534 and by binding analysis of a revertant of d4-534, which has recovered chemoresponse to folate by a second site mutation (White and Van Houten, unpublished results).

The establishment of a relationship between binding and chemoresponse is an important step in the description of the chemosensory system for folate. It is now clear that folate-specific binding sites exist and that binding to these sites somehow results in membrane electrical changes. We are currently isolating folate binding proteins from membranes of normal and mutant cells in an effort to establish whether a subset is involved in chemoreception and to determine the nature of these chemosensory pathway components.

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