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Correlations between cyclic AMP binding and chemoreception in *Paramecium*

Robert Smith, Robin R. Preston, Stephanie Schulz, Michael L. Gagnon and Judith Van Houten

Department of Zoology, University of Vermont, Burlington, VT (U.S.A.)

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Paramecium tetraurelia **is attracted to cyclic AMP, which probably, as other attractants, signifies the presence of food. Attraction to cyclic AMP was specific, saturable, and, therefore, likely to be receptor-mediated. In these studies, we measured the binding of cyclic [^{3}H]AMP to whole cells and found it to be saturable, reversible, and displaying specificity similar to that of attraction. An HPLC method of separating nucleotides was devised and used to determine that external cyclic AMP was degraded in the absence of IBMX, a phosphodiesterase inhibitor, and that cyclic AMP was taken into the cells in small amounts. Since binding and attraction were subsequently measured in the presence of IBMX, it was cyclic AMP and not a degradation product that served as the attractant stimulus for *Paramecium*.**

Introduction

Paramecium tetraurelia, a ciliated unicell, responds behaviorally to some of the chemicals in its environment. For example, paramecia are attracted to folate and acetate [1], which probably signal the presence of food (bacteria) to *Paramecium* in its natural environment. Adenosine 3′,5-(cyclic)monophosphate (cAMP) also is an attractant [2], and the natural source of this attractant may also be bacteria [3].

In these studies, an HPLC assay for cAMP was developed and used to demonstrate that external cAMP was hydrolyzed to AMP unless the phosphodiesterase inhibitor IBMX was present. Cells were attracted to cAMP in the presence of IBMX, suggesting that it was cAMP and not a degradation product that served as an attractant stimulus for *Paramecium*.

It is likely that external chemical stimuli are detected by binding to receptors on the surface of the cell [4] and that this binding is transduced into electrical information in the receptor cell. It has been demonstrated that there are specific cell-surface folate-binding sites that were associated with a specific and saturable chemoresponse to folate [5]. Similarly, attraction to cAMP showed characteristics of a receptor-mediated response, and therefore we set about studying cAMP surface-binding sites, some of which should correspond to putative cAMP chemoreceptors. Radiolabeled cAMP was used to measure stimulus binding, and the kinetics of this binding were compared with electrophysiological and behavioral responses of *P. tetraurelia* to cAMP. Cells bound [^{3}H]cAMP in a specific, saturable manner and 5′AMP, an inhibitor of attraction, also inhibited binding and cAMP-induced hyperpolarization.

Abbreviations: IBMX, isobutylmethylxanthine; TLC, thin-layer chromatography.

Correspondence: J. Van Houten, Department of Zoology, Univeristy of Vermont, Burlington, VT 05405, U.S.A.

Half-maximal behavioral and electrical responses occurred at approx. 1 mM, which was approximately 4-times the K_D for binding. The relationship of this binding to chemoresponse is discussed.

Attractants of *Paramecium* typically cause a membrane hyperpolarization [6], which results in altered ciliary beating and swimming behavior. Presumably binding of stimuli to surface receptor sites precedes the hyperpolarization, which is produced possibly by transport of the attractant stimulus into the cell to act as a second messenger or indirectly by a change in membrane electrical properties. The HPLC assay demonstrated that cAMP was taken into the cells in small amounts.

Materials and Methods

Culturing. Paramecium tetraurelia, stock 51-s, was grown at 28° C in Cerophyl medium (rye grass extract, buffered with Na_2HPO_4 and supplemented with stigmasterol) [7], inoculated with *Klebsiella pneumoniae* 24 h before use.

Behavioral assay. The behavioral responses of *P. tetraurelia* to chemicals were measured using a modified T-maze assay [8]. Unless otherwise stated, buffers for cells contained 1 mmol/l $Ca(OH)_2$, 1 mmol/l citric acid and 1.3 mmol/l Tris (pH 7.00) with added salts indicated.

Binding assay. Whole cell binding was assayed as instantaneous binding as described by Schulz et al. [5], with the exception that incubation solutions contained varying amounts of unlabeled cAMP, 1 mM 3-isobutyl-1-methyl-1-xanthine (IBMX), and 31 nM [^{3}H]cAMP (added 10 μl/ml of [^{3}H]cAMP, 26–27 Ci/mmol, 1 μCi/μl). Buffers used were the same as for behavioral assay above. In this and most of the following procedures, the cells were resuspended for 60 min in a 2 mM NaCl buffer containing 2 mg/ml streptomycin and $2.2 \cdot 10^3$ units/ml penicillin in order to kill bacteria that could take up or break down the compounds in the incubation solution. Protein was assayed by the Bio-Rad method with γ-globulin standard. Competition binding studies followed the same procedure described above with the exception that 2 mM $Na_2$5′AMP or 4 mM NaCl was added to all solutions. Binding data in Figs. 1 and 5 were plotted using the MLAB program for kinetic analysis [9].

HPLC assay of cAMP. The Waters SAX 10 μm High-Pressure Liquid Chromatograph (HPLC) cartridge used for these experiments could not be exposed to citrate. Thus, all buffers used with cells for HPLC contained only 1 mM $CaCl_2$, 3 mM KCl and 1 mM Tris (pH 7.0), unless stated otherwise. Methods of treatment of cells and extraction of cAMP were modified from procedures described by Nelson (Refs. 10, 11 and personal communication). Cyclic AMP fractions from Dowex columns (Bio-Rad AG 50W-4X 200–400 mesh H form) [11] were pooled and lyophilized. The samples were dissolved in a total volume of 1 ml of 7 mM KH_2PO_4/7 mM KCl (pH 4.0) and filtered through a Gelman Acro LC13 45 μm HPLC sample filter.

Cell extracts were analyzed on a Waters 510 HPLC equipped with a SAX Radial PAX 10 μm cartridge and a 2 μm in-line filter. 50 μl samples or standards were eluted with an isocratic mobile phase (7 mM KH_2PO_4/7 mM KCl (pH 4.0)) at a flow rate of 3 ml/min. Cyclic AMP in mobile-phase standard solution was routinely monitored at 254 nm using a Waters 441 detector. The standard eluted in one peak at 5 min. Standard solutions of 500 μM cAMP with 500 μM 5′AMP, cGMP, 5′GMP or IBMX, in the solution for mobile phase above, were also analyzed using 254 nm absorbance. Concentrations of cAMP from cell extracts were determined from areas under the profile peaks using a Zeiss integrator and compared to the areas under the peaks of known cAMP concentrations. The internal cAMP level was $1.5 \cdot 10^{-16}$ mol/cell under hyperpolarizing conditions, which compares favorably with Nelson's value of $4.8 \cdot 10^{-16}$ mol/cell [10].

To determine whether cAMP enters the cells, cells from 500 ml of culture were incubated in buffer with about 61 nM [^{3}H]cAMP (20 μl of [^{3}H]cAMP in 9 ml buffer) for 30 min, washed twice by centrifugation, and analyzed for cAMP content as described above. 250 μl of the Dowex column eluate was applied to the HPLC column as above. Duplicate samples from each 0.6 ml column aliquot were analyzed by liquid scintillation counting in a Beckman LS7000 instrument.

The HPLC assay was also used to monitor

extracellular breakdown of [^{3}H]cAMP to [^{3}H] 5′AMP with time. 1 liter of late log phase culture was centrifuged and the pellet was resuspended in buffer for HPLC and with antibiotics as for the binding assay above. One aliquot of cells was pelleted by centrifugation and resuspended at a concentration of $2 \cdot 10^5$ cells per ml in 10 ml of buffer for HPLC containing about 30 nM [^{3}H] cAMP (10 μl of [^{3}H]cAMP), 1.5% sucrose and 1 mM IBMX. (The sucrose was included to prevent swelling caused by IBMX.) The second aliquot of cells was treated identically but resuspended in incubation buffer without IBMX. 1-ml samples of cells were taken from the incubation buffers at 10 s and 30 min, and filtered using Gelman 45 μm filters. 25 μl samples of the filtrate were analyzed for [^{3}H]cAMP and [^{3}H]5′AMP using the Waters 510 HPLC system described previously. The elutant was collected and analyzed by liquid scintillation counting as above.

TLC assay for cAMP. All buffers for cells were the same as for the behavioral assay. The thin-layer chromatography (TLC) assay was used to monitor extracellular breakdown of [^{3}H]cAMP to [^{3}H] 5′AMP with time. 1 liter of late log phase culture was centrifuged, and the pellet was resuspended in antibiotics and 2 mM NaCl buffer. One aliquot of cells was pelleted by centrifugation, and resuspended at a concentration of $2 \cdot 10^5$ cells/ml in 10 ml of 2 mM NaCl buffer containing 1.5% sucrose and 1 mM IBMX. The second aliquot of cells was treated identically but resuspended in a solution without IBMX. The treatment of both aliquots of cells was identical in all subsequent procedures. After 5 min, 10 μl [^{3}H]cAMP was added to each incubation buffer to bring the cAMP concentration to 30 nM. 1-ml samples of cells were taken from the incubation buffers at 10 s and 25 min and filtered through Gelman 45 μm filters. 10-μl duplicate samples of filtrate were spotted on EM Precoated Silica Gel 60 F-254 TLC sheets. 10 μl of 10 mM cAMP and 10 mM 5′AMP were spotted on top of the samples to locate the nucleotides. The chromatography was carried out as described by Flouret and Hechter [13]. The spots were visualized using a UVSL-25 mineral light and identified by their R_F values. The silica was scraped off each spot, dissolved overnight in 3 ml of scintillation fluid and analyzed by liquid scintillation counting.

Electrophysiology. Membrane potential in varying cAMP concentrations was measured as described in Refs. 6 and 14.

Results

Cyclic AMP acts externally as a chemoreception stimulus

Cyclic AMP could be separated from cGMP, 5′AMP, 5′GMP and IBMX using HPLC (Fig. 1a). Cyclic AMP added to cell extracts eluted in the same HPLC fraction as cAMP in buffer (Fig. 1b). External [^{3}H]cAMP was degraded by the cells over the course of 30 min, but it was not detectably degraded when 1 mM IBMX was present (Fig. 1c). Within 30 min, a small amount of [^{3}H]cAMP was taken up by the cells or at least not washed off the cells in two centrifugations. After incubation of cells with 61 nM [^{3}H]cAMP, approx. $5.5 \cdot 10^{-21}$ mol [^{3}H]cAMP/cell ($3.3 \cdot 10^3$ molecules/cell) were found in the trichloroacetic acid-soluble cell fraction (Fig. 1d).

Binding of cAMP to whole cells

Binding of cAMP was measured as 'instantaneous binding' using a centrifugation assay. The amount of [^{3}H]cAMP associated with whole cells following centrifugation through a wash solution was measured as a function of time. The cpm values were extrapolated to time zero and this value was used as a measure of instantaneous binding of [^{3}H]cAMP to whole cells [5]. IBMX, a phosphodiesterase inhibitor [15], was included in the cAMP incubation solution as a precaution against phosphodiesterase hydrolysis of cAMP. However, IBMX causes cells to swell after 30 min. Although sucrose alleviated the swelling and was used in longer experiments, such as the HPLC assays above, we preferred to reduce incubation time instead of adding sucrose to the incubation solutions for the binding assays. Consequently, in assays of binding, the cells were incubated in IBMX for 5 min or less and showed no change in shape, size or viability and binding was measured at time zero and not at equilibrium. In each of the binding experiments, the amount of [^{3}H]cAMP was small and constant (about 31 nM) and the amount of unlabeled cAMP was varied. Fig. 2 indicates that there was low, nonspecific binding

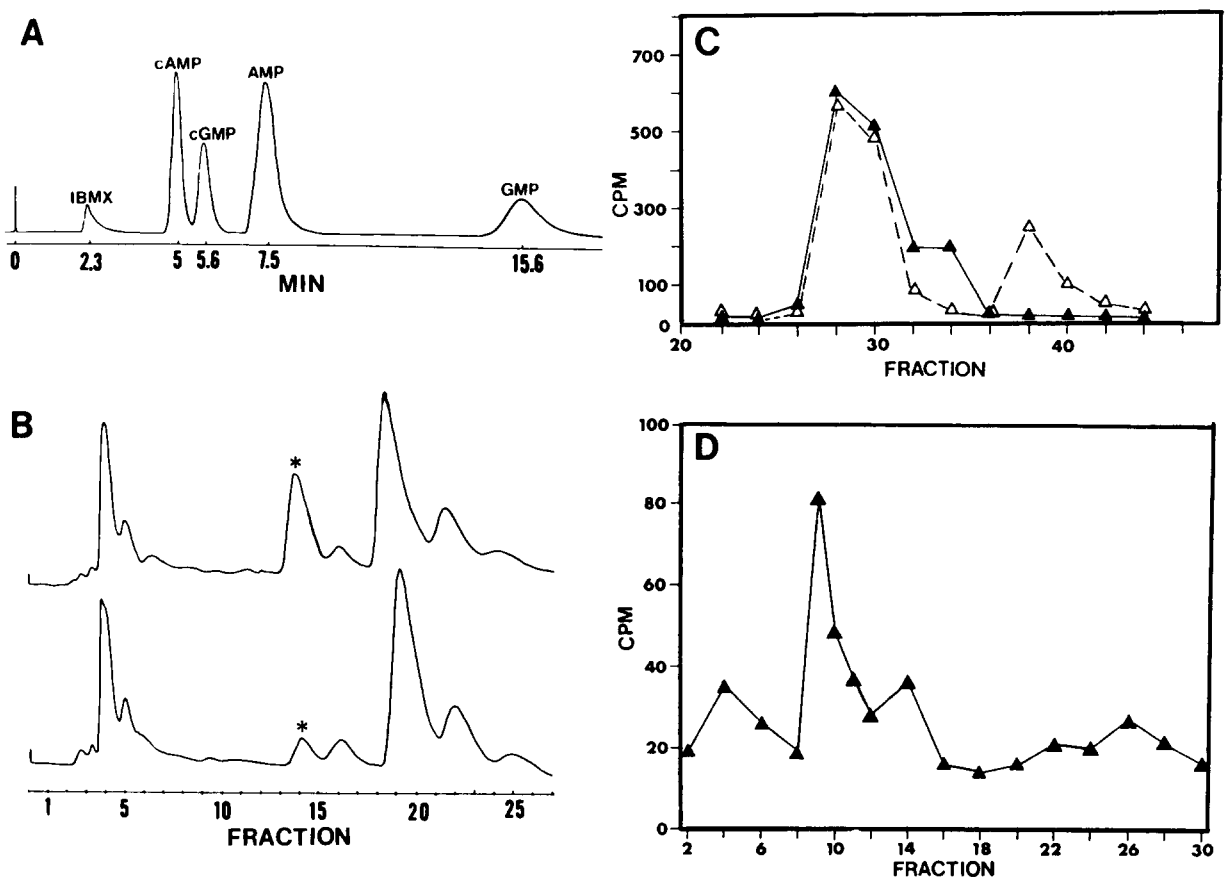

Fig. 1. HPLC profiles. (A) Combined HPLC 254 nM *A* profiles of 500 μM cAMP mixed with 500 μM AMP, GMP, cGMP or IBMX in buffer. (IMP, not included on these profiles, had a retention time of 10 min.) (B) Cyclic AMP added to cell homogenate elutes in the same fraction as cAMP in buffer. Bottom profile is trichloroacetic acid-soluble fraction of homogenate; top profile is soluble fraction of homogenate with added cAMP. Asterisk denotes the peak at 5 min. retention time for elution of cAMP in buffer. (C) HPLC ^{3}H profile of external medium with added [^{3}H]cAMP and with IBMX (———) or without IBMX (- - - - - -). Fraction 27 corresponds to 5 min and fraction 38 to 7.5 min retention times, characteristic of elution times for cAMP and AMP, respectively. The HPLC fractions thought to contain cAMP and AMP were analyzed by TLC to verify that the ^{3}H cpm originated from cAMP or AMP. The shoulder on the cAMP peak in IBMX did not always occur. (D) HPLC ^{3}H profile of trichloroacetic acid-soluble fraction of cell homogenate. Cells were incubated for 30 min in [^{3}H]cAMP in buffer prior to harvesting. Fraction 10 corresponds to 5 min retention time in (A). (Fraction size differs in (C) and (D).)

at high concentrations of unlabeled cAMP (no less than 1 mM) and half-maximal binding, approx. 250 μM, agreed fairly well with both the half-maximal behavioral chemoresponse, approx. 1 mM (Fig. 3), and the cAMP-induced hyperpolarization that is associated with chemoreception (Fig. 4). The number of binding sites was estimated to be $5.6 \cdot 10^9$/cell.

Adenosine 5′-monophosphate (5′AMP) inhibited chemoattraction to cAMP (Table I). Therefore, AMP should have inhibited binding of cAMP to sites involved in chemoreception. Competition binding experiments are shown here as dissociation curves (Fig. 5) with 2 mM $Na_2$5′AMP or control 4 mM NaCl added to all solutions. The binding of [^{3}H]cAMP in the presence of 2 mM $Na_2$5′AMP was reduced to a low, nonsaturable level. Alternatively, 4 mM NaCl had no significant effect on binding and the control curve in Fig. 4 was comparable with that at lower ionic strength

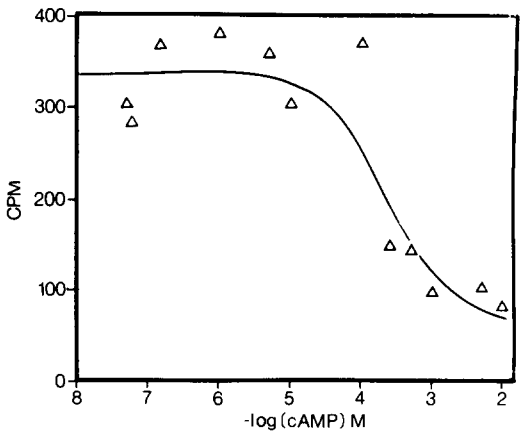

Fig. 2. Binding of [^{3}H]cAMP in varying amounts of unlabeled cAMP. Binding was measured by the centrifugation assay described in Materials and Methods. Half-maximal displacement occurs at approx. 300 μM. Data are averages of 1–5 experiments, which are done in duplicate. Line was calculated using the MLAB program [9].

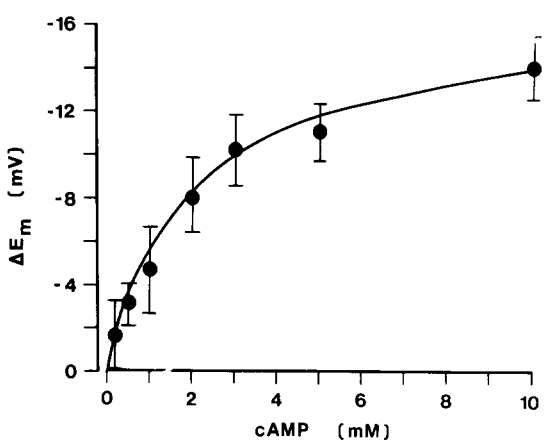

Fig. 4. Electrophysiological measurements of cAMP-induced hyperpolarization. Differences in membrane potential between those in NaCl and those in comparable amounts of Na-cAMP are plotted vs Na-cAMP concentration. Data are averages from three or more cells. Bars are 1 S.D.

(Fig. 1). Dissociation curves of [^{3}H]cAMP bound in the presence of 0.5 mM cAMP were intermediate between those in control 4 mM NaCl and 2 mM $Na_2$5′AMP (data not shown).

Competition binding data from binding in the presence of 2 mM $Na_2$5′AMP or 4 mM NaCl (Fig. 5) were corrected for nonspecific binding by subtracting the NaCl control value at 10^{-3} M unlabeled cAMP and were plotted in a Scatchard transformation (Ref. 16, and data not shown). This transformation was not completely appropriate for nonequilibrium binding data, but served to demonstrate that binding in the presence of the attraction inhibitor, 5′-AMP, resulted in a line with no measurable slope, which was char-

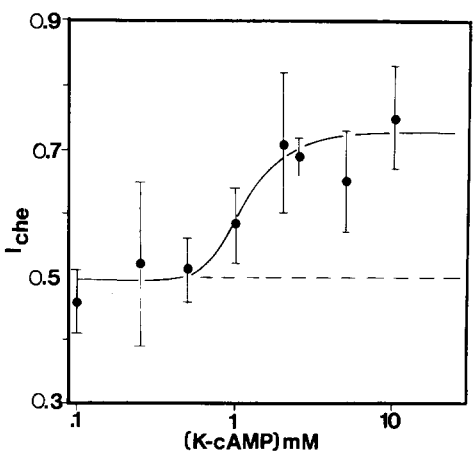

Fig. 3. T-maze measurements of chemoresponse to cAMP. Attraction of K-cAMP in comparable amounts of KCl measured in T-maze assays. $I_{che} > 0.5$ indicates attraction; $I_{che} < 0.5$ indicates repulsion. Data are averages of three or more T-mazes ± 1 S.D.

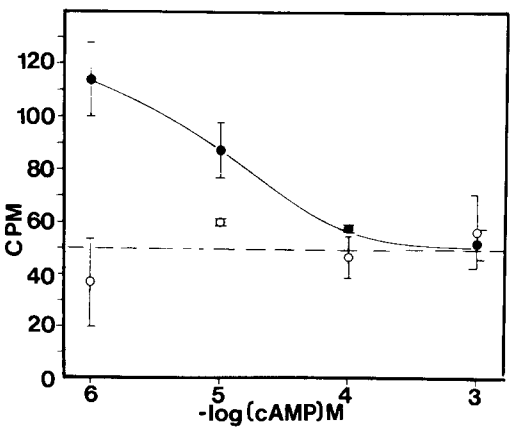

Fig. 5. Binding of [^{3}H]cAMP in presence of Na_2AMP or NaCl. Binding was measured as in Fig. 2, with the exception that 2 mM Na_2AMP (○) or 4 mM NaCl (●) were included in all solutions. Data are averages of 4–8 data points from two or three experiments, which are done in duplicate. Bars represent 1 S.D.

TABLE I

INHIBITION OF ATTRACTION BY AMP

Data are averages of n T-mazes ± 1 S.D. Each of the results with AMP as competitor is significantly different from the control by the Mann-Whitney U Test.

Test soln.	Control soln.	T-maze I_{che}	n
[a] 1 mM K-cAMP + 5 mM KCl	6 mM KCl	0.71 ± 0.13	10
1 mM K-cAMP + 5 mM K-5′AMP	1 mM KCl + 5 mM K-5′AMP	0.49 ± 0.14	8
[a] 2 mM Na-cAMP + 2 mM NaCl	4 mM NaCl	0.79 ± 0.04	3
2 mM Na-cAMP + 2 mM Na-5′AMP	2 mM NaCl + 2 mM Na-5′AMP	0.67 ± 0.05	3
[a] 2.5 mM K-cAMP + 5 mM KCl	7.5 mM KCl	0.64 ± 0.04	6
2.5 mM K-cAMP + 5 mM K-5′AMP	2.5 mM KCl + 5 mM K-5′AMP	0.55 ± 0.06	3

[a] Controls for experiments that follow. See Fig. 3 for definition of I_{che}.

acteristic of an inhibitor of specific binding. A proper range of points was taken into consideration for a kinetic analysis and linear transformations of binding data. The data were plotted as suggested by Hollenberg and Nex Ø [17] and cyclic AMP concentration values were taken from the slope as well as the plateau of this graph insuring an adequate range of ligand concentrations.

Mutant shows non-specific binding

A mutant (cyc^{-1}) that does not detect cAMP normally was isolated among cells that failed to remain in the high concentration end of a linear gradient of cAMP. Late log phase mutant cells were not attracted to cAMP ($I_{che} = 0.52 \pm 0.04$), while normal cells were attracted to cAMP ($I_{che} = 0.78 \pm 0.16$). (See Fig. 3 for definition of I_{che}.) The mutation was recessive and segregated in a Mendelian fashion, unlinked to a marker gene for resistance to copper ($\chi^2 = 3.96$, 3df, $0.5 > P > 0.2$). Late log phase cyc^{-1} cells showed nonspecific, nonsaturable binding in displacement experiments similar to those of Fig. 2 (Table II).

TABLE II

BINDING OF [^{3}H]cAMP TO NORMAL AND Cyc^{-1} CELLS

Normal cell data are averages from 1–7 experiments. Cyc^{-1} data are averages from 3–7 experiments. Binding at 10^{-3} M was considered nonspecific and used to correct other data for nonspecific binding in order to calculate the % total binding at a given concentration that was nonspecific. Binding at $1 \cdot 10^{-6}$ M was arbitrarily set at 100%. [^{3}H]cAMP in all cases was about 31 nM.

Unlabeled cAMP (M)	% Total binding that is specific	
	normal cells	Cyc^{-1}
$5 \cdot 10^{-3}$	2	–
10^{-3}	0	0
$5 \cdot 10^{-4}$	13	–
$2.5 \cdot 10^{-4}$	14	–
$1 \cdot 10^{-4}$	52	−5
$1 \cdot 10^{-5}$	55	8
$5 \cdot 10^{-6}$	70	–
$1 \cdot 10^{-6}$	75	1
$1 \cdot 10^{-7}$	72	–

Discussion

Paramecia detect cAMP and populations of cells accumulate in solutions of cAMP. Other attractants (including folic acid and acetate) are clearly products of bacterial metabolism and are likely to be cues to paramecia that their foodstuff is in the vicinity [1]. Similarly cAMP can be found in the vicinity of *Escherichia coli* [3], where it may also function as a food cue for paramecia. This is in contrast to chemoresponse in *Dictyostelium discoideum*, in which folic acid is thought to be the indicator of food (bacteria) to vegetative amoebae [18], while cAMP is a stimulus for aggregation for multicellular slug development [19]. *Dictyostelium* shows maximal chemotactic response to folic acid and cAMP at different times in development, whereas paramecia seem to use both folic acid and cAMP concurrently as food-indicating stimuli. *Dictyostelium* has exoenzymes that degrade the chemotactic signals [18] and thereby maintain a spatial gradient for the cell to follow. The cAMP-

degrading activity we detected upon incubation of cells with labeled cAMP may serve a similar function to that in *Dictyostelium*, but our concern with the degrading activity was to inhibit it in order to study the effects of known, unchanging amounts of cAMP. We have only indirect evidence for a cAMP-degrading exoenzyme, but upon visual inspection of cells there were no noticeably damaged or lysed cells that could have been the source of the activity from intracellular locations.

Cyclic AMP is secreted by *Tetrahymena* [20], another ciliate. However, paramecia were not noticeably attracted to each other by diffusible cues [21] and there was no evidence of cAMP in extracellular buffer to which no cAMP was added (data not shown). Therefore, it was most likely that cAMP acted as a food cue and not as an intercellular signal.

If cAMP were a food cue like folic acid, it should act externally by binding to surface membrane receptors and this binding should somehow be transduced into the hyperpolarization that is characteristic of attractant stimuli [1,6]. The binding should show the same specificity and perhaps half-maximal value as the behavioral response and hyperpolarization [21]. We found that cAMP bound to paramecia in a saturable, specific way, albeit with a relatively low affinity ($K_D = 250$ μm). In contrast, the half-maximal values for chemoresponse and hyperpolarization were approx. 1 mM. It is not uncommon to encounter differences between the binding and chemoresponse dose-response curves [22], but we feel that there is fair agreement between the curves considering the amount of variability that is inherent in the measurements of both behavior and low-affinity binding. Binding was reduced to a low, nonspecific level by 5′-AMP but not by Cl. The chemoresponse to cAMP also was inhibited by 5′AMP, but high ionic strength alone interfered with chemoresponse to cAMP and therefore it was not possible to include an ideal, large excess of potential inhibitor in the solutions. However, it was clear that 5′AMP inhibits both chemoresponse behavior and binding of [^{3}H]cAMP.

Binding was measured in the presence of IBMX, which did not interfere with behavioral chemoresponse to cAMP or other attractants [23] and therefore should not have interfered with the binding of [^{3}H]cAMP that was associated with chemoreception. However, after 30 min, IBMX caused the cells to swell. Therefore, binding was measured as instantaneous binding by extrapolation to time zero of measurements of [^{3}H]cAMP bound over 5 min. There were no detectable effects of IBMX within this short time, but instantaneous binding may have been an underestimate of the binding capacity, but not K_D, of the cell. Since both chemoresponse behavior and binding were measured in the presence of IBMX, cAMP and not a degradation product must have been both stimulus and bound ligand.

Attractants of *P. tetraurelia* have generally been active in the 0.1–1 mM range [1] and cAMP appears to be no exception. It can be reasoned that the pond is a chemically noisy environment and exquisite sensitivity would not serve paramecia well in their hunt for food. We have previously established that a bacterial fermentation product and attractant, lactate, can reach millimolar amounts close to the fermenting bacteria (Van Houten, J., unpublished results), but we have no similar information about cAMP. The K_m of ciliary adenylate cyclase for ATP is reported to be 0.2 mM [24] and may indicate fairly low affinity in other aspects of the *Paramecium* cAMP system.

Attractant stimuli like cAMP typically hyperpolarize the cell [6]. The ionic mechanism of this hyperpolarization is not yet clear, but it does not depend on external K, or Na [23]. The uptake of an anionic form of the stimulus could account for the hyperpolarization in some attractants, although this was not the mechanism of the cAMP-induced hyperpolarization since cAMP had no net charge at pH 7. The HPLC measurements of [^{3}H]cAMP showed that cAMP may have entered the cell and, therefore, cAMP could have been acting as a second messenger inside the cell. Considering that IBMX did not potentiate or inhibit chemoresponse [23], it was not likely that internal cAMP was serving as part of the transduction pathway. However, cAMP and other nucleotides have been implicated in the control of ciliary beating [10,25,26,27] and the role of cyclic nucleotides in chemoresponse will be investigated further.

Binding and chemoresponse to cAMP showed similar kinetics and specificity. Additionally, a

conditional mutant of *Paramecium* showed both defective chemoresponse to and totally non-specific binding of cAMP. Therefore, the low-affinity binding we measured showed correlations with chemoresponses of *Paramecium* and it was indeed cAMP and not its degradation products to which paramecia responded.

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