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Studies of the Cyclic Adenosine Monophosphate Chemoreceptor of *Paramecium*

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Summary. A doublet of proteins (~48,000 M_r) from the *Paramecium* cell body membrane fits several criteria for the external cAMP chemoreceptor. These criteria include: (*i*) selective elution from a cAMP affinity column, matching a specificity that could be predicted from the behavioral response and whole-cell binding; (*ii*) binding to wheat germ agglutinin indicating the presence of carbohydrate moieties indicating surface exposure; and (*iii*) selective inhibition of the intact cells' chemoresponse to cAMP by antibodies against the doublet. Additional evidence for the existence of a receptor, in general, comes from selective elimination of the cAMP chemoresponse by photoaffinity labeling of whole cells with 8-N_3-cAMP. The doublet proteins are not identical to the regulatory subunit of a cAMP-dependent protein kinase from *Paramecium,* the *Dictyostelium* cAMP chemoreceptor, or the 42–45 kDa range proteins related to the large surface glycoprotein in *Paramecium*. The doublet proteins are not readily separable and, as in *Dictyostelium,* may represent two different covalent modification states of the same protein. Amino acid analysis indicates that the proteins are similar, but does not distinguish between the possibilities of proteolysis and covalent modification. Once cloned, this doublet may prove to be only the fifth external, eukaryotic chemoreceptor to be identified.

Key Words chemoreceptor · cyclic AMP · *Paramecium* · sensory transduction

Introduction

The sensory pathways of taste and smell in metazoans and chemo-taxis and -kinesis in unicellular, eukaryotic organisms begin at surface membrane chemoreceptors or perhaps, in some cases, at the membrane itself (Anholt, 1987; Lancet & Pace, 1987). Studies of signal transduction in olfaction (Snyder, Sklar & Pevsner, 1988), gustation (Kinnamon, 1988), and chemotaxis (Devreotes & Zigmond, 1988) are filling in the details of intracellular events following stimulus binding to external receptors, while studies of the chemoreceptors themselves have been limited for most systems to binding measurements. As a consequence, our understanding of intracellular signaling is outstripping our understanding of receptors. The clear identification of external chemoreceptor proteins from among the proteins of the surface membrane has remained elusive, in part because of the relatively low affinities of these receptors, lack of pharmacological tools such as bungarotoxin that played a role in studies of the acetylcholine receptors, and lack of a source highly enriched in receptors. There are several candidates for olfactory chemoreceptors (Novoselov, Krapivinskaya & Fesenko, 1988; Price & Willey, 1988; Vogt, Prestwich & Riddiford, 1988), but successful identification has come only in the studies of unicellular model systems and even with these systems only four receptors have been clearly identified and cloned: a cAMP[1] receptor from *Dictyostelium discoideum* (Klein et al., 1988), the two yeast mating factor receptors (Burkholder & Hartwell, 1985; Nakayama, Miyajima & Arai, 1985; Hagen, McCafrey & Sprague, 1986), and the resact receptor from sea urchin spermatozoa (Singh et al., 1988).

Those few chemoreceptors identified to date are very different in primary sequence from each other and the four receptors fall into three different motifs of intracellular signaling. Therefore, gaining insights into chemoreception by understanding one representative receptor will not be possible and it remains for still other external chemoreceptors to be identified and characterized before any generalizations can be made. We describe here evidence for a fifth, external chemoreceptor, that of a cAMP receptor in *Paramecium*. Paramecia are attracted to cAMP and other stimuli, probably as indicators of the presence of bacteria as food (Smith et al., 1987). The attraction is specific for 3′,5′-cAMP: 5′ AMP acts as a partial agonist, while cGMP is not inhibitory (Smith et al., 1987). As with other attractants, there is saturable,

[1] *Abbreviations:* 8-N_3-cAMP, 8-azido-cAMP; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IOAc, iodoacetate; OAc, acetate; and cAMP, 3′,5′-cyclic adenosine monophosphate.

specific binding of ligand to the cell body rather than to the ciliary surface and this binding is transduced into a hyperpolarization of the membrane potential (Smith et al., 1987). A change in membrane potential is correlated with a change in ciliary beating that, in turn, translates into a change in population behavior, i.e., attraction (Van Houten, 1979; Preston & Van Houten, 1987). A doublet of cell body membrane proteins of ~48,000 M_r appears to comprise the receptor that mediates this chemoresponse. We present here the evidence for the cAMP chemoreceptor.

Materials and Methods

Culture of *P. tetraurelia*

Strain 51-s (sensitive to killer) was cultured at 28°C in wheat grass (Pines, Inc.) medium inoculated with *Klebsiella pneumoniae* and supplemented with stigmasterol and harvested by centrifugation (Smith et al., 1987; Sasner & Van Houten, 1989).

Membrane and Cilia Preparations

Plasma membranes were prepared by a modification of the method of Bilinski, Plattner and Tiggeman (1981). Cells in early stationary phase (8,000–10,000 cells/ml) were harvested by centrifugation for 2 min at 350 × *g* (IEC-HNSII centrifuge). The cell pellet was washed twice by centrifugation for 2 min in homogenization medium (20 mM Tris-maleate, pH 7.8, 1 mM Na_2EDTA) and resuspended in ice-cold homogenization medium plus leupeptin (2.2 μg/ml), pepstatin (1 μg/ml) and phenyl-methyl-sulfonyl fluoride (PMSF, 1 mM). 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 centrifuged for 5 min at 1240 × *g* (Beckman J2-21 centrifuge), and the pellet was washed with ice-cold homogenization medium with inhibitors by vigorous mixing for 30 sec. The washes were continued (6–8 times) until the supernatant was clear.

Enzyme assays of 5′ nucleotidase, glucose-6-phosphatase, succinate dehydrogenase and acid phosphatase (methods of Aronson & Touster, 1965; methods of Lee & Lardy, 1965; Linhardt & Walter, 1965; described in Sasner & Van Houten, 1989) demonstrated that his preparation was enriched in plasma membrane and depleted of cilia, mitochondria, and endoplasmic reticulum. Whole cilia were prepared by the methods of Adoutte et al. (1980).

Behavioral Assays

Behavioral assays were T-maze assays (Van Houten, 1978; Van Houten, Martel & Kasch, 1982). All solutions for behavior assays contained 1.3 mM Tris base, 1 mM $Ca(OH)_2$, 1 mM citric acid, pH 7.2 with other salts as indicated. An index of chemoresponse (I_{che}) was measured as the number of cells accumulating in the arm of the T-maze with test solution divided by the total number of cells distributing in the control and test arms. $I_{che} = 0.5$ indicated neutral or no response; $I_{che} >$ or < 0.5 indicated attraction and repulsion, respectively. Competition assays, in which potential competitor compounds are tested for their interference with the attraction to cAMP, were described in detail previously (Van Houten, 1978).

Affinity Chromatography

Cyclic AMP-agarose affinity column matrix was stored in Buffer 1 (50 mM Tris, 2.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM EGTA, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.05% NaN_3, 0.02% isobutyl methyl xanthine (IBMX) pH 7.2). The column was prepared for use by washing with 10 bed volumes (100 ml) of a low pH buffer (0.1 M acetate, 0.05 M NaCl, pH 4.0) alternating with 10 bed volumes of a high pH buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and repeating 4–5 times. The column was then washed for 10 bed volumes with dH_2O and 10 bed volumes of Buffer 1. The plasma membrane proteins were solubilized in 2 ml of 50 mM Tris, 50 mM KCl, 5 mM $MgCl_2 \cdot 6H_2O$, 1 mM EGTA pH 7.4 with 1% Triton X-100, 1 mM PMSF, 2.2 μg/ml leupeptin and 1 μg/ml pepstatin. The solubilized proteins were centrifuged at 6750 × *g* for 10 min and the supernatant was applied to the column. After 1 hr of equilibration, the column was washed consecutively with 3 bed volumes of Buffer 1 containing (*i*) 20 mM KCl, (*ii*) 200 mM KCl, followed by (*iii*) 20 mM K-cAMP or other compound of interest with 200 mM KCl. All subsequent washes of the column were with 200 mM KCl in Buffer 1 with or without additional compounds as indicated.

The column fractions were dialyzed against water, lyophilized, and solubilized in sample buffer for gels or other buffers for other purposes. Alternatively, fractions were concentrated by centrifugation with Amicon Centriprep concentrators.

SDS Gels

SDS polyacrylamide gels were made as 8–12% gradients or homogeneous 8% gels (Laemmli, 1970). Gradient gels generally were run in large (8 × 16 cm) or mini-gel (10 × 10 cm) format and the homogeneous gels in mini-gel or micro-gel (3 × 3 cm) format. The micro-gels were run in an apparatus specifically designed and constructed for us by the laboratory of V. Neuhoff. Gels were stained with Coomassie brilliant blue (R-250 or G-250) or alternatively with a Pierce Chemical silver stain kit. Sample buffer for SDS gels contained 4% (vol/vol) SDS, 0.002% (wt/vol) bromphenol blue, 20% (vol/vol) glycerol, 1.5% (wt/vol) Tris base, and 10% (vol/vol) β-mercaptoethanol.

Detection with Lectins

SDS gels of fractions eluted from the affinity column with cAMP were electroblotted onto nitrocellulose (Towbin, Staehelin & Gordon, 1979). The blot was then incubated with wheat germ agglutinin conjugated with biotin (2 μg/ml) in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0). After washing in TBST, the blot was incubated with avidin conjugated with alkaline phosphatase (1.7 U/ml) in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). Substrates nitro-blue tetrazolium (NBT, 150 μg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 330 μg/ml) were added to the AP buffer and production of violet-blue color was considered an indication of proteins with N-acetylglucosamine moieties (Bayer, Ben-Hur & Wilchek, 1987). One ng of ovalbumin was used as positive control, and similarly, 1 ng of serum albumin (66 kDa) was used as a negative control.

Photoaffinity Labeling

Cells from 100 ml of culture were centrifuged and resuspended in 1 ml buffer for behavioral assays (*see* Materials and Methods) with 0.25 mM cAMP or 8-N_3-cAMP and exposed to ultra violet light (254 nm mineral lamp) at a distance of 5.7 cm for 1 min. This time and distance were empirically determined to be optimal for irradiation without lethality. Cells were centrifuged and resuspended in Buffer 1 with NaCl as control solution for T-maze assays. T-maze assays measured responses to attractants 2.5 mM Na-cAMP, 5 mM NaOAc, or 2.5 mM Na_2-folate relative to comparable amounts of NaCl (2.5 or 5 mM) in buffer. These concentrations of stimuli are known to elicit maximal chemoresponse (Van Houten, 1978; Smith et al., 1987).

Efficiency of photoaffinity labeling was monitored by irradiation of two spots of 8-N_3-cAMP on cellulose TLC sheets with one spot covered followed by chromatography with butanol : acetic acid : water mobile phase of 5 : 2 : 3 (as suggested by Amersham). The nucleotides were visualized with ultraviolet light. The irradiated ligand was covalently bound to the chromatography plate and did not migrate with the spot that was shielded from irradiation.

Covalent linking of 0.6 mCi of 8-N_3-[^{32}P]-cAMP (67 Ci/mmol) to whole cells was carried out as above with the exception that cells from 1 liter of culture were harvested and resuspended in 2.4 ml of 2 mM NaCl in Buffer 1 with isotope. Immediately after a 5-min exposure to UV light, cells were harvested and their plasma membranes isolated. These membranes were solubilized in sample buffer and immediately applied to a large format SDS polyacrylamide gel. The gel was subsequently dried and exposed to x-ray film at −70°C. for varying amounts of time. The film (Kodak X-Omat) was developed and analyzed by densitometry (Hoefer).

Antibody Production

White, New Zealand rabbits were inoculated with Freund's complete adjuvant with emulsified acrylamide gel strips containing the 48-kDa proteins excised from acrylamide gels. The amount of protein injected into four sites was estimated from planimeter measurements of peaks from densitometry scans of the gel before excision of the bands (using low molecular weight markers as standards). Approximately 200 μg of doublet protein was used for each inoculation or monthly boost. Two weeks following each inoculation or boost, blood samples were taken for monitoring antibody titer. ELISAs (enzyme linked immunosorbent assays; Engvall & Perlmann, 1972) were carried out using *Paramecium* cell body membranes as antigen (1 μg protein/well). One μg of cAMP-binding proteins from cAMP affinity column elutions was used in ELISA assays of antibody titer. ELISAs were carried out with horseradish peroxidase conjugated anti-rabbit antibodies (ICN) as secondary antibody. Sera were diluted with phosphate-buffered saline with 10% Tween, and controls with no primary, no secondary, no antigen or preimmune serum were run in parallel (Table 1).

Immunoblots

SDS polyacrylamide gels of cAMP column fractions, whole plasma membranes and whole-cell homogenate were electroblotted onto nitrocellulose membranes. The membranes were cut into replicate sets each including lanes of unstained and prestained molecular weight markers and lanes of membrane proteins. One set was stained with amido black (0.1%) for total protein. Other sets were blocked in TBS (10 mM Tris, 150 mM NaCl, pH 8) with 0.5% nonfat dried milk, incubated with primary antibody (dilution in TBS with 0.05% nonfat dried milk), washed in TBS with milk (Jagus & Pollard, 1988) and incubated in secondary anti-rabbit antibody conjugated with alkaline phosphatase. After washing in TBS with milk, substrates NBT and BCIP were added at 150 and 330 μg–ml in AP buffer and color was allowed to develop for 5 or more min. Development was stopped by washing in water. Only blots without nonspecific reactions with secondary antibody alone are presented here.

Amino Acid Analysis

Cyclic AMP elution fractions were electrophoresed and electroblotted onto polyvinylidene difluoride membranes (PVDF) according to Matsudaira (1987) with the exception that the blotting time was increased to 2 hr and 0.1% EDTA was added to the transfer buffer. The blots were lightly stained with Coomassie blue R-250 and analyzed at the University of California, Riverside, CA, protein analysis facility for total amino acids.

Iodination

Surface labeling of whole cells with [^{125}]NaI was carried out using the iodo-bed technique of Pierce Chemical. Three liters of cells were harvested, washed in Dryl's buffer (1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 mM Na-citrate, 1.5 mM $CaCl_2$, pH 7), resuspended in 1 ml Dryl's buffer, exposed with gentle rocking to 1 mCi [^{125}]NaI (added to the cells in 0.4 Dryl's buffer) and 10 Pierce iodo-beads for 25 min. The cells were then harvested, cell body membranes prepared and proteins separated on gels as described above. The dried gel is exposed to x-ray film for varying amounts of time.

Serotyping Cells

Antisera against cells expressing A or B types of large surface glycoprotein (courtesy of J. Preer; Preer, 1959) were diluted 1 : 20 with the buffer for behavioral assays (*see* Materials and Methods), and cells were incubated in diluted sera or buffer alone for 25 min. The cells' motility was monitored during this time. Cells cease to swim when the appropriate anti-glycoprotein antibodies bind to their surfaces (Preer, 1959).

Materials

Secondary antibodies conjugated with alkaline phosphatase were from ICN; alkaline phosphatase substrates were procured from Promega; ampholytes were from LKB and Pharmacia; and nitrocellulose for blotting was purchased from Gelman. All other chemicals and reagents were supplied through Sigma Chemical. Isotopes were from Amersham ([^{125}]NaI).

Results

Behavioral Tests Show Similar Specificity for cAMP

Cells are attracted to 3′,5′-cAMP (Table 1; Smith et al., 1987). To test the specificity of this attraction, we included potential competitors in *both* the test

Table 1.

Test solution	Control solution	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] 2.5 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] 2.5 mM Na-cAMP + 5 mM NaCl	7.5 mM NaCl	0.88 ± 0.02	3
2.5 mM Na-cAMP + 5 mM Na-cGMP	2.5 mM NaCl + 5 mM Na-cGMP	0.76 ± 0.08	3
[a] 2.5 mM Na-cAMP + 5 mM NaCl	7.5 mM NaCl	0.89 ± 0.01	3
2.5 mM Na-cAMP + 5 mM Na-OAc	2.5 mM NaCl + 5 mM Na-OAc	0.60 ± 0.05	3

An I_{che} value >0.5 indicates attraction; <0.5 indicates repulsion; = 0.5 indicates no response. Data are averages ± SD.
[a] Denotes control for experiment that follows.

and control arms of the T-maze. Competitors, either agonists or antagonists, would be expected to reduce the index of chemoresponse (I_{che}) to neutral by binding to the receptor and making it impossible for the cell to detect the gradient of the cAMP in the T-mazes. When 5′AMP was included in both control and attractant solutions of the T-maze, it interfered with attraction. Likewise, the binding of [^{3}H]-cAMP that correlates with chemoresponse (Smith et al., 1987) was disrupted by 5′AMP. Therefore, 5′AMP appears to be an agonist of attraction to 3′,5′-cAMP as it interfered with cAMP response, and it had attractant properties of its own (I_{che} = 0.74 ± 0.01 for attraction to 5 mM K_2AMP *vs.* 10 mM KCl, n = 3).

A compound structurally related to cAMP, 3′,5′-cGMP, did not interfere with attraction to cAMP when included in two times excess (Table 1), reinforcing the results in controls that ionic or osmotic strength alone was not the cause of the interference by 5′AMP. Surprisingly, Na-OAc, a structurally unrelated attractant stimulus, had inhibitory effects on attraction to cAMP at a ratio of 2 : 1 OAc to cAMP.

The behavioral data predicted that 5′AMP and, to some extent, OAc should elute the cAMP chemoreceptor from the cAMP affinity matrix; cGMP should not. The results of the affinity chromatography (*below*) were consistent with this prediction.

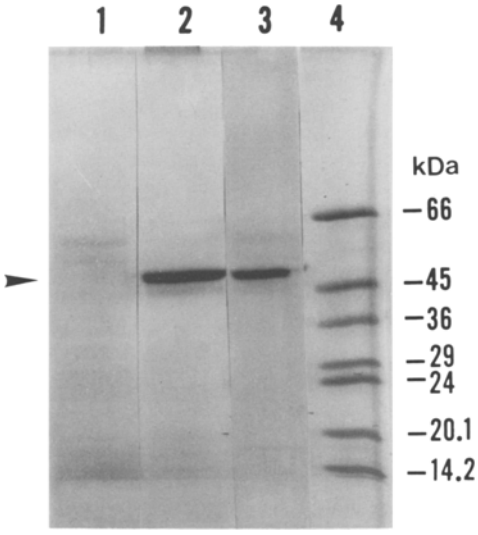

Fig. 1. Lanes from an SDS PAGE 8–12% gradient gel stained with Coomassie blue. Lane *1*, proteins eluting with 3 bed volumes each of 200 mM KCl from the cAMP-agarose affinity column; lane *2*, proteins eluting with 3 bed volumes of 20 mM K-cAMP in 200 mM KCl; lane *3*, proteins eluting with subsequent 3 bed volumes of K-cAMP; and lane *4*, molecular weight markers. The band of interest is at 48,000 M_r between the top two molecular weight markers of 66,000 and 45,000

One Major Band of Protein Is Specifically Eluted with cAMP from Affinity Columns

Cell body membranes were solubilized and loaded onto cAMP-agarose affinity columns. The columns were washed extensively with low and high KCl buffers before elution with 20 mM cAMP in the high salt buffer. As shown in Fig. 1, when the column yielded little protein with the salt wash (lane *1*), the ligand cAMP was able to displace protein that is displayed on SDS gels (lanes *2* and *3*). The protein consistently eluting with cAMP had an approximate molecular weight of 48,000 on SDS PAGE. Other proteins were not always present or eluted with KCl as well as cAMP. Cyclic GMP did not elute protein of 48 kDa that cAMP clearly could displace (Fig. 2*A*, lanes *2–4*), while 5′AMP did elute this protein (Fig. 2*B*, lanes *3–9*). The attractant K-OAc eluted some protein of apporoximately 48 kDa (Fig. 2*C*, lanes *2–4*), but after extensive washing with KOAc (Fig. 2*C*, lanes *2–4*), cAMP still displaced a relatively large amount of protein (Fig. 2*C*, lanes *5–8*). When the order of elutions was reversed, cAMP first

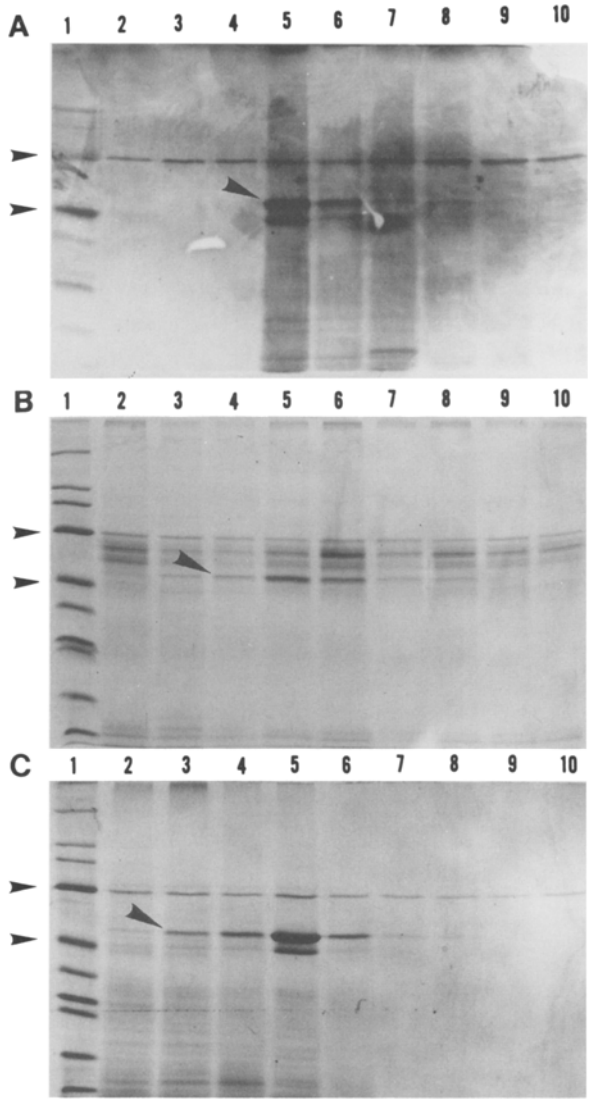

Fig. 2. Three SDS PAGE 8–12% gradient gels of fractions from cAMP affinity columns. In all cases lane *1* contains molecular weight markers. The markers of 66,000 and 45,000 M_r are marked with arrows. (Molecular weight markers in order are 205, 116, 97.4, 66, 45, 36, 29, 24, 20.1, and 14.2 kDa.) All lanes contain proteins from 3 bed volumes of elution. (*A*) In lanes *2–4* are proteins from fractions eluting with 20 mM K-cGMP in 200 mM KCl; in lanes *5–8* each are proteins eluting with 20 mM K-cAMP in 200 mM KCl; and in lanes *9–10* are proteins eluting with 200 mM KCl alone. (*B*) Lanes *2–4* contain proteins eluting with 200 mM KCl; lanes *5–8* contain proteins eluting with 20 mM 5′ AMP in 200 mM KCl; and lanes *9–10* contain proteins eluting with 200 mM KCl. (*C*) Lanes *2–4* contain proteins eluting with 20 mM KOAc in 200 mM KCl; lanes *5–8* contain proteins eluting with 20 mM K-cAMP in 200 mM KCl; and lanes *9–10* contain proteins eluting with 200 mM KCl

displaced all the 48-kDa protein and KOAc subsequently eluted no more (*data not shown*).

The protein of 48 kDa that eluted from the cAMP affinity column appeared to comprise a single, broad band on SDS Page (Figs. 1 and 2). However, when the midrange of the gel was better resolved, the 48-kDa region protein appeared as a closely banding doublet, usually with the upper band more concentrated (Figs. 3 and 6). Both protein bands of the doublet showed the same specificity. It was not possible to selectively elute one without the other with the above-mentioned ligands.

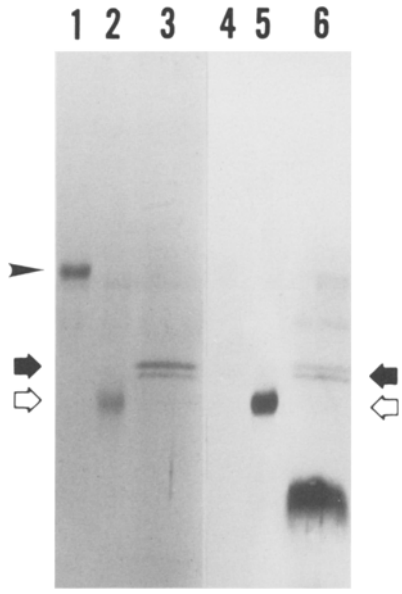

Fig. 3. Results of wheat germ agglutinin binding to electroblots. Lanes *1–3* are stained for total protein with 0.1% amido black; lanes *4–6* are developed with wheat germ agglutinin-biotin and avidin-alkaline phosphatase. Lanes *3* and *6* are proteins eluting from cAMP affinity column with cAMP elution. Large filled arrows point to 48-kDa doublet. Large open arrows point to ovalbumin (45 kDa, 1 ng each in lanes *2* and *5*) as a positive control. Note some multimers of ovalbumin are evident. Small filled arrowhead points to 66-kDa protein in lanes *1* and *4* that contain serum albumin (66 kDa, 1 ng each lane) as a negative control

Doublet Proteins Are Glycosylated

Electroblots of gels of the cAMP elution fractions from the affinity column when incubated with wheat germ agglutinin-biotin complex, followed by avidin-alkaline phosphatase and substrates, showed color development over the doublet of 48-kDa proteins (Fig. 3, lane *6*) as well as over the positive control (lane *5*) and other minor proteins from the cAMP affinity column elution (lane *6*). The negative control, serum albumin, showed no color development (lane *4*). We expect, therefore, that both proteins of the doublet contain N-acetylglucosamine.

Table 2.

Pretreatment	Chemoresponse		
	Test stimulus	I_{che}	n
2.5 mM Na-cAMP	2.5 mM Na-cAMP	0.66 ± 0.05	25
0.25 mM 8-N_3-cAMP	5 mM Na-cAMP	0.50 ± 0.06	14
0.25 mM 8-N_3-cAMP	2.5 mM Na_2folate	0.62 ± 0.07	11
0.25 mM 8-N_3-cAMP	5 mM NaOAc	0.66 ± 0.06	12

Data are averages of n T-mazes ±1 SD. T-maze assays were used to test chemoresponse. Test stimuli and control solutions were balanced for salt (*see* Materials and Methods).

8-N_3-cAMP Inhibits Chemoresponse

When cells were irradiated in the presence of 0.25 μM 8-N_3-cAMP, a photoaffinity probe, they specifically lost their chemoresponse to cAMP. Responses to other stimuli remained within normal ranges (Table 2). We took these results to be further evidence for a surface-exposed cAMP receptor. In keeping with these results, it should have been possible to radiolabel the receptor with ^{32}P-8-N_3-cAMP and, indeed, when cell body membrane proteins from irradiated cells were displayed on gels and autoradiographed, there was a band of 48 kDa riding on a high background of labeled protein (*data not shown*).

The Doublet Proteins are not Immunologically Similar to Related Proteins

The size of the proteins of the doublet was close to that of the *D. discoideum* cAMP receptor, the *Paramecium* cAMP-dependent protein kinase regulatory subunit (M. Hochstrasser & D.L. Nelson, *personal communication*), and some surface proteins related to the large surface glycoprotein (Eisenbach, Ramanthan & Nelson, 1983). Rabbit antiserum against the *D. discoideum* receptor (Klein et al., 1987; antiserum courtesy of P. Devreotes) at very high titer showed no recognition of the *Paramecium* doublet on immunoblots (1 : 66 dilution of primary antibody and 1 : 1000 dilution of secondary antibody with TBS with milk; *data not shown*). Similarly, ascites fluid with monoclonal antibodies against the protein kinase regulatory subunit recognized proteins of 44, or 44 and 48 kDa in cell body and ciliary membranes, respectively (Fig. 4, lanes *6* and *7*), as expected, but did not recognize the cAMP doublet (Fig. 4, lane *8*). Rabbit antiserum against the large, surface glycoprotein recognized proteins in the range of 42–45 kDa on blots of total membrane proteins. However, this antiserum did not recognize the doublet of cAMP binding protein (1 : 500 dilution of primary antibody and 1 : 1000 dilution of secondary antibody with TBST; *data not shown*).

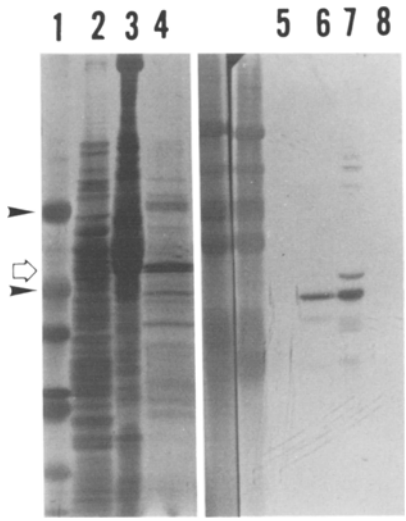

Fig. 4. Immunoblot using monoclonal antibodies against the regulatory subunit of *Paramecium* cAMP-dependent protein kinase. The lanes are two identical sets with lanes *1–4* stained with 0.1% amido black and lanes *5–8* developed with monoclonal antibody. Lanes *1* and *5* are molecular weight markers; lanes unnumbered in center are prestained molecular weight markers used to line up blots; lanes *2* and *6* are total cell body membranes; lanes *3* and *7* are total ciliary proteins; and lanes *4* and *8* are heavily concentrated cAMP affinity column fractions. Doublet at 48 kDa is noted with open arrow. Antiserum was diluted 1:50, and secondary anti-mouse antibodies with alkaline phosphatase were diluted 1:500 using TBS with milk

Antibodies Against the cAMP Receptor Doublet Inhibit cAMP Chemoresponse

Slices of SDS gels with the protein doublet were emulsified in Freund's complete adjuvant and injected into rabbits to develop an antiserum. ELISAs with *Paramecium* cell body membranes or cAMP

Table 3.

Pretreatment	Test stimulus	I_{che}	n
None	Na-cAMP	0.73 ± 0.07	18
Preimmune serum		0.67 ± 0.06	15
Immune serum		*0.50 ± 0.04	16
None	Na-lactate	0.79 ± 0.04	8
Preimmune serum		0.69 ± 0.07	8
Immune serum		0.65 ± 0.06	8
None	NH_4Cl	0.94 ± 0.02	3
Preimmune serum		0.91 ± 0.02	3
Immune serum		0.94 ± 0.02	3
None	Na-OAc	0.82 ± 0.08	11
Preimmune serum		0.72 ± 0.10	12
Immune serum		0.69 ± 0.12	11

Data are averages of n T-mazes ± 1 SD. The T-mazes responses to Na-cAMP after pretreatment with immune serum (*) are statistically significantly different from the responses with preimmune serum or no pretreatment as determined by the Mann-Whitney U test. Within each group of data for each stimulus, all other combinations of responses showed no significant differences. T-mazes compared 2.5 mM Na-cAMP with 2.5 mM NaCl; 5 mM of each of the other stimuli with 5 mM NaCl.

column elution fractions as antigen were used to monitor the increasing titer of the antiserum. After four months, the titer reached its peak and was used to treat whole cells. Cells incubated in a 1 : 40,000 dilution of antiserum showed no chemoresponse to cAMP in amounts that should elicit maximal chemoresponse behavior (Smith et al., 1987), while cells incubated in a 1 : 40,000 dilution of preimmune serum showed a slight decrease in motility, but no statistically significant loss of attraction to cAMP (Table 3). The same cells that were treated with serum and were unable to respond to Na-cAMP showed attraction responses to Na-lactate, NH_4Cl, and Na-OAc within normal ranges of response with or without preincubation in preimmune serum. However, the results of attraction to Na-OAc ranged from no attraction to normal, giving an unusually large standard deviation for normal cells.

Anti-cAMP Receptor Antiserum Recognizes Proteins of 48 kDa

The doublet of proteins of 48 kDa alone was recognized on blots of cAMP elution fractions (Fig. 5). Elution proteins were not detected by the preimmune serum. When the anti-cAMP receptor serum was used in immunodetection of blots of solubilized cell body membranes, one prominent band of protein at 48 kDa was recognized along with several minor bands (*data not shown*). Buffers containing milk were necessary to block electroblots of total cell body membrane proteins in order to eliminate nonspecific reactions of the secondary antibodies alone. However, the time required for color development of these blots led us to believe that the antiserum will not be sufficiently sensitive for many techniques involving nitrocellulose, such as plaque lifts, perhaps because of loss of epitopes by denaturation of the proteins on the nitrocellulose membrane.

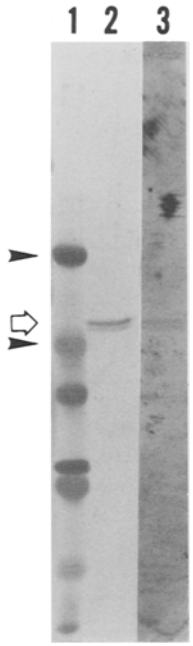

Fig. 5. Immunoblot using polyclonal antibodies against the 48-kDa protein of *Paramecium*. Lanes *1* and *2* are stained for total protein with 0.1% amido black and lane *3* is developed with polyclonal antibody. Lane *1* contains molecular weight markers. (The 66- and 45-kDa markers are identified by small filled arrowheads.) Lanes *2* and *3* are concentrated cAMP affinity column fractions. Doublet at 48 kDa is identified with large open arrow. Antiserum was diluted 1:50, and secondary anti-rabbit antibodies with alkaline phosphatase were diluted 1 : 1000 using TBS with milk

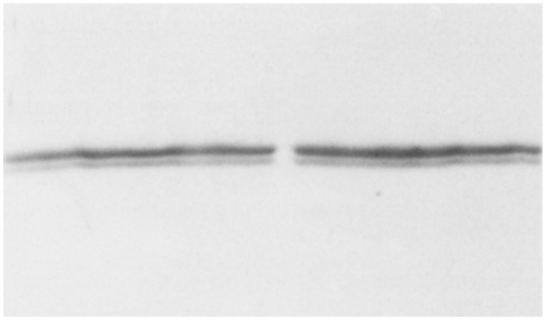

Fig. 6. Electroblot of cAMP elution fraction showing doublet of proteins stained with Coomassie blue

Amino Acid Analysis of the Doublet

Cyclic AMP elution fractions were electrophoresed and blotted onto PVDF membranes (Fig. 6) for total amino acid analysis (Table 4). The amino acid profiles of the two bands of the doublet were similar, although not identical.

Table 4.

Amino acid	Upper band	Lower band
	Residues/Molecule	
ASX	23	47
GLX	35	45
SER	37	31
GLY	58	56
HIS	10	9
ARG	22	19
THR	27	25
ALA	51	41
PRO	20	16
TYR	23	19
VAL	24	22
MET	5	4
ILE	25	28
LEU	42	36
PHE	16	17
LYS	34	32
Total	452	447

Discussion

We present here evidence for a doublet of cAMP-binding proteins that is derived from the cell body membrane of *Paramecium* and satisfies criteria for the cAMP chemoreceptor. First, the doublet of proteins (approximately 48 kDa in apparent molecular mass) is eluted from cAMP affinity columns with cAMP and 5'AMP, but not with cGMP. Assuming that the interaction occurs at the level of receptor binding these elutions are predicted from the inhibition of behavioral response to cAMP by 5'AMP and lack of inhibition by cGMP. K-OAc, a structurally unrelated stimulus, elutes a fraction of the doublet but clearly the majority of the protein is eluted with cAMP only. Again, the tests of inhibition of behavior predict that OAc could have an effect on the cAMP-binding protein that is the receptor.

Second, photoaffinity labeling of whole cells with 8-N_3-cAMP specifically inhibits attraction to cAMP and provides evidence (in addition to radioligand binding [Smith et al., 1987]) that there are, indeed, surface-exposed receptors. The assumption here is that the ligand is covalently bound to its receptor, among other proteins, and that consequently the cell is unable to detect new gradients of cAMP and respond accordingly in the T-maze behavioral assay. Protein of approximately 48 kDa molecular mass is covalently labeled with 8-N_3-[^{32}P]-cAMP on whole cells among other cell body membrane proteins. This is no proof that this labeled protein has a relation to the receptor, because under the conditions used, all proteins that bind cAMP specifically or nonspecifically would be labeled. Ideally, excess competing cAMP or cGMP would be included during the labeling to determine which peaks contain proteins that bind cAMP preferentially and specifically, as expected for the receptor. Unfortunately, the cost is prohibitive to use the label even at 10-fold less than the K_D and, therefore, these experiments could not be done.

Third, the doublet proteins are glycoproteins and, therefore, are surface exposed (Sturgess, Moscarello & Schachters, 1978), as expected for a receptor. The carbohydrate moiety was revealed to be N-acetylglucosamine, by binding of wheat germ agglutinin to the doublet. (Since the similar experiments with concanavalin A gave nonspecific results, we do not know whether α-D-mannose and α-D-glucose are represented in the doublet.) The glycoprotein tests also serve to verify that the doublet proteins are accessible at the cell surface, as would be expected for a receptor, since surface glycoproteins have the carbohydrate moiety exposed to the outside, not the inside of the cell (Sturgess et al., 1978). Attempts to demonstrate surface exposure by iodination of the doublet by labeling whole cells with [^{125}]NaI failed, indicating only that tyrosines and secondarily serines were not available for covalent labeling (*data not shown*).

Fourth, anti-serum produced against the doublet selectively eliminates chemoresponse to cAMP in whole cells. The dilution sufficient to eliminate chemoresponse is high (1 : 40,000), and there appears to be little nonspecific effect on motility. Other

chemoresponses clearly are intact. Our interpretation is that the antibodies bind to and occlude the cAMP-binding sites on the receptor and, as in photoaffinity crosslinking, prevent the receptor from detecting new gradients of stimulus. This very specific inhibition of chemoresponse is, perhaps, the strongest evidence that the doublet includes the receptor.

Considering that OAc elutes a small subset of the doublet from affinity columns and inhibits cAMP chemoresponse, there may be an epitope in common between the OAc and cAMP receptors and this epitope could be the OAc-binding site on the OAc receptor. However, the chemoresponse to OAc by anti-serum treated cells is not statistically significantly different from the response of preimmune treated cells, although the average response is lower and the range and standard deviation are very large. Therefore, it seems unlikely that the two receptors are the same molecule with different binding sites for stimuli. More likely, there may be regions of primary or tertiary structure shared by separate receptors. In support of this presumption is a mutant with a single site mutation that very specifically affects OAc attraction and resistance to IOAc, but not chemoresponse to cAMP (*unpublished results*).

The doublet of protein is very reminiscent of the doublet of 40 and 43 kDa that comprise the *D. discoideum* cAMP surface receptor (Klein et al., 1988). In *Dictyostelium,* the same protein moiety changes apparent molecular weight with increasing phosphorylation as part of the receptor desensitization process (Vaughan & Devreotes, 1988). It is very likely that the doublet of *Paramecium* surface protein is one protein with differing amounts of covalent modification or proteolysis making slight changes in mobility on gels. No method of separating the proteins on the basis of affinity, glycosylation or pI has yet been found. (Two-dimensional electrophoresis demonstrates that a protein of the appropriate molecular weight focuses at pH 6.6, but the second protein of the doublet has not yet been resolved.) The total amino acid analyses are similar and, because there are some amino acid differences expected to account for molecular weight differences, are consistent with either scenario. A more definitive determination of their relationship awaits antibody production against the upper band of the doublet or cloning of their respective genes.

Since the doublet binds cAMP it might share epitopes with the *D. discoideum* cAMP receptor. To test this, anti-serum against the *Disctyostelium* receptor was used on immunoblots, with no recognition of the *Parmecium* doublet. Similarly, monoclonal antibodies against another cAMP-binding protein from *Paramecium,* the regulatory subunit of the cAMP-dependent protein kinase, did not recognize the doublet. Additionally, the receptor doublet is not identical to the surface proteins that are antigenically related to the large surface glycoprotein of *Paramecium* and that come close to the doublet in apparent molecular weight (42–45 kDa). There was no recognition of the cAMP receptor doublet from cells expressing surface glycoprotein A using anti-A antiserum on immunoblots. Therefore, the doublet of cAMP-binding proteins is not identical to other binding proteins or surface proteins previously described.

Adenine compounds were first screened for their ability to inhibit chemoresponse to another stimulus, folate, because it was reported that adenine could share folate transport in some cells (*see* Schulz et al., 1984, for discussion). Cyclic AMP subsequently was found to have attractant properties of its own. The natural source of cAMP for paramecia could be bacteria (Makman & Sutherland, 1964), in which case, cAMP probably signals the presence of food to the paramecia. It is also possible that not 3′,5′-cAMP but 2′,3′-cAMP is the naturally occurring stimulus for which receptors evolved because 2′,3′-cAMP is just as potent an attractant as the 3′,5′-cAMP isomer (T. O'Reilly & J. Van Houten, *unpublished observations*). Other aquatic organisms respond to adenine nucleotides (Carr & Thompson, 1983), and, indeed, the preference for ATP or AMP seems to be a function of preference for live or dead, and therefore, decaying flesh (Zimmer-Faust & Martinez, 1988). However, in addition to *Paramecium,* only *Dictyostelium* has been shown to respond to cAMP as an attractant stimulus, and in the case of the slime mold amoebae, folic acid serves to signify the presence of bacteria and cAMP serves to bring cells together for the process of developing into a slug (Devreotes & Zigmond, 1988). For paramecia, both folic acid and cAMP appear to signify food.

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