EVIDENCE FOR A PRONOUNCED SECRETION OF CYCLIC AMP BY *TETRAHYMENA*

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ABSTRACT

The unicellular eukaryote *Tetrahymena pyriformis* secretes significant amounts of cyclic AMP into its external medium. Cells transferred from growth medium into any of the following three different non-nutrient media: (*a*) 5 mM phosphate buffer containing 47 mM NaCl and 1 mM $MgSO_4$, (*b*) 10 mM Tris, or (*c*) 1.3 mM Tris containing 1 mM citrate and 1 mM $Ca(OH)_2$, released to the outside almost 60–80% of the total cyclic AMP produced during 2–5 h of incubation. Tris-citrate-Ca^{+2} medium was chosen for further experiments because of its minimal nonspecific interference in the cyclic AMP radioimmunoassay.

The identity of the secreted material recognized as cyclic AMP by radioimmunoassay was confirmed by demonstrating its almost complete hydrolysis with commercial beef heart phosphodiesterase. Furthermore, the radioimmunoassay-active material in the concentrated medium co-chromatographed on paper with [^{3}H]cyclic AMP, as judged by assay of the eluted material.

After resuspending cells in Tris-citrate-Ca^{2+} medium, the extracellular concentration of cyclic AMP rose steadily over a 5-h period, reaching a level equivalent to ~35–50 pmol cyclic AMP/10^6 cells vs. an internal cyclic AMP quantity at 5 h of 8–10 pmol/10^6 cells. After 5 h, the level of extracellular cyclic AMP reached a plateau. There was no degradation or uptake of external cyclic AMP by the cells during this period.

The unicellular eukaryote *Tetrahymena pyriformis* has been shown to vary its intracellular concentration of cyclic AMP under different physiological conditions (6, 27). *Tetrahymena* has also been reported to contain an adenylate cyclase that responds in vitro to β-adrenergic agonists. Our own recent analyses (15) produced evidence suggesting that agents that depolarize the plasma membrane can raise internal cyclic AMP levels. In the course of this work, we also discovered that *Tetrahymena* secretes cyclic AMP into its medium. Because the secretion of cyclic AMP has been detected in several cell types (2, 4, 8, 10, 12, 13, 18, 19) and is known to serve a regulatory function in some cases (8, 14), we initiated the present study to characterize the extrusion of cyclic AMP by *Tetrahymena*.

MATERIALS AND METHODS

Culture Conditions for Normal Growth

Unless specified otherwise, *T. pyriformis* NT-1 was grown with shaking in medium containing 2% proteose peptone, 0.2% yeast extract, 0.5% glucose, and a trace of chelated iron (23) at 28°C and allowed to reach the mid-logarithmic phase of growth (1×10^5 to 2×10^5 cells/ml).

Starvation Conditions

A measured volume (100–1,000 ml) of the above cell suspension (depending on the number of cells required for a given experiment) was centrifuged at 800 *g* at 4°C in a Sorvall RC-2B refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) for 5 min. After immediate aspiration of the supernate, the cells were pooled and washed in 200 ml of inorganic medium consisting of 47 mM NaCl and 1 mM $MgSO_4$ in 5 mM potassium phosphate buffer, pH 7.2 (9).

After recentrifugation, the cell pellets were resuspended in ~5 ml of the desired test medium and diluted with more of the medium to yield a cell density of ~1 × 10^5 cells/ml. This cell suspension was then incubated with shaking at 25°-26°C. In experiments involving Tris-citrate-Ca^{+2} buffer, the cell pellets were first resuspended in 5 ml of inorganic medium (9) and then diluted with the required quantity of Tris-citrate-Ca^{+2} medium. This procedure prevented difficulties that were experienced in dispersing cells from the pellet when the Ca^{+2} containing medium was added directly to them.

Non-nutrient media used in this report for the incubation of cells were: (*a*) 5 mM potassium phosphate buffer, pH 7.2, containing 47 mM NaCl and 1 mM $MgSO_4$ (9), (*b*) 10 mM Tris-HCl, pH 7.3, and (*c*) 1.3 mM Tris, 1 mM citric acid, and 1 mM $Ca(OH)_2$, pH 6.5 (24).

Sample Preparation for Cyclic AMP Assay

After incubating cells in the starvation medium for various times as indicated in the text, samples were prepared for cyclic AMP determination as follows:

A required aliquot of cell suspension (100–200 ml) was centrifuged at 1,460 *g* for 10 min at 4°C. The supernate, after aspiration into a clean Buchner flask, was recentrifuged to remove any residual cells. A measured volume of cell-free supernate was heated to 80°C for 7 min and then lyophilized.

Cells from the above centrifugation were resuspended in 5 ml deionized water and treated with 5 ml of cold 12% TCA. The cell suspension was sonicated for 2 min with a probe type sonicator set for maximum output and left at 4°C for 2–3 h. The acid-precipitable material was discarded after centrifugation in a clinical centrifuge at 150 *g* for 4 min. The acid-soluble material in the supernate was washed four times with 4 vol of water-saturated diethyl ether. After removing traces of ether from the aqueous sample by heating it briefly, the sample was lyophilized.

The lyophilized samples were reconstituted with an appropriate volume (generally 1–2 ml) of 0.05 M sodium acetate buffer, pH 6.2, and stored frozen for assay.

Cyclic AMP Measurement

Cyclic AMP was measured by the method of Steiner et al. (22), with a cyclic AMP radioimmunoassay kit (New England Nuclear, Boston, Mass.). Aliquots (200 μl) of the reconstituted samples and related standards were measured in duplicate.

Paper Chromatography of Cyclic AMP

A 200-μl aliquot of the reconstituted cell supernate was co-chromatographed with 5 μl (17,800 cpm) of tracer [^{3}H]cyclic AMP on Whatman No. 1 paper, using the solvent system 1.0 M ammonium acetate: ethanol 30:70 vol/vol (20). As a standard, an equal amount of [^{3}H]cyclic AMP, mixed with authentic carrier cyclic AMP was chromatographed on the same paper.

The location of the cyclic AMP standard was detected with a shortwave UV lamp. The corresponding region (4 × 2.5 cm) of the sample lane as well as equal areas above and below this spot were cut out. A blank area was also obtained from the standard lane. The paper strips were extracted individually with 10 ml deionized water (three extractions of 3 ml each followed by a final wash with 1 ml). Each combined extract was centrifuged to remove paper fibers, lyophilized, reconstituted with 0.05 M sodium acetate buffer, and assayed for cyclic AMP. An aliquot was also counted for [^{3}H]cyclic AMP.

Phosphodiesterase Assay

Commercial beef heart phosphodiesterase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) at a concentration of 10 mg/ml, sp act, 0.25 U/mg at 25°C. A suitable aliquot of the cell supernate was treated with 50 μl (0.125 U) of phosphodiesterase for 60 min at 28°C. The reaction was terminated by heating the sample for 7 min at 80°C. The contents of the tube were centrifuged and the supernate was used for cyclic AMP measurement. A suitable blank was prepared by incubating the cell supernate with heated phosphodiesterase under identical conditions.

RESULTS

During experiments designed mainly to measure intracellular cyclic AMP levels in *Tetrahymena* (15), we frequently detected a cyclic AMP-like substance in the proteose peptone growth medium left after centrifuging aliquots of cells. At the time, our efforts to characterize this substance were frustrated by technical problems, not the least of which was a high and variable background value detected with the standard cyclic AMP radioimmunoassay technique. However, because of the potential significance of the finding, we have modified our experimental approach, redirecting attention to the behavior of cells transferred from proteose peptone medium into several simpler media, including those shown in Table I. The radioimmunoassay background was very substantially re-

TABLE I

Extrusion of Cyclic AMP by Tetrahymena during Incubation in Various Media

Cell medium	Degree of medium concentration before assay	"Cyclic AMP" in medium after incubation with cells	"Cyclic AMP" in control medium not incubated with cells
		pmol/200 μl	*pmol/200 μl*
Proteose peptone	None	90	80
K phosphate buffer, NaCl, $MgSO_4$	50	62	4.3
Tris-citrate-Ca^{2+}	50	52	1.8
10 mM Tris	50	58	24

Cells were centrifuged and resuspended in one of the above media as described in Materials and Methods. After incubation for 2–5 h, the medium was recovered by centrifugation and was, in most cases, concentrated by lyophilization. The lyophilized residue was reconstituted in a smaller volume of acetate buffer, as indicated in column 2, and 200-μl aliquots were assayed for cyclic AMP. Control medium was prepared by concentrating an equal amount of the respective medium that had not been exposed to cells and reconstituting it with acetate buffer as done with the corresponding cell supernate.

duced in these media, as compared with that in proteose peptone, and a surprisingly large amount of cyclic AMP-like material was released during a 2- to 5-h incubation with cells.

After establishing that secretion did occur in each of these commonly used media, we chose to use the Tris-citrate-Ca^{2+} medium for all additional experiments reported here, as this medium evoked the least amount of interference in the radioimmunoassay. It was also of special interest because it has been employed for chemokinesis studies of ciliates (24–26).

Logarithmic phase *Tetrahymena* cells transferred to the Tris-citrate-Ca^{2+} medium rapidly decreased their rate of cell division so that no significant cleavage was detectable after 3-4 h. However, these cells appeared normal by phase microscopy and, on occasion, were observed to remain viable for several days. Under conditions similar to those used by Van Houten for *Paramecium* (25), the *Tetrahymena* cells displayed strong chemokinetic behavior.

Secretion of cyclic AMP-like material into the Tris-citrate-Ca^{2+} medium in quantities comparable to those shown in Table I was confirmed in several similar experiments. The amounts released were always at least twice and sometimes as much as five to seven times the amounts retained within the cells (an average of 9 pmol/10^6 cells).

To show that the high level of putative cyclic AMP was not merely an artifact caused by the sample preparation and assay procedures, authentic cyclic AMP was added to the Tris-citrate-Ca^{2+} medium (not exposed to cells) before concentration, reconstitution, and assay. In one such test, an aliquot of the reconstituted medium calculated to contain 12.5 pmol cyclic AMP was estimated by radioimmunoassay to contain 15.5 pmol cyclic AMP.

The radioimmunoassay procedure used for quantitative cyclic AMP determination is recognized as one of the most specific and accurate methods presently available for measuring this nucleotide (7). However, because secretion of cyclic AMP by *Tetrahymena* has not been reported previously, additional criteria were employed to establish the compound's identity. An aliquot of the concentrated medium from *Tetrahymena* incubations was chromatographed on paper alongside an authentic cyclic AMP standard. To each sample was added a tracer amount of [^{3}H]cyclic AMP. Essentially all of the radioimmunoassay-positive material and all of the ^{3}H counts chromatographed together in both the sample lane and the standard lane (Table II). The R_f value of the *Tetrahymena*-derived sample and its co-chromatographed [^{3}H]cyclic AMP was slightly lower than that of the standard cyclic AMP (and its accompanying [^{3}H]cyclic AMP) run in the adjacent lane, but this difference seems to be caused by interference by materials in the medium. The ^{3}H material in both lanes, R_f ~0.49, was well separated from other nucleotides of interest, e.g., 5′-AMP, R_f 0.22; cyclic GMP, R_f 0.20.

TABLE II

Paper Chromatographic Co-migration of [^{3}H]Cyclic AMP with Active Material Secreted by Tetrahymena

Area eluted	Recovered [^{3}H]-cyclic AMP	Recovered cyclic AMP (by radioimmunoassay)
	cpm	*pmol*
Lane A		
Cyclic AMP standard	14,300	500
Blank	120	<0.25
Lane B		
Cyclic AMP-containing area*	13,300	90
Blank 1	250	2.8
Blank 2	340	<0.25

Approximately 1 μg of an authentic cyclic AMP standard was applied to each of two positions (lanes A and C) on Whatman No. 1 chromatographic paper. A 200-μl aliquot of concentrated medium from 5-h starved *Tetrahymena* was spotted in lane B, between the two standards. Then, after applying a tracer amount (17,800 cpm) of [^{3}H]cyclic AMP to each of the three spots, they were chromatographed, and appropriate areas were eluted and assayed as described in Materials and Methods.

* This material and its co-chromatographed [^{3}H]cyclic AMP were recovered at a slightly lower position than was the immunologically active material in lane A.

As a further characterization of the secreted material, its sensitivity to beef heart phosphodiesterase was tested. Cell-free supernate from 200 ml cell suspension incubated in Tris-citrate-Ca^{2+} medium for 5 h was concentrated and reconstituted in 2 ml of acetate buffer as described in Materials and Methods. Aliquots of this sample were processed as follows:

An aliquot was assayed in duplicate for cyclic AMP without any further treatment. A second aliquot (400 μl) was incubated with 50 μl (0.125 U) of phosphodiesterase for 60 min, heated (80°C for 7 min) to stop the reaction, then centrifuged, and aliquots of the supernate were assayed. The

third aliquot (400 μl) was incubated under identical conditions with heat-killed enzyme, centrifuged, and assayed. Radioimmunoassay gave the following results: sample 1 (control), 26 pmol cyclic AMP/0.1 ml; sample 2 (phosphodiesterase-treated), 2.0 pmol cyclic AMP/0.1 ml; sample 3 (treated with heat-denatured phosphodiesterase), 32 pmol cyclic AMP/0.1 ml. It is apparent from the above data that incubation with active phosphodiesterase destroyed over 90% of the extracellular cyclic AMP present in the medium.

We concluded from the foregoing experiments that *Tetrahymena* cells are indeed capable of secreting cyclic AMP into their surrounding medium. Tests were next conducted to determine the rate of cyclic AMP appearance in the medium and to correlate the levels of intracellular and extracellular cyclic AMP. These results are illustrated in Fig. 1.

It is apparent from the figure that cyclic AMP extrusion was rapid during the first few hours after resuspending cells in the Tris-citrate-Ca^{2+} buffer.

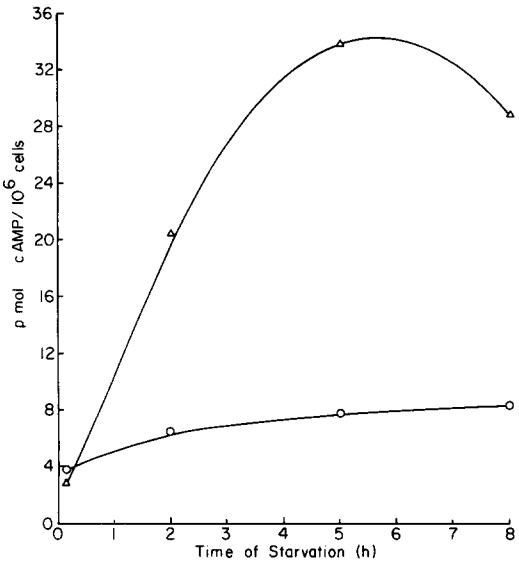

FIGURE 1 Cells in mid-log phase were centrifuged, pooled, and resuspended in 1 l of starvation medium to yield a cell density of ~100,000 cells/ml. 200 μl of [^{3}H]cyclic AMP marker was added and, after mixing, 200-ml aliquots of cell suspension were distributed into each of four Erlenmeyer flasks and incubated with shaking. The flasks were harvested at 0, 2, 5, and 8 h as described in Materials and Methods. An aliquot of the reconstituted sample was assayed for cyclic AMP and also counted for [^{3}H]cyclic AMP. ○, cyclic AMP in cells. △, cyclic AMP in the medium.

By approximately 5 h after the cells were placed in this medium, the quantity of extracellular cyclic AMP was nearly five times greater than that retained by the cells.

The experiment shown in Fig. 1 also included a test for the destruction or cellular uptake of extracellular cyclic AMP. A tracer amount of [^{3}H]cyclic AMP was added to the suspending medium at 0 time, giving a final concentration of 1,400 cpm/ml. Radioactivity was measured in the reconstituted concentrates of samples taken at periodic intervals. The values of recovered ^{3}H were 3,900; 4,042; 3,654; and 3,592 cpm/0.1 ml at 0, 2, 5, and 8 h, respectively. Even after 8 h, almost all the recovered radioactivity migrated with authentic cyclic AMP on paper chromatograms. Thus under these conditions, there was no appreciable uptake of cyclic AMP by the cells nor was there any significant destruction of cyclic AMP in the medium by extracellular phosphodiesterases.

The pattern of cyclic AMP appearance in the medium indicated that some physiological response (perhaps starvation) initiated by cell resuspension in Tris-citrate-Ca^{2+} medium caused an increased production and active extrusion of the cyclic nucleotide. But the experiment did not prove that cyclic AMP secretion was spontaneous and continuous during that period. Alternatively, cyclic AMP might accumulate in some type of extrusion organelle (11) capable of being explosively discharged by the trauma of our final centrifugation.

The possible existence of an easily discharged cyclic AMP compartment was tested by comparing the extracellular cyclic AMP level in identical 50-ml aliquots of 4.5-h starved cells. One aliquot was centrifuged at 1,460 *g* for 10 min at 25°C, while the other was centrifuged at <60 *g* for 0.5 min at 25°C. The latter cells were not pelleted, but were merely concentrated in the lower half of the centrifuge tube. Recoveries of extracellular cyclic AMP were: high speed supernate: 49.7 pmol/10^6 cells; low speed supernate: 52.6 pmol/10^6 cells. Likewise, the effect of low temperature on cyclic AMP release was examined by chilling aliquots of cells to 4°C for 10 min and comparing cyclic AMP in their medium with cyclic AMP in medium of equivalent but unchilled cell suspensions. The supernate after low speed centrifugation contained, in the case of chilled cells, 36.6 pmol/10^6 cells, and for unchilled cells, 36.9 pmol/10^6 cells. The results of these tests argue against the likelihood of massive cyclic AMP discharge during sample prepa-

ration. On the other hand, we have upon occasion found indications of small decreases in intracellular cyclic AMP during chilling and centrifugation. This may result from intracellular phosphodiesterase activity.

Does extracellular cyclic AMP have a significant biological function in *Tetrahymena*? A definitive answer cannot be provided at this time. We have begun to investigate its possible role in several physiological processes, including phagotrophy, chemokinesis, and sexual conjugation. Our preliminary findings indicate that while cyclic AMP apparently does, under certain conditions, have a distinct effect upon the latter two processes, unidentified variables in the test systems must be more adequately controlled before firm conclusions are reached. Efforts to develop more reproducible test systems are underway.

DISCUSSION

The extrusion of intracellular cyclic AMP into the surrounding medium, first demonstrated in pigeon erthrocytes (5), has now been widely observed (2, 4, 10, 12, 13, 18, 19), but in most cases neither the mechanism of extrusion nor the biological significance of the process is understood. The only clear insight into the physiological role of extracellular cyclic AMP comes from work with certain cellular slime molds, such as *Dictyostelium discoideum*, in which external cyclic AMP behaves as a primary messenger for cellular morphogenesis and also serves as a powerful chemoattractant (8).

Characterization of cyclic AMP secretion in higher organisms, including most cultured vertebrate cells, has shown that extrusion of the nucleotide is preceded by its intracellular accumulation in response to action by epinephrine or some other catabolic hormone (4, 5, 19). While one can envision the extrusion process in these systems as being one of several mechanisms for swiftly reducing the internal cyclic AMP level to its basal value, the secretion of cyclic AMP by certain lower organisms (10, 18) cannot be so logically explained. These organisms may routinely secrete almost 90% of the cyclic AMP they produce into the medium, especially during periods of starvation (8, 18). This phenomenon frequently occurs without any marked intracellular cyclic AMP accumulation (10, 18). The results presented above illustrate that *Tetrahymena* falls into this latter category.

It is tempting to speculate that extracellular cyclic AMP has some functional significance in *Tetrahymena*. An attempt to determine the function of external cyclic AMP in *Polysphondylium* was equally frustrating (10). Providing exogenous cyclic AMP to *Polysphondylium* amoeba did not produce the dramatic effect seen in *Dictyostelium*, but instead had only marginal effects, such as increasing slightly the onset of aggregation. In view of the very complex interactions known to take place among the cyclic nucleotides, phosphodiesterases, Ca^{2+}, calmodulin, and other regulatory factors participating in these systems, it seems likely that a systematic study may well reveal a function for external cyclic AMP.

One feature common to most of the lower organisms thus far examined is the enhanced secretion of cyclic AMP under experimental conditions leading to a cessation of growth, e.g., attaining high cell densities or being deprived of nutrients (10, 12, 14, 18). In such cases, external cyclic AMP may serve as a signal of impending unfavorable conditions. It is common under these circumstances to observe morphological changes, such as aggregation in slime molds (14) or heterocyst formation in *Anabena* (12).

Analogous behavior has been demonstrated in *Tetrahymena*. For example, starvation transforms cells into a morphologically distinct phenotype designated as the dispersal form (16, 17). We have observed the appearance of this form during the period 3–5 h after the onset of starvation, when extracellular cyclic AMP reaches a significant concentration (S. G. Nandini-Kishore and G. A. Thompson, unpublished results). Several other physiological responses of *Tetrahymena* are also induced by starvation. Sexual conjugation between compatible mating types requires preincubation of the cells for several hours in 10 mM Tris (3, 28) or a similar nutrient-free medium (1). Chemokinesis (in the related ciliate, Paramecium) is induced by resuspending cells in Tris-citrate-Ca^{2+} medium (25, 26). In these and related investigations, we are unaware of any published inquiry into possible fluctuation in extracellular cyclic AMP levels.

Although in our system, cyclic AMP concentration changes accompany starvation, we have not proved a direct cause and effect relationship. The straightforward approach of comparing growing and starving cells is inexpedient because the growth medium interferes with cyclic AMP measurement. We are exploring the other means for clarifying the role of starvation in cyclic nucleotide metabolism.

Because of the key physiological role played by

external cyclic AMP in *Dictyostelium*, we feel that its possible participation in the control of *Tetrahymena* behavior must also be investigated. Efforts are currently underway in our laboratory to confirm the action of external cyclic AMP in promoting developmental changes in this ciliate.

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