Cyclic AMP is involved in sexual reproduction of *Chlamydomonas eugametos*

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When *plus* and *minus* mating type gametes of *Chlamydomonas eugametos* were mixed, a rapid transient increase in the amount of cAMP was observed with a maximum at 20 s after the start of the sexual agglutination reaction. The transient increase only occurred within the cells and was also exhibited when cell suspensions of single mating type were presented with isolated flagella of the other mating type. Cyclic AMP-dependent protein kinase and cyclic AMP-phosphodiesterase activities were found in cell homogenates. Since the rise in cAMP concentration preceded all known morphological and physiological changes in the cells that prepare them for fusion, it might be a primary response, induced by sexual agglutination.

Chlamydomonas *Sexual reproduction* *Agglutination* *Signalling* *Cyclic AMP* *Protein kinase*

1. INTRODUCTION

Sexual reproduction in *Chlamydomonas* proceeds in steps. When gametes of opposite mating type (mt^+ and mt^-) are mixed, they agglutinate via their flagellar surfaces. Agglutination induces the formation of an intracellular signal, which causes the outgrowth of a papilla at the anterior end of the cell body. The gametes eventually fuse in pairs via their papillae. The nature of the signal that triggers papilla formation is unknown. The agglutination receptors which are involved in sexual agglutination are thought to cluster and to be transported to the tips of the flagella, which results in a change in the ultrastructure of the tips [1]. It was found that papilla formation was strongly correlated with this change in the tip ultrastructure, which suggests that a signal is evoked in the tips of the flagella, and relayed to the flagellar bases where the papilla is formed. Others [2,3] presented evidence suggesting that the intracellular release of Ca^{2+} triggers papillar outgrowth.

Here, we present data indicating that sexual agglutination induces a rapid, transient 30-fold increase in intracellular cyclic AMP (cAMP) concentration. Our data suggest that cyclic AMP may be a primary response after mt^+ and mt^- flagella make contact.

2. MATERIALS AND METHODS

2.1. *Cell culture*

C. eugametos, strains UTEX 9 (mt^+) and UTEX 10 (mt^-) (Algal Culture Collection, University of Texas at Austin) were cultivated in petri dishes as in [4]. Sexually active gamete suspensions were obtained by flooding 2–3-week old agar plates with distilled water or 10 mM Hepes buffer (pH 7.2) contaiing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (HMC buffer). Sexual agglutination between cells of opposite mating type was initiated by mixing mt^+ and mt^- cells 1:1. Flagella were isolated as in [4].

2.2. *cAMP determination*

Intracellular cAMP concentrations were determined in samples, obtained after centrifuging mating cells through a layer of silicone oil (AV 200/AV20; Goldschmidt, Amsterdam) into 50 µl of 3 M perchloric acid. An aliquot of the acid layer was neutralized with ice-cold 3.5 N KOH, contain-

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ing 1.5 N $KHCO_3$, centrifuged for 1 min at 10000 × *g* at 4°C and assayed as described below. Extracellular medium was lyophilized and dissolved in a small volume of distilled water before measuring the cAMP concentration. Samples were used either the same day or stored at −20°C. Two competition binding assays were used for determining cAMP concentration. In one assay we used the regulatory subunit of cAMP-dependent protein kinase I from bovine muscle [5] as a cAMP binding protein, as in [6]. In the other assay a highly specific antibody against cAMP was used [7], obtained from New England Nuclear (Dreieich).

2.3. *Enzyme activities*

Cell homogenates were prepared as follows. Cell suspensions of approx. 2×10^9 cells (cell density varying between 1 and 5×10^7 cells·ml^{-1}) were centrifuged for 10 min at 2000 × *g* and the remaining cell pellet dissolved in approx. 5 ml of homogenization buffer containing 10 mM Hepes, 250 mM sucrose, 7 mM $MgCl_2$, 2 mM EDTA, 10 mM 2-mercaptoethanol (pH 7.6), at 4°C. An equal volume of glass beads (Ø 0.5 mm) was added and the suspensions were mixed for 5 min on a whirl mixer, at 4°C, to disrupt all cells completely. Fractionation of a crude cell homogenate in a soluble and a membrane fraction was achieved by centrifugation for 30 min at 40000 × *g*, at 4°C. The pellet was washed once in 2 ml homogenization buffer. All fractions were assayed for phosphodiesterase activity, at 0.5 μM cAMP, as in [8]. Protein was determined as in [9].

cAMP-dependent protein kinase activity in a fractionated soluble cell fraction was determined using the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly as an artificial phosphate acceptor [11]. The soluble cell fraction was fractionated by chromatography on DEAE–cellulose (DE-52). The column was equilibrated with homogenization buffer without sucrose. After sample application the column was eluted with about 4 column volumes of homogenization buffer without sucrose and subsequently eluted with a 0–0.5 M NaCl gradient in the same buffer. The obtained fractions were assayed for cAMP-dependent protein kinase activity as in [10].

2.5. *Materials*

Hepes, kemptide, IBMX (3-isobutyl-1-methylxanthine), and Dowex 1X2 were obtained from Sigma (St Louis, MO), [γ-^{32}P]ATP from New England Nuclear and [5′,8-^{3}H]cAMP from The Radiochemical Centre (Amersham). Beef heart phosphodiesterase was supplied by Boehringer (Mannheim), DEAE–cellulose (type DE 52) by Whatman (Maidstone), 3′,5′-cyclic AMP by Serva (Heidelberg) and perchloric acid, theophylline and EDTA were supplied by British Drug Houses (Poole). 2-Mercaptoethanol was from Fluka (Buchs).

3. RESULTS AND DISCUSSION

3.1. *Mating-induced changes in cAMP concentration in* C. eugametos *gametes*

Fig.1 shows the changes in cAMP concentration in a cell suspension after mixing mt^+ and mt^- gametes. The intracellular cAMP concentration rose sharply and reached a peak after approx. 20 s, followed by a decrease: The cAMP concentration did not regain the low pre-agglutination level, even after several minutes. Maximum cAMP concentration varied between 20 and 40 pmol cAMP per 10^8

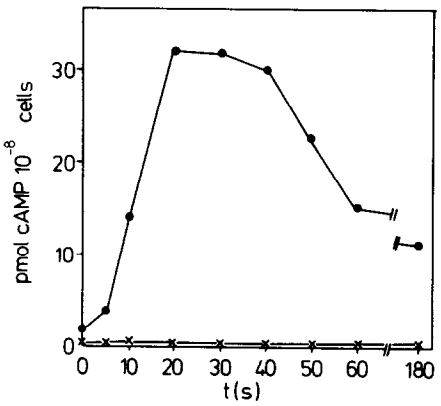

Fig.1. Changes in the concentration of cAMP in a suspension of mixed mt^+ and mt^- gametes of *C. eugametos*. The agglutination reaction was started by mixing 125 μl mt^+ cells with 125 μl mt^- cells (cell densities: 0.20 and 0.13 × 10^8 cells·ml^{-1}, respectively). The agglutination reaction was terminated by separation of the cells from the extracellular medium by centrifugation through a layer of silicone oil. The mating competence of the cells was 75%. Intracellular cAMP concentration (●—●) and extracellular cAMP concentration (×—×) were determined using a radioimmunoassay for cyclic AMP [7].

Table 1

Competition of endogenous cAMP in two competition binding assays for cAMP using as binding proteins: the regulatory subunit of cAMP-dependent protein kinase I of bovine muscle and a specific antiserum against cAMP

	% binding[a]	
	Protein kinase I	cAMP antiserum
H_2O	100.0 (± 3.2)	100.0 (± 1.2)
1.25 nM cAMP	79.9 (± 1.2)	40.1 (± 0.4)
Cell sample[b]	82.0 (± 2.7)	9.6 (± 0.6)
Cell sample + PDE[c]	100.0 (± 1.9)	74.3 (± 1.2)
Cell sample + PDE + 2 mM IBMX	82.1 (± 3.2)	12.3 (± 0.2)
Cell sample + 2 mM IBMX	82.0 (± 1.2)	11.5 (± 1.0)

[a] Values represent means of two separate determinations

[b] Cell samples were obtained as follows: mt^+ cells were mixed with an equal amount of mt^- cells and after 20 s the suspension was centrifuged through silicone oil into a layer of 3 M perchloric acid. An aliquot was neutralized and tested for cAMP in both competition binding assays

[c] Cell samples were preincubated with 0.6 units/ml PDE in either the presence or absence of IBMX, for 1 h at 20°C. Before assaying, the enzyme activity was destroyed by addition of 3 M perchloric acid

cells, whereas the basal level of cAMP in free swimming gametes was approx. 0.5–2 pmol per 10^8 cells. These values showed some variability, possibly dependent on the sexual competence and the physiological conditions of the cells. The observed changes in cAMP concentration were exclusively intracellular. The concentration of cAMP in the extracellular medium was constant at about 0.03 nM (fig.1).

The following evidence showed that what was formed was indeed cAMP (see table 1): (i) it was active in a ^3H- or ^{125}I-labelled cAMP competition binding assay using two different cAMP binding proteins: the regulatory subunit of bovine cAMP-dependent protein kinase and a highly specific antibody against cAMP, respectively; (ii) it was destroyed by beef heart phosphodiesterase in the absence of the phosphodiesterase inhibitor IBMX, but not in its presence (2 mM) (table 1).

3.2. *Mating type specificity*

To determine whether the cAMP concentration was increased in both mating types during sexual agglutination, gametes from one mating type were mixed with isolated flagella of the opposite mating type, as in [4]. Fig.2 shows a rapid, transient increase in intracellular cAMP concentration in cells of both mating types, although the level in mt^+ gametes was usually found to be higher than that in mt^- gametes. No rise in cAMP concentration was detected when cells were mixed with flagella of the same mating type. This strongly suggests that

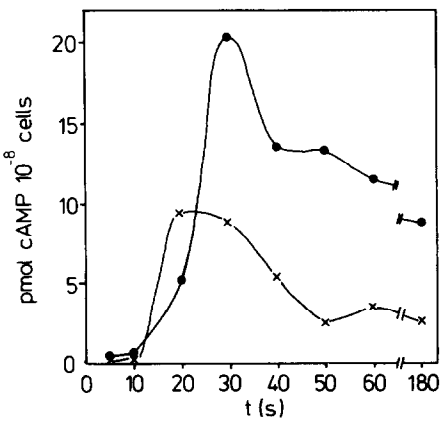

Fig.2. Changes in the intracellular concentration of cAMP in one mating type incubated with isolated flagella of the other mating type: 250 µl mt^+ cells (0.39×10^8 cells·ml^{-1}), were mixed with 15 µl isolated mt^- flagella (●—●); 250 µl mt^- cells (0.50×10^8 cells·ml^{-1}) mixed with 15 µl isolated mt^+ flagella (×—×). cAMP concentrations were determined as described in fig.1.

Table 2

cAMP phosphodiesterase activity in crude cell homogenates

	cAMP phosphodiesterase activity		
	(pmol·min^{-1}·mg^{-1} protein)	Particulate (%)	Soluble (%)
Mating cells	15.0	54	46
Mating cells + IBMX (2 mM)	0.05	n.d.	n.d.
Mating cells + theophylline (5 mM)	0.10	n.d.	n.d.
mt^+ cells	17.8	n.d.	n.d.
mt^- cells	15.8	n.d.	n.d.

n.d., not determined

during normal sexual agglutination, a rapid transient increase in intracellular cAMP concentration occurs in both mating types.

3.3. *Phosphodiesterase activity in cell homogenates*

Table 2 shows that cAMP-phosphodiesterase activity was present in crude cell homogenates. Enzyme activity in a homogenate of mixed cells was about the same as in single cells. About 50% of the enzyme activity was particulate. No activity was found in the extracellular medium, nor were intact cells capable of hydrolyzing extracellular cAMP, excluding the presence of phosphodiesterase activity on the cell surface. The enzyme activity was effectively inhibited by 2 mM IBMX or 5 mM theophylline.

3.4. *cAMP-dependent protein kinase activity*

If cAMP is an intracellular signal, it is likely to exert its action by activating a cAMP-dependent protein kinase. To demonstrate that cAMP-dependent protein kinase activity was present in *C. eugametos* gametes, the soluble cell fraction of a suspension of mixed gametes was chromatographed on DEAE–cellulose to separate cAMP-dependent and cAMP-independent protein kinase activities. Fig.3 shows that cAMP-dependent protein kinase activity is present. Two peaks of cAMP-dependent protein kinase activity were found. One bound only weakly to the DEAE–cellulose, and was eluted with a peak at fraction 14. A second peak of activity was eluted with 0–0.5 M NaCl.

3.5. *Concluding remarks*

Here we present evidence for a transient increase in intracellular cAMP concentration after mixing cells of opposite mating type. The cAMP response is rapid, reaching its maximum after about 20 s. Furthermore, we show that *C. eugametos* cells possess cAMP-phosphodiesterase and cAMP-dependent protein kinase activity, in addition to adenylate cyclase activity as demonstrated in [12]. Obviously, all activities for cAMP metabolism and

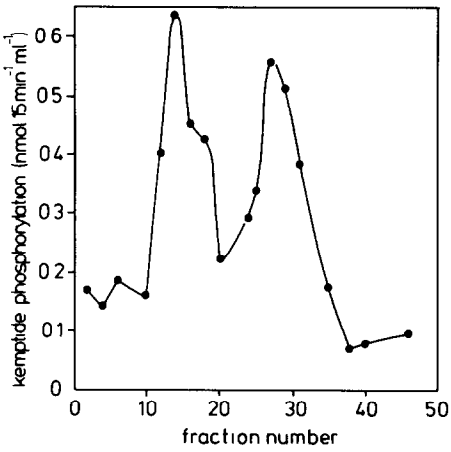

Fig.3. cAMP-dependent protein phosphorylation of the soluble fraction of mating cells (isolated from 3×10^8 cells) after fractionation on DEAE–cellulose. The heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide) was used as an artificial substrate in the cAMP-dependent protein kinase assay. A linear 0–0.5 M NaCl gradient was started at fraction 14 and finished at fraction 48. The values represent the difference of kemptide phosphorylation in the presence and absence of 30 μM cAMP.

cAMP detection are present in these cells. These data suggest that cAMP is an early intracellular signal after sexual interaction, preceding all presently known biochemical and ultrastructural changes induced by mating. This possibility is being further investigated.

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