Cyclic AMP as an intraspheroidal differentiation signal in *Volvox carteri*

R. Gilles, R. Moka, C. Gilles and L. Jaenicke

Institut für Biochemie, Universität Köln, An der Bottmühle 2, 5000 Köln 1, FRG

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The action of the macromolecular inducer glycoprotein on sexual reproduction in the green alga *Volvox carteri* can be modified by altering the external (intraspheroidal) cAMP concentration. Direct proof for the presence of cAMP in the spheroids is given. Protein binding assay and HPLC-fluorimetric analysis independently demonstrate the existence of cAMP in the matrix, cells, and culture medium. Its concentration is higher in sexual cultures, pointing to a transmitting function in sex induction. The presence in the matrix of other members of a protein phosphorylation system suggests an induction-specific signal cascade in this plant.

Cyclic AMP *Differentiation* *Extracellular matrix* *Sexual induction* Volvox carteri

1. INTRODUCTION

Sexual reproduction in *Volvox carteri*, a dioecious multicellular green alga, involves cascade-like steps of signal amplification. The first event seems to be a very frequent (1.1×10^{-4}) sex^c mutation which turns the algae sexual without any external stimulus [1]. A male sexual constitutive mutant (sex^c_m) will form sperm packets, and – on their maturation – a pheromone is released into the culture medium. This sexual inducer, a 30-kDa glycoprotein, is a species-specific signal which synchronizes sexual reproduction [2,3]. The threshold concentration of the pheromone is extremely low (6×10^{-17} M), so that a single sexual male spheroid will induce sexuality in the whole culture. Less than 3000 molecules of inducer per spheroid suffice for the biological response [4]. This calls for efficient conversion and amplification of the initial inducing signal down to the genome. There is evidence that the basic pheromone is first concentrated into the extracellular matrix which contains acidic binding molecules and that no direct interaction with the plasma membrane of the gonidia occurs [4]. Even though only differentiation of the gonidia is visibly affected by the pheromone (they will form sexual spheroids rather than asexual spheroids), the somatic cells also react to the signal. Immediately after addition of the sex inducer to the culture medium, synthesis of a phosphoprotein (pp290) in the matrix largely made by the somatic cells is changed [5]. As the whole organism responds to the pheromone, it makes sense that the primary signal, first bound in the extracellular matrix, is transformed into an intraspheroidal second message which then leads to intracellular reactions. A candidate for this second messenger is cAMP, since we have found that experimental changes of the extracellular cAMP level affect sexual induction. High levels of cAMP, as caused by addition of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), block sexual induction, while lowering the level, by the addition of mammalian PDE, stimulates inducer-independent sexual induction [4].

2. EXPERIMENTAL

2.1. *Culture conditions*

V. carteri f. *nagariensis*, strain HK-10 and the sterile 'Poona' male strain are gifts from Dr R.C. Starr, Austin, TX. The $sex^?$ strain is a spontaneous

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mutant. The conditions for axenic culture and growth have been described [3].

2.2. *cAMP analysis*

Cultures were grown to a density of 10^6 spheroids/l; after 10 days (5 generations) the spheroids were harvested by filtration through 50 μm nylon net and mechanically disrupted in a Yeda press [4]. Cells and matrix were separated by low-speed centrifugation. Spheroidal fractions and medium were vacuum-dried and extracted with 80% (v/v) ethanol. After centrifugal clarification, extracts were either analysed by the standard protein binding assay (Boehringer, Mannheim) [6] or treated with 0.1% (w/v) chloroacetaldehyde, prepared as in [7], at 80°C for 45 min [8]. The etheno derivatives were separated on an anion-exchange (AX-300, Kontron) high-performance liquid chromatography (HPLC) column using a linear gradient of 10–100 mM Na_2HPO_4, pH 6 (1 ml/min) generated by a microcomputer-controlled dual pump HPLC system (Beckman). Concentrated fractions containing etheno-cAMP were loaded on a reversed-phase (RP 18, LiChrosorb 5 μm, Merck) HPLC column and eluted by a linear gradient of 0–70% methanol in 10 mM acetate, pH 4 (1 ml/min). Etheno-cAMP was detected by its fluorescence at 420 nm (E_{ex} = 300 nm) (Shimadzu fluorescence spectrometer, RF 530).

3. RESULTS AND DISCUSSION

Evidence for the occurrence of cAMP in *Volvox* previously was only circumstantial [4]. Here we prove directly its existence in *Volvox* cultures and its concentration changes in sexual induction by radioisotopic assay and chromatographic identification. By the standard protein binding assay [6] the cAMP concentration in the algae is 450 pmol/10^6 individuals (65 pmol/g fresh wt); in the culture filtrate it is 180 pmol/l. These values vary with the age and density of the culture but are within a range reported for other species of algae [9]. After separation of *Volvox* spheroids into cells and intraspheroidal matrix, 40% (180 pmol) of the cAMP is found in the matrix compartment. Whereas a high matrix concentration of cAMP seems to be required for its function as an intraspheroidal messenger, the importance of cAMP in the medium is unknown. We assume that the cells release cAMP into the acidic extracellular matrix, from which it diffuses into the medium unrestrained by any membranes.

It is well known that plant cells may contain interfering substances that mimic cAMP in the protein binding assay [10]. Indeed, we have encountered such troubles in *Volvox* preparations, but purification of cAMP from extracts with charcoal and Dowex 1 column chromatography [11] as well as controls by preincubating extracts with PDE reduced these blanks. A quantitative and sensitive direct assay for cAMP, not fraught with interference, is HPLC combined with the fluorimetric detection of cAMP as its strongly fluorescent etheno derivative [8]. Aliquots of the pre-purified extracts were treated with chloroacetaldehyde and loaded on a reversed-phase column. The etheno-cAMP was eluted by a linear gradient of acetate/methanol as a single symmetrical peak (fig.1); its height is proportional to concentration over the range 0.3–10^4 pmol. The fluorimetric detection has therefore a lower limit in the order of 10^{-13} mol/injection (20–100 μl). Using this analytical technique, we find cAMP in all 3 fractions of *Volvox* cultures – cells, matrix and

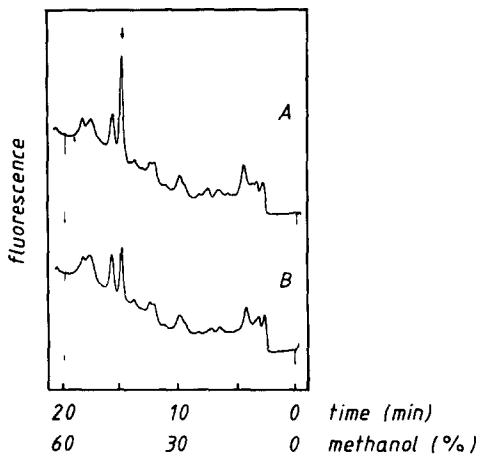

Fig.1. Quantitative determination of cAMP in *Volvox* by HPLC separation on a reversed-phase column and fluorimetric detection of its etheno derivative as described in the text. Pre-purified medium without (B) and with (A) co-injection of 1.5 pmol authentic etheno-cAMP (arrow); cells and matrix fractions give comparable results.

medium – in concentrations very close to those estimated from the protein binding assay (table 1).

The demonstration of the intra- and extracellular existence of cAMP provides the basis to investigate its physiological function in induction. If cAMP is a mediator for sexual induction, its concentration should differ in sexual and asexual cultures. Furthermore, strains with mutant sexual behavior should have altered cAMP levels. As seen from table 1, this is indeed the case. The cAMP concentration is significantly higher in sexual than in asexual cultures. From the in vivo experiments mentioned above, we concluded that a high level of cAMP inhibits sexual induction. In agreement, sexual cultures – which, unless mating occurs, will return to an asexual subsequent cycle even in the presence of inducer – have high cAMP concentrations, as shown for the female strain HK-10. In the non-inducible sterile 'Poona' male strain, the 10–20-fold higher concentration of cAMP again might be the reason for their sterility. This as well as the very low level of cAMP in the matrix of cAMP of the sexual constitutive *sex*$^{c}_{f}$ strain which is sexual (inducible) in each cycle, further corroborates this assumption. These data on the presence of cAMP in sexual, asexual, and mutated spheroids strongly suggest a physiological role in *Volvox* differentiation; moreover, ongoing investigations point to dynamic changes in cAMP concentration under sexual and asexual conditions, as predicted.

Table 1

Distribution of cAMP in different strains of *Volvox carteri*

Strain	Cyclic AMP (pmol)		
	Matrix	Cells	Medium
HK-10 (asexual)	170	280	220
HK-10 (sexual)	400	1800	530
'Poona' male (sterile)	610	21000	33000
sex$^{c}_{f}$ (constitutive sexual)	170	1300	610

Data refer to 10^6 algae and 1 l medium. Mean of 3 independent estimations by HPLC/fluorimetric analysis as described in the text

Up to now, we have not been able to demonstrate unambiguously the compartment – cells or matrix – in which cAMP acts as a messenger. It might be that cAMP is only an intracellular messenger in *Volvox* as in higher animals, but many observations show the extracellular matrix as the functional compartment in which cAMP primarily operates. It would be difficult to explain otherwise, how PDE, added from the outside, could affect the intracellular cAMP concentration to trigger induction. The most conclusive argument, however, is the finding that cAMP is one component of a very complex sexual induction cascade operating in the intraspheroidal matrix. In this compartment we have identified the binding sites for the pheromone [4]; a glycoprotein, the phosphorylation of which reflects sexual induction [5]; PDE and protein kinase activity [12]. In this set of macromolecules cAMP might be the linker: binding of the inducer alters the intraspheroidal cAMP concentration by regulation of a matrix PDE; this, in turn, changes the activity of a protein kinase which phosphorylates the major matrix phosphoprotein(s). At present such a signal cascade is still speculative, but *Volvox* obviously is a promising organism in which a key function of cAMP in the plant kingdom may be studied.

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