Effect of cAMP Level on Thallus Growth and Gametangia Development in Macroalga *Chara vulgaris* (Charales, Charophyta)

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cAMP is a second messenger which plays a regulatory role in a wide variety of biological processes in organisms ranging from prokaryotes to higher eukaryotes, but knowledge of its role in macroalgae and vascular plants is limited. We modified cAMP levels in the macroalga *Chara vulgaris* thallus and studied the effects on thallus growth and gametangia development: db-cAMP (permeable analog of cAMP), adenylate cyclase (AC) activator, forskolin and theophylline (cAMP phosphodiesterase (PDE) inhibitor) were used to elevate cAMP levels, and the AC inhibitors 2'-dAdo and 2'-d3'-AMP were used to decrease them. The results suggest that in *Chara vulgaris* the cAMP pathway may regulate both vegetative thallus growth and gametangia development, and that these effects may depend on this second-messenger level. Elevated cAMP stimulated thallus growth and delayed gametangia development; decreased cAMP inhibited thallus growth and accelerated maturation of both antheridia and oogonia. These results suggest that the cAMP pathway participates in regulation of developmental processes in *Chara vulgaris* and that thallus growth and gametangia development require different cAMP levels in cells.

Key words: Antheridia development, cyclic AMP, cAMP level-modifying substances, *Chara vulgaris*, oogonia development, thallus growth.

INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cAMP) is a ubiquitous and important cellular mediator of extracellular signals in organisms ranging from prokaryotes to higher eukaryotes. It is synthesized by adenylate cyclases (ACs) (Kamenetsky et al., 2006) and hydrolyzed by cAMP phosphodiesterases (PDEs) (Conti and Beavo, 2007; Omori and Kotera, 2007). Elevated levels of cAMP induce or modify cellular processes, while reduced levels of this messenger can switch off the cAMP signal. A number of studies have demonstrated that in mammals this second messenger plays a key role in the regulation of many cell processes, acting mainly by activating cAMP-dependent protein kinases (PKAs) and also directly regulating ion channels and Rap guanine exchange factors (Epacs) (Kopperud et al., 2003).

Unlike in the animal kingdom, in which the cAMP pathway is well characterized, in plants there is insufficient information about this second-messenger system, although in plants the presence of cAMP, enzymes for its synthesis and hydrolysis, and some other substances participating in cAMP the pathway have been identified. It is thought that in higher plants the cAMP pathway does not have a universal regulatory role as in animal systems (Assmann, 1995; Newton et al., 1999; Trewavas et al., 2002; Newton and Smith, 2004; Kaplan et al., 2007).

In algae the role and course of the cAMP pathway has been examined mainly in unicellular ones. Work in which endogenous cAMP levels were altered and cAMP level-modifying substances were applied indicates that in unicellular algae this second messenger is involved in regulating the progress of the cell cycle (Aline et al., 1984; Tong et al., 1991; Sakuanrungsirikul et al., 1996; Mohabir and Edmunds, 1999) and performs an essential role in mating processes (Pan and Snell, 2000; 2002), gravitaxis (Lebert et al., 1999; Streb et al., 2002), phototaxis (Ntefidou et al., 2003) and flagellum formation and motility (Rubin and Filner, 1973; Jayaswal et al., 1991; Gaillard et al., 2006).

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In unicellular algae the presence of cAMP, PDE and AC is widely known, and other substances participating in the cAMP pathway have been found, including heterotrimeric G-protein (Korolkov et al., 1990; Gromes and Zetsche, 1992; Calenberg et al., 1998), small G-protein (Fabry et al., 1992; Hable et al., 2008), PKA (e.g., Carre and Edmunds, 1993; Dawson et al., 1996; Kiriyama et al., 1999; Leighfield et al., 2002; Gopal et al., 2012), A-kinase anchoring protein (AKAP) (Gaillard et al., 2001; 2006) and cAMP response element (CRE) in promotors of some genes (Uchida et al., 2004; Ohno et al., 2012).

In unicellular algae, life cycle and developmental processes may require a change in the cAMP level. In the vegetative phase of growth algae cells contain high cAMP levels, and applying cAMP-elevating substances stimulates culture growth. In *Chlorella fusca*, Berchtold and Bachofen (1977) reported that exogenous cAMP and its analog db-cAMP (dibutyryl cAMP) had a positive effect on chlorophyll synthesis and culture growth rate. Adding db-cAMP to the medium increased intracellular cAMP levels and stimulated culture growth in *Crypthecodinium cohnii* (Lam et al., 2001). In *Chlamydomonas*, fluctuations of intracellular cAMP were correlated with growth, division and morphological changes (Sharaf and Rooney, 1982).

In experiments on the moss protonema the cAMP level has been shown to be significant to developmental processes: a cAMP level-dependent effect on protonema growth and development was observed in *Funaria hygrometrica*, and the presence of cAMP and PDE was demonstrated in protonema cells (Handa and Bressan, 1977; Sharma and Johri, 1982). The endogenous level of cAMP was significantly higher in chloronema than in caulonema cells (Handa and Johri, 1976; 1979), and adding cAMP to the medium stimulated chloronema formation and proliferation (Handa and Johri, 1979; Johri, 2008).

Investigations of *Micrasterias thomasiana* var. *notata* indicated that cAMP may function as a signal simultaneously stimulating cell proliferation and repressing their differentiation into gametes and the formation of zygotes (Imaizumi and Doida, 1995). This effect was observed after medium was supplemented with cAMP and several chemicals that raise the intracellular level of cAMP.

cAMP has been detected in the red macroalgae *Porphyra leucosticte* (Segovia et al., 2001), *Porphyra umbicans* (Newton et al., 1995) and *Gelidium sesquipedale,* and the green macroalga *Ulva rigida* (Gordillo et al., 2004), but little is known about the role of the cAMP signaling pathway. In these algal species light induced a pronounced increase in cAMP levels in the thallus (Segovia et al., 2001; Gordillo et al., 2004). In *Gelidium sesquipedale* and *Ulva rigida*
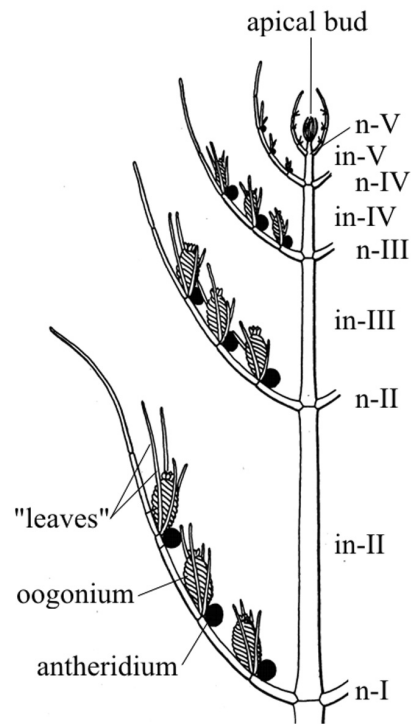
Fig. 1. Scheme of apical parts of *Chara vulgaris* thallus. Nodes (n) and internodes (in) of the main axis are numbered from the youngest to the oldest parts of the thallus.

the cAMP levels in thallus cells correlated positively with light intensity and photosynthetic activity (Gordillo et al., 2004). In a comparison of cAMP levels and thallus growth between *Ulva rigida* and *Gelidium sesquipedale*, the fast-growing *Ulva rigida* had significantly higher endogenous cAMP than the slower-growing *Gelidium sesquipedale* (Gordillo et al., 2004).

The relationship between cAMP levels, gametophyte growth and gametangia development has not been investigated in macroalgae. Here were report our study of the relation between cAMP levels and the course of these processes. We used indirect methods, applying substances that modify the cAMP level and assessing their affect on thallus growth and gametangia development in the multicellular alga *Chara vulgaris* (Chlorophyta, Characeae). This macroalga is an attractive model for studying developmental process regulation because it follows a typical basipetal gradient of development and because all developmental stages of the vegetative and reproductive organs can be observed on an individual plant (Fig. 1). New thallus segments (nodes and internodes) of the main axis are formed and gradually separate from the apical bud. Lateral branches (laterals) consist of 1–4 internodes and nodes, but their growth is limited. The mitotic divisions that increase the number of thallus cells take place in the apical bud area. The growth of

thallus cells usually is completed in the fifth segment of the main axis.

Oogonia and antheridia are formed on the nodes of the laterals (Fig. 1). Their development starts from initial cells in the apical bud area; their maturation, that is, the breakup of antheridia with the release of spermatozoids and the formation of oogonia ready for fertilization, usually takes place in the fifth node of the main axis. Fertilized oogonia rapidly convert into oospores, manifested in the color change from light green to dark brown.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Chara vulgaris L. thalli were collected from a pond in the Botanical Garden in Łódź. After two days of adaptation to laboratory conditions, plants showing similar morphology and having apical parts of thalli with five nodes were selected and cultivated in water (control material) or in media with the cAMP level-modifying substances. In each experimental variant, 15 morphologically similar plants were placed in 100 ml beakers. The plants were cultivated in laboratory conditions (280 $\mu Em^{-2}s^{-1}$, LD 16:8, 20–22°C) in pond water (pH 7.2) which was sterilized by autoclaving and then subjected to aseptic aeration. All the initial preparation as well as the whole experimental procedure were performed in aseptic conditions. The plants were surface-sterilized by immersion in a mixture of 0.1% acetone chloroform (1,1,1-trichloro-tert-butanol) and 0.1% soap for 15 min. The db-cAMP, theophylline, 2'-dAdo and 2'-d3'-AMP were dissolved in sterile water. Forskolin was dissolved in DMSO (dimethyl sulfoxide) and diluted to the final concentration with deionized water. The chemicals were purchased from Sigma.

All cAMP-modifying substances were used at concentrations of 20 or 100 μM. These concentrations were established in preliminary experiments in which a broad range of their effects was tested. The plants were cultivated for nine days, and the incubation solutions were changed every 3 days to maintain the concentrations of the tested substances, and also to maintain the medium composition because it is known that algae can release cAMP and other substances to culture medium (Bressan et al., 1980; Bressan and Handa, 1980; Francko and Wetzel, 1980; Gilles et al., 1985; Francko, 1989).

In previous experiments we found that *Chara vulgaris* antheridial filament cells react quickly (within a few hours) and strongly to cAMP level-modifying substances (A. Domańska, unpublished data). In the present study we chose 9-day continuous incubation because their effect on morphological changes of the thallus, and a substantial part of

gametangia development, can be observed within this period (Godlewski and Kwiatkowska, 1980).

MEASUREMENT OF THALLUS GROWTH AND GAMETANGIA DEVELOPMENT

The nodes and internodes of the plants were numbered at the start of the experiments (Fig. 1). After 9-day cultivation the material was fixed in ethanol/acetic acid (3:1 v/v) for 2 h and then washed and kept in 70% ethanol. The growth of the main axis of the thallus was determined from the number of new nodes formed and from internode length. The length of laterals and their "leaves" (large cells developed in nodes of laterals) was also measured. The laterals of the experimental plants had three nodes with four "leaves". The larger pair of "leaves" of the distal node was always measured (Fig. 1).

The effect of cAMP level-modifying substances on antheridia development in subsequent nodes was assessed from the number of ruptured antheridia releasing spermatozoids. The numbers of antheridia at the beginning and end of the experiment were compared. The number of cell divisions leading to the spermatid stage was determined from the number of spermatids in one antheridial filament. Counts were made from squash preparations of antheridia stained with Feulgen's method.

The effect of the used substances on oogonia development was evaluated in the subsequent nodes on the basis of the number of fertilized oogonia, which was diagnosed on the basis of their color change from light green to dark brown.
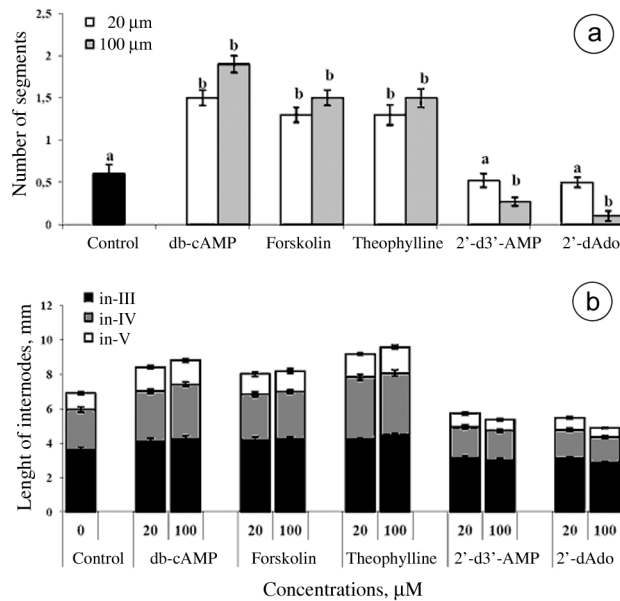
STATISTICS

Each experimental variant comprised 15 plants (n = 15) and was replicated three times. Similar results were obtained in all three independent experiments. The significance of differences between the experimental variants was checked by ANOVA followed by Tukey's test. All statistical analyses were made using Statistica ver. 10 (StatSoft Inc.).

RESULTS

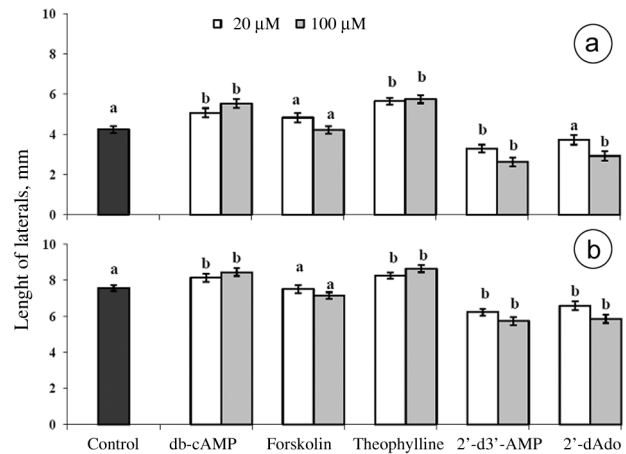### EFFECT OF cAMP LEVEL-MODIFYING SUBSTANCES ON VEGETATIVE GROWTH OF THALLUS

To assess the effect on thallus growth of the cAMP-increasing substances db-cAMP, forskolin and theophylline, and of the cAMP-decreasing substances 2'-dAdo and 2'-d3'-AMP, we compared the control and 9-day-treated plants in regard to the following parameters: number of newly formed segments, internode length of main axis, length of laterals and length of "leaves."

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Fig. 2. Effect of cAMP level-modifying substances on main axis growth. (**a**) On number of newly-formed segments (nodes and internodes), (**b**) On internode length. Values are means ± SE. Values bearing different letters differ significantly from the control at P < 0.05 by ANOVA followed by Tukey's test.

Fig. 3. Effect of cAMP level-modifying substances on length of laterals (**a**) in node No. V5, (**b**) in node 4. Values are means ± SE. Values bearing different letters differ significantly from the control at P < 0.05 by ANOVA followed by Tukey's test.

The results show that the presence in media of substances that modify the cAMP level in cells affected *Chara vulgaris* thallus growth. Generally, cAMP-increasing substances stimulated and cAMP-decreasing substances inhibited thallus growth (Fig. 2). They affected both development of new segments and the longitudinal growth of internodes. During culture the control plants developed ~0.6 new main axis segments on average (Fig. 2a); those incubated with 100 µM solution of db-cAMP, forskolin or theophylline developed 2–3 times more segments (Fig. 2a). In contrast, 2'-dAdo and 2'-d3'-AMP at both concentrations significantly reduced the number of newly formed main axis segments (Fig. 2a). Incubation with db-cAMP, forskolin and theophylline also stimulated the longitudinal growth of main axis internodes, while 2'-dAdo and 2'-d3'-AMP significantly reduced their length (Fig. 2b). Measurements of all five internodes of the cultivated apical parts of the thallus indicated that the oldest ones (internodes 4 and 5, Fig. 1) reacted less to the tested substances (data not shown) than the younger internodes did (internodes 1–3, Fig. 1).

The laterals grew only in length, without producing new segments. Length measurements of the laterals of nodes 1 and 2 showed that these parts of the thallus reacted in a manner similar to the main axis: db-cAMP and theophylline elongated and 2'-dAdo and 2'-d3'-AMP shortened them versus the control (Fig. 3). The "leaves" reacted similarly:

100 µM db-cAMP and theophylline increased while 2'-dAdo and 2'-d3'-AMP diminished their growth rate versus the control (Fig. 4).

EFFECTS OF cAMP LEVEL-MODIFYING SUBSTANCES ON GAMETANGIA DEVELOPMENT

Development of the oogonia and antheridia formed on the nodes of the laterals starts in the apical bud area. Their maturation (i.e., breakup of antheridia, release of spermatozoids and formation of oogonia ready for fertilization) usually takes place on the fifth node of the main axis, counting from the apex (apical bud). We compared the control and 9-day-treated plants in terms of the number of mature antheridia and mature oogonia (oogonia which after fertilization developed into oospores) calculated for nodes 4 and 5 of the main axis (Fig. 1).

The cAMP level-altering substances affected the development of both types of gametangia. The control plants had 23% disintegrated antheridia in node 4 and 70% in node 5. Incubation with db-cAMP, forskolin and theophylline at 100 µM concentration reduced the percentage of percentage of mature antheridia by about half in node 4 and by ~40% in node 5 versus the control. The presence of 2'-dAdo and 2'-AMP-d3' in the media significantly increased the number of disintegrated antheridia, more than doubling it in node 4 and raising it by ~25% in node 5 versus the control (Fig. 5). The effects on oogonia maturation were similar: cAMP-elevating substances increased and cAMP-lowering substances decreased the number of fertilized oogonia (Fig. 6). In the control material, node 4 had 4.2% fertilized oogonia (oospores) and node 5 had ~60% oospores. The
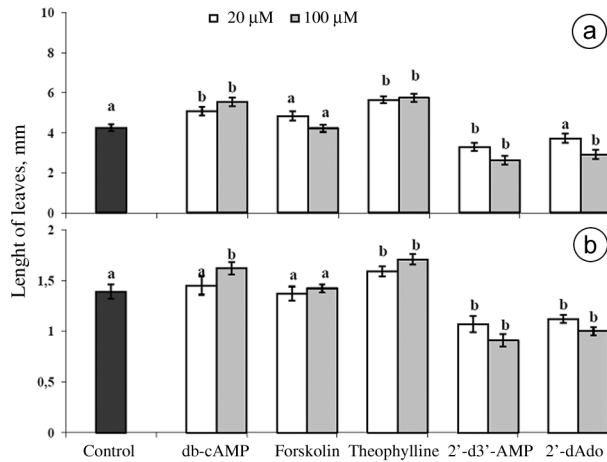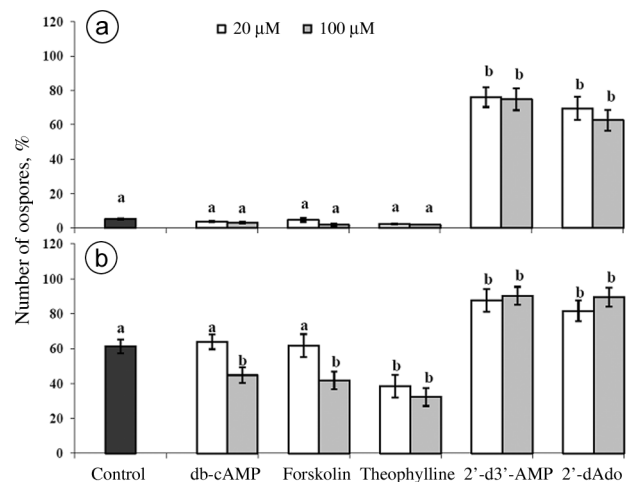
Fig. 4. Effect of cAMP level-modifying substances on the length of "leaves" (**a**) in node 5, (**b**) in node 4. Values are means ± SE. Values bearing different letters differ significantly from the control at P < 0.05 by ANOVA followed by Tukey's test.

cAMP-elevating substances db-cAMP, forskolin and theophylline had little effect on the number of fertilized oogonia in node 4 but significantly affected the number in node 5, at 100 μM concentration reducing the number in node 5 by 25–45% versus the control (Fig. 6). Adding dAdo and 2'-d3'-AMP to the medium had the opposite effect: they both increased the number of fertilized oogonia, by more than twofold in node 4 and by ~40% in node 5 versus the control (Fig. 6).

Several dozen antheridial filaments are formed during the development of *Chara vulgaris* antheridia. Two consecutive phases lead to spermatozoid formation: first the proliferative phase, with synchronous division of antheridial filament cells leading to the spermatid stage, and second the spermiogenesis phase, leading to the formation of spermatozoids. The number of spermatids in antheridial filaments depends on the number of mitotic divisions. In *Chara vulgaris* the spermatid stage usually is achieved after six divisions, ultimately producing 64 spermatids in a filament (Olszewska and Godlewski, 1972). To determine the effect of the tested substances on the number of cell divisions leading to the spermatid stage, we counted the spermatids in antheridial filaments in squash preparations of antheridia of nodes 3 and 4 of plants fixed at the end of the experiment (after nine days of culture). In the control material the antheridial filaments had ~60 spermatids on average, meaning that in most filaments this stage was achieved after six cell divisions. The tested substances did not affect the number of spermatids in filaments, except in the treatment with forskolin at 100 μM, where the antheridial filaments had 44 spermatids on average (Fig. 5).
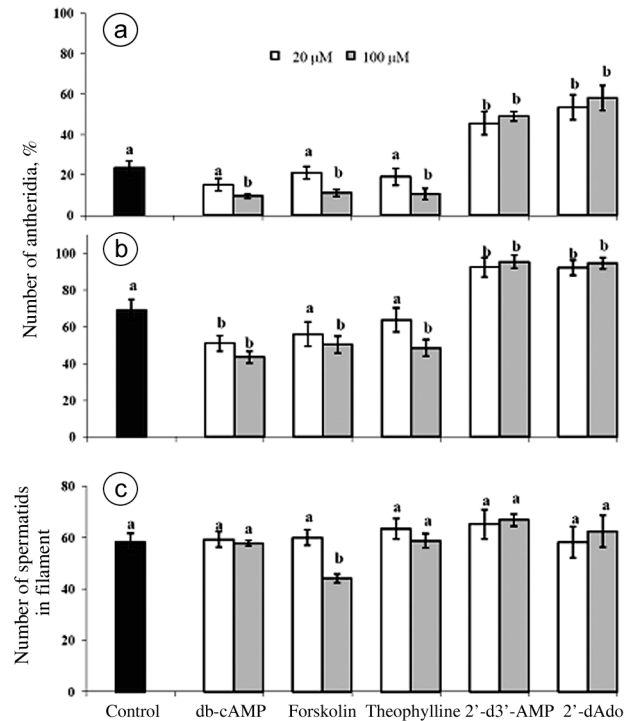
Fig. 5. Effect of cAMP level-modifying substances on antheridia development. (**a**) On number of mature antheridia in node 4, (**b**) On number of mature antheridia in node 5, (**c**) On number of spermatids per antheridial filament. Values are means ± SE. Values bearing different letters differ significantly from the control at P < 0.05 by ANOVA followed by Tukey's test.

Fig. 6. Effect of cAMP level-modifying substances on oogonia maturation. (**a**) On number of fertilized oogonia in node 4, (**b**) On number of fertilized oogonia in node 5. Values are means ± SE. Values bearing different letters differ significantly from the control at P < 0.05 by ANOVA followed by Tukey's test.

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DISCUSSION

In this work we studied the effects of cAMP level-modifying on *Chara vulgaris* thallus growth and gametangia development. We applied five substances that exert different effects on the cAMP level in cells. Three of them elevate it: db-cAMP is a permeable analog of cAMP, forskolin is an AC activator, and theophylline is a cAMP PDE inhibitor. Two of them lower it: 2'-dAdo and 2'-d3'-AMP are AC inhibitors. The effects of these substances cell levels of cAMP are well documented in animals, algae and higher plants (e.g., Imaizumi and Doida, 1995; Dessauer et al., 1999; Yeung, et al., 1990; Domańska et al., 2009). In our comparison of the effects of these substances after 9 days of continuous incubation, all three cAMP-elevating substances had similar effects, and both cAMP-lowering substances also exerted similar effects; this uniformity lends credence to the results.

Our findings suggest that in the macroalga *Chara vulgaris* the cAMP signaling pathway participates in the regulation of both thallus growth and gametangia development. The clear effects of specific activators and inhibitors suggest that AC and PDE are present in *Chara vulgaris* cells. Generally the presence of cAMP-elevating substances in the culture medium stimulated vegetative growth of the thallus but delayed the maturation of antheridia and oogonia, whereas cAMP-lowering substances showed the opposite effects.

The stimulatory effect of cAMP-elevating substances on thallus growth was manifested in the formation of a greater number of segments (nodes and internodes) in the main axis, increasing the size of internodes, laterals and "leaves." Formation of new segments in the main axis of the *Chara* thallus takes place in the apical bud area and is determined by mitotic divisions. The positive effects of cAMP-elevating substances and negative effects of those lowering it indicate that cell proliferation in the apical bud depends on the level of cAMP in cells. cAMP-elicited stimulation of cell proliferation has been observed in unicellular algae, for example in culture of *Chlorella fusca* (Berchtold and Bachofen, 1977), *Crypthecodinium cohnii* (Lam et al., 2001) and *Chlamydomonas* (Sharaf and Rooney, 1982), where fluctuations of intracellular levels of cAMP were correlated with growth and cell division.

Internode cells of the main axis of *Chara* achieve gigantic size (up to several cm), and the cells of laterals and "leaves" also are large. The growth of these large cells is connected with endopolyploidization of DNA and repeated fragmentation of endopolyploid nuclei (Shen, 1967; Foissner and Wasteneys, 2000). Recently we observed that substances that raise the cAMP level can increase DNA content and the number of nuclei in these syncytial cells, and that substances that lower it show the opposite effect (M. Godlewski and A. Domańska, unpublished data).

The effect of cAMP level on thallus growth has not been investigated in macroalgae before, but studies of lower plants yield certain information. cAMP level-dependent effects on the growth and development of protonema of the moss *Funaria hygrometrica* have been shown; cAMP and PDE are present in protonema cells (Handa and Bressan, 1977; Sharma and Johri, 1982) and the endogenous concentration of cAMP is significantly higher in chloronema than in caulonema cells (Handa and Johri, 1976; 1979). Adding cAMP to the medium stimulated chloronema formation and proliferation (Handa and Johri, 1979; Johri, 2008).

In our experiments the effect of cAMP level-modifying substances on gametangia development was opposite to the effect on thallus growth. cAMP-lowering substances inhibited thallus growth but accelerated the maturation of both oogonia and antheridia; the effect of cAMP-elevating substances was the reverse. This conclusion is based in part on observations that in the presence of cAMP-lowering substances the completion of antheridia development and of oogonia fertilization took place on laterals younger than in the control, whereas in the presence of cAMP-elevating substances they both took place on laterals older than in the control.

In *Chara* the process of spermatozoid formation consists of two phases: successive synchronous divisions of antheridial filament cells leading to the spermatid stage (spermatogenesis), and transformation of spermatids into spermatozoids (spermiogenesis). In the control the cells achieve spermatid stage in filaments containing 64 cells, that is, usually after six cell cycles (Olszewska and Godlewski, 1972). The spermatid counts from filaments of the control and treated plants indicate that the cAMP level-modifying substances did not significantly affect the number of cell divisions leading to the spermatid stage. This suggests that the effect of these substances on antheridia development is connected with the second phase, spermiogenesis.

We did not examine the role of cAMP levels in gametangiogenesis and fertilization of oogonia in *Chara vulgaris* but we might offer some suggestions on the basis of studies of other organisms such as algae and mammals, though *Chara* is a haplobiont and these processes occur in it without meiosis. In *Chara vulgaris* the transformation of spermatids into spermatozoids (spermiogenesis) is a complicated process which includes reduction of spermatid nucleus size (Olszewska and Godlewski, 1973; Kwiatkowska, 1996), its elongation, and

exchange of histones with protamine-like proteins (Popłońska, 2002; Kwiatkowska and Popłońska, 2003), and construction of the flagellum. A probable similarity between *Chara vulgaris* and mammals in the role of the cAMP pathway in regulating spermiogenesis is the replacement of DNA-binding histones by protamines. In mammals the promoter region of the protamine gene contains CRE (cAMP response element) regulated by the transcription factor CREM (cAMP-responsive element modulator) (Blocher et al., 2003). The cAMP pathway in mammals may participate in the regulation of several other genes expressed in spermatids, as they have CRE in promotors (Kistler et al., 1994; Zhou et al., 1996). The delayed *Chara vulgaris* antheridia development we observed after treatment with cAMP-elevating substances may also be due to the effect on flagellum formation. In *Chlamydomonas reinhardtii*, elevated cAMP decreased the formation and motility of flagellae (Jayaswall et al., 1991).

In the macroalga *Chara vulgaris*, cAMP level-modifying substances affected both thallus vegetative growth and gametangia development. Our results suggest that these effects depend on this second-messenger level. cAMP-elevating substances stimulated thallus growth, while cAMP-lowering substances accelerated the maturation of antheridia as well as oogonia. Apparently the cAMP signaling pathway participates in the regulation of developmental processes in *Chara vulgaris*.

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