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Changes in Intracellular cAMP Level and Activities of Adenylcyclase and Phosphodiesterase during Meiosis of Lily Microsporocytes

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ABSTRUCT. **In the yeasts, *Saccharomyces cerevisiae* and *Schyzosaccharomyces pombe*, reduction of intracellular cyclic adenosine monophosphate (cAMP) is known to trigger the sporulation processes by activating various meiosis specific genes. In order to ascertain whether a similar mechanism is operative in higher plants, we carried out preliminary studies on lily microsporocytes. Measurement of cAMP levels as well as the activities of adenyl cyclase and phosphodiesterase in somatic cells and different stages of meiosis, and arrest of its in protoplasts cultured under conditions of high cAMP provided direct evidence that similar phenomena occur in plant meiocytes as earlier documented in yeasts.**

In both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, a decrease in the intracellular cAMP level has been recognized as a trigger for the initiation of meiosis. This conclusion is based on the following: 1. intracellular cAMP concentration becomes reduced after transfer to sporulation media (1); 2. disruption of the adenylcyclase (the enzyme responsible for cAMP synthesis) gene (*cyr1*), resulting in production of cells with little cAMP, creates cells which are viable but highly derepressed for meiosis (2); 3. stimulation of cAMP degradation by overexpression of *pde1*, which encodes a phosphodiesterase homologue, reduces cAMP levels and suppresses inhibition of meiosis (1, 3); 4. addition of a high concentration of cAMP into sporulation media inhibits mating and meiosis (4); and 5. constitutive expression of the catalytic domain of *S. cerevisiae* adenyl cyclase in *S. pombe* results in meiosis (1). Despite the accumulation of reports indicating that intracellular cAMP levels are important for induction of sexual development and sporulation in yeasts, information in this area is totally lacking for higher eukaryotes. Meiosis in lily microsporocytes proceeds synchronously and the cells and their protoplasts can be cultured *in vitro*. Since the protoplast membrane can incorporate cAMP and nucleic acids for a short period of time after preparation (manuscript submitted) this system was chosen for investigation. As a result we observed meiotic retardation after adding cAMP to the culture media. Additional measurement of intracellular levels of cAMP, and adenyl cyclase and phosphodiesterase activities in this preliminary study indicated the presence of similar physiological mechanisms in plant meiocytes as in yeasts.

MATERIALS AND METHODS

Microsporocytes of *Lilium* hybrids, chiasmatic cultivar "Enchantment" and achiasmatic cultivar "Black Beauty", were prepared as described earlier (5). Young anthers obtained from buds smaller than 8 mm. in length and calli grown on 1% agar-Murashige-Skoog media (6) supplemented with 2 mg/l 2,4-D under light were used as sources of somatic cells.

cAMP assay. The quantitation of cAMP was carried out using an assay kit (code TRK 432) according to the protocol provided by the supplier (Amersham). Briefly, microsporocytes and tissues (anthers or calli) were homogenized in a glass tissue grinder in 70% ethanol followed by sonication in Eppendorf centrifuge tubes. After centrifugation at 12,000 rpm for five minutes in a microfuge, the clear supernatant was separated. The pellet was washed once with 70% ethanol, centrifuged again and the combined supernatant was then dried at −55°C under a nitrogen stream. All samples to be compared were assayed simultaneously using the same assay batch of kit. Values were the averages taken from three measurements. The amount of protein was determined according to the method of Lowry *et al.* (7).

cAMP phosphodiesterase assay. The enzyme activity was measured by the one-step assay procedure (8) using [^{3}H] 3′,5′-AMP and snake venom. Briefly, the cells or tissue were homogenized in about 10 times volume of 40 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 4 mM 2-mercaptoethanol with a tissue grinder and sonicator, followed by centrifugation at 12,000 × g for 10 min. The supernatant (50 μl) was mixed in an Eppendorf tube, with an equal volume of twice concentrated reaction mixture, consisting of 1 part of [^{3}H] cAMP (200,000 cpm /assay), 2 parts of 50 mM cold cAMP in 40 mM Tris-HCl, pH 8.0, 2 parts of 10 mM 5′ AMP in 40 mM Tris-HCl, pH 8.0 and 5 parts of 40 mM Tris-HCl pH 8.0. The incubation was

started by addition of snake venom (10 μl of a 1 mg/ml solution) and immersion in a 30°C water bath for 10 min. The reaction was stopped by addition of 0.8 ml of resin slurry (Bio-Rad Laboratories AG 1 × 2, 200 mesh, two volumes of water and one volume of resin). After 10 min. the resin settled down and an aliquot was taken out and counted in scintillation vial with 10 ml of scintillator. For the control, cell homogenate heated to 95°C for 10 min. was used.

AMP cyclase assay. The activity of adenyl cyclase was measured according to method C using the published procedure (9). Briefly, microsporocytes and somatic tissues ground in a tissue-grinder in 10 times volume of 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA, were further sonicated after addition of NaCl to 1 M followed by high speed centrifugation at 20,000 rpm for 60 min. The resultant pellet was resuspended in 2.2 M sucrose in Tris-EDTA overlaid by a small amount of 0.3 M sucrose and centrifuged. The membranous material, localized at the top of the 2.2 M sucrose under the 0.3 M sucrose due to its light density, was collected carefully, diluted with Tris-EDTA and pelleted through 0.5 M sucrose. The pellet was resuspended in a small amount of 5 mM Tris-HCl (pH 7.5) with aid of sonication and aliquots were used to determine protein by the Lowry method (5). The reaction mixture (100 μl) consisted of the following final concentrations given in parentheses: Tris-HCl (pH 7.5) (5 mM), $MgCl_2$ (1 mM), creatine phosphate (4 mM), creatine phosphokinase (20 unit /ml), cAMP (0.2 mM), α-^{32}ATP (0.2 mM, 100 cpm/pmole), glucagon (0.2 mM) and 2.5–25.0 μg protein. Incubation was carried out at 30°C for 5 min. and stopped by addition of 100 μl of 2% SDS, 40 mM ATP and 1.4 mM cAMP at pH 7.5. Fifty μl of [^{3}H] cAMP (20,000 cpm) was then added as a marker. For measurement of background, membrane fraction heated at 95°C for 10 min. was used. To each reaction tube was added 0.75 ml of 50 mM Tris-HCl, pH 7.5, followed by mixing and decanting into Dowex 50 AGW × 4 (0.4 × 15 cm). The pass through fraction and two successive 1 ml washes were discarded. Three ml of H_2O were added onto the column and the elutes were collected. To each 3 ml fraction, 0.2 ml of 1.5 M imidazole-HCl, pH 7.2 was added and decanted into columns (0.4 × 15 cm) containing 0.6 g neutral alumina. The radioactivity of eluents was measured in scintillation counter with 12 ml Aquasol. Production of cAMP was calculated from the ^{32}P activity.

Formation of protoplasts. Microsporocytes precultured overnight in White's media as described earlier (9) were resuspended in 2% cellulase Onozuka 565-N, 1% macerozyme R-10, 0.35 M sucrose and incubated at room temperature for 15 to 25 min. with occasional stirring. When the microsporocyte filaments could be broken to form a cell suspension by up-down movement through a Pasteur pipette, the incubation was terminated by addition of White's media with 0.35 M sucrose and the protoplasts were collected by centrifugation (1,000 rpm., 2 min.). After washing they were subjected to *in vitro* cAMP treatment.

Progress of meiosis and chromosome behavior. Treatment of protoplasts with cAMP, caffeine and other chemicals was carried out by adding the chemicals to the culture media (White's media with 0.35 M sucrose) and changing media every 7 days. Meiotic stages were observed under a microscope after fixation (3 : 1 mixture of ethanol and acetic acid with 0.3 M sucrose) and staining with acetocarmin.

RESULTS

Intracellular level of cAMP during meiosis. During meiosis, the level of intracellular cAMP in lily micrisporocytes was determined to be in the range of 0.1 pmole/mg protein to 0.5 pmole/mg protein (Fig. 1). In lily callus cells, it was in the range of 0.6 pmole/mg protein to 0.9 pmole/mg protein with an average value of 0.75 of a pmole/mg protein, this range being found in young anthers in which meiocytes were not yet differentiated. In the microspore products of meiosis, it showed an average value of 0.8 of a pmole/mg protein, this cAMP level being lower in meiotic than in somatic or haploid germ cells. In the present experiment, we could not pinpoint the time of reduction of cAMP level but speculate that it occurs during the G_2 period of premeiotic interphase or the late premeiotic Sphase, since the cAMP level of total anther tissue showed no change until micrisporocytes become separable from tapetal tissue. After tapetal cell degradation, when the micrisporocytes become free from the anther wall, in late premeiotic S or G_2, a reduction of total cAMP was observed, probably due to the lower level in meiocytes. During

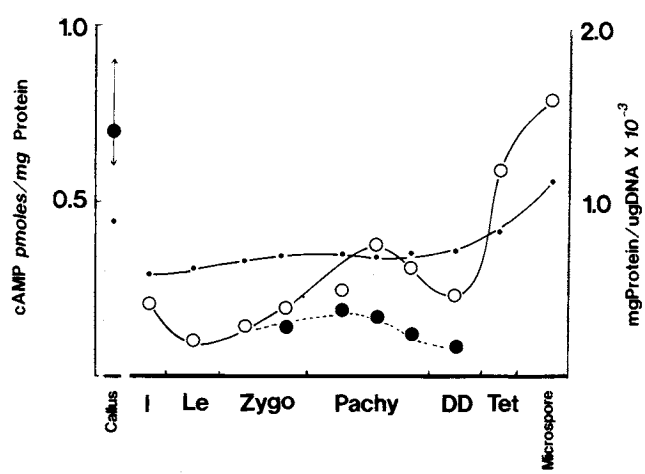

Fig. 1. The level of cAMP in lily microsporocytes.
Callus: actively growing calli under lighted conditions. Meiotic stages on the horizontal axis are I: premeiotic interphase, Le: leptotene, Zygo: zygotene, Pachy: pachytene, DD: diplotene-diakinensis but mixed with metaphase and anaphase cells, Tet: Tetrads mixed with division II. Changes of protein content per unit DNA are indicated by (●——●), cAMP levels in pmoles/mg protein are indicated for normal cultivar Enchantment (○——○) and achiasmatic Black Beauty (●----●) microsporocytes.

this period, morphological features of cells in the anther wall remain the same, including the mitotic frequency.

The appearance of a minor increase in cAMP in zygotene and pachytene which may have some relation with phenomena like chromosome condensation, homologue pairing and recombination can not be ignored. The increase was up to four times the minimum leptotene value, with about 50% of the high value found in mitotic cells, and was reproducible. In Black Beauty, however, this increase was much less pronounced (30% of the normal, about 0.2 cAMP pmoles/mg protein). The alterations in cAMP levels mentioned above are all expressed in terms of mg protein. However, assessed on the basis of DNA or per cell, the results were still the same.

Activity of cAMP phosphodiesterase and adenylcyclase. The activity of cAMP phosphodiesterase was measured in normal chiasmatic lily and achiasmatic Black Beauty meiocytes. As shown in Fig. 2, phosphodiesterase activity was much higher (2.2×10^3 pmoles cAMP hydrolysis/mg protein/10 min.) at the beginning of meiosis than in somatic cells decreased to less than one fifth at the end of zygotene and remaining at low levels until the microspore stage. The phosphodiesterase activity in the Black Beauty was essentially the same as in the normal chiasmatic micrisporocytes. The activity of adenylcyclase was high, at about 80 to 90 pmoles cAMP synthesis/mg protein/10 min., in premeiotic interphase, tetrads, and microspores as well as in somatic cells. High activity was also observed during zygotene and early pachytene (Fig. 2). A good fit for the temporal sequence in change of adenylcyclase and intracellular cAMP was obtained. The absence of adenylcyclase and cAMP peaks during zygotene/pachytene in the achiasmatic Black Beauty should be noted in relation to homologous pairing and probable DNA recombination.

Effect of exogenous cAMP on meiotic progress. Unlike in yeast, there is at present no available method to alter the intracellular cAMP level by introducing or removing responsible genes in lily micrisporocytes. We therefore attempted to increase the level by simply adding cAMP to the culture media. However, micrisporocytes as well as microspores and somatic cells did not incorporate cAMP from the culture medium even in the presence of concentrations as high as 20 mM of cAMP and thus the amount of intracellular cAMP did not increase. The addition of ^{3}H-cAMP (1 μc/nmole/ml) to the medium also indicated no incorporation of cAMP into cells even after 72 hours culture. The use of dilute concentrations of digitonin or Triton x100, or nonidet P-40 did not alter the permeability of cell membranes to cAMP. The use of organic solvents often used for prokaryotes killed micrisporocytes. In contrast to the micrisporocyte case its protoplasts did incorporate radioactive cAMP with significant increase in intracellular levels (up to a high of 3.5 pmols/mg protein) after 24

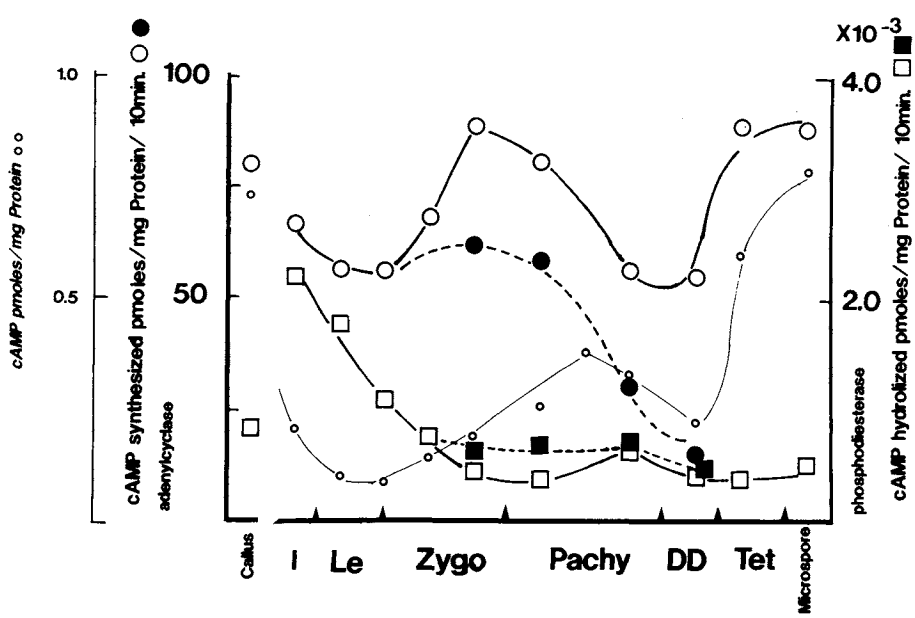

Fig. 2. Activities of adenylcyclase and cAMP phosphodiesterase.
Adenylcyclase activity (circles) and cAMP phosphodiesterase activity (squares) in normal chiasmatic (○—○, □—□) and achiasmatic lily microsporocytes (●----●, ■----■) were measured. The thin line with small open circles represents cAMP pmoles/mg protein, shown for comparison. Meiotic stages on the horizontal axis are the same as in Fig. 1.

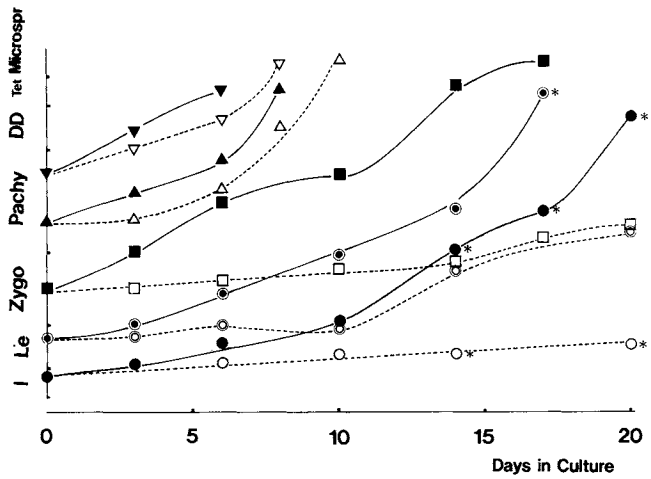

Fig. 3. Progress of meiosis in protoplasts prepared from lily microsporocytes cultured in the presence of high concentrations of cAMP (10 mM) and caffeine (5 mM).
The stages when the protoplasts were exposed to cAMP are indicated on the vertical axis, as in Fig. 1. Cytological observations were made at the desired intervals as described earlier (5). Since a loss of synchrony became evident, the progress of the majority of protoplasts was followed. For the samples denoted by asterisks, the stage of the most advanced group was recorded. Symbols were given to identify each line and controls without cAMP were indicated by solid ones. The presence of 5 mM caffeine alone did not demonstrate any influence (data not shown).

hours. Transformation into protoplasts was associated with only slight loss of intracellular cAMP. We therefore cultured protoplasts in the presence of 10 mM cAMP together with 5 mM caffeine (presence of caffeine increases permeability to cAMP), and observed meiotic stages every 3 to 5 days. The results of our observations are summarized in Fig. 3. The addition of cAMP, with concomitant induction of higher levels of intracellular cAMP, stopped or slowed down the meiotic process in all stages before pachytene (Fig. 3). If premeiotic interphase protoplasts were exposed to high exogenous concentrations of cAMP, they remained in interphase. If the treatment was started in leptotene or zygotene, the shift to the zygotene-pachytene interval occurred. Treatment started during pachytene caused only a slight slowing down of meiosis. After pachytene, exogenously given cAMP exerted practically no effect on meiosis. Thus the findings indicated that protoplasts can not enter into meiosis and pass through the pairing stage of meiotic prophase unless the intracellular cAMP level is reduced in premeiotic interphase or leptotene. Once through pachytene a high level of cAMP does not disturb meiosis even without the reduction of cAMP level observed during diplotene-diakinesis and division II in normal chiasmatic micrisporocytes.

DISCUSSION

It is well established that cAMP is indispensable for the cell cycle, particularly in the early G_1 phase (11), with a high level or cAMP moving the cell cycle from G_1 to S. Sulfur starvation in yeast, induces a shift in cell cycle from G_1 to G_0 which appears due to a reduction in cAMP level (10). In the case of nitrogen starvation, it has been shown that a decrease in intracellular cAMP triggers the initiation of sporulation in *S. cerevisiae* and both sexual development and sporulation in *S. pombe*. The present results clearly showed that this molecular message plays a similar role in the higher plant, *Lilium*. A reduction of cAMP thus appears to bring about activation of a series of specific genes whose products are responsible for carrying out the earlier phase of meiosis.

However, the level of cAMP at different stages of meiosis has not been measured previously. Using lily microsporocytes, in which meiosis occurs synchronously and each stage is distinguishable under the microscope, we were able to demonstrate that an increase of cAMP level coincides with chromosomal pairing and recombination followed by a second reduction, which is in essential agreement with studies in yeasts (1, 11). The fact that with unstable pairing and reduced or no chiasma, as in the case in Black Beauty, no increase of cAMP occurs suggests the requirement of a minor cAMP peak at this stage of normal meiosis.

Our observations on adenyl cyclase and phosphodiesterase activities suggest that the former has the dominant responsibility for controlling intracellular cAMP levels. This requires confirmation by further molecular studies of adenyl cyclase in the lily.

We have demonstrated in the present study that protoplasts are permeable to exogenous cAMP, and that the presence of 5 mM caffeine in the culture medium, which helps uptake of cAMP, is only associated with minimal abnormality, i.e. less than 10% protoplasts had chromosomal fragmentation, producing micronuclei in tetrads. The inhibition of meiosis observed using this model system with exogenously supplied cAMP in the early meiotic process may directly reflect processes operating in yeast sporulation. The apparent decrease in inhibitory influence after late pachytene is a newly observed phenomenon whose further elucidation is clearly of importance for understanding the background mechanisms underlying effects of cAMP and how its expression is regulated.

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