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ON THE PRESENCE OF ADENOSINE 3', 5' -CYCLIC MONOPHOSPHATE IN MOSS (FUNARIA HYGROMETRICA).

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SUMMARY. Partially purified nucleotide fraction of moss containing [14C] -labelled putative adenosine 3', 5' -cyclic monophosphate (cAMP) and marker authentic [3H] -cAMP was characterized by chemical deamination and also by the enzymatic hydrolysis with beef heart cyclic nucleotide phosphodiesterase. A significant conversion of marker authentic [3H] -cAMP into [3H] -inosine 3', 5' -cyclic monophosphate (cIMP) and [3H] -5' adenosine monophosphate was observed by respective treatments. In contrast, the [14C] -labelled putative cAMP from control and theophylline-treated moss tissue was insensitive to chemical deamination and enzymatic hydrolysis. Apparently, the [14C] -labelled product which comigrates with authentic [3H] -cAMP does not represent true cAMP. Both the methods employed for characterization of the labelled putative cAMP were sensitive enough to detect picomole quantities of authentic [3H] -cAMP. Lack of detectability of pre-labelled [14C] -cAMP in our preparations implies that the tissue may contain authentic cyclic AMP below the picomole levels. Thus, the attributed physiological role to adenosine 3', 5' -cyclic monophosphate in moss tissue appears somewhat skeptical.

INTRODUCTION

Cyclic AMP, a mediator of several animal hormonal responses (1), has been detected in the entire animal kingdom and also in the prokaryotes (2). The presence of cAMP in higher plants, however, continues to be a controversial issue (3-5). The evidence in support of the occurrence of cAMP is reported in the leaf tissue of Phaseolus vulgaris by mass spectrometric analysis (5). Other workers claim, that this cyclic nucleotide was present below the threshold level and is of no physiological relevance in higher

Abbreviations: 5' AMP - Adenosine 5'-monophosphate, cAMP - Adenosine 3', 5' -cyclic monophosphate, ATP - Adenosine triphosphate, cIMP - Inosine 3', 5' - cyclic monophosphate, PDE - Cyclic nucleotide phosphodiesterase, DPM - Disintegrations per minute, PCA - Perchloric acid, Rf - Relative flow.

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plants (3,4, 6-8). Among the lower plants, cAMP has been reported in Chlamydomonas reinhardtii (9,10), Dictyostelium discoideum (11) and Aspergillus nidulans (12). Recently, Handa and Johri have claimed the presence of cAMP in moss (Funaria hygrometrica) (13) and suggested that it played a regulator role in cellular differentiation (14). They isolated a nucleotide which activated protein kinase, and in this respect, was indistinguishable from authentic cAMP. However, no further attempt was made to characterize this putative cAMP. The protein kinase assay by itself, cannot be considered a decisive evidence for the absolute identification of cAMP (10) and the above claim could at best be considered a stimulatory effect of putative cAMP on protein kinase. The objective of the present investigation was to provide a rigorous proof for the presence of authentic cAMP in moss tissue. Only then, is one justified in assigning a physiological or a metabolic role to this cyclic nucleotide in Funaria. This study has revealed that the [14C]-labelled putative cAMP, isolated from moss tissue, showed no resemblance to authentic [3H]-cAMP, when it was subjected to chemical deamination and enzymatic hydrolysis.

MATERIALS AND METHODS

Theophylline, beef heart cyclic nucleotide phosphodiesterase, inosine 3',5'-cyclic monophosphate, adenosine 3',5'-cyclic monophosphate and adenosine-5'-monophosphate were purchased from Sigma Chemicals, U.S.A. and radioactive [3H]-cAMP and adenine -[8-14C] were supplied by the Isotope Division, Bhabha Atomic Research Centre, Trombay, India.

Moss protonemal filaments and gametophytic tissue were cultured in liquid low calcium medium (14) at 25±2°C in continuous light (3500+100 lux). The protonemal (10-15 day-old) and gametophytic (30-40 day-old) cultures were administered labelled adenine -[8-14C](15 μCi per experimental set; sp. act. 54 mCi/mmole) + chloramphenicol (50 μg/ml) for 72 hr. In another set of experiments, theophylline (10 mM) was added along with labelled [14C] -adenine to the moss cultures. The tissue (0.3 - 1.0 g) was harvested after 72 hr and rinsed (x2) with chilled Tris-HCl buffer (50 mM, pH 7.6). Authentic [3H]- cAMP (10 μCi, sp. act, 10.1 Ci/mmole) was added during homogenization of the tissue. The homogenate was centrifuged in cold at 15,000 xg for 15 min. The clear supernatant was precipitated with cold perchloric acid (PCA) to a final concentration of 5%. The PCA - precipitable

fraction was removed by centrifugation (12,000 xg, 10 min.). The pH of the supernatant was adjusted to 7.0 with alkali. The average recovery of authentic [3H]- cAMP in this supernatant was 61%. The neutralized fraction (7.0 ml) was dried at 37°C and the residue was resuspended in distilled water (one ml). Authentic unlabelled cAMP (4.8 µmoles) was mixed with this fraction. The preparation was then fractionated by ascending paper chromatography on Whatman No. 1 (solvent system - ammonium acetate 1M: ethanol 95%; 3:7) for the isolation of [14C]-labelled product which comigrated with [3H]-cAMP. The zone containing authentic [3H]-cAMP together with [14C]-labelled putative cAMP was eluted with an aliquot (20 ml) of Tris-HCl buffer (50 mM; pH 7.6). The partially purified fractions (Fraction I) were further processed and subsequently characterized by enzymatic and chemical procedures.

Hydrolysis by cyclic nucleotide phosphodiesterase :

The nucleotide pool in the above fraction was adsorbed on purified activated charcoal (4 mg/ml). The charcoal pellet was eluted with 3% ammonia prepared in 50% ethanol. The eluant was dried completely and redissolved in distilled water (one ml) . This charcoal fraction, (Fraction II) was subjected to enzymatic hydrolysis with cyclic nucleotide phosphodiesterase (PDE). The incubation mixture comprised sample Fraction II (100 µl), $MgSO_4$ (2 µmoles), PDE (0.1 enzyme unit in 0.5 mg protein) and Tris-HCl buffer (7.5 µmoles; pH 7.6) in a final volume of 250 µl. The mixture was incubated for 3 hr at 30°C. Boiled enzyme (PDE) served as control. The reaction was terminated by immersing the assay tubes in boiling water for 3 min. and then instantly chilled. The denatured precipitated protein was removed by centrifugation. An aliquot (0.1 ml) from the enzyme -treated fraction was subjected to ascending paper chromatography for product analysis. Authentic cAMP and 5' AMP were used as markers for locating the relative position of the two labelled nucleotides on the chromatograms. Two cm long strips were cut out serially from the chromatograms for scanning the radioactivity of [3H]- and [14C]-label.

Chemical deamination by nitrous acid :

The following procedure was followed for the deamination of [3H] -cAMP and [14C]-labelled putative cAMP product. An aliquot (0.35 ml) of Fraction I was mixed with acetic acid (0.6 ml, 2N) and sodium nitrite (3.4 mmoles) in a total volume of 950 µl. The reaction was allowed to occur for 48 hr at 25°C and finally terminated by dilution with H_2O to 4.0 ml. The nucleotide pool in deaminated and undeaminated fractions was adsorbed on activated charcoal(4 mg/ml). The charcoal pellet fraction was eluted with 15 ml of 3% ammonia prepared in 50% ethanol. The eluant was evaporated to dryness and the residue was resuspended in distilled water (one ml). The samples were mixed with carrier unlabelled cAMP and inosine 3', 5' -cyclic monophosphate (cIMP) and electrophoresed (5.25 hr) on Whatman 3 mm using ammonium formate buffer (50mM; pH 3.0). The radioactivity of [3H]and [14C]-label was measured in one cm long strips from electropherograms in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The protonemal and gametophytic tissue of moss was administered [14C]- adenine (72 hr) with a view to label the endogenous pool of ATP. It

was argued, that if the system has the enzymatic potential to metabolize labelled[14C]-ATP to[14C]-cAMP, then it should be possible to accumulate sufficient amount of[14C]-labelled putative cAMP, which could be characterised later. Accordingly, the tissue incubated with[14C]-adenine was harvested and authentic[3H]-cAMP (10 μCi) was added during homogenization in order to monitor the recovery and purity of the cyclic nucleotide. The nucleotide preparation, obtained by perchloric acid (PCA 5%) precipitation of the homogenate, was fractionated by ascending paper chromatography. By this method, it was possible to separate[14C]-labelled putative cAMP (Rf = 0.36) from the bulk of[14C]-adenine (Rf = 0.68). The[14C]-labelled nucleotide fraction which comigrated with authentic [3H]-cAMP, was eluted from the chromatogram (Fraction I) and further purified by adsorption on activated charcoal. The nucleotide fraction, eluted from the charcoal pellet was hydrolyzed by cyclic nucleotide phosphodiesterase (PDE) and the products analyzed by paper chromatography. The results of this experiment are presented in Fig. 1A, B. A substantial conversion of authentic [3H]-cAMP to[3H]-5' AMP by PDE treatment was observed in the partially purified fractions prepared from protonemal and gametophytic tissue. In contrast, the[14C]-labelled putative cAMP remained virtually unaffected and there was no migration of the [14C]-label into the region of 5' AMP. Boiled PDE, kept as control, showed no migration of[3H]- and [14C]-label to the region of 5' AMP. In another experiment, theophylline (10 mM) was added along with[14C]-adenine to protonemal cultures of moss. Theophylline is known to increase the endogenous level of cAMP by inhibiting the activity of cyclic nucleotide phosphodiesterase (15). In this case too, the partially purified fraction, when subjected to hydrolysis by PDE showed substantial conversion of[3H]-cAMP to [3H]-5'AMP, while[14C] -labelled putative cAMP remained unaffected (Fig. 1C). Thus, no modulation of the endogenous pool of cAMP was observed by theophylline treatment of

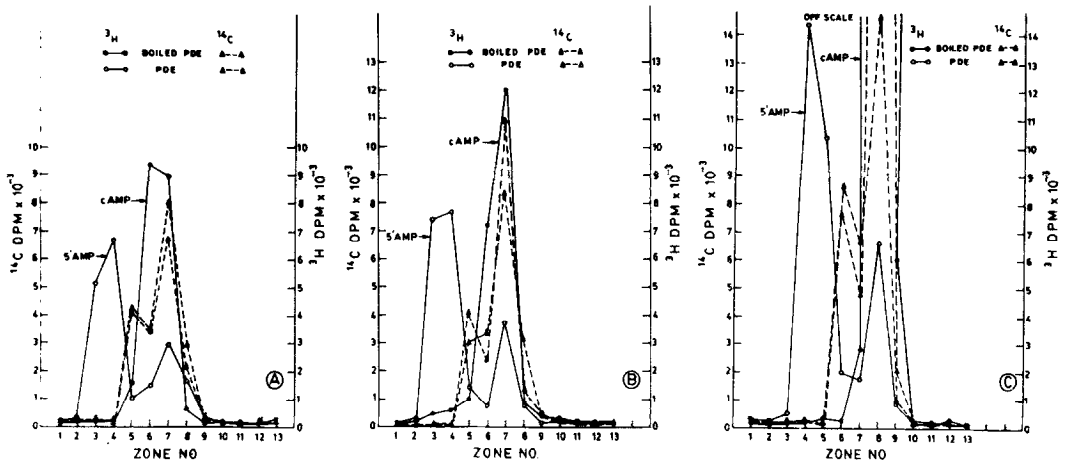

Fig. 1. Chromatographic profiles of [3H]- and[14C]- labelled nucleotide fractions following hydrolysis by beef heart cyclic nucleotide phosphodiesterase. A: protonemal filaments, B: gametophytic tissue and C: protonemal filaments treated with theophylline (10 mM).

moss cultures. The partially purified fractions used for enzymatic analysis repeatedly showed a small peak activity of[14C]-label between the cAMP and 5' AMP zones (Fig. 1 A, B, C), irrespective of treatment with active or boiled PDE. The fact that PDE treatment did not increase this peak activity rules out its import in the present investigation.

In other experiments, instead of scanning the entire chromatogram, the radioactivity of [14C]- and [3H]-label was measured in the UV spots of cAMP and 5' AMP in PDE-treated samples. There was a 50-80% conversion of authentic[3H]- cAMP to[3H]- 5' AMP, whereas the per cent conversion of [14C]-labelled putative cAMP to[14C]-labelled 5' AMP was only 1-3%. This suggested that the bulk of[14C]-labelled product co-migrating with authentic [3H]-cAMP did not represent true cAMP. Chromatographically separated authentic[3H]-cAMP together with[14C]-labelled putative cAMP (Fraction I) was also subjected to chemical deamination by nitrous acid, which converts authentic cAMP to cIMP. After deamination, the preparations were fractionated by paper electrophoresis for the separation of two nucleotides. The[3H]-

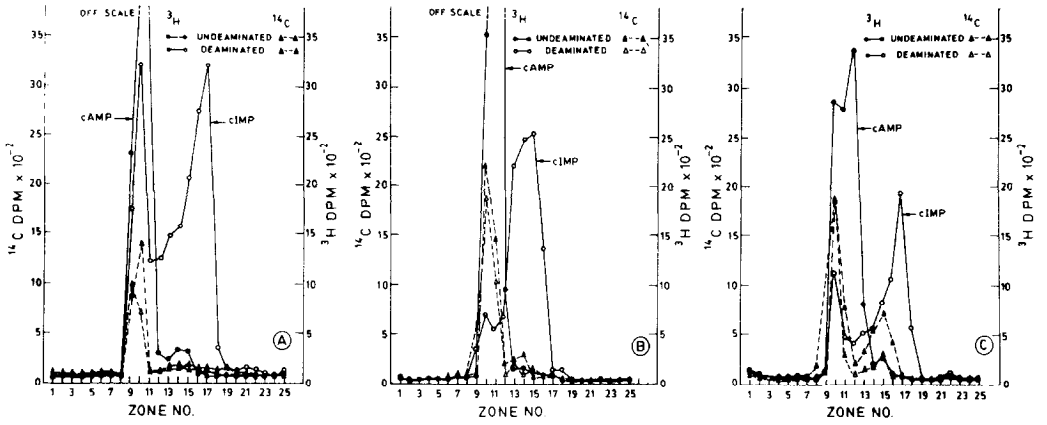

Fig. 2. Electrophoretic profiles of [3H]- and [14C]- labelled nucleotide fractions following deamination by nitrous acid. A: protonemal filaments, B: gametophytic tissue, C: gametophytic tissue treated with theophylline (10 mM).

and[14C]-label in the undeaminated samples were restricted to the zone of authentic cAMP. Deamination resulted in the diminishing of[3H]-cAMP peak with a concomitant appearance of the [3H]-label in the authentic cIMP zone. In contrast, the[14C]-label did not migrate to the zone of cIMP (Fig. 2A, B). This indicated that the[14C]-labelled putative cAMP did not undergo any deamination. Furthermore, the[14C]-labelled putative cAMP, isolated from theophylline-treated gametophytic tissue, did not undergo any deamination (Fig. 2C). We have also tested the presence of cAMP in another moss (Dicranella coarctata) by the use of labelling technique. The[14C]-labelled product which comigrated with authentic [3H]- cAMP was isolated from the protonemal tissue and subjected to deamination. Once again, the[14C]-labelled putative cAMP showed no conversion into[14C]-cIMP. Thus, the[14C]-labelled putative cAMP was clearly distinguishable from authentic[3H]-cAMP by chemical and enzymatic analysis.

The methods employed in the present study were sensitive enough to detect one picomole of authentic [3H]-cAMP in our nucleotide preparations. Thus it appears that the levels of cAMP in moss tissue are perhaps too low to

be detected by these procedures. This then raises a question, whether the presence of cAMP below the picomole level has any physiological relevance in the moss system. It may be recalled that in animal systems, where cAMP has been shown to have a physiological role, it exists at concentrations of 100-500 picomoles/g tissue (4). Also the level of this cyclic nucleotide is modulated by the addition of theophylline (15). However, no cAMP was detected in our system, following the addition of theophylline to moss cultures.

Thus our characterization data of[14C]-labelled putative cAMP, tentatively suggests that cAMP may not have any physiological role in the cellular differentiation of moss. We consider that the moss tissue contains som nucleotide which comigrates with authentic[3H]-cAMP and possibly interferes with the protein kinase assay.

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