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Cyclic Adenosine 3′:5′-Monophosphate in Axenic Rye Grass Endosperm Cell Cultures[1]

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ABSTRACT

Cyclic adenosine 3′:5′-monophosphate (cAMP) was extensively purified from rye grass (*Lolium multiflorum*) endosperm cells grown in axenic suspension culture. The cAMP was purified by neutral alumina and anion and cation exchange chromatography. The cAMP was quantitated by means of a radiochemical saturation assay using a beef heart cAMP-binding protein and also by an assay involving activation of beef heart protein kinase. The cAMP levels found (corrected for recovery of tracer cyclic 3′,5′-[8-^{3}H]AMP included from the point of sample extraction) ranged from 2 to 12 pmol/g fresh weight. The material purified from rye grass cultures was indistinguishable from authentic cAMP with respect to chromatography in two cellulose thin layer systems, behavior on dilution in both the saturation and protein kinase activation assays, and rates of degradation by a mammalian cAMP phosphodiesterase. The cAMP from rye grass cultures was completely degraded by a mammalian cAMP phosphodiesterase, and 1-methyl-3-isobutylxanthine inhibited such degradation. The protein kinase activation and saturation assays gave essentially the same values for the cAMP content of axenic rye grass culture extracts. Material satisfying the above criteria for identity with cAMP was also isolated from the culture medium. The increase observed in medium cAMP levels during culture growth provides evidence for the synthesis and secretion of cAMP by rye grass endosperm cells in suspension culture.

The occurrence of cyclic AMP in higher plants is currently a matter of some controversy. While there have been many claims for the presence of cAMP[3] in higher plants, the adequacy of the evidence advanced has been questioned on many grounds (2, 15). Ambiguities in previous studies derive from the presence of plant compounds that interfere with biochemical cAMP assay systems, the sensitivity of the assay systems used, the possibility of microbiological contamination, and insufficiency of criteria satisfied to support identity of the extracted plant substance with cAMP. While reported estimates of the cAMP content of higher plants range up to 10^4 pmol/g fresh wt (14, 22) other groups, employing sensitive detection and characterization procedures, have reported that cAMP, if present at all, occurs at levels of less than 1 to 10 pmol/g fresh wt (1, 6, 13).

In a recent study we estimated minimum cAMP levels of 1 and 2 to 6 pmol/g fresh wt in *Kalanchoë daigremontiana* and *Agave americana*, respectively (5). Identification of plant material as cAMP involved satisfaction of a more exacting set of criteria than in previous higher plant studies. The criteria satisfied included co-chromatography in a variety of thin layer systems, identical behavior in saturation and protein kinase assays, and identical rates of phosphodiesterase-catalyzed degradation of the plant material and authentic cAMP (5). The microbiological contamination of the *K. daigremontiana* and *A. americana* leaves was at least 10^3 times too low to account for the observed cAMP as being due to intracellular bacterial cAMP (5). Nevertheless a formal (albeit unlikely) possibility exists that long term, continuous secretion of cAMP from the microflora present was responsible. We have now eliminated this residual ambiguity by the analysis of higher plant cells in axenic liquid suspension culture. In the present paper we present evidence for the occurrence of intra- and extracellular cAMP in axenic rye grass endosperm cell cultures.

MATERIALS AND METHODS

Growth of Rye Grass Endosperm Suspension Culture. The *Lolium multiflorum* Lam. endosperm cell culture was the line described by Norstog *et al.* (19) and was grown in liquid suspension culture at 26 C on an orbital shaker using the methods and medium described by Smith and Stone (24). The cells were grown in 250-ml conical flasks containing 100 ml of a modified White's liquid medium containing 0.4% (w/v) Difco yeast extract (24). Sucrose (4%, w/v) was routinely used as the carbon source in the growth medium.

Extraction and Isolation of cAMP from Rye Grass Cell Cultures. Cells were collected by filtration without further washing on a sintered glass funnel. The cells were rapidly weighed and suspended in 75 ml (about 3 volumes) of ice-cold 0.4 M $HClO_4$ containing 0.8 pmol [8-^{3}H]cAMP. The suspension was homogenized using an Ultra Turrax blender at top speed for 1 min. To ensure complete disruption of the cells the homogenate was then subjected to three cycles of freezing in an ethylene glycol-methanol bath (−30 C) and thawing. Insoluble debris was removed by filtration through GF/C filter paper. The filtrate was neutralized with 10 M KOH to pH 7.5, made 50 mM with respect to Tris (Cl^-) and the $KClO_4$ precipitate removed by centrifugation.

When the culture medium was processed, 3 ml of 72% $HClO_4$ was placed in the receiving flask of the filtration apparatus, the filtered medium collected and then tracer [8-^{3}H]cAMP added (1 pmol/flask). The acidified medium was neutralized to pH 7.5 with 10 M KOH and the precipitate removed by centrifugation. Polysaccharides, which interfere with the subsequent column chromatography, were precipitated by the addition of 4 volumes of ethanol, removed by centrifugation, and the supernatant taken to dryness in a rotary evaporator. The residue was redissolved in 60 mM Tris (Cl^-) (pH 7.5).

Cyclic AMP was purified from rye grass cell and medium extracts using a modification of our previously described procedure (5). This involved initial batchwise treatment with alumina followed by anion and cation exchange chromatography and was less time-consuming than the previous method. The batchwise procedure was found to be as effective as column chromatography

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[3] Abbreviation: cAMP: cyclic adenosine 3′:5′-monophosphate.

for removal of substances absorbing at 260 nm and for recovery of a high percentage of tracer [8-^{3}H]cAMP. In all of the following purification steps, solutions were made up in glass-redistilled H_2O. Dry neutral alumina was added to the neutralized tissue extracts (1 g of alumina/g fresh wt of tissue or 1 g of alumina/50 ml of the original medium) and the suspension stirred at room temperature (22 C) for 15 min. The alumina was removed from the tissue extract by filtration through a sintered glass funnel and the retained alumina washed with 1 volume of 0.6 M Tris (Cl^-) (pH 7.5). The entire fraction not absorbed to alumina was then applied to a column (1.1 cm diameter) containing AG 2 anion exchange resin (0.5 ml of resin/g fresh wt of plant tissue or 0.5 ml of resin/50 ml of medium extracted). The column was washed with 2 column volumes of water and the cAMP was then eluted with 50 mM HCl and 3-ml fractions collected. The cAMP emerged soon after the pH of the eluate dropped to the pH of the eluant. The exact position of the tracer [8-^{3}H]cAMP was determined by counting aliquots from each fraction. The fractions containing [8-^{3}H]cAMP were pooled, concentrated to 5 ml, and made 0.1 N with respect to HCl. The cAMP fraction was then applied to a column (0.9 cm diameter) containing 2 ml of AG 50W cation exchange resin which had been preequilibrated immediately prior to use with at least 10 ml of 0.1 N HCl. The column was eluted with a further 5 ml of 0.1 N HCl followed by 1.5 ml of H_2O. Cyclic AMP was then eluted with 5 ml of H_2O and the cAMP fraction concentrated by freeze-drying. The purification procedure resulted in recoveries of about 30% of tracer [8-^{3}H]cAMP.

Thin Layer Chromatography. Plant fractions containing cAMP (and tracer [8-^{3}H]cAMP) were chromatographed on cellulose thin layer sheets as described by Goldberg and O'Toole (11) using either solvent A (isopropyl alcohol-aqueous NH_3-H_2O, 14:3:3, v/v) or solvent B (isobutyric acid-aqueous NH_3-H_2O, 57:4:39, v/v). After ascending chromatography, the chromatograms were dried and cut into 1-cm segments which were subsequently eluted with 2 ml of 50% (v/v) ethanol. Each eluate was taken to dryness and the residue redissolved in a buffer solution appropriate to the subsequent characterization procedures.

Saturation Assay for cAMP. The saturation assay was modified from that previously described (5) to reduce nonspecific interference and to lower the detection limit of the assay substantially. We have found that BSA reduces interference in the saturation assay caused by the inhibitory material encountered in extracts of *K. daigremontiana*. Accordingly, BSA (1.25 mg/ml final concentration) was routinely included in the saturation assay. The detection limit of the saturation assay was lowered by a factor of about 10 by conducting the assay in a final volume of 20 μl instead of the 200-μl volume used previously (5).

All components of the assay were dissolved in assay buffer containing 50 mM Tris(Cl^-), 4 mM EDTA (Na^+) (pH 7.5). Before each saturation assay an aliquot of cAMP-dependent protein kinase was diluted in assay buffer containing 5 mg of BSA/ml so that in the assay conditions used about 30% of [2,8-^{3}H]cAMP was bound to the binding protein in an assay containing no added unlabeled cAMP. Prior to each series of assays an aliquot of [2,8-^{3}H]cAMP was dried at room temperature in a stream of N_2 and then redissolved in assay buffer. To each assay tube was added in the following order: 10 μl of unlabeled cAMP (of either standard or unknown concentration), 5 μl of [2,8-^{3}H]cAMP (0.8 μCi/ml; 38 Ci/mmol) and 5 μl of appropriately diluted cAMP-dependent protein kinase. The solution was incubated for at least 90 min at 0 C before addition of 125 μl of charcoal suspension (0.33% [w/v] Norit GSX and 2% [w/v] BSA in assay buffer) and centrifugation at 2,850*g* for 10 min at 0 C. A 100-μl aliquot of the supernatant (containing protein-bound cAMP) was counted in 4 ml of scintillation fluid A containing an additional 0.3 ml of H_2O. A standard curve relating bound [2,8-^{3}H]cAMP to added unlabeled cAMP (0.025–3.2 pmol) was determined for every series of assays. A typical standard curve is shown in Figure 2.

Protein Kinase Activation Assay. Cyclic AMP concentration was estimated using the assay procedure of Mayer *et al.* (18) and the cAMP-dependent protein kinase obtained from beef heart. The reaction mixture was incubated for 90 min at 30 C and then processed as described by Mayer *et al.* (18). The paper discs were counted in 5 ml of scintillation fluid B.

Degradation of cAMP by a Cyclic Nucleotide Phosphodiesterase. Degradation of cAMP by beef heart cyclic nucleotide phosphodiesterase was conducted essentially as described previously (5). TLC on polyethyleneimine cellulose was used to separate 5'-[^{3}H]AMP from [^{3}H]cAMP (5).

Chemicals. Cyclic [2,8-^{3}H]AMP (specific radioactivity: 38 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were obtained as described previously (5). Scintillation fluid A, used for counting the radioactivity of aqueous samples, contained 1 volume of Triton X-114, 3 volumes of xylene, 0.3% PPO (2,5-diphenyloxazole), and 0.02% dimethyl-POPOP. Scintillation fluid B containing 0.5% PPO and 0.03% dimethyl-POPOP in toluene was used for counting paper discs from protein kinase assays. The counting efficiency was the same for all samples within each radiochemical experiment. The AG 50W-X8; (200–400 mesh) and AG 2-X8; (200–400 mesh) ion exchange resins were obtained from Bio-Rad Laboratories and were washed extensively before use as described previously (5).

Detection of Bacterial Contamination of Rye Grass Suspension Cultures. Every flask of rye grass cells used in this study was examined immediately before harvest for the presence of bacteria or fungi by plating a 1-ml aliquot of the cell suspension onto nutrient agar or yeast extract agar plates and the plates incubated at 26 C for 36 hr. Very rarely was contamination detected, and every flask showing contamination was discarded. Occasionally, additional aliquots were plated onto indicator plates containing glucose eosin methylene blue agar, yielding the same results as with the media used routinely. No bacterial colonies were observed when the endosperm cells were allowed to settle in the suspension culture and the supernatant plated onto yeast extract agar.

The nutrient media used to detect bacterial contamination of the suspension cultures were: (*a*) nutrient agar (2.5% w/v nutrient broth, 1.5% w/v agar); (*b*) yeast extract agar (0.75% w/v yeast extract, 2.8% w/v agar); and (*c*) glucose eosin methylene blue agar (1% w/v glucose, 3.75% w/v eosin methylene blue agar).

RESULTS

Purification of Cyclic AMP from Rye Grass Cultures. The procedures employed previously to purify cAMP from *A. americana* and *K. diagremontiana* (5) were effective in purifying cAMP from rye grass extracts. A large amount of non-cAMP material that interferes with the saturation assay (equivalent to 250 pmol of cAMP/g fresh wt) was removed in the batchwise alumina treatment step. Cyclic AMP eluting from the final AG 50W column was well separated from any material inhibitory to the saturation assay. Thus, in such an elution while 50 pmol of cAMP eluted with tracer [8-^{3}H]cAMP, inhibitory material equivalent to 1 pmol of cAMP in the saturation assay was eluted both before and after the cAMP fractions (data not shown). As is described in detail below, further analysis of the cAMP fraction from the AG 50W step failed to reveal any evidence of material interacting with the saturation assay other than cAMP. The yields of [8-^{3}H]cAMP in the purification procedure described were about 30%.

Thin Layer Chromatography of cAMP from Rye Grass. The putative cAMP from rye grass endosperm cells exactly cochromatographed with tracer [^{3}H]cAMP in two TLC systems (Fig. 1, A and B). The putative cAMP from the 12-day rye grass culture medium also exactly co-migrated with [^{3}H]cAMP on TLC (Fig. 1C). No significant amounts of material interacting in the saturation assay were evident elsewhere on these chromatograms.

Interaction of cAMP from Rye Grass with Beef Heart cAMP-

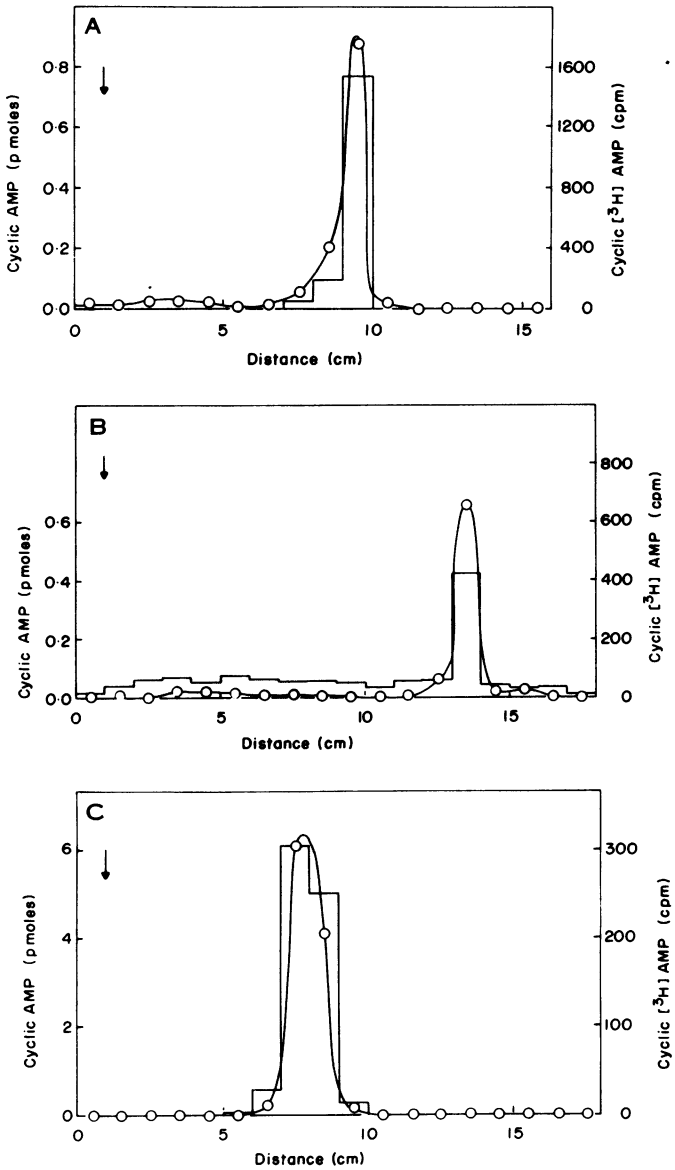

FIG. 1. Cellulose TLC of cAMP purified from rye grass. The cAMP fraction obtained from the AG 50W column step was chromatographed on a cellulose thin layer using either solvent A or solvent B. An aliquot of the eluate from each segment was counted to determine tracer [8-^{3}H]cAMP and an aliquot was assayed for cAMP using the saturation assay. In all of the chromatographic profiles illustrated the amount of [8-^{3}H]cAMP accounted for less than 2% of the cAMP estimated using the saturation assay. ○: [8-^{3}H]cAMP; ——: cAMP estimated using the saturation assay; arrows indicate center of origin. A: cAMP from rye grass cells chromatographed in solvent A; B: cAMP from rye grass cells chromatographed in solvent B; C: cAMP from the medium of rye grass cells chromatographed in solvent A.

binding Protein. The interaction of cAMP from rye grass with the cAMP-binding protein from beef heart was examined over a 500-fold range of concentration in the standard saturation assay. Figure 2 shows that the dilution curve obtained with the cAMP-containing fraction from rye grass can be exactly superimposed upon the dilution curve obtained with authentic cAMP. The cAMP-containing fractions purified from the rye grass culture medium also behaved like authentic cAMP on dilution in the saturation assay. This suggests that the putative cAMP from rye grass cultures interacts with the beef heart cAMP-binding protein in the same way as does authentic cAMP. The dilution curves of the partially purified plant cAMP fractions in the saturation assay system are quite different from the dilution curve obtained with non-cAMP inhibitory material from *K. daigremontiana* (5).

Activation of Beef Heart Protein Kinase by cAMP from Rye Grass. The activation of beef heart cAMP-dependent protein kinase by cAMP-containing fractions from rye grass was examined over a 5-fold range of concentrations. The degree of activation was proportional to the amount of the partially purified cAMP fraction added, and the dilution curve could be exactly superimposed upon the authentic cAMP dilution curve (Fig. 3). Thus, the putative cAMP from rye grass not only binds to the protein kinase but this binding also effects changes leading to activation of the protein kinase.

In both binding to and activation of the beef heart protein

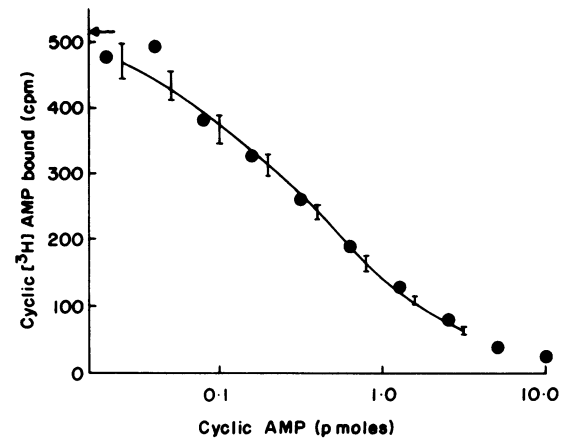

FIG. 2. Interaction of cAMP from rye grass cells with a cAMP-binding protein from beef heart. Cyclic AMP was purified from rye grass cells up to and including cellulose TLC employing solvent A. The cAMP spot was eluted, dried, and redissolved in 240 μl of assay buffer. An aliquot of this sample was carried through a series of nine 2-fold dilutions and 10-μl aliquots were assayed in the saturation assay. Each point of the cAMP standard curve was determined in quadruplicate. Arrow indicates the amount of [2,8-^{3}H]cAMP bound in the absence of added cAMP. ●: cAMP from rye grass (mean of duplicate determinations); ——: cAMP standard curve. Error bars indicate one SD about the mean of each point for the cAMP standard curve.

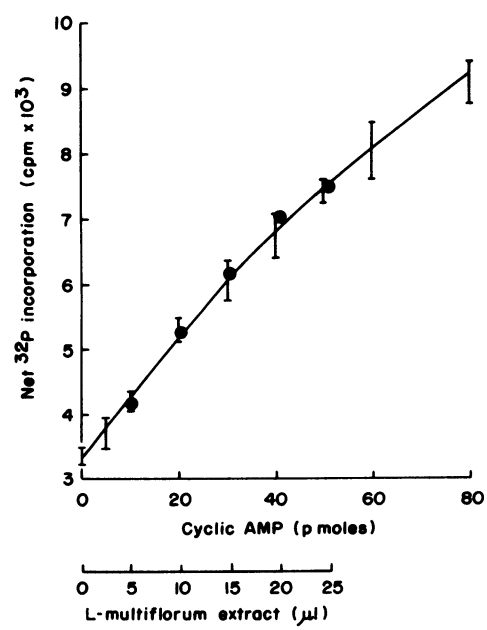

FIG. 3. Interaction of cAMP from rye grass with cAMP-dependent protein kinase. Cyclic AMP was purified from rye grass cells up to and including chromatography on cellulose thin layers using solvent A. The cAMP fraction was redissolved in H_2O and various aliquots added to the protein kinase activation assay. ●: cAMP from rye grass (mean of duplicate determinations); ——: authentic cAMP standard curve (indicating one SD about the mean).

kinase, the putative cAMP from rye grass is quantitatively indistinguishable from authentic cAMP (Figs. 2 and 3). The cAMP content of 12-day cultured ryegrass endosperm cells was estimated using both the protein kinase activation assay and the saturation assay, yielding values of 12 ± 1.3 and 14 ± 2 pmol/g fresh wt (mean ± SD) respectively (samples used for these estimates were purified by the standard procedure up to and including TLC on cellulose in solvent A—see Fig. 1A. Thus, both assay systems yield essentially the same result within experimental error.

Degradation of cAMP from Rye Grass by a Cyclic Nucleotide Phosphodiesterase. Cyclic AMP purified from rye grass cell culture medium was degraded by a beef heart cyclic nucleotide phosphodiesterase at the same rate as authentic cAMP in the same conditions. The putative cAMP purified from the medium was completely degraded by the phosphodiesterase, and this degradation was prevented in the presence of 1 mM 1-methyl-3-isobutylxanthine, a specific inhibitor of the phosphodiesterase (Fig. 4). The progress of this degradation was monitored using both the protein kinase activation assay (Fig. 4A) and the saturation assay (Fig. 4B). The kinetics of cAMP disappearance as determined by either assay system was the same as the kinetics of degradation of authentic [^{3}H]cAMP in the same conditions (Fig. 4). Similarly, putative cAMP from 12-day cultured rye grass endosperm cells was >95% degraded by beef heart cyclic nucleotide phosphodiesterase and such degradation was completely inhibited by inclusion of 1-methyl-3-isobutylxanthine (1 mM) in the reaction mixture. While the absolute rate of degradation of cAMP from rye grass cells was slower than that of authentic cAMP, these rates were the same when corrected for the relative rates of [^{3}H]cAMP disappearance in the same conditions. This suggests that observed differences in the rates of phosphodiesterase-catalyzed hydrolysis of plant cAMP and authentic cAMP may derive from traces of phosphodiesterase inhibitors in the purified plant extracts, as was found for extracts from *K. daigremontiana* (5).

Levels of cAMP in Rye Grass Endosperm Cell Cultures. All samples used for these determinations had been purified by the standard procedure up to and including TLC on cellulose in solvent A. Cyclic AMP was estimated in rye grass cells to be at levels of 3 ± 1, 6.1 ± 2.1, and 14 ± 2 pmol/g fresh wt (mean ± SD of at least four replicates) for cells grown for 9, 10, and 12 days, respectively, after subculturing. The cAMP level found in the medium of 12-day-old cultures was 11 ± 1.9 pmol/ml (mean ± SD of four replicates). Prior to inoculation, 40 pmol of cAMP (derived from the yeast extract nutrient) were present in the medium in each flask. Twelve days after inoculation, the medium contained 925 pmol of cAMP (as compared to a total of 360 pmol of cAMP in 30 g of endosperm cells in the culture). The value for the amount of cAMP secreted into the medium during a 12-day growth period (885 pmol) is considerably underestimated since extracellular cAMP can be degraded during growth of the cells. Thus, less than 1% of tracer [2,8-^{3}H]cAMP included in an initial inoculum could be absorbed to charcoal after 12 days of culture growth. Brewin and Northcote (7) have shown that intact soybean cells in liquid suspension culture can hydrolyze external cAMP.

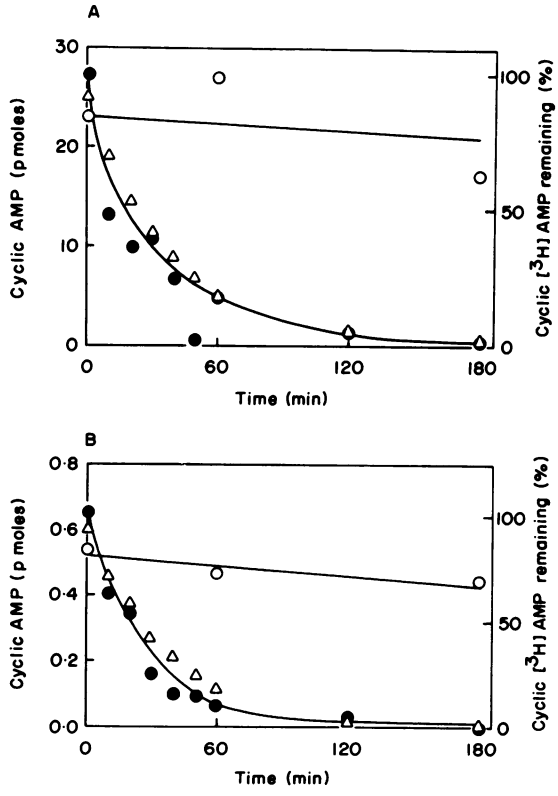

FIG. 4. Degradation of cAMP from rye grass by cyclic nucleotide phosphodiesterase. A reaction mixture containing cAMP purified from the medium of the rye grass cells as shown in Figure 1C was degraded by beef heart phosphodiesterase in the presence or absence of 1 mM 1-methyl-3-isobutylxanthine. Aliquots removed from the reaction mixture at various times were assayed to estimate the cAMP remaining. The cAMP was measured using either the protein kinase activation assay (A) or the saturation assay (B). Cyclic [2,8-^{3}H]AMP (1.5 μCi/ml) was also degraded in the presence of the rye grass extract as described previously (5). ○ and ●: pmol of cAMP (present in [A] the protein kinase activation assay or [B] the saturation assay) after degradation in the presence or absence of 1-methyl-3-isobutylxanthine, respectively. △: per cent of original [2,8-^{3}H]cAMP remaining in reaction mixture containing rye grass extract.

DISCUSSION

The material partially purified from rye grass endosperm cell cultures fulfilled a multiplicity of necessary criteria for identity with authentic cAMP. (*a*) The plant substance co-purified with [^{3}H]cAMP on TLC in two systems (Fig. 1, A, B, and C). (*b*) Dilution curves for the plant substance and authentic cAMP in both the saturation and protein kinase activation assays were superimposable (Figs. 2 and 3). Estimates of cAMP content of rye grass cultures by both assays gave the same value. (*c*) The plant substance was degraded by a beef heart cyclic nucleotide phosphodiesterase at the same rate as authentic cAMP and its disappearance could be monitored using either the saturation assay or the protein kinase activation assay (Fig. 4). Such degradation was prevented by 1-methyl-3-isobutylxanthine (a specific inhibitor of the enzyme), demonstrating that disappearance of the substance was due to the catalytic activity of the cyclic nucleotide phosphodiesterase (5).

Microbiological contamination of the suspension cultures used for analysis was not detectable after plating on several nutrient media, showing that the cAMP accumulation during growth of the cultures was due to the endosperm cells. The specific radioactivity of the tracer [8-^{3}H]-cAMP was the same as that used previously (5) and was added at the point of tissue extraction at the level of only about 0.02 pmol/g fresh wt, *i.e.* added tracer cAMP did not contribute significantly to the observed levels.

Accordingly, we believe that the results presented here provide compelling evidence for the occurrence of cAMP of nonmicrobiological origin in higher plant cells. While rigorous chemical identification (not possible in this study) would be desirable, identification of the plant substance as cAMP in the present analysis involved satisfaction of a more exacting set of biochemical and chromatographic criteria than in previous studies. The function of cAMP as a "second messenger" in animal cells involves its interaction with cAMP-dependent protein kinase (mediation of the primary hormonal signal) and its degradation by cyclic nucleotide phosphodiesterase (to render transient the intracellular consequences of the hormonal signal). With respect to beef heart proteins, the plant substance is functionally indistinguishable from cAMP in terms of binding to the protein kinase regulatory subunit, protein kinase activation, and cyclic nucleotide phosphodiesterase-catalyzed degradation.

A major problem in biochemical analyses for cAMP in plants has derived from the large amounts (relative to cAMP levels) of plant material that is inhibitory in the cAMP-binding saturation assay. Thus, in the detailed studies by Bressan *et al.* (6) and Handa and Johri (12) the levels of cAMP determined by the saturation assay were 7 to 20 times the levels determined by the protein kinase assay in some cases, and the presence of non-cAMP inhibitory material was apparent from chromatographic separations. In both of these studies a cation exchange chromatography step was employed in the purification of the cAMP fraction—the samples in both cases being applied to and eluted from the Dowex 50 or Aminex MS in H_2O (6, 12). This procedure was found to be ineffective for separation of inhibitor and cAMP on AG 50W (Dowex 50) in our previous studies with *K. daigremontiana*; however chromatography on 0.1 N HCl was found to retard cAMP sufficiently to permit complete separation from inhibitory material (unpublished data). This latter procedure was also employed for the purification of cAMP from rye grass cells on AG 50W. The *K. daigremontiana* inhibitor that was resolved from cAMP on AG 50W (5) binds to alumina (Ashton and Polya, unpublished data) and was clearly not completely removed at the first purification step by the quantity of alumina used (0.5 g/g fresh wt). Accordingly, in the present study 1 g of alumina/g fresh wt was employed to ensure virtually complete removal of inhibitory material (about 250 pmol of "cAMP equivalent"/g fresh wt) from rye grass extracts. While the saturation assay inhibitor(s) encountered in rye grass, *K. daigremontiana* (5), moss protonema (12), and a variety of higher plants (1, 6) are not necessarily similar chemically, the procedures used in the present study have been effective in resolving such inhibitors from cAMP in extracts from several higher plants and may be useful in further biochemical estimations of higher plant cAMP levels. Unfortunately, the saturation assay appears to be considerably more sensitive to such inhibitors than the protein kinase activation assay (6, 12), while it is analytically the more sensitive assay. Given the very low levels of cAMP in higher plants, application of effective purification procedures of the kind described here are required before unambiguous saturation assays—including highly sensitive radioimmuno assays (8)—can be applied to cAMP-containing fractions from plants.

In *L. multiforum* endosperm cells grown for about 14 days in suspension culture, the cytoplasm is present as a narrow peripheral layer with a width approximately 2% of the cell radius (17). In these cells the cytoplasm occupies about 5% of the intracellular volume. If all of the rye grass cAMP is localized in the cytoplasm the cAMP concentration (12 pmol/g fresh wt) will be about 0.2 μM. This is comparable to cAMP levels in some animal cells (3).

The level of cAMP found in the medium of 12-day axenic cultures (10 nM) is too great to be accounted for by the release of cAMP from dead cells (equivalent to an additional 75 g fresh wt/culture flask over the 30 g fresh wt present as intact cells). Moreover, the net amount of cAMP in the medium at 12 days considerably underestimates the amount of cAMP entering the medium during a 12-day growth period since cAMP in the medium is degraded. Accordingly, the data suggest that cAMP is secreted from the rye grass cells and that a concentration gradient of 0.2 μM inside to 0.1 μM outside the cell may occur in 12-day-old cultures. The secretion of cAMP has been described for both microorganisms (10) and animal cells (9). However, while secretion of cAMP from bacteria represents a major mechanism for actively reducing intracellular cAMP concentrations (10), low and high K_m cyclic nucleotide phosphodiesterases, rather than secretion, are largely responsible for this function in animal cells (3). Higher plant cyclic nucleotide phosphodiesterases have high K_m values for cAMP (4, 7, 16), and it is possible that secretion of cAMP from higher plant cells may provide an additional means of regulating intracellular cAMP levels. Extracellular phosphodiesterases have been demonstrated in *Dictyostelium discoideum* (23), animal (20), and fungal (21) systems, in all of which cAMP is secreted. Extracellular cAMP is also hydrolyzed by intact soybean cells in suspension culture (7), suggesting that such hydrolysis is the eventual fate of cAMP secreted by higher plant cells.

There is a multiplicity of proteins present in higher plants that have a high affinity for cyclic nucleotides (2). In addition, a multiplicity of enzymes capable of catalyzing the hydrolysis of cyclic nucleotides is present in higher plants (4, 7, 16). The evidence presented in this paper for the occurrence of cAMP in higher plants at 0.2 μM concentration suggests that the interactions of cAMP with these proteins are functional rather than fortuitous. The lack of convincing demonstrations of adenylate cyclase in cell-free extracts from higher plants may derive from very low levels of this enzyme in higher plants. Thus, ignoring degradation of cAMP, a minimum adenylate cyclase level of only 0.3 pmol of cAMP synthesized/hr · g fresh wt can account for the observed cAMP synthesis by rye grass endosperm cells in suspension culture.

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