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Artefactual Origins of Cyclic AMP in Higher Plant Tissues

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ABSTRACT

A highly sensitive radioimmunoassay has been used to determine the levels of adenosine 3′,5′-cyclic monophosphate (cAMP) in five higher plants (*Lactuca sativa, Helianthus annuus, Oryza sativa, Pinus pinaster, Nicotiana tabacum*). Particular attention was paid to the three main sources of errors in the characterization of cAMP in plants: presence of interfering substances in plant tissues; possible artefactual formation of cAMP from endogenous ATP during extraction, purification, and assay; and microbial origin of cAMP. In all the tested tissues, the cAMP level was below the detection limit of 0.5 picomole per gram fresh weight, a value much lower than those reported for similar materials of the same species in many previous studies. This result is not in favor of cAMP-dependent regulations in higher plants.

Cyclic AMP is present in animal tissues and in many microorganisms such as bacteria, fungi, and green algae. Its occurrence in higher plants and its role in the regulation of their metabolism are still a matter of debate. The main evidence supporting the existence of the cAMP[1] system in plant tissues is based on cAMP estimations, but a large diversity exists among reported determinations of cAMP in higher plants: for example, in the case of tobacco callus, published values range from 900 (15) to less than 0.5 pmol·g^{-1} (2). On the other hand, there is only scant evidence for enzymes involved in cAMP metabolism (9). Most data in this field can be found in reviews (9) and (16).

The problems most often encountered in cAMP determination are the very low amount of this cyclic nucleotide in plants as compared to animal tissues, and the occurrence of compounds that can interfere with true cAMP during the assays. The most sensitive assay of cAMP is RIA, which allows the detection of cAMP amounts as low as 10 fmol (8). Although many compounds may interfere, the possibility of performing the assay over a 10-fold dilution scale allows the assignment of the tested compound to true cAMP, when consistent results are obtained at different dilutions. Other problems concern the origin of the detected cAMP; cAMP may be formed by contaminating microorganisms (4) or by chemical cyclization from ATP.

In the present work, we used the RIA procedure of Cailla *et al.* (7, 8) to check the presence of cAMP in different plants chosen among gymnosperms, monocotyledons, and dicotyledons. Great care was taken to ensure the elimination of interfering compounds and the absence of *de novo* synthesis of cAMP during the assay, together with maximal recovery of endogenous cAMP. In all tissues, the cAMP level was below the detection limit of the assay: the discrepancy between these results and published ones on similar materials is discussed in the light of potential sources of errors.

[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; RIA, radioimmunoassay.

MATERIALS AND METHODS

Biological Material

Lettuce (*Lactuca sativa* L., cv Val d'Orge) was obtained from Société Clause (France). Lettuce seeds were sterilized by soaking for 20 min in commercial sodium hypochlorite (150 g of chlorine per L). After extensive washing with sterile water, seeds were either immediately frozen in liquid nitrogen or germinated for 2 h at 20°C.

Sunflower (*Helianthus annuus* L., cv Rodeo) was obtained from Cetiom (France). Different materials were used: immature seeds were removed from plant heads 7 and 14 d after self-pollination in a growth cabinet and nondesiccated mature seeds after 28 d; these seeds, as well as mature dry seeds, were soaked for 15 min in one-fourth diluted commercial sodium hypochlorite, dehusked, and further sterilized in one-tenth hypochlorite dilution; young seedlings were obtained 3 and 8 d after the beginning of germination under sterile conditions.

Rice (*Oryza sativa* L., var Cigalon) was obtained from Station d'Amélioration des Plantes (INRA Montpellier, France). Dehusked dry seeds were sterilized as described for lettuce seeds. Embryos, and seedlings obtained after germination of decontaminated seeds for 48 h at 25°C, were also used.

Commercial seeds of *Pinus pinaster* Sol. (INRA, Laboratoire d'Amélioration des Arbres Forestiers, Bordeaux, France) were used. Pine needles were produced by *in vitro* culture of cloned shoots (17).

Tobacco callus (*Nicotiana tabacum* L, cu White Burley, tumoral strain grown on KNOP medium) was a generous gift from Professeur A. Lutz, Bordeaux.

Controls for bacterial and fungal contaminations were performed for all plant materials as described by Raymond and Pradet (19).

Preparation of Crude Extract

Triplicate samples of plant materials were frozen in liquid nitrogen and stored at −20°C for less than 3 d. They were ground at 0 to 3°C in 1 M perchloric acid (1 mL/100 mg fresh weight), with an Ultra-Turrax homogenizer, three times for 10 s at maximum speed. When needed, standard cAMP was added just after nitrogen evaporation, and ATP was mixed with the perchloric solution. The homogenate was centrifuged for 30 min at 17,000*g* in a SS34 Sorvall rotor at 0°C. The supernatant was carefully neutralized by dropwise addition of 1 M potassium hydroxide with constant rapid stirring to avoid a local rise in pH. The $KClO_4$ precipitate was removed by centrifugation. This crude extract was freeze-dried for direct cAMP measurement or used for further purification.

Preparation of Purified Extract

The crude extract was applied to a column of neutral alumina (aluminium oxide 90 active, neutral, Merck, 1 g/100 mg of sample initial weight). Cyclic AMP was immediately eluted with distilled water and the eluate freeze-dried.

Radioimmunoassay

We used the radioimmunoassay procedure of Cailla *et al.* (7, 8), as modified by Volker *et al.* (23). The dry residue after freeze-drying was taken up in 1 M perchloric acid; 0.1 mL of a 9 M KOH and 0.25 M EDTA solution was added to 0.5 mL of the perchloric extract; EDTA is needed to chelate Mg^{2+}. The precipitate (mainly $KClO_4$) was pelleted, and 0.15 mL of the supernatant was added to 6.75 mg of succinic anhydride. The succinylated sample was diluted six-fold with H_2O. Final pH was between 5 and 6. Radioactive tracer (2′-O-succinyl cAMP [^{125}I]iodotyrosine methyl ester in 0.1 M citrate buffer, pH 6.2) was added, and the mixture was dialyzed against anti-cAMP antibodies dissolved in the same buffer. The standard curve and determinations were performed as in (23). B/T is the ratio of bound to total iodinated tracer in the antibody chamber; B_0 is the B/T value in the absence of cAMP or any interfering compound. Figure 1A shows the standard curve used in the various assays. This curve is reliable for $(B/T)/B_0$ values between 0.15 and 0.85. When the $(B/T)/B_0$ value is lower than 0.15, the concentration is above 2 nM in the succinylated and diluted sample, and the sample must be further diluted. When the $(B/T)/B_0$ value is higher than 0.85, the cAMP concentration is too low (below 0.07 nM in the succinylated, diluted sample). This detection limit corresponds to 0.5 pmol of cAMP per g fresh weight, when the extract of 1 g of sample is taken up in 1 mL of perchloric acid. Each value was determined in triplicate. Results are expressed in pmol of cAMP per g of starting material.

cAMP Hydrolysis by Phosphodiesterase

PDE (EC 3.1.4.17; crude complex from bovine heart, Sigma) was used to hydrolyze cAMP by incubation for 60 min at 25°C in 0.5 mL of a medium containing 3.3 nkat of PDE, 0.15 mL of sample, 1 mM $MgCl_2$, and 3.3 mM Tris-HCl (pH 7.4). The reaction was stopped by addition of perchloric acid to 1 M final concentration. Residual cAMP was determined by RIA as described above.

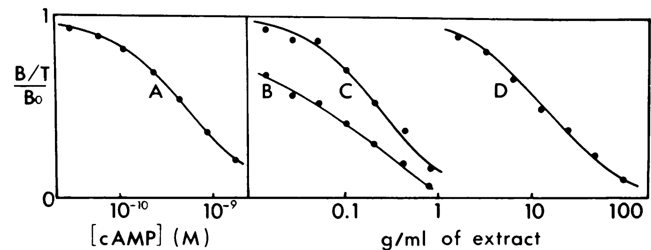

Figure 1. Effect of purification and succinylation of samples on the determination of cAMP in lettuce seeds by RIA. Normalized semilogarithmic plot of the B/T ratios *versus* cAMP concentrations (curve A) or extract concentrations (curves B, C, and D). The B/T and B_0 values were determined as described under "Materials and Methods." The cAMP scale (curve A) refers to the concentration of cyclic AMP in 100 μL of the succinylated and diluted samples added to the iodinated tracer. The extract concentrations (curves B, C, and D) correspond to the weight of the sample the freeze-dried extract of which was taken up in 1 mL of 1 M perchloric acid. Curve B, succinylated crude extract; curve C, succinylated purified extract; curve D, purified extract without succinylation.

Table I. *Effect of Dilution on cAMP-like Content Determined by RIA of Crude and Purified Extracts of Lettuce Seeds*

Concentration[a]	Cyclic AMP-like[b]	
	Crude extract	Purified extract
g/mL	*pmol/g*	
1	[c]	8.5
0.5	[c]	8.5
0.25	25	8.8
0.125	30.6	7.7
0.062	39	[d]
0.031	61.7	[d]

[a] Concentration is the weight of dry seeds whose freeze-dried extract was taken up in 1 mL of 1 M perchloric acid for RIA. [b] cAMP-Like content is expressed as pmol of cAMP per g of dry seeds. [c] Concentration too high to be determined. [d] Concentration too low to be determined.

RESULTS

Determination of cAMP in Lettuce Seeds

Validity of the Assay and Pitfalls

The cAMP content was determined in extracts of sterilized intact lettuce seeds. A cAMP-like response was obtained with the crude extract (Table I). However, inconsistent results were obtained with successive dilutions (Table I): the shape of the dilution curve (Fig. 1B) was different from that of the standard curve (Fig. 1A), indicating the presence of interfering compounds. After purification of the extracts on a neutral alumina column (24), the RIA determination gave consistent results of about 8.5 pmol/g for successive dilutions of the extract (Table I, last column), leading to a dilution curve with the same shape as the standard curve (Fig. 1C).

Two controls were performed to assess whether true cAMP

was detected in the extract. The first took advantage of the increase in sensitivity by about two orders of magnitude caused by the succinylation of the nucleotide (7). Figure 1D shows the dilution curve obtained with a nonsuccinylated purified extract; the shape of the dilution curve and its displacement on the concentration axis in relation to that of the succinylated extract were similar to those observed with cAMP (7). The purified extract was also subjected to PDE hydrolysis. Most of the cAMP-like response of the extract was abolished; the small residual response was equivalent to about 1 pmol of cAMP per g of seed, when tested with the concentrated extract. Under the same conditions, an internal standard of cAMP was totally hydrolyzed. We therefore concluded that out of the 8.5 pmol/g of cAMP-like response measured under our assay conditions, 7.5 pmol corresponded to authentic cAMP.

An important pitfall in cAMP measurement could also be its artefactual formation from ATP during the extraction or assay procedures. Cyclization of ATP occurs under alkaline conditions, in the presence of divalent cations (6). Magnesium ions are not regarded as an efficient catalyst of the reaction, but under alkaline conditions 6 mM $MgCl_2$ catalyzes the cyclization of 1/12500 of 1 mM ATP in 10 min (23). This phenomenon may occur during the neutralization of the perchloric extract, because of local alkalinization, or during the succinylation step which has to be performed at alkaline pH. It can be prevented by EDTA at a concentration high enough to chelate all of the Mg^{2+} (23). To detect any chemical synthesis of cAMP from ATP during extraction or assay, 500 nmol of ATP per g of lettuce seeds were added during the extraction step. This amount is similar to that in germinating seeds and 10 times higher than that in dry seeds (19). No increase in cAMP could be detected by the radioimmunoassay. Therefore, the cAMP detected in purified seed extracts was not formed by chemical synthesis from ATP.

Microbial Origin of cAMP

We examined the possibility that, despite seed sterilization, cAMP detected in the purified extract might be of microbial origin, being either released by microorganisms and absorbed by seed teguments, or still be present in dead microorganisms (4). A lettuce 'seed' is in fact an achene, composed of two functional parts: living tissues (embryo and endosperm) and dead tissues (pericarp and testa) that have an entangled structure and are in close contact with microorganisms (about 10^6 colony-forming units per g of dry seeds). The amount of cAMP was therefore determined in sterilized lettuce seeds deprived of their external tegument. No cAMP was detected in the extracts (Table II). If present, its content was below the detection limit of 0.5 pmol/g, instead of the 7.5 pmol/g determined in whole seeds. This last amount therefore appears to be localized in the dead tissues of the achene.

Recovery of cAMP

We checked that the low level of cAMP in lettuce seed extracts did not arise from losses during extraction. To avoid cAMP degradation inside the tissues, seeds had been frozen in liquid nitrogen immediately after decontamination, and homogenization was performed at 0 to 3°C in perchloric acid, which is efficient for cAMP extraction (7). A further advantage of perchloric acid as an extracting agent is the low solubility of potassium perchlorate (0.1 M) which allows the removal of most of the acid by addition of KOH, without any loss of cAMP. On the other hand, cAMP is lost during the diethylether extraction of trichloracetic acid (8). No interference of $KClO_4$ was detected in our RIA (7, 8). The cAMP recovery after the extraction and purification procedure was tested with a cAMP internal standard (2 pmol/g of dry seeds) added to the samples as soon as liquid nitrogen was evaporated, in order to avoid uncontrolled losses. Recoveries at the 'purified extract' step were within the range of 83 to 89%, with a mean value of 85%. It was concluded that lettuce seeds contain about 9 pmol $cAMP \cdot g^{-1}$, all or most of which is in the dead part of the seed.

Table II. *Cyclic-AMP Detection in Various Plant Tissues*

Plant	Tissue	cAMP detected	
		This work	Published values (ref)
		pmol/g	
Lettuce	Dry seeds[a]	8.5	
	2 h imbibed seeds	8.5	
	2 h imbibed seeds without external tegument	<0.5	
	6 h imbibed seeds		100 (18)
	14 h imbibed seeds		<2 (5)
	Seedlings		265 (12)
Pine	Embryos	<0.5	
	Needles	<0.5	60 (25)
Rice	Embryos	<0.5	
	Seedlings	<0.5	
	Plumules		42 (13)
Tobacco	Callus	<0.5	140 (12)
			900 (15)
			<0.5 (2)
			100 (11)
	Pith parenchyma		0.9 (2)
Sunflower	Callus		12 (21)
	7 d embryos	<0.5	
	14 d embryos	<0.5	
	Mature seeds	<0.5	
	Dessicated mature seeds	<0.5	
	3 d seedlings	<0.5	
	8 d seedlings	<0.5	

[a] Seeds were soaked for only 20 min in hypochlorite.

Determination of cAMP in Other Plant Tissues and at Various Developmental Stages

The occurrence of cAMP was also tested in different materials from four other higher plants: rice, pine, sunflower, and tobacco, at different stages of development (Table II). An internal standard of cAMP (2 pmol/g) was added to duplicate frozen samples of all these materials. The percentage of re-

covery, about 85%, was identical to that found with lettuce seeds. Pine and tobacco were cultivated under sterile conditions, and samples of tissues were frozen directly after removal from the culture tube. No cAMP was detected in either tobacco callus or pine needles or embryos. Similarly, no cAMP was detected in rice tissues, either embryos frozen immediately after decontamination, or seedlings after 48 h of germination. We also studied sunflower seeds at different stages of maturation or germination in order to reveal increases in the cAMP level that could take place during the physiological changes occurring in the transition from the embryonic state to the greening seedling. At no time was cAMP detected. If present, cAMP was below the detection limit of 0.5 pmol/g fresh weight.

DISCUSSION

Most reviews on the presence of cAMP in higher plants have emphasized that, owing to its low concentration, numerous compounds interfere with the analysis of the nucleotide, leading to a lack of specificity of detection methods (3, 9, 14). The radioimmunoassay, based on the displacement of an iodinated cAMP derivative from specific antibodies, is especially sensitive to such interfering compounds. In the present work, two controls were used to test for the presence or the absence of such interfering substances, *i.e.* the shape of the displacement curve, and the increase in sensitivity caused by sample succinylation.

With unpurified extract of lettuce seeds, the shape of the dilution curves did show the presence of interfering compounds, thus preventing the quantification of cAMP. After purification on an alumina column, most of the interfering compounds were apparently eliminated, and a response equivalent to 8.5 pmol cAMP$\cdot g^{-1}$ was found, most of which was sensitive to hydrolysis by PDE. We verified that this compound was not formed by a chemical transformation of ATP to cAMP. However, we also found that this amount of cAMP could not be detected in the living tissues of the seed; this indicates that it probably results from contamination of the external teguments by microorganisms, in spite of seed sterilization by hypochlorite.

Similarly, no cAMP was detected above the detection limit of 0.5 pmol/g of tissue in any of the other tissues studied, although the recovery of internal standard of cAMP was higher than 83% in all cases. In similar materials of the same species, higher levels of cAMP have been reported (Table II). The absence of cAMP in sterilized and dehusked germinating lettuce seeds and in tobacco callus is in agreement with results of other authors (2, 5) but differs from the higher values (100–250 pmol$\cdot g^{-1}$) found in our previous study (18) on similar materials. In this latter work, cAMP was very probably formed from the ATP present in plant tissues during the first chromatographic step, which used an alkaline mobile phase (isopropanol/NH_4OH/H_2O, 70:15:15: v/v). In the same chromatography system with 2 μmol of pure ATP, about 1 nmol of cAMP was formed, as tested by the protein kinase activation and the bioluminescence assays (P Raymond, unpublished results). Volker *et al.* (23) also have emphasized the importance of the formation of cAMP from ATP under alkaline conditions in the production of overestimated results. The production of spurious cAMP during extraction could annihilate the advantages of otherwise very specific techniques, such as mass spectrometry. We therefore suggest that any claim to the presence of cAMP in living tissues should include controls performed by adding ATP during the initial steps of the extraction procedure. Only such controls can rule out any artefactual formation of cAMP during the neutralization of acid extracts, purification steps, or the assay itself.

The possibility that cAMP of microbial origin contaminates plant extracts has been stressed by different authors; the amount of cAMP in culture media can be much larger than the intracellular content of bacteria (22). Sterilization procedures kill microorganisms but do not necessarily eliminate the cAMP previously released and absorbed by the plant tissues. Lettuce seeds allowed an estimation of this source of contamination because the external tegument can be removed. Most of the cAMP in these seeds was associated with the external, dead teguments.

When the three sources of error described above (presence of interfering compounds, chemical synthesis from ATP, and microbial contamination) were avoided, no cAMP was detected at a detection limit of 0.5 pmol$\cdot g^{-1}$ in any of the plant tissues studied. This value is two to three orders of magnitude lower than the basal content of animal tissues (50–250 pmol$\cdot g^{-1}$). It corresponds to a 0.6 nM concentration, assuming that there is an even distribution of the cyclic nucleotide in the tissues. This low value questions the possible role of cAMP in plant metabolism. The K_d for cAMP of the cAMP-binding proteins studied in plants is close to 0.2 μM (9), which is of the same order as the K_d for cAMP of the cAMP-dependent protein kinases in animal tissue. Evidence for cAMP-dependent phosphorylation of proteins has recently been provided (10); in this system, 1 μM cAMP does not provide full activation of the kinase, which suggests that the K_a is of the same order of magnitude as the K_d. If cAMP is present in the plant tissues studied, its concentration seems too low to allow any significant effect of this nucleotide, at least through interaction with these cAMP-binding proteins. After viral infection of plant tissues, an increase of cAMP at very low concentrations (20) and an activation of cAMP synthesis *in vitro* (1) have been reported. These findings, if confirmed, leave open the question of the functional role of low concentrations of cAMP in higher plants.

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