CONTENT OF 3',5' CYCLIC AMP AND CYCLIC AMP PHOSPHODIESTERASE IN DORMANT AND ACTIVATED TISSUES OF JERUSALEM ARTICHOKE TUBERS

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Received February 1,1974

SUMMARY. Using an highly sensitive and specific radioimmunoassay for 3',5' cyclic AMP, we have detected this cyclic nucleotide in partially purified extracts obtained from tuber tissues of Jerusalem artichoke. The level of cyclic AMP found in dormant tubers was very high compared with those of sprouting tubers and rapidly declines when dormant tissue slices are activated by incubation in aerated water. Cyclic AMP phosphodiesterase seems to play an important role in these changes of cyclic AMP content.

INTRODUCTION. Adenylate cyclase and cyclic AMP phosphodiesterase activities have been detected in dormant tubers of Jerusalem artichoke (1,2) and a relationship of gibberellic acid to adenylate cyclase has been observed (1). However, the presence of 3',5' cyclic AMP (cyclic AMP) in these tissues as well as the possible role of adenylate-cyclase-cyclic AMP phosphodiesterase system in the control of the level of cyclic AMP remain to be established.

Therefore, using an highly sensitive and specific radioimmunoassay for cyclic AMP, we have tried to detect cyclic AMP in dormant tubers of Jerusalem artichoke as well as in sprouting tubers and in slices excised from dormant tubers and activated in aerated water. The results show that cyclic AMP occurs in Jerusalem artichoke tuber tissues and that its level is much higher in dormant than in sprouting or activated tuber tissues. Cyclic AMP phosphodiesterase seems to play an important role in these changes of cyclic AMP content.

MATERIALS AND METHODS. Tubers of Jerusalem artichoke (Helianthus tuberosus) were harvested in November and stored in coldroom at 4°C. Cylindrical slices of homogeneous medullary parenchyma were excised transversely to the axis of tubers. The slices were briefly washed in cold distilled water, blotted and weighed. For each experimental condition, four slices (50 mg fresh weight each) were incubated at 25°C in 3 ml of distilled water under aeration. All operations were carried out under sterile conditions.

Phosphodiesterase actitivity assay: phosphodiesterase activity was assayed, as previously reported (1), on the undialysed 105,000 x g supernatant of tissue homogenates.

(^{3}H)cyclic AMP degradation: slices of dormant tubers were incubated, as before described, in the presence of 10^{-5}M unlabelled cyclic AMP and 0.15 μCi/ml (^{3}H)cyclic AMP (spec.act.2.65 Ci/mmole). At different times of incubation the slices (200 mg initial fresh weight) were removed, washed three times with 10 ml of distilled water containing 10^{-5}M cyclic AMP and then homogenized in 5% TCA. Each homogenate was centrifuged at 13,000 rpm x 20 min. at 4°C. An aliquot of supernatant was dissolved in Instagel and counted for the determination of total acid soluble radioactivity; another was passed over a 3 ml column of BioRad AG 50 W-X8 (200-400 mesh,H^+form) previously washed with distilled water (3). The column was eluted with distilled water and fractions of 2 ml were collected. An aliquot(0.2 ml) of each fraction was chromatographed on PEI cellulose precoated plates in presence of cyclic AMP, cyclic IMP, 5'-AMP, 3'-AMP and adenosine as incorporated standards. The chromatograms were developed as previously reported (1,2) using isopropanol-water-ammonium hydroxide 7:2:1. The spots detected under U.V. light were scraped from the plates and counted in Instagel.

Cyclic AMP assay: samples of tissues (1 g each) were homogenized with 1 ml of cold 5% TCA. (^{3}H) cyclic AMP (7,000 cpm) was added to estimate the recovery and the homogenates were centrifuged at 13,000 rpm for 20 min at 4°C. Each supernatant was chromatographed on a 3 ml column of BioRad AG 50 W-X8 (200-400 mesh, H^+form) as before described. Cyclic AMP eluted between 8 and 12 ml. Cyclic AMP-containing fractions were passed through a 4 ml column of neutral alumina (4) previously washed with distilled water. The column was eluted with distilled water. Cyclic AMP eluted between 4 and 12 ml. Parallel experiments with cold ATP, 5'-AMP, 3'-AMP and 2',3' cyclic AMP indicated that this purification procedure separated completly cyclic AMP from these nucleotides. Recovery of cyclic AMP ranged between 70-90%. The cyclic AMP-containing eluats were lyophilized. The lyophilized was dissolved in 0.2 ml of citrate-phosphate buffer, pH 6.2, and its content of cyclic AMP measured by radioimmunoassay (5) according to D'Armiento et al.(6).

RNA and protein synthesis: to measure the rate of RNA and protein synthesis samples of tissue (200 mg fresh weight) were incubated, as before described, with either 1 μCi/ml uridine 5(^{3}H)-5'monophosphate(spec.act. 14 Ci/mmole) or 1 μg/ml cold L-leucine and 1 μCi/ml L-leucine 4,5(^{3}H)(spec.act. 38 Ci/mmole). After incubation, the slices were rinsed three times, blotted and homogenized in cold 10% TCA (w/v=1:4). The homogenates were centrifuged and the precipitates were treated for extraction of RNA and protein according to Sacher (7). Radioactivity incorporated was measured by dissolving appropiate aliquots of RNA and protein in Instagel and by counting in a liquid scintillation spectrometer.

TABLE I

Month of the assay	Physiological state of tubers	Cyclic AMP content pmoles/g fresh tissue
December	Dormant	60
January	Dormant	60
February	Dormant	62
March	Dormant	60
April	Sprouting	25
September	Sprouted	25

TABLE I. Cyclic AMP content in dormant, sprouting and sprouted tubers of Jerusalem artichoke. Cyclic AMP was extracted as described in MATERIALS AND METHODS and assayed by radioimmunoassay according to D'Armiento et al.(6).

RESULTS. As shown in Table I, cyclic AMP occurs in Jerusalem artichoke tubers. Its concentration depends on the physiological state of the tissues. Dormant tubers contain about 3 fold more cyclic AMP than sprouting tubers. The decline of cyclic AMP level seems to occur rapidly at the sprouting time, because no significant changes are observed during the dormancy period. Furthermore, when slices excised from dormant tubers are incubated in aerated water, the level of cyclic AMP rapidly declines within 15 minutes of incubation, the shortest incubation time studied, and reaches the lowest value within two hours. Afterwards, the level remains steady (Fig.1). Fig.1 shows also that the decline of cyclic AMP content occurs before the increase in RNA and protein synthesis.

In order to clarify the role of cyclic AMP phosphodiesterase in the observed changes of the cyclic AMP content, we followed the fate of (^{3}H)cyclic AMP supplied to slices during the incubation; moreover we measured the phoshodiesterase activity of cell-free extracts obtained from slices incubated at different times. As shown in Table II, a very small aliquot of total radioactivity found in tissue extracts was identified as cyclic AMP; on the contrary, the bulk of radioactivity was identified as (^{3}H)5'-AMP and (^{3}H)3'-AMP, the reaction products of phosphodiesterase present in these tissues (1,2). No (^{3}H)5'-AMP or (^{3}H)3'-AMP was found in the medium after 1 hour of incubation (unpublished results), indicating that cyclic AMP degradation occurred after (^{3}H)cyclic AMP penetration.

When we measured the phosphodiesterase activity of the crude extracts, we

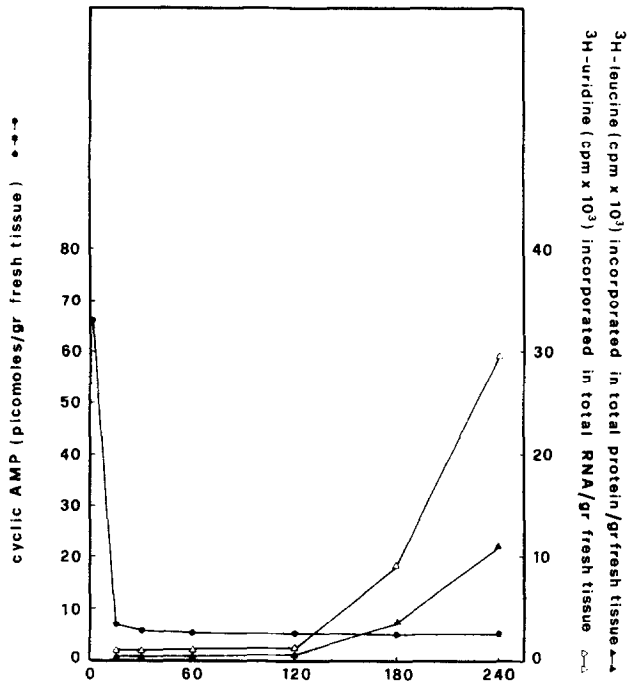

Fig.1. Cyclic AMP content of slices excised from dormant tubers of Jerusalem artichoke and incubated in aerated water. Cyclic AMP was assayed by radioimmunoassay according to D'Armiento et al.(6). Parallel experiments were carried out to measure the rate of RNA and protein synthesis.

observed a significant increase within 15 minutes of incubation; the activity continued to increase for up to 60 minutes of incubation (Table III).

DISCUSSION. Although both adenylate cyclase and cyclic AMP phosphodiesterase have been detected in Jerusalem artichoke(1,2) as well as in other plant tissues (8,9,10,11), the presence of cyclic AMP in higher plants is still debated. Recently, Raymond et al.(12) have extracted from some plant tissues and purified a compound chromatographically identical to cyclic AMP. Since this compound gave a positive response on two enzymatic assays for cyclic AMP, i.e. bioluminescence and activation of protein kinase, the Authors concluded that it was cyclic AMP.

The results reported here give additional evidence for the presence of cyclic AMP in higher plants. Using an highly sensitive and specific radioimmunoassay for cyclic AMP, we have detected this cyclic nucleotide in the Jerusalem artichoke tuber. The amount we have detected in dormant tubers is almost of the same magnitude of those found by Raymond et al. (12) in other parenchymal plant tissues using different assay techniques.

TABLE II

Incubation (min)	Total acid soluble radioactivity (cpm/g fresh weight)		(^{3}H)cyclic AMP (cpm/g fresh weight)		(^{3}H)5'-AMP + (^{3}H)3'-AMP (cpm/g fresh weight)	
	0°C	30°C	0°C	30°C	0°C	30°C
15	1750	2200	1013	1200	640	960
30	1760	2880	800	1080	800	1680
60	1960	4200	1112	1240	680	2660
120	2020	8100	1100	1000	700	6900

TABLE II. (^{3}H) cyclic AMP degradation. Slices excised from dormant tubers of Jerusalem artichoke were incubated at 25°C or at 4°C in presence of 10^{-5}M unlabelled cyclic AMP and 0.15 µCi/ml (^{3}H)cyclic AMP. At different times of incubation, four slices (200 mg initial fresh weight) were removed, washed and then homogenized in 5% TCA. After centrifugation, an appropiate aliquot of supernatant was withdrawn for the determination of total acid soluble radioactivity; another was treated for identification of labelled nucleotides as described in MATERIALS AND METHODS.

Furthermore, we have observed that the level of cyclic AMP in dormant tubers is very high compared with those of sprouting tubers and rapidly declines when dormant tissue slices are activated by incubation in aerated water.

It is well known that dormancy break, occurring when tuber sprouts as well as slices excised from dormant tubers are incubated in aerated water, results in a dramatic increase in metabolism, the main and early features of which are higher rates of RNA and protein synthesis (13,14). Our present results suggest that cyclic AMP can be involved in the physiological mechanism of the dormancy break. It cannot be definitely determined at the present time if the observed lowering of the cyclic AMP content in both sprouting and activated tuber tissues is a pre-requisite for the dormancy break or an effect of the increased metabolism of activated tissues. We favor the former hypothesis because the rapid decline of cyclic AMP level greatly precedes the increase of RNA and protein synthesis. Therefore, if our assumption is correct, cyclic AMP could act as "a brake" on the metabolism of dormant tubers and the rapid decline of cyclic AMP content could rapresent a switch from dormancy to active metabolism. A

Table III

Time (min)	Total activity (mUnits)	Specific activity (mUnits/mg protein)
0	42	6.0
15	75	10.7
30	90	13.8
60	100	17.0

TABLE III. Phosphodiesterase activity of crude extracts obtained from slices excised from dormant tubers of Jerusalem artichoke and incubated in aerated water. Phosphodiesterase activity was assayed on the undialysed 105,000 x g supernatant of tissue homogenate (1). The reaction mixture contained 40 mM acetate buffer, pH 5.4, 5mM $MgCl_2$, 0.1 mM cyclic AMP and (^{3}H)cyclic AMP (2 x 10^4 cpm). The reaction was run at 37°C and started by adding an aliquot of enzyme. The final volume was 0.4 ml. At the end of the incubation, the reaction was stopped by heating the tube to 100°C for 3 min and cyclic AMP of the reaction mixture recovered as reported elsewhere (1,2). The amount of cyclic AMP hydrolyzed was calculated on the basis of the difference between the amount added to the reaction mixture and that recovered. One unit of phosphodiesterase is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmole of cyclic AMP per 60 min in a standard reaction mixture.

function of "brake" to slow fibroblas growth has been suggested by Otten et al. (15) and it seems correlated with a "pleiotypic" function of cyclic AMP (16).

The fact that the rapid decline of cyclic AMP in water-activated slices is accompanied by a corresponding increase in cyclic AMP phosphodiesterase activity, together with the observation that cyclic AMP supplied to slices is rapidly degraded in vivo to 3'-AMP and 5'-AMP, which are the reaction products of Jerusalem artichoke phosphodiesterase (1,2), indicate that this enzyme may be responsible for the lowering of cyclic AMP level. If the increase in phosphodiesterase activity observed during tissue slices incubation is an enzyme activation or de novo synthesis remains to be elucidated. However, we have previously suggested (2) that phosphodiesterase in dormant tubers could be greatly inhibited in vivo by inorganic

phosphate. Thus, the possibility that the enzyme activity is modulated during the dormancy break by intracellular changes in inorganic phosphate can be envisaged.

Studies on the possible relationship of cyclic AMP to plant hormones have shown that exogeneous cyclic AMP does not mimic fully the plant hormone effects and that high concentrations of cyclic AMP are needed to obtain some hormone-like effect. Among the possible reasons for this are poor penetration through cell membranes and rapid destruction by phosphodiesterase. Our results indicate that cyclic AMP penetrates in the plant cells, but it is rapidly degraded by phosphodiesterase. Thus, cyclic AMP derivatives having a greater resistence to the action of phosphodiesterase are needed in order to investigate the role of cyclic AMP in plant metabolism.

ACKNOWLEDGEMENTS. This work was supported by Grant CT73.00222.06 form Consiglio Nazionale delle Ricerche, Italia. The Authors thank dr. Ira PASTAN, Labor. of Molecular Biology, NIH, Bethesda, USA, for valuable criticism in reading the manuscript.

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