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PROPERTIES OF A MULTIFUNCTIONAL 3′,5′-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *LACTUCA* COTYLEDONS: COMPARISON WITH MAMMALIAN ENZYMES CAPABLE OF HYDROLYSING PYRIMIDINE CYCLIC NUCLEOTIDES

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Key Word Index—3′,5′-Cyclic nucleotide phosphodiesterase; cyclic AMP; cyclic GMP; cyclic CMP; *Lactuca sativa*; lettuce cotyledons; germination.

Abstract—Hydrolysis of 3′,5′-cyclic nucleotides by this *Lactuca* multifunctional cyclic nucleotide phosphodiesterase yields the corresponding 5′-nucleotides. With 2′,3′-cyclic nucleotides, the point of cleavage is affected by the nature of the base. Equimolar amounts of 2′- and 3′-AMP are produced from 2′,3′-cyclic AMP but four times more 3′-CMP than 2′-CMP is produced from 2′,3′-cyclic CMP. With 2′,3′-cyclic GMP, 2′-GMP is the sole product. The presence of one cyclic nucleotide affects the rate of hydrolysis of another. With 3′,5′-cyclic nucleotide substrates, mixed-type inhibition was exhibited by other 2′,3′- and 3′,5′-cyclic nucleotides. During hydrolysis of 3′,5′-cyclic AMP, K_i values of 16 and 6 μM were obtained with 3′,5′-cyclic GMP and 2′,3′-cyclic AMP, respectively. Coupled with the K_m of 0.73 mM for 3′,5′-cyclic GMP and that of 1.12 mM for 2′,3′-cyclic AMP, the low K_i values suggest that more than one binding site is available for each nucleotide. Hill coefficients of 1.26, 0.88 and 0.91, for 3′,5′-cyclic AMP, 3′,5′-cyclic GMP and 3′,5′-cyclic CMP, respectively, together with the linear nature of the Hill plots obtained, indicate that when a single substrate is present, there is no cooperative effect between the sites. The properties of the *Lactuca* enzyme are compared with those of other plant and mammalian cyclic nucleotide phosphodiesterases and the significance of the occurrence of this enzyme in *Lactuca* is considered.

INTRODUCTION

Although the natural occurrence of cyclic AMP in higher plants was unequivocally established several years ago, its functions have yet to be elucidated (for reviews see [1, 2]). The presence of cyclic GMP has now also been demonstrated in higher plants [3, 4] as has the existence of the enzymes responsible for its synthesis and degradation [4]. In mammals a third endogenous cyclic nucleotide, cytidine 3′,5′-cyclic monophosphate (cyclic CMP) has been shown to occur [5–7]. This nucleotide, which may also be an intracellular regulator [8], occurs together with a cytidylate cyclase [9] and at least two phosphodiesterases capable of cyclic CMP hydrolysis [10–16]. While one of these phosphodiesterases displays absolute specificity for 3′,5′-cyclic CMP [14–16] as substrate, the other was active both with pyrimidine and purine cyclic nucleotides, and with their 2′,3′- and 3′,5′-isomers [10–14]. An enzyme we have recently partially purified from *Lactuca* cotyledons [17] has also been found to hydrolyse both purine and pyrimidine cyclic nucleotides and differs in a number of properties from other plant phosphodiesterases. The present study aimed to investigate further the properties of this enzyme and to compare them with those of the multifunctional and cyclic CMP specific phosphodiesterases isolated from mammalian tissues. It was hoped that such a comparison would show whether the phosphodiesterase from *Lactuca* bears any closer similarity to these recently discovered mammalian enzymes than to other plant phosphodiesterases and indicate if the possible involvement of cyclic CMP in cell proliferation in mammals should also be considered in relation to higher plant cells.

RESULTS AND DISCUSSION

The phosphodiesterase fraction obtained at the affinity chromatography stage of purification was active over the pH range 4.5–8.5, with a sharp maximum at pH 5.5. Under the incubation conditions described in the Experimental, the activity of the enzyme increased gradually up to a maximum at 60°, above which it sharply declined. The observed activity optimum at pH 5.5 represents a lower pH than that generally observed with mammalian cyclic AMP and cyclic GMP phosphodiesterases but is well within the range previously observed with higher plant phosphodiesterases (see [1, 2]). In comparison with the mammalian cyclic CMP phosphodiesterase with its pH optimum at 7.2–7.4 [14–16] and the mammalian multifunctional phosphodiesterase of pH optimum 7.0 [10–13] the *Lactuca* enzyme again has its optimum at a lower pH. However, when cyclic CMP is the substrate, all three of these enzymes display a sharpness of the pH maxima not usually observed with other phosphodiesterases. The plant enzyme also differs widely from the two mammalian phosphodiesterases in remaining active at much higher temperatures.

Isoelectric focusing of the *Lactuca* phosphodiesterase fraction from the affinity chromatographic stage of

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purification showed that a number of proteins were still present (Fig. 1). Elution of these proteins from the electrophoretic gel yielded only one band exhibiting phosphodiesterase activity. This protein had an isoelectric point at pH 4.5 which corresponds closely to that of the mammalian cyclic CMP specific phosphodiesterase at pH 4.2–4.4 [14–16] and of the multifunctional phosphodiesterase at pH 4.6 [10–13].

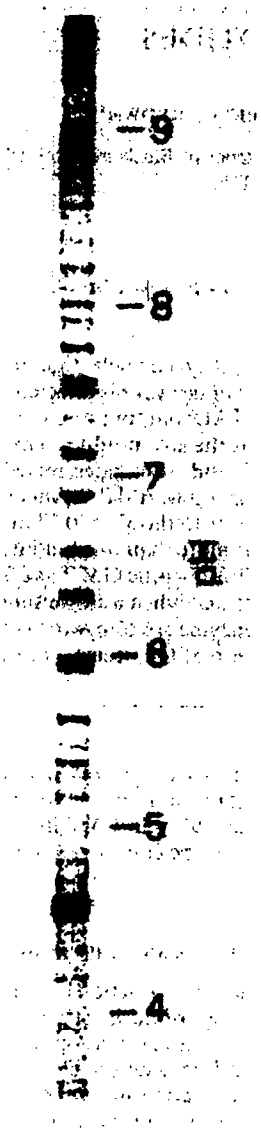

Fig. 1. Isoelectric focusing of the cyclic nucleotide phosphodiesterase from *Lactuca* cotyledons. The preparation had been purified to the Affi-Gel Blue affinity chromatography stage. Further details are given in the text.

The unusual characteristics of the mammalian multifunctional phosphodiesterase are its ability to hydrolyse both purine and pyrimidine cyclic nucleotides, and to hydrolyse both 3',5'- and 2',3'-cyclic nucleotide substrates [10–13]. Investigation of the *Lactuca* enzyme showed that it also was capable of hydrolysing 2',3'-cyclic nucleotide substrates at each stage of its purification (Table 1). In all but one case, the relative activity of the enzyme with the 2',3'-cyclic nucleotide substrates was lower than with 3',5'-cyclic nucleotides and further decreased significantly after affinity chromatography on Affi-Gel Blue. The residual 2',3'-cyclic nucleotide phosphodiesterase activity was also found, together with the 3',5'-cyclic nucleotide phosphodiesterase activity, to occur only in the protein band located at p*I* 4.5 after isoelectric focusing. This indicates that a single protein in the preparation was responsible for the hydrolysis of the two isomers of each of the cyclic nucleotides tested as substrate. While the ability to hydrolyse 2',3'- and 3',5'-cyclic nucleotides within the same order of magnitude of activity is common to both the *Lactuca* and pig liver multifunctional phosphodiesterases, these enzymes are significantly different to the cyclic CMP phosphodiesterase isolated from rat liver. With 2',3'-cyclic CMP and other 2',3'- and 3',5'-cyclic nucleotides [16] as substrate, the latter enzyme has less than 1% of the activity obtained with 3',5'-cyclic CMP.

Kinetic examination of the enzyme at a series of substrate concentrations produced the K_m and V_{max} values in Table 2. These again illustrate the broad specificity of the *Lactuca* enzyme with K_m values of a similar order of magnitude for all six substrates, and the values of V_{max} also being of a similar order of magnitude for all six substrates. The K_m values obtained with each of the six substrates are at least an order of magnitude lower than those reported for the cyclic CMP specific phosphodiesterase [14–16] but are more comparable with the data for the multifunctional enzyme from pig liver which has a reported K_m for cyclic CMP of 128 μM [10–14]. The V_{max} values obtained with the *Lactuca* enzyme are 1–2 orders of magnitude lower than the activities reported for the two mammalian phosphodiesterases [10–16]; this probably only reflects the partial purity of the *Lactuca* enzyme relative to the homogeneity of the others, rather than any basic difference in enzymic properties. The similarity between the *Lactuca* phosphodiesterase and the pig liver multifunctional phosphodiesterase [10–14] became further evident when the products of hydrolysis of the six cyclic nucleotide substrates were determined (Table 3). With 3',5'-cyclic nucleotide substrates, the major product in

Table 1. Hydrolysis of 3',5'- and 2',3'-cyclic nucleotide substrates by the *Lactuca* cotyledon phosphodiesterase

Enzyme preparation*	Phosphodiesterase activity (nmol/min/mg of protein)					
	3',5'-cAMP	3',5'-cGMP	3',5'-cCMP	2',3'-cAMP	2',3'-cGMP	2',3'-cCMP
$(NH_4)_2SO_4$ ppt.	5.3	3.2	4.9	3.4	2.8	2.5
Gel filtration fraction	35.8	10.1	27.2	24.1	12.3	8.7
Affinity chromatography fraction	106	45.1	94.1	27.5	3.8	15.7

*Enzyme fractions taken at each of the main stages in the purification procedure [17].

Table 2. Kinetic parameters of the *Lactuca* phosphodiesterase with 3',5'-and 2',3'-cyclic nucleotide substrates. The conditions under which these were determined are described in the Experimental

Substrate	K_m (mM)	V_{max} (nmol/min/mg protein)
3',5'-cAMP	0.93	312.1
3',5'-cGMP	0.73	110.9
3',5'-cCMP	0.67	221.0
2',3'-cAMP	1.12	71.6
2',3'-cGMP	0.85	14.3
2',3'-cCMP	0.74	45.4

Table 3. Products of the hydrolysis of 3',5'- and 2',3'-cyclic nucleotide substrates by the *Lactuca* phosphodiesterase. The experimental conditions are described in the text

Substrate	Products formed (nmol/min/mg protein) 2'-Nucleotide	3'-Nucleotide	5'-Nucleotide
2',3'-cAMP	9.7	11.3	—
2',3'-cGMP	4.1	n.d.*	—
2',3'-cCMP	3.8	14.4	—
3',5'-cAMP	—	18.2	56.7
3',5'-cGMP	—	9.9	25.4
3',5'-cCMP	—	13.1	61.3

*None detected.

each case is the corresponding 5'-nucleotide, indicating that irrespective of the base present the *Lactuca* phosphodiesterase preferentially cleaves the 3'-bond. This is similar to the action of the mammalian phosphodiesterases, e.g. the pig liver enzyme liberates no detectable 3'-nucleotide, but contrasts with several previous reports of plant phosphodiesterases [18, 19] in which the major product of 3',5'-cyclic AMP hydrolysis is 3'-AMP. With the 2',3'-cyclic nucleotide substrates, the position of attack of the *Lactuca* phosphodiesterase is affected by the nature of the base present. Approximately equal amounts of 3'- and 2'-AMP are produced from 2',3'-cyclic AMP but almost four times as much 3'-CMP as 2'-CMP is produced from 2',3'-cyclic CMP, while 2'-GMP is the sole product from 2',3'-cyclic GMP. This again is similar to the data for the pig liver enzyme [10–13] in which equimolar quantities of 3'- and 2'-AMP are liberated from 2',3'-cyclic AMP but 2'-GMP and 3'-CMP are the major products from 2',3'-cyclic GMP and 2',3'-cyclic CMP, respectively.

With the *Lactuca* phosphodiesterase, the presence of one cyclic nucleotide was found to have an effect upon the hydrolysis of another. As would have been anticipated, the presence of equimolar amounts of unlabelled cyclic GMP or CMP inhibited the hydrolysis of radiolabelled cyclic AMP, and presence of unlabelled cyclic GMP or AMP inhibited the hydrolysis of cyclic CMP (Table 4). However, hydrolysis of cyclic GMP was slightly but reproducibly stimulated in the presence of cyclic AMP (13% above control value), and was almost doubled in the presence of an equimolar concentration of cyclic CMP. This indicates that a single site for all the cyclic nucleotide

Table 4. Effect of the presence of 3',5'-cyclic nucleotides on the *Lactuca* phosphodiesterase activity

Additions	Enzymic activity as percentage of the respective control activity: [8-^{3}H]-Labelled substrate 3',5'-cAMP	3',5'-cGMP	3',5'-cCMP
None (control)	100	100	100
3',5'-cAMP	—	113	72
3',5'-cGMP	64	—	82
3',5'-cCMP	73	197	—

All substrates and additions were at a final concentration of 2.5 mM. Other conditions were as described in the text. The control (100%) values for each substrate are given in Table 1.

substrates is unlikely, and further investigation confirmed this view. The hydrolysis of each of the three 3',5'-cyclic nucleotides was found to be subject to mixed-type inhibition by other cyclic nucleotides including 2',3'- and 3',5'-isomers. With the hydrolysis of 3',5'-cyclic AMP, K_i values of 16 and 6 μM were obtained for 3',5'-cyclic GMP and 2',3'-cyclic AMP, respectively. These low K_i values, considered in relation to the K_m of 0.73 mM for 3',5'-cyclic GMP and 1.12 mM for 2',3'-cyclic AMP, suggest that more than one binding site is available for each cyclic nucleotide. This is in contrast to the cyclic CMP phosphodiesterase [14–16] and the multifunctional phosphodiesterase from pig liver [10–13] both of which appear to have only one binding site. With the latter enzyme, the K_m and K_i values for cyclic nucleotides are closely similar and kinetic data indicate that each substrate is a competitive inhibitor of another.

While the presence of one cyclic nucleotide has an effect on the hydrolysis of another by the *Lactuca* phosphodiesterase, when only one substrate is present there is an absence of any cooperative effect between the sites. This is indicated by the Hill coefficients of 1.26, 0.88 and 0.91 for 3',5'-cyclic AMP, GMP and CMP, respectively, and by the linear nature of the plots obtained of V_{max}/V_0 against $1/[S]$ (Fig. 2) [20].

Although many of the kinetic properties of the *Lactuca* phosphodiesterase show greater similarity to those of the pig liver multifunctional phosphodiesterase than to those of any other plant or mammalian phosphodiesterase so far reported, its higher M_r (61 000–63 000 compared to 33 000 for the pig liver enzyme), plurality of binding sites and the mixed-type inhibition of one substrate by another, indicate significant differences between them. Nevertheless, the many similarities that do exist between the two enzymes and which also include their subcellular distribution, in which 85% of the recovered *Lactuca* phosphodiesterase activity is in the soluble phase (Table 5), a situation comparable to that of the pig liver enzyme, suggest that knowledge of the properties of one enzyme may be of value in interpreting those of the other. In this context, it is relevant to consider the increase in phosphodiesterase activity during germination of the seeds and early growth of *Lactuca* seedlings (Fig. 3) alongside the reported depressed levels of activity of the multifunctional phosphodiesterase in foetal, young and rapidly proliferating mammalian tissues [10] and the

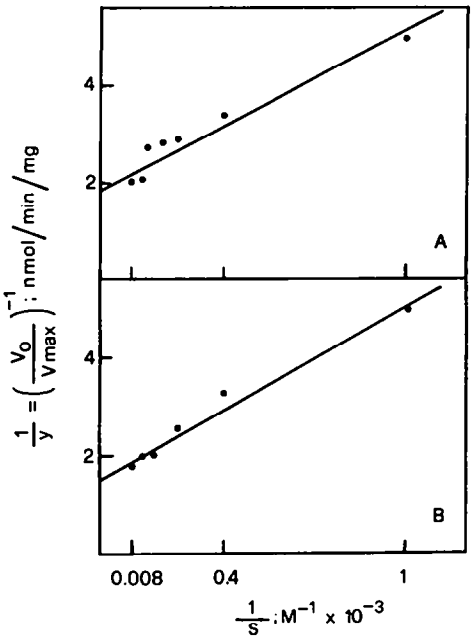

Fig. 2. Plots of V_0/V_{max} against $1/[S]$ for (a) 3',5'-cyclic CMP and (b) 3',5'-cyclic AMP, as substrates of the cyclic nucleotide phosphodiesterase from *Lactuca* cotyledons. The enzyme preparation had been purified to the affinity chromatography stage.

Table 5. Cyclic nucleotide phosphodiesterase activity of centrifugal fractions obtained from *Lactuca* cotyledon homogenates

Fraction	Total activity (nmol/min/ml of suspension)
1500 *g* pellet	0
10000 *g* pellet	0.63
30000 *g* pellet	0
100000 *g* pellet	94.0
100000 *g* supernatant	520.0

Enzymic activity was determined using 2.5 mM 3',5'-cAMP as substrate. Each pellet was suspended in 1 ml of buffer for assay.

suggestion that cyclic CMP has a regulatory role in the proliferation process [8]. There is an increase in both the total and specific activities of the enzyme within *Lactuca* seedlings while the concentration of soluble protein is decreasing, suggesting either that synthesis of the enzyme is occurring or that it has a much slower turnover than the majority of proteins present.

The major conclusion to be drawn from these data is that, in *Lactuca* at least, one of the major factors affecting the hydrolytic breakdown of a particular cyclic nucleotide is the presence of other cyclic nucleotides. Presence of the 2',3'-cyclic nucleotide isomers as intermediates in RNA catabolism by plants is already established, as is the existence of 3',5'-cyclic AMP and cyclic GMP [2]. The natural occurrence of 3',5'-cyclic CMP has only recently been unequivocally demonstrated in mammals [5–7] and no information relating to higher plants in this respect is yet available. While a role for cyclic CMP in mammalian tissues has not yet been elucidated, the enzymes responsible for its synthesis and for initiation of cyclic CMP-dependent protein kinase activity appear distinct from those involved with cyclic GMP and cyclic AMP, and although there exists a multifunctional phosphodiesterase which hydrolyses all three of these 3',5'-cyclic nucleotides at one site, there is also a distinct cyclic CMP-specific phosphodiesterase. Similarly, the cyclic AMP and cyclic GMP cyclases and phosphodiesterases, and the cyclic AMP- and cyclic GMP-dependent protein kinases of mammalian tissues are distinct entities. In contrast, not only is hydrolysis of cyclic nucleotides in plants more complex as observed here, involving neither simple competitive, non-competitive or uncompetitive inhibition, but also the cyclic nucleotide-sensitive protein kinases of plants are each responsive to more than one cyclic nucleotide, including 3',5'-cyclic IMP [21]. It would thus appear that the cyclic nucleotide system of higher plants is very different from and even more highly integrated than that of mammals and in order to understand it fully, our earlier suggestion [2] that further studies should involve simultaneous consideration of cyclic AMP and cyclic GMP may well have to be extended to include other cyclic nucleotides.

EXPERIMENTAL

Materials. Radioactively labelled cyclic nucleotides and other chemicals were obtained from the sources previously listed [17]. Seeds of *Lactuca sativa* cv Arctic King were surface sterilized, germinated and their cotyledons detached and homogenized at 4° as previously described [17].

Assay of cyclic nucleotide phosphodiesterase activity. The procedure used routinely in this work was essentially a modification of that of ref. [17]. Enzyme samples, each containing 0.8–100 ng of protein were assayed in a reaction mixture containing 0.025 μCi of the appropriate [8-^{3}H]-labelled nucleoitide. Details of the composition of the incubation medium and incubation conditions were previously described [17]. The radioactivity of the incubation products was measured by scintillation counting. Samples (0.5 ml) were counted in 5 ml portions of OptiPhase RIA scintillation fluid (LKB, South Croydon). The scintillation counter was programmed for automatic quench control and operated at a pre-set error of 0.2%. Counting efficiency was 35%.

Determination of protein concentrations. Samples containing above 20 μg of protein per ml were assayed for protein content by the procedure of ref. [22]. Lower concentrations were measured by the methods of [23, 24].

Extraction and purification of the cyclic nucleotide phosphodiesterase. The phosphodiesterase activity was extracted from detached *Lactuca* cotyledons, purified to the affinity chromatography stage and concentrated as previously described [17].

Determination of pH and temperature optima. The activity of the phosphodiesterase was determined between pH 4.0–9.1 at increments of 0.3, using 1.25 mM [^{3}H]-3',5'-cyclic AMP as substrate. The buffers employed were 50 mM Na cacodylate–HCl (pH 4.0–7.1), 50 mM HEPES (pH 6.8–7.6) and 50 mM Tris–HCl (pH 7.0–9.1). The activity of the phosphodiesterase was determined at temperatures between 15 and 70° at 5° increments also using 1.25 mM [^{3}H]cyclic AMP as substrate.

Determination of isoelectric point. Protein samples (5–10 μl),

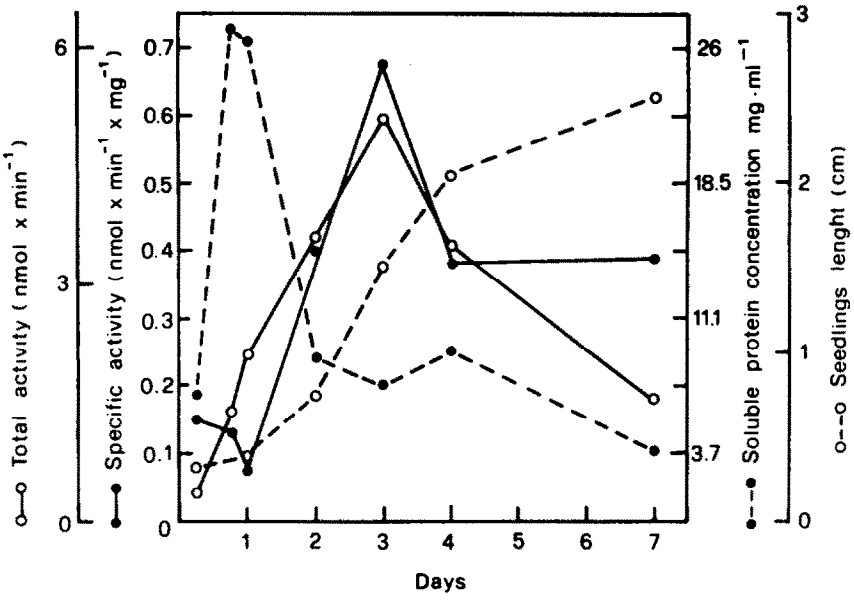

Fig. 3. Cyclic nucleotide phosphodiesterase activity and protein content of *Lactuca* seedlings during early growth. Seeds were set to germinate on Day 0.

obtained at the affinity chromatography stage of purification, were placed as bands onto commercially prepared layers (LKB Ltd., Croydon, U.K.) of polyacrylamide gel containing ampholyte with a pH range of 3.5–9.5. After isoelectric focusing, each gel was cut in half lengthwise, and the separated proteins on one half fixed and stained as previously described [16]. The replicate half of the gel was cut into pieces (5 × 5 mm) each of which was separately eluted and the phosphodiesterase activity determined as described earlier [17] using [^{32}P]cyclic AMP as substrate.

Substrate specificity and kinetics. The phosphodiesterase activity of the preparation after ammonium sulphate precipitation, gel filtration and affinity chromatography [17] was separately determined against [^{32}P]-labelled 3',5'-cyclic AMP, GMP and CMP and against [^{32}P]-labelled 2',3'-cyclic AMP, GMP and CMP. In each case, a final substrate concentration of 0.5 mM was used and it contained 2×10^5 d.p.m. All other assay conditions were as previously described [17].

For determination of K_m and V_{max}, the phosphodiesterase activity of the fraction obtained at the affinity chromatography stage of purification was determined at substrate concentrations between 0.1 and 2 mM for each of the six [^{32}P]-labelled cyclic nucleotides.

Analysis of the products of hydrolysis. The standard reaction mixture (final vol. 100 μl) consisted of 40 mM Tris buffer (pH 6.5), containing $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), $FeCl_2$ (0.5 mM), 1–5 μg of the enzyme preparation from the affinity chromatographic stage of purification and 2×10^5 to 3×10^5 d.p.m. of one of the six cyclic nucleotides at a substrate concentration of 0.5 mM. Incubation was at 30° for 75 min. The reaction was stopped by addition of 1 ml ice-cold H_2O and the products were separated by the method of Helfman *et al.* [12, 13]. Spots corresponding to standards run at the same time were scraped off, eluted in 1 M HCl containing 25% EtOH, and counted as above.

Effects upon cyclic nucleotide hydrolysis of the presence of other cyclic nucleotides. Effects were initially examined by determining the phosphodiesterase activity, in the otherwise standard assay procedure, with the appropriate 3',5'-cyclic nucleotide as the radioactive substrate (1.25 mM) in the separate presence of each of the other two 3',5'-cyclic nucleotides at a final concentration of 2.5 mM. The effects of 2',3'-cyclic AMP and 3',5'-cyclic GMP on cyclic AMP phosphodiesterase activity were investigated further using the standard assay procedure with [^{3}H]-labelled 3',5'-cyclic AMP as substrate at a series of concentrations between 0.1 and 2.5 mM in the presence of a series of concentrations, first of 3',5'-cyclic GMP and then of 2',3'-cyclic AMP between 0.1 and 2.5 mM. Lineweaver–Burk plots [25] were constructed and secondary plots of $1/v$ against [I] were used to calculate K_i values for 3',5'-cyclic GMP and 2',3'-cyclic AMP. Appropriate controls were included to ensure that any effects of 2',3'-cyclic nucleotides on the nucleotidases were monitored: however, no such effects were observed.

Hill plot and Hill coefficient. Plots of V_0/V_{max} against 1/[S] were constructed with the data obtained from the K_m determination with 3',5'-cyclic AMP, 3',5'-cyclic GMP and 3',5'-cyclic CMP as substrates. Hill coefficients were determined by replotting the data as a Hill plot [20, 26] of log [S] against log $(V_0/V_{max}) - V_0$.

Subcellular distribution of phosphodiesterase activity. Cotyledons (7–8 g) were detached and gently macerated with an ice-cold mortar and pestle in 40 mM Tris–HCl buffer (pH 7.4) containing 0.3 M sucrose. The homogenate was filtered through a double layer of Miracloth (Calbiochem-Behring) and the filtrate made up to a total volume of 30 ml with the buffered sucrose solution. Polyvinylpyrrolidone (Polyclar AT; BDH plc) was then added at the rate of 0.5 g/g fr. wt tissue. After 15 min, the suspension was centrifuged at 200 *g* for 2 min, the pellet discarded and the supernatant centrifuged at 1500 *g* for 20 min. This time, the pellet was retained for examination of phosphodiesterase activity and protein content. The supernatant was centrifuged at 10000 *g* for 30 min and after resuspension in 1 ml of 40 mM Tris–HCl buffer, pH 7.4, the pellet was examined for phosphodiesterase activity and protein content. This procedure was repeated at 20000 *g* for 30 min and the pellet examined for phosphodiesterase activity and protein content. The supernatant

was then centrifuged at 100000 *g* for 90 min and to this supernatant was added solid $(NH_4)_2SO_4$ to give 98% saturation. This was stirred at 4° for 1 hr then centrifuged at 20000 *g* for 15 min. The $(NH_4)_2SO_4$ precipitate and the resuspended 100000 *g* pellet were separately tested for phosphodiesterase activity and protein content after resuspension in buffer.

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