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ALLOSTERIC REGULATION OF PHOSPHODIESTERASE FROM *PORTULACA* CALLUS BY cGMP AND PAPAVERIN

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Key Word Index—*Portulaca grandiflora*; Portulacaceae; phosphodiesterase; allosteric interconversion; papaverin; cAMP; cGMP.

Abstract—Three fractions of phosphodiesterase activity capable of hydrolysing cyclic 3′,5′-AMP and cyclic 3′,5′-GMP were purified from *Portulaca* callus. Hydrolysing bis-(*p*-nitrophenyl)-phosphate, two fractions showed linear Lineweaver–Burk plots. One fraction showed positive cooperativity. This fraction can be activated competitively by blue dextran, indicating a possible allosteric regulation by nucleotides, demonstrated by changing from being positively cooperative, to following Michaelis–Menten kinetics by cGMP and papaverin. cGMP triggers an enzyme highly active against 3′,5′cAMP and 3′5′cGMP, and papaverin triggers high activity against 2′,3′cAMP, demonstrated by two separate enzyme fractions.

INTRODUCTION

Cyclic nucleotides are of major importance in the regulation of growth and metabolism in bacteria [1], the slime mold *Dictiostelium discoideum* [2] and animal cells [3–7]. However, considerable doubt exists about their occurrence and their physiological role in higher plants [8, 9]. Recently, more evidence has been found for the existence of the 'cAMP-system' in higher plants [10–13]. A possible participation of this system has also been suggested in the regulation of betacyanin accumulation [14]. In the betacyanin forming callus of *Portulaca grandiflora*, var. JR [15, 16], animal phosphodiesterase (PDE)-inhibitors, like theophylline and papaverin, as well as cAMP itself influenced the DOPA and betacyanin accumulation, accompanied by a characteristic variation of nucleotide concentration [14]. If this effect is induced by inhibiting PDE, PDE must be present and capable of rapidly changing intracellular cyclic nucleotide concentration. PDE, capable of hydrolysing 3′,5′-cAMP has been detected in various higher plant tissues [9] and partially purified PDE was demonstrated [11, 17–24]. These enzymes have acid pH optima, do not require bivalent cations for activity and are insensitive to methylxanthins. These properties are in contrast to those of phosphodiesterases from eukaryotic organisms [25].

The present work deals with a preliminary study on the behaviour of the partially purified cyclic phosphodiesterase from callus of *P. grandiflora*, var. JR. To compare with the enzyme behaviour, the content of 2′,3′ and 5′AMP and GMP, as well as cAMP and cGMP of the callus, cultivated on media containing different concentrations of papaverin was determined.

RESULTS

Extraction and partial purification

The *Portulaca* callus phosphodiesterase was capable of hydrolysing cyclic nucleotides (cAMP,cGM) as well as bis-(*p*-nitrophenyl)-phosphate (BNP) in agreement with refs. [22, 26, 28]. Therefore, the hydrolysis of this artificial substrate was used routinely. At first, the enzyme of step 2 (Table 1) was characterized. The Line-

Table 1. Partial purification of cyclic phosphodiesterase from callus of *Portulaca grandiflora*, var. JR

	Protein (mg/ml)	Activity (μmol *p*-nitrophenol/10 min)	Specific activity (μmol/mg)	Total activity (μmol/mg × total ml)	Purification (fold)
Homogenate	22.3	0.34	0.015	0.93	1
Phosphocellulose	6.02	4.03	0.67	8.35	44.6
$(NH_4)_2SO_4$ ppt.	3.45	4.10	1.18	5.9	78.7
Dialyse (16 hr)	3.28	4.49	1.37	13.4	91.3
Dialyse (65 hr) and Sephadex G-200					
F1	0.71	0.20	0.28	3.79	18.7
F2	1.21	1.80	1.49	8.43	99.3
F3	1.30	0.11	0.09	1.47	6.0

The activity was tested as described under Experimental with 2 mM bis-(*p*-nitrophenyl)-phosphate and the release of *p*-nitrophenol (μmol) in 10 min at 65° is reported. Values are corrected for sampling losses.

weaver–Burk plot (L-B.-plot) was linear with K_m = 2.5 mM for BNP. The enzyme hydrolyses this artificial substrate between pH 4 and 6 with strongly marked maxima at pH 4.3, 4.65, 5.05 and 5.5, which are in the range of cyclic phosphodiesterases from higher plants [11, 17–20, 22, 23]. The temperature optimum was at 65°, much higher than in pea seedlings (40°) [17]. There was a shoulder at 45°.

For further characterization, partial purification was necessary. The results of a typical preparation are presented in Table 1. Starting from the 40 to 70% $(NH_4)_2SO_4$ precipitation, a 99.3-fold increase in purification was achieved. Phosphatase activity could not be separated completely from the diesterase.

Positive cooperativity

As known from research on animal and human objects, mercaptoethanol prevents interconversion of different 'activity states'. Therefore we dialysed the enzyme solution for 65 hr against the routinely used buffer (Tris–HCl, 10 mM, pH 7.4) without mercaptoethanol. Tested with 0.5 mM cAMP-[8-^{3}H] as substrate, the enzyme showed relative high specific activity (1.2 μmol/mg). BNP used as substrate, the L-B.-plot indicates positive cooperativity which can be characterized by a Hill plot [34]. This plot is a convenient method to obtain evidence for positive cooperativity, indicated by coefficients (n_H) greater than one (Fig. 1). The n_H coefficients of our enzyme changed from 1.4 for 0.1–1.0 mM, to 3.9 for BNP greater than 1 mM. If dialysed against the routinely used buffer containing mercaptoethanol (4 mM), there was no cooperativity detectable. This indicates that the cooperativity is not an artificial phenomenon.

When the 65 hr dialysed enzyme was fractionated by column chromatography on Sephadex G-200, like soya bean callus [18] and potato tubers [21], 3 peaks of protein concentrations with enzyme activity (F1, F2, F3) were obtained (Table 1). The L-B.-plots of fraction 1 (K_m:0.17 mM) and fraction 3 (K_m:1.5 mM) were linear (n_H = 1, Fig. 1) with BNP. The dissociated form catalyses the hydrolysis faster than the associated form (K_m: 2.5 mM). Fraction 2 shows positive cooperativity (Fig. 2). In the range of 0.1–0.5 mM BNP, the L-B.-plot is linear (n_H = 1), and in the range of 0.75–2 mM, the L-B.-plot shows positive cooperativity (n_H = 5.8). Dependent on the amount of BNP the Michaelis–Menten kinetic shows linearity or positive cooperativity (Fig. 1).

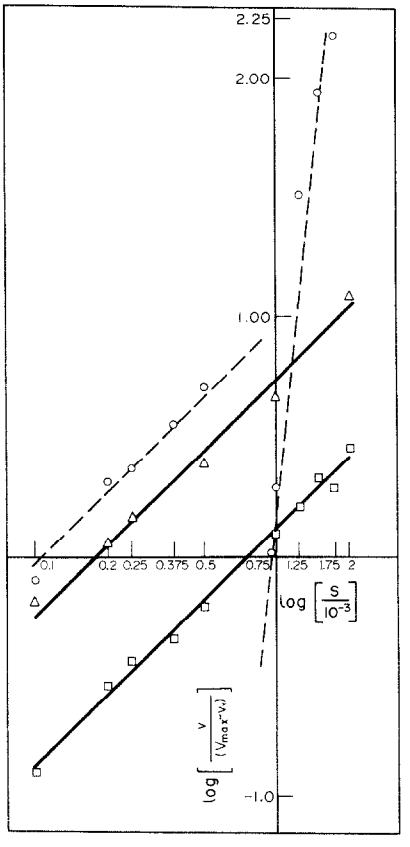

Fig. 1. Hill-plot of the three fractions F1, F2, F3, demonstrating the linearity (n_H = 1) of F1 and F3 as well as the positive cooperativity (n_H = 5.8) of F2 hydrolysing cAMP in the range of 0.75–2 mM bis-(*p*-nitrophenyl)-phosphate, as routinely used specific substrate, replacing cAMP. In the range of 0.1–0.5 mM artificial substrate, the Lineweaver–Burk plot of F2 is linear. The activity is characterized by the release of *p*-nitrophenol (400 nm). One μmol *p*-nitrophenol per min under the assay conditions is equivalent to 16 extinctions (E) units. F1 △——△; F2 ○– – –○; F3 □———□.

Allosteric influences

All 3 fractions were also active against cyclic GMP-[8-^{3}H]. In contrast to the relatively weak activity of F3 against cAMP (K_m: 1.5 mM) and cGMP (K_m: 2.1 mM), F1 shows reversed kinetic behaviour (K_m: cAMP 0.17 mM; cGMP 0.2 mM). F2 hydrolyses both nucleotides without any preference. A possible allosteric regulation of this fraction is demonstrated by the addition of cGMP (1 μM, 10 μM) to the enzyme reaction mixture hydrolysing BNP. The enzyme is changed from being positively cooperative to being fully active and following MM-kinetics (n_H = 1; K_m = 0.3 mM) (Fig. 3). A competitive activation of F2 by blue dextran (0.1; 1; 2 μm) (Fig. 4), one of the characteristics for enzymes influenced by nucleotides [35, 36], demonstrates the possibility of a super-secondary structure, called the dinucleotide fold.

When the enzyme solution was dialysed for 65 hr against the buffer containing papaverin (10^{-3} M) without mercaptoethanol, only one peak with enzyme activity could be detected after chromatographic separation. The L-B.-plot was linear and the K_m (7 mM) for BNP is similar to the K_m (6.6 mM) of the only extractable protein peak with enzymatic activity isolated from callus, cultured on a medium containing papaverin [14] (Fig. 3). This value agrees with the K_m expected for F2 at low (<0.5 mM) substrate concentration, whereas the low K_m (0.3 mM) of the cGMP regulated enzyme resembles that one expected for high (>0.5 mM) substrate concentrations (Fig. 3).

Substrate specificities

As shown in Table 2, the two fractions 1 and 3 show different activities against the cyclic nucleotides used. Against 3′,5′-cAMP and 3′,5′-cGMP, the hydrolysing rate of fraction 3 is only 30 and 26%, respectively of the hydrolysing capacity of fraction 1 (100%) against 3′,5′-cAMP. Against 2′,3′-cAMP, the situation is reversed (F1: 18%; F3: 120%). Treated with cGMP, fraction 2

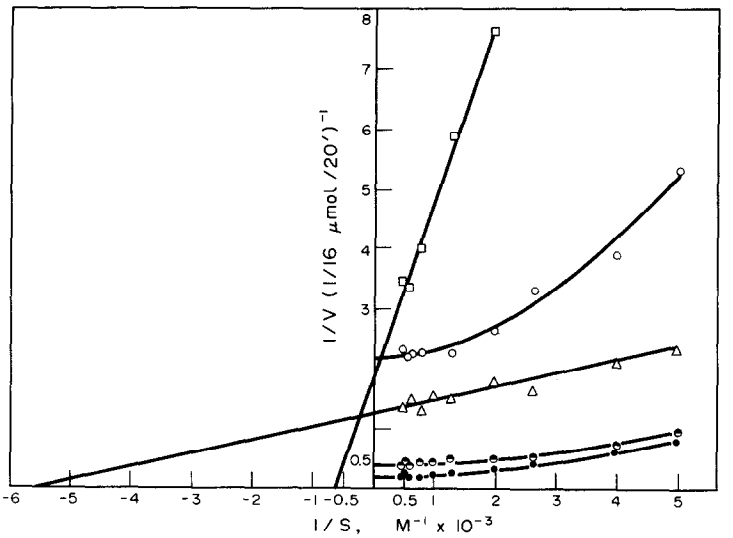

Fig. 2. Lineweaver–Burk plot of fractions F1, F2, and F3 of the partially purified enzyme after dialysis for 65 hr and separation by column chromatography on Sephadex G-200. The influence of different concentrations of enzyme protein on the enzyme activity is demonstrated. The activity is characterized by the variation of the release of *p*-nitrophenol by the enzyme reaction. One µmol *p*-nitrophenol/min is equivalent to 16 extinction units. [F1 100 µl △——△; F2 100 µl ●——●; F3 100 µl □——□; F2 50 µl ◓——◓; F2 10 µl ○——○.]

becomes highly active against 3',5'-cAMP (94%) and cGMP (82%), accompanied by very low activities against 2',3'-cAMP. The enzyme from papaverin-treated callus demonstrates the reversed situation (3',5'-cAMP: 40%; 3',5'-cGMP: 33%; 2',3'-cAMP: 112%).

Situation in the callus

As shown by enzyme extraction and kinetic studies, papaverin induces a very inactive phosphodiesterase against 3',5'-cAMP. The situation in the callus (Table 3), indicated by the content of 2'-, 3'- and 5'-AMP, respectively GMP as well as the cellular concentration of cAMP and cGMP, reflects the same situation. With rising concentrations of papaverin (10^{-5}–10^{-8} M), the content of cAMP (10^{-8} M: +19%; 10^{-5} M: +80%) and cGMP (10^{-8} M: +22%; 10^{-5} M: +275%) as well as the mononucleotides 2',3' and 5'AMP and GMP rises simultaneously. This indicates that the enzyme activity in the callus is changed from having high activities against 3',5'-cAMP and cGMP and low activities against 2',3'-cAMP to the reversed situation. The accompanied accumulation of 5'AMP as well as 5'GMP in spite of increasing concentrations of cAMP and cGMP, indicates further activities hydrolysing products containing 5'AMP or 5'GMP in the callus.

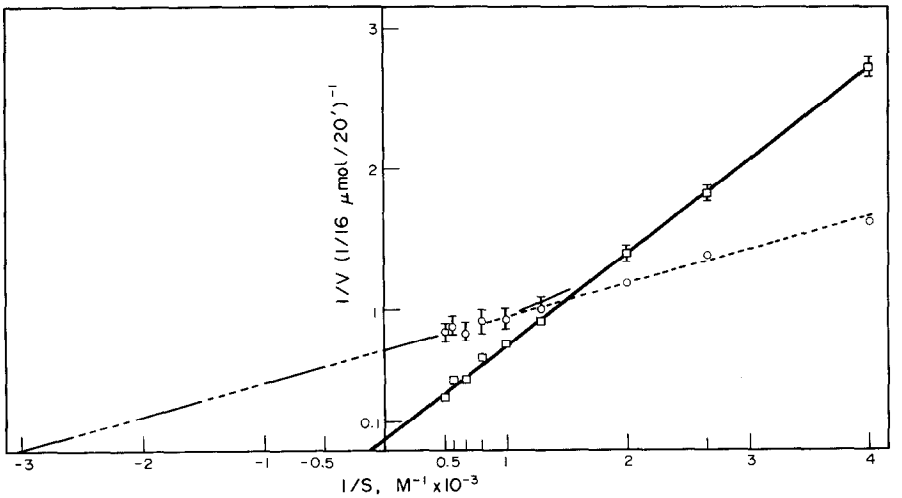

Fig. 3. Lineweaver–Burk plot of fraction F2 of the partially purified enzyme after dialysis for 65 hr and separation by column chromatography on Sephadex G-200. [Uninfluenced (I——I) and under the influence of 1 µM cGMP (○– – –○) and 1 µM papaverin (□——□).]

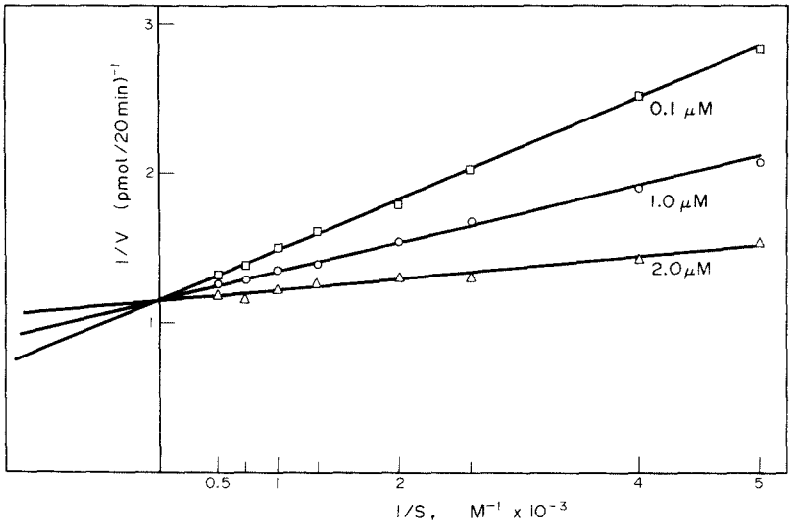

Fig. 4. Influence of blue dextran (0.1, 1.2 μM) on the activity of F2, demonstrating the competitive activation. The activity was tested with cAMP-[8-^{3}H] as described in the Experimental. [0.1 μM blue dextran □——□; 1.0 μM blue dextran ○——○; 2.0 μM blue dextran △——△.]

Further characterizations

In agreement with other investigators [10–13, 18, 22] and in contrast to ref. [38], there was little influence of different concentrations of papaverin and theophylline (inhibition at 10–20%), NADH, NH_4NO_3, EDTA, $CuCl_2$ and $MgCl_2$ (no influence) on the activity of the crude extract and the separated fractions. Obviously, the influence of papaverin on the enzyme structure is not detectable within the reaction time. NADPH has one inhibiting concentration (10^{-2} M: 27%). 5'AMP and 5'-ADP triggers a decreasing inhibition in respect to the concentration used. Maximum inhibition is reached at 10^{-2} M (90 and 99%, respectively).

Table 2. Substrate specificities of the phosphodiesterase fractions out of *Portulaca* callus

Substrate	Activity in %			
	F1	F3	F2	
	untreated		treated with	
			papaverin (10^{-5} M)	cGMP (1 μM)
3',5'-cAMP	100	30	40	94
3',5'-cGMP	92	26	33	82
2',3'-cAMP	18	120	112	22

Rates of hydrolysis, determined as described in Experimental, with 4 mM final concentration for all substrates. Rates are given relative to that for 3',5'-cAMP (100%) by fraction 1.

DISCUSSION

Cyclic nucleotide phosphodiesterase activity has been reported in various higher plants [11, 17, 18, 22, 24, 33, 38, 39]. Several authors interpreted their results as indicating a regulatory role by cyclic nucleotides in plants. Others [17, 39] suggest that the properties of their enzyme differ so markedly from their animal and bacterial counterparts, that they are probably concerned with the hydrolysis of cyclic 2',3'-cAMP during RNA

Table 3. Influence of papaverin on the accumulation of cAMP and cGMP as well as the mononucleotides 2',3',5' AMP and GMP in the callus of *Portulaca grandiflora*, var. JR

M	AMP 2'	AMP 3'	AMP 5'	cAMP	GMP 2'	GMP 3'	GMP 5'	cGMP	cAMP/cGMP
		total extinction		pmol/10 mg		total extinction		pmol/10 mg	—
untreated callus	5.4	2.25	0.92	7.2	1.72	0.71	0.29	2.27	3.17
Papaverin									
10^{-5}	+78	+20	+297	+80	+110	+28	+416	+275	1.53
10^{-6}	+43	+12	+212	+66	+ 64	+16	+244	+120	2.39
10^{-7}	+19	+ 6	+120	+43	+ 45	+11	+171	+ 54	2.94
10^{-8}	+ 9.5	+ 1	+ 42	+19	+ 34	+ 8	+123	+ 22	3.09

The table shows the content of cAMP and cGMP (pmol/10 mg), the cAMP/cGMP relationship as well as the content of 2', 3' and 5' adenosine- and guanosine-monophosphate in total extinction in relation to 1 g lyophilized callus, and the variation (%) induced by different concentrations of papaverin compared with the untreated callus. Each value is the average of 50 Petri dishes from two separate experiments.

catabolism. For example, in contrast to animal phosphodiesterases, the enzyme obtained from pea seedlings [17] has been reported to have acidic pH optimum [5, 4] and greater activity towards cyclic 2',3'-cAMP than the 3',5'-isomer.

The facts presented demonstrates an enzyme from callus of *P. grandiflora*, var. JR, hydrolysing 3',5'-cAMP, 3',5'-cGMP and 2',3'-cAMP. The kinetic data are intermediate between those reported for the enzymes from pea seedlings [17] and potatoes [22]. It seems to consist of 3 'activity states', only detectable after dialysis for 65 hr against the buffer without mercaptoethanol. All activity states can hydrolyse all 3 nucleotides, but with different affinities. This separation can be prevented by mercaptoethanol and by papaverin. Papaverin induced a very inactive phosphodiesterase against 3',5'-cAMP and 3',5'-cGMP but with high activity against 2',3'-cAMP.

The hydrolysis of BNP by F2 is activated by cGMP. BNP replaces cAMP as well as cGMP, therefore cGMP can be both activator and substrate. Competitive activation by blue dextran indicates an allosteric mode of activation of fraction 2 by this nucleotide (cGMP) [35, 36]. Hydrolysing BNP, F2 shows positive cooperativity at higher substrate concentrations. The extracted phosphodiesterase can be changed, probably allosterically, by papaverin, the artificial substrate, as well as by cGMP in its hydrolysing activity.

The changed activity, triggered by papaverin, is also shown by the cAMP and cGMP content in spite of an increasing content of 2'- and 3'- as well as 5'-AMP or GMP in the callus. Our enzyme is probably interconvertable from high 3',5'-cAMP and/or 3',5'-cGMP activity towards a high 2',3'-cAMP hydrolysing activity. This supports the assumption [9, 17], that phosphodiesterase participates in the degradation of *m*RNA, as demonstrated by acid phosphodiesterase from tobacco cells [40, 41]. The phosphodiesterase from *Portulaca* callus is able to hydrolyse the cyclic nucleotides 3',5'-cAMP and cGMP and/or 2',3'-cAMP, depending on the cellular environment. The simultaneously increasing content of 5'-AMP and 5'-GMP, perhaps derived from hydrolysed *m*RNA, demands further investigation.

EXPERIMENTAL

Enzyme preparation. Internodal callus [15, 16] was taken after the first passage, frozen at −20°, lyophilized and the enzyme extracted from 6 g callus with Tris–HCl (10 mM, pH 7.4) containing sorbitol (0.5 M) and 2-mercaptoethanol (4 mM). Also callus cultivated on a medium containing papaverin (10^{-3} M) [14] was extracted. The supernatant after centrifugation (20000 *g*, 20 min) was concd by ultrafiltration (Amicon, PM 10) and the bulk of the enzyme purified and separated from acid phosphatase by substrate affinity chromatography on cellulose phosphate [26]. The column was equilibrated with 0.01 M Tris–HCl, pH 7.4 and the separation ensured by passing a linear gradient of buffer (0.01–0.5 M Tris–HCl). After concn by ultrafiltration, the enzyme was characterized (step 2, Table 1) and followed by $(NH_4)_2SO_4$ (40–70%) precipitation. After centrifugation (20000 *g*, 20 min), the pellet was resuspended in Tris–HCl (10 mM, pH 7.4) and dialysed (16 hr) against the same buffer containing 2-mercaptoethanol. 100 µl of the enzyme were tested before starting the dialysis. Solid was again removed by centrifugation (12000 *g*, 20 min). After concn (Amicon), 100 µl were tested and the bulk of the enzyme soln was dialysed against the above mentioned buffer without 2-mercaptoethanol for 65 hr, followed by chromatography on Sephadex G-200 with 300 ml of Tris–HCl (0.01 M, pH 7.4) buffer. The column was connected to an absorbance monitor (ISCO Model UA-5). Each fraction was concd (Amicon) and tested.

Nucleotide extraction. 2',3'- and 5'-AMP and GMP as well as cAMP and cGMP were extracted from callus after homogenization in cold $HClO_4$ (0.6 M). After centrifugation at 20000 *g* for 15 min and filtration of the supernatant through glass wool, the pH was adjusted to 7 with 5 N KOH when nucleotides were extracted and to 7.5 with Tris–HCl (50 mM) when cyclic nucleotides (AMP and GMP) were extracted [13]. Before adding on the used column, the pre-purified extract was centrifuged again (10000 *g*, 15 min). After standing cold (30 min), the neutralized soln was centrifuged again.

Separation. Cyclic- and polyphosphate nucleotides from the callus or from enzymatic expts were separated on a DEAE-Sephadex A-25 (medium, chloride-form) column with a linear gradient of ammonium carbonate (0–0.4 M) [42]. The separation of the mononucleotides AMP, GMP, CMP and UMP was ensured as described earlier [14]. After collecting the fractions containing the AMP and GMP and concn by lyophilization, the 2',3' and 5' mononucleotides of AMP and GMP were separated by stepwise elution with (0.01, 0.15, 1.3 N) HCO_2H from individual Dowex 1 × 8 200–400 mesh columns [43]. The columns were calibrated with known nucleotides and monitored at 260 nm.

To determine substrate specifity, the hydrolysing rates of the distinct fractions were determined, the resulting products separated as described above, losses taken in consideration and given relative to the rates of hydrolysis of 3',5'-cAMP (100%) by fraction 1.

We purified our extract on an Al_2O_3-column prewashed with 0.06 M Tris–HCl [13, 44, 45]. After elution of adenosine by H_2O [46], *ca* 90% of cAMP or cGMP is extractable by 0.6 M Tris-HCl (pH 7.5). Now the cyclic nucleotides are fixed on Dowex 1 × 8 (Cl^-, 100–200 mesh) and cAMP cluted by 0.05 N HCl and cGMP by 0.5 N HCl [44]. The pH of the eluted fraction was adjusted to 7.5 with Tris–HCl, concd on a rotatory evaporator (30°) and resuspended in 0.6 M Tris–HCl. To separate further unknown interfering substances, cAMP was purified again on Dowex 50 WX 8 (H^+, 200–400 mesh) and cGMP on Sephadex G-10, with NH_4OH and H_2O prewashed column. The first 3 ml of the eluted fractions were concd under vacuum and resuspended in 1 ml acetate-buffer (0.05 M, pH 5.6). The separation was done twice and losses, determined using cAMP-[8-^{3}H] and cGMP-[8-^{3}H], were taken in consideration.

Tests. Non-radiochemical assays to determine rates of hydrolysis of 2',3'-cAMP,3',5'-cAMP and 3',5'-cAMP by the different fractions of the isolated enzyme were carried out in the same assay medium described later but with the inclusion of 4 mM substrate. Fraction 2 (F2) contains 10^{-5} M papaverin and 1 µM cGMP.

Protein concn was determined by UV absorbance at 280 and 260 nm [27]. Enzyme activity was tested at 65° (Table 2), pH 4.3 for 20 min. 0.5 mM [8-^{3}H]-adenosine 3',5'-cyclic monophosphate (sp. act. 26 Ci/mmol) and 0.5 mM [8-^{3}H]-guanosine 3',5'-cyclic monophosphate (sp. act. 21 Ci/mmol) were used as substrate. Routinely BNP was used as artificial substrate [22, 26, 28]. The reaction was started by adding 100 µl enzyme soln and terminated by adding 0.1 M NaOH [21]. Phosphatase activity was measured simultaneously by using *p*-nitrophenylphosphate [22]. The release of *p*-nitrophenol ($\varepsilon_{400\,nm}$ = 16000 l./Mcm) was measured at 400 nm [31]. The radioactive reaction mixture was applied to an Al_2O_3 column, equilibrated with 10 mM Tris–HCl, pH 7.4. The first 3 ml eluted from the column were collected and the radioactivity counted [11]. The amount of cyclic nucleotides hydrolysed was calculated on the basis of the difference between the amount added to the reaction mixture and those recovered from the column. Rate of recovery was tested by using the above radioactive substances (80–90%).

Before testing the cAMP and cGMP content using the radio isotope dilution test with G-3,5-MP- and A-3,5-MP-binding

protein $(NH_4)_2SO_4$ precipitation technique, the cyclic nucleotides were succinylated [45]. 2'-*O*-succinyl derivates have a much greater affinity for their specific antibodies than do the cyclic nucleotides. Succinylation was carried out by one-step addition of premixed reagents. It was made by mixing 25 vols of succinic anhydride (200 mg/ml) in Me_2CO with 9 vols of $N(Me)_3$. The reagent was made up immediately before use. The cyclic nucleotides (100 µl) were derivatized by adding 20 ml of the premixed reagent and mixed for 1–2 min. The radioactivity was determined in 3 g PPO, 0.2 g POPOP, 700 ml toluene, 300 ml Triton X-100.

Further substances and methods. The buffer used for pH dependence determination consists of (a) 1N HOAc/NaOH (pH 3.6–5.6) and (b) 0.2 Tris–Maleate/NaOH (pH 5.2–8.6) [32]. A possible influence of papaverin (10^{-3}–10^{-8} M), theophylline (10^{-2}–10^{-9} M), NH_4NO_3, NADH and NADPH (10^{-2}–10^{-11} M), 5'-ADP and 5'-AMP (10^{-1}–10^{-10} M), $CuCl_2$ and $MgCl_2$ (10^{-2}–10^{-8} M) as well as cyclic guanosine (1 µM, 10 µM) and blue dextran (0.1,1,2 µM) on the enzyme activity was tested. For determination of the influence of different concns (10^{-5}–10^{-8} M) papaverin on the cAMP, cGMP and 2',3' as well as 5'-AMP and -GMP, callus was cultivated as described earlier [14] on a medium containing papaverin.

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