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Author(s)	Wood, HN; Lin, MC; Braun, AC
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The Inhibition of Plant and Animal Adenosine 3':5'-Cyclic Monophosphate Phosphodiesterases by a Cell-Division-Promoting Substance from Tissues of Higher Plant Species

(adenylate cyclase/cytokinesins/theophylline/*Vinca rosea*/crown-gall tumor)

HENRY N. WOOD, MICHAEL C. LIN*, AND ARMIN C. BRAUN

The Rockefeller University, New York, N.Y. 10021

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ABSTRACT One member of a new class of cell-division-promoting factors, that has been given the trivial name of cytokinesin I, is a potent inhibitor of adenosine 3':5'-cyclic monophosphate phosphodiesterases of both plant and animal origin. Since an adenylate cyclase has been demonstrated in this study to be present in plant cells, the results suggest that cytokinesin I may be exerting its biological effects in promoting division in cells of higher plant species as a regulator of adenosine 3':5'-cyclic monophosphate.

Two members of a new class of cell-division-promoting factors, having partition coefficients, or *K* values, of 1.9 (cytokinesin I) and 2.7 (cytokinesin II) in butanol-water after 500 transfers in countercurrent distribution, have been isolated from crown-gall tumor tissue of *Vinca rosea* L. These substances play a central role in the development of a capacity for autonomous growth of plant-tumor cells, since their continued synthesis by the tumor cells keeps those cells dividing persistently. Normal cells of the type from which the tumor cells were derived do not commonly synthesize these substances and require an exogenous source of a 6-substituted adenylate cytokinin, such as kinetin (6-furfurylaminopurine) or a cell-division factor of the kind described here, if growth accompanied by cell division is to occur. It was found, however, that if normal cells are grown in the presence of kinetin they synthesize a compound that is indistinguishable in its physical, chemical, and biological properties from cytokinesin I produced by the tumor cells in the absence of an exogenous source of kinetin or other 6-substituted adenylate cytokinin. This finding has led to the suggestion that kinetin may activate the synthesis of cytokinesin I in normal cell types, and that it is that substance, rather than the 6-substituted adenylate cytokinins, that is directly involved in promoting division in cells of higher plant species (1). The distribution among dicotyledonous plant species and the biological activity of the cytokinesins in several different test systems have been reported (2).

Recent studies with low-resolution mass spectrometry, nuclear magnetic resonance, neutron activation analysis, and UV spectroscopy in acid, neutral, and alkaline solutions have suggested that the chromophore of cytokinesin I consists of a 3,7-dialkyl-2-alkylthio-6-purinone (3). Earlier chemical studies had shown glucose, but not ribose, to be present as the sugar

moiety. Cytokinesin II was found to be a very similar type of compound, having all of the major components of cytokinesin I.

Since the cytokinesins appear to be purinone derivatives having cell-division-promoting activity, and since the methylxanthines are known to be phosphodiesterase inhibitors with at least one, theophylline, possessing cell-division activity in the tobacco pith assay, it was of interest to learn whether the cytokinesins, like certain of the methylxanthines, also functioned as inhibitors of adenosine 3':5'-cyclic monophosphate (cAMP) phosphodiesterases and, hence, possibly promoted cell division by regulation of cAMP.

MATERIALS AND METHODS

The methods used to grow the *V. rosea* tumor cells and the biological assay of cell-division activity have been described (4). The culture medium for testing theophylline contained, in addition, 2% glucose and 2.5% acid-hydrolyzed casein (pH 6.0). The cell-division factor, cytokinesin I, was isolated as described (3).

Preparation of enzymes

cAMP Phosphodiesterase. Crown-gall tumor cells of *V. rosea* (100 g) were broken in a Waring blender at 4°C in the presence of an equal volume of 1 mM Tris·HCl (pH 7.5)-2 mM MgCl₂-2 mM dithiothreitol. The resultant brei was centrifuged at 6596 × *g* in a Sorvall centrifuge for 15 min to remove cell-wall debris. The clear supernatant was brought to either 45 or 80% saturation with (NH₄)₂SO₄ and allowed to stand at 4°C. Both fractions gave similar results. The resultant precipitate was removed, dissolved in a minimum of the Tris·HCl buffer, and dialyzed against this buffer. 3-ml quantities of the retentate were placed on 90 × 1.5-cm columns containing Bio-Gel A-5M, 50-100 mesh. The phosphodiesterase appeared as a peak centered at an elution volume of 91 ml; the void volume of the column was 40 ml. The monoesterase activity was significantly reduced. Phosphodiesterase was prepared from normal *V. rosea* tissue in an identical manner. These preparations were unstable, particularly after column chromatography.

Isoelectric Focusing. Ampholine (pH 3-10) was mixed with a sucrose gradient (0-50%) in a 110-ml electrofocusing column (5). The final ampholine concentration was 2%. The ampholine and electrofocusing column were obtained from LKB Productor AB, Stockholm, Sweden. *V. rosea* phosphodiester-

*Present address: Section on Membrane Regulation, NIAMD, National Institutes of Health, Bethesda, Md. 20014

ase was introduced into the column by addition to the dense sucrose solution before gradient formation. Electrofocusing was performed at 700 V and about 14 mA for 48 hr at 4°C. Fractions of 1.0–1.2 ml were collected at the end of the electrofocusing run, with the aid of a peristaltic pump.

cAMP Phosphodiesterase from Bovine Brain. For the preparation of soluble diesterase (6), the brain cortex was homogenized with nine volumes of 0.05 M Tris·HCl (pH 7.6)–5 mM MgCl₂ at 0°C in a Teflon–glass homogenizer. The homogenate was centrifuged at 30,000 × *g* for 20 min at 2°C. The supernatant was used as the source of enzyme.

Demonstration of Plant Adenylate Cyclase. *V. rosea* tumor cells were ground in a mortar with about one-third their volume of Tris·HCl buffer, as described above. The broken cells were centrifuged in a Sorvall centrifuge at 6596 × *g* for 15 min. The supernatant was discarded and the precipitate was taken up in an equal volume of a digitonin solution (7). The membrane preparation was then sonicated in an M.S.E. apparatus for 15–20 min at 18–20 kHz. After centrifugation in a clinical centrifuge to remove solid particles, the supernatant was assayed directly. The enzyme was then concentrated by means of dialysis against powdered sucrose.

Enzyme assays

Chromatographic Assay for cAMP Phosphodiesterase. The reaction mixture consisted of 0.05 mM Tris·HCl (pH 7.6)–5 mM MgCl₂–1 mM dithiothreitol–0.2 mM cAMP–2 μl of [8-¹⁴C]cAMP, ammonium salt (10 μCi/ml, 39.6 Ci/mol) obtained from New England Nuclear Co. The enzyme preparations were added in 5- to 50-μl aliquots, and the reaction was run at 30°C for 20 min. The 100-μl reaction mixture was boiled for 3 min, cooled, and 10 μl of 0.2 mM 5'-AMP and 10 μl of 0.2 mM adenosine were added as carriers. The volume of the final mixture was 120 μl. A 50-μl aliquot was, in each instance, chromatographed on Whatman 3-mm paper by the method of

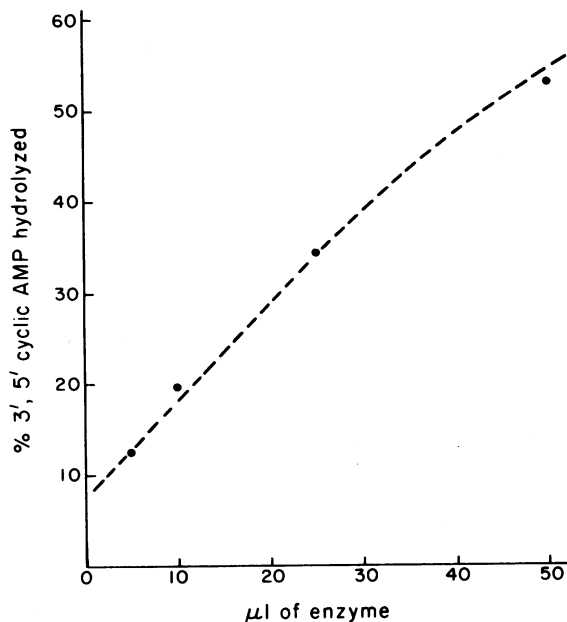


FIG. 1. The hydrolysis of cAMP by increasing amounts of cAMP phosphodiesterase isolated from normal *Vinca rosea* cells.

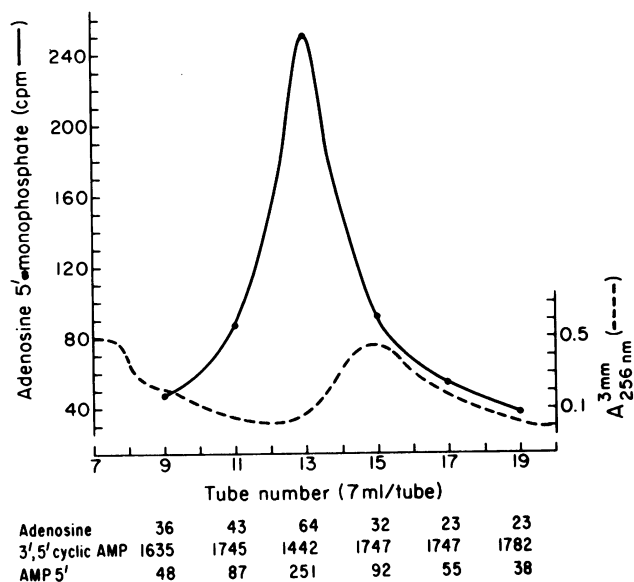


FIG. 2. Separation on a Bio-Gel A-5M column of cAMP phosphodiesterase activity from *Vinca rosea* tumor cells. Assay values for phosphodiesterase product (5'-AMP), phosphomonoesterase product (adenosine), and cAMP are indicated for every second tube.

Hirata and Hayaishi (8), with 1 M ammonium acetate–95% ethanol 3:7 as solvent. The *R_f* values were 0.15 for 5'-AMP, 0.46 for cAMP (3':5'), and 0.62 for adenosine. The spots were detected under UV light, cut out, and placed in vials containing 15 ml of Liquifluor scintillation fluid (New England Nuclear Co.). The radioactivity was measured with a Packard liquid scintillation counter, model 3375.

Chromatographic Assay for Adenylate Cyclase. To 50 μl of reaction mixture, containing 0.15 M Tris·HCl (pH 7.6)–15 mM MgCl₂–30 mM NaF–3 mM dithiothreitol–3 mM ATP–0.1 μCi of [α-³²P]ATP, was added 100 μl of enzyme solution. The solution was incubated at 30°C for 30 min and the reaction was stopped by immersion in a boiling-water bath for 3 min. To the cooled solution were added 10 μl of 2 mM cAMP as carrier and 50 μl each of 1 M ZnSO₄ and 1 M Na₂CO₃. The well-shaken mixture was allowed to stand 1 hr at room temperature; the precipitate, containing most of the nucleotides except cAMP, was centrifuged for 10 min in a clinical centrifuge. A 50-μl aliquot was applied to Whatman 3-mm paper and chromatographed with a solvent of isopropyl alcohol–water–ammonium hydroxide 7:2:1. The spots were identified with UV light, cut out of the chromatogram, and counted as indicated above.

Column Chromatographic Assay for cAMP Phosphodiesterase. Diesterase activity was also determined by an automated column chromatographic method† that measured the formation of 5'-AMP produced from radioactive cAMP. Briefly, the enzyme, 10–50 μl containing 5–100 μg protein, was added to 0.5 ml of the substrate mixture, containing 50 mM Tris·HCl (pH 7.6)–5 mM MgCl₂–2 mM dithiothreitol–10 mM NaF–0.2 mM cAMP (with about 5000 cpm of ¹⁴C). After 10 min of incubation at 35°C, the reaction was terminated by

† Lin, M. C. & Moore, S., manuscript in preparation.

TABLE 1. Membrane adenylate cyclase assay

	cAMP formed (cpm)
100 μ l of sonicated extract	12.0
100 μ l of 10-fold concentrate of sonicated extract	117.0

immersion in a boiling-water bath for 2 min. Then 0.7 ml of 0.2 mM 5'-AMP was added as a carrier. 1-ml samples were injected into a column chromatographic system continuously monitored by a scintillation counter equipped with a flow cell. The radioactivity of the 5'-AMP peak was measured.

RESULTS

Demonstration of cAMP phosphodiesterases

The cAMP phosphodiesterases were isolated, as described above, from both normal and tumor cells of *V. rosea*. Fig. 1 shows the effects of different concentrations of crude enzyme, prepared as a fraction 45%-saturated with $(\text{NH}_4)_2\text{SO}_4$ from normal cells, on the hydrolysis of cAMP. These studies demonstrate increased hydrolysis of cAMP with increased enzyme concentrations. Similar results were obtained with *V. rosea* tumor cells. The crude enzyme preparation, nevertheless, contained significant monoesterase activity, as evidenced by the conversion of 5'-AMP to adenosine. Purification of the crude enzyme preparation from *Vinca* tumor cells by the use of Bio-Gel A-5M columns significantly reduced monoesterase activity, as shown in Fig. 2. A single peak of cAMP phosphodiesterase was present at an elution volume of 91 ml.

The cAMP phosphodiesterase fractions from three columns were combined, and concentrated 10-fold by dialysis against powdered sucrose. The biologically active preparation was then subjected to electrofocusing and, after 48 hr, two bands of cAMP phosphodiesterase activity were found, one at pH 4.0 and the other at pH 7.0. Much of the phosphodiesterase protein precipitated during the electrofocusing, but enough activity remained on the column to be assayed. Thus, at least two cAMP phosphodiesterases are present in extracts of *V. rosea* tumor cells.

Demonstration of adenylate cyclase

Having established the presence of cAMP phosphodiesterases in both normal and tumor cells of *V. rosea*, we determined whether an adenylate cyclase was also present in these cells. Such an enzyme was found. As indicated in *Methods*, sonication in the presence of digitonin was necessary to liberate the adenylate cyclase from the membrane and wall fractions of the cells. Table 1 shows that a low, but reproducible, synthesis of cAMP was obtained from $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. When such a preparation was concentrated 10-fold by means of dialysis against powdered sucrose, the enzymatic activity was increased about 10-fold. Thus, the synthesis of cAMP from $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ has been demonstrated in extracts from these tissues.

Inhibition of cAMP phosphodiesterases by cytokinesin I

The demonstration of both cAMP phosphodiesterases and adenylate cyclase in *V. rosea* tissues permitted an examination of the question whether the cell-division-promoting factor, cytokinesin I, acted as an inhibitor of the phosphodiesterases and thus possibly as a regulator of cAMP. These studies were

done with preparations of enzyme from *V. rosea* tumor prepared from the 80%-saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate. The assay was performed by the column chromatographic technique described in *Methods*. Results of those studies, which are summarized in Table 2, demonstrate that cytokinesin I is indeed a potent inhibitor not only of plant but also of animal (bovine brain) cAMP phosphodiesterases. In contrast, zeatin riboside, a typical 6-substituted adenylate cytokinin, is a rather poor inhibitor of those enzymes. The known phosphodiesterase inhibitor, theophylline, on the other hand, was an excellent inhibitor of bovine-brain cAMP phosphodiesterase, but it was less effective as an inhibitor of these enzymes derived from plants.

Since electrofocusing techniques had indicated the presence of at least two distinct cAMP phosphodiesterases in the tumor cells, and since increasing concentrations of cytokinesin I inhibit the phosphodiesterases in an essentially linear manner up to about 50% inhibition, and since further increases in the concentration of the inhibitor do not cause further inhibition, the results suggest that only one of the two phosphodiesterases found may be concerned with the division process and be specifically inhibited by cytokinesin I. This question is now being investigated further.

THEOPHYLLINE AS A CELL-DIVISION-PROMOTING FACTOR

Since cytokinesin I appeared to be a purinone derivative and an excellent inhibitor of cAMP phosphodiesterases, as well as a potent cell division-promoting factor, it was of interest to learn whether the methylxanthines, which are purinones and known to inhibit the phosphodiesterases, are also cell-division-promoting factors. When three methylxanthines were tested for cell-division-promoting activity in the tobacco pith system, it was found that theophylline (1,3-dimethylxanthine), and to a lesser extent theobromine (3,7-dimethylxanthine), but not caffeine (1,3,7-trimethylxanthine), was effective as a cell-division-promoting factor. The quantity of theophylline required to promote division in tobacco pith cells in the presence of an auxin was about 400-times more than is required with a substance such as kinetin to obtain a comparable response in our bioassay (4). The cell-division-promoting activity of theophylline was, nevertheless, real and reproducible. The results of this study are summarized in Fig. 3.

TABLE 2. Results of cAMP phosphodiesterase inhibition studies

cAMP phosphodiesterase inhibitors	<i>Vinca</i> tumor phosphodiesterase(s)		Bovine-brain phosphodiesterase	
	Concentration, mM	% Inhibition	Concentration, mM	% Inhibition
Zeatin riboside	0.29	16	0.33	12
Theophylline	0.30	18	0.20	32
Cytokinesin I	0.29	57	0.29*	48

* The concentration of cytokinesin I was calculated from the absorbance of weighed cytokinesin I standards. The molecular weight was derived from the sulfur content and the assumption of 1 mole of sulfur in the molecule. Neutron activation analysis indicated a sulfur content of 4.35%.

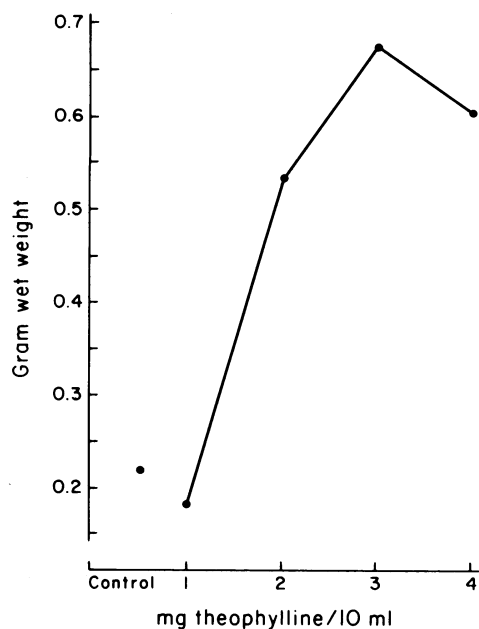


FIG. 3. The growth-response curve of tobacco pith explants to increasing amounts of theophylline.

DISCUSSION

A cell-division-promoting factor, which has been found to play a central role in the development of a capacity for autonomous growth of the plant tumor cell, has been shown to be a potent inhibitor of both plant and animal (bovine brain) cAMP phosphodiesterases. A membrane-bound adenylate cyclase has also been demonstrated in this study to be present in plant cells. These findings suggest that members of this new class of naturally occurring cell-division factors may be exerting their biological effects as regulators of cAMP.

Physical and chemical studies have indicated that members of this class of cell-division-promoting factors are purinone derivatives that contain alkyl groups, sulfur, and glucose as the sole sugar moiety (3). They thus differ from the 6-substituted purine cytokinins isolated from certain tRNAs of bacterial, plant, and animal origin in that the purinone derivatives described here contain glucose rather than ribose, which is present in all tRNA. Although both the 6-substituted adenylate cytokinins and the purinone derivatives promote cytokinesis in cells of higher plant species, they appear to function at different levels in metabolism. Our earlier studies suggested that the *N*⁶-monosubstituted adenylate cytokinins activate the synthesis of the purinone derivatives in normal cell types and it is those substances, rather than the adenylate cytokinins, that are directly involved in the promotion of cell division (1). The fact, moreover, that the purinone derivatives are potent inhibitors of the cAMP phosphodiesterases, while

zeatin riboside is a rather poor inhibitor of these enzymes, suggests that these two types of compounds act in different ways to promote cytokinesis. Goren and Rosen (9) have recently shown that adenine, adenosine, guanine, and guanosine inhibit beef-heart phosphodiesterase about 10%. This figure approximates that found by us when the inhibitory effects of zeatin riboside were tested against animal and plant phosphodiesterases. None of the compounds tested by Goren and Rosen are known to promote division in tobacco pith cells when used at physiological concentrations. That the biological activity of the purinones and the 6-substituted adenyl cytokinins may differ strikingly in other systems is evidenced particularly by differences in the ability of the two types of compounds to initiate "bud" formation in the moss species, *Funaria hygrometrica* (2).

Since the classical cytokinins include, by definition, only those compounds that are either naturally occurring or synthetic *N*⁶-monosubstituted adenyl derivatives, having the same types of biological activity as does kinetin, it is clear that the purinone derivatives described here should not be classified as cytokinins (10). We are, therefore, proposing the new generic term, *cytokinesins*, for the purinone derivatives. Two naturally occurring members of this class of compounds have now been isolated in pure form, and partially characterized chemically (3). The first of these, which has a partition coefficient of 1.9 in butanol-water in a 500-tube countercurrent distribution, has been given the trivial name cytokinesin I. The second member of this group, having a partition coefficient of 2.7 in the same solvent, will be called cytokinesin II.

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