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Competitive Inhibition of Beef Heart Cyclic AMP Phosphodiesterase by Cytokinins and Related Compounds

(cyclic AMP metabolism/intracellular cyclic AMP concentration)

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ABSTRACT **Two cytokinins and four related analogs, none of which is a cyclic ribonucleotide, have been shown to act as competitive inhibitors of the high K_m cyclic-AMP phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) activity from beef heart. Weak inhibition of the low K_m cyclic AMP phosphodiesterase activity was also observed, suggesting a possible mechanism for regulation of intracellular cyclic AMP levels by the exogenously added compounds. In addition to the kinetic data, obtained on the six inhibitors in four different heterocyclic series, 15 other cytokinins and related compounds have been shown to inhibit the high K_m cyclic AMP phosphodiesterase activity at single concentrations of substrate and inhibitor. Heterocycles such as adenosine and 7-amino-3-methylpyrazolo[4,3-*d*]pyrimidine, which lack the *N*-substituent, were inactive as cyclic AMP phosphodiesterase inhibitors. The observed inhibition of cyclic AMP phophodiesterase supports prior observations which implicate exogenously added cytokinins in cyclic AMP metabolism.**

Cytokinins were first isolated as plant factors responsible for the promotion of cell division and growth (1, 2). The compounds, which are typically N^6-substituted adenine and adenosine derivatives, occur at the purine, ribonucleoside, and ribonucleotide levels in plants, as well as in the transfer RNAs of most forms of life (2–5). In spite of the widespread natural occurrence of cytokinins and the diverse metabolic effects which they are now known to promote in both plants and animals (1–13), relatively little has been learned about their mechanism of action at the molecular level. This prompted the design and synthesis of a class of potent anticytokinins, structurally related to the cytokinins, in the hope that the antimetabolites might extend the study of cytokinins to new biological systems and provide useful information pertinent to the mechanism of cytokinin action (14–17).

On the basis of the similar effects obtained with N^6-(Δ^2-isopentenyl)adenosine and dibutyryl cyclic AMP, the cytokinins have been postulated to mediate their effects in phytohemagglutinin-treated human lymphocytes by involvement in cyclic AMP metabolism (13). This postulate was consistent with the finding that the cytokinin *trans*-zeatin ribonucleoside was inhibitory to the cyclic AMP phosphodiesterases from beef brain and crown-gall tumor cells, the latter of which was also shown to contain detectable adenylate cyclase activity, suggesting that the cytokinins might function by raising the intracellular level of cyclic AMP (18). If operative in mouse fibroblasts this phenomenon might also explain the observed growth inhibition of such cells by cytokinins (S. M. Hecht and R. B. Frye, in preparation), since it has been shown that there is an inverse relationship between intracellular cyclic AMP concentration and growth in fibroblasts (19).

To further explore the apparent involvement of exogenously added cytokinins in cyclic AMP metabolism, we have investigated the kinetics of interaction of certain cytokinins and related compounds with the cyclic AMP phosphodiesterase activity from beef heart. This enzyme preparation has been shown to have both high and low K_m activities (20, 21), both of which were utilized in the present experiments.

MATERIALS AND METHODS

Cyclic [^{3}H]AMP, specific activity 33.2 Ci/mmol, was obtained from New England Nuclear Corp. Snake venom phosphodiesterase (*Crotalus adamanteus*) and beef heart cyclic AMP phosphodiesterase were both obtained from Sigma Chemical Co. The cyclic AMP phosphodiesterase was shown, on the basis of a v versus v/S plot of initial velocity data, to contain both high and low K_m activities, comparable to those described in ref. 20. Also verified was the absence of contaminating activities, e.g., 5′-nucleotidases and phosphatases, which could have affected the results reported here.

Synthesis of Heterocycles. The syntheses of compounds *1* (22), *2* (23), *3* (24), *4* (25), *5* (26), *6* (27), *7* (28), *8* (16), *9* (14), *10* (16), *12–18* (F. Skoog, R. Y. Schmitz, S. M. Hecht, and R. B. Frye, in preparation), *19–21* (29), *22* and *23* (S. M. Hecht, R. B. Fry, D. Werner, S. D. Hawrelak, F. Skoog, and R. Y. Schmitz, in preparation), have been reported. The synthesis of formycin cyclic 3′:5′-monophosphate (*11*) (30, 31) was carried out as follows:

To 53 mg (0.2 mmol) of formycin was added 3 ml of acetonitrile and 0.1 ml (0.72 mmol) of pyrophosphoryl chloride (32). The solution was stirred at 0° for 2 hr, diluted with 10 ml of ice water and extracted with ether. The aqueous layer was adjusted to pH 5 with aqueous sodium hydroxide, treated with 7.5 ml of 0.5 M barium acetate solution and centrifuged. The supernatant was purified by chromatography on a DEAE-cellulose column (1.8 × 20 cm), elution with a linear gradient of ammonium bicarbonate (2 liters total volume; 0 → 0.3 M) at a flow rate of 150 ml/hr. The appropriate fractions were pooled and desalted by repeated evaporation of portions of water to afford formycin 5′-monophosphate as a white solid, yield 61 mg (87%); λ_{max} (pH 1) 304 nm (shoulder), 295 (ϵ =

Abbreviations: Dibutyryl cyclic AMP, N^6,$O^{2'}$-(dibutyryl)adenosine cyclic 3′:5′-monophosphate; cyclic AMP, adenosine cyclic 3′:5′-monophosphate; *trans*-zeatin ribonucleoside, N^6-(4-hydroxy-3-methyl-*trans*-2-butenyl)adenosine; 8-bromo cyclic AMP, 8-bromoadenosine cyclic 3′:5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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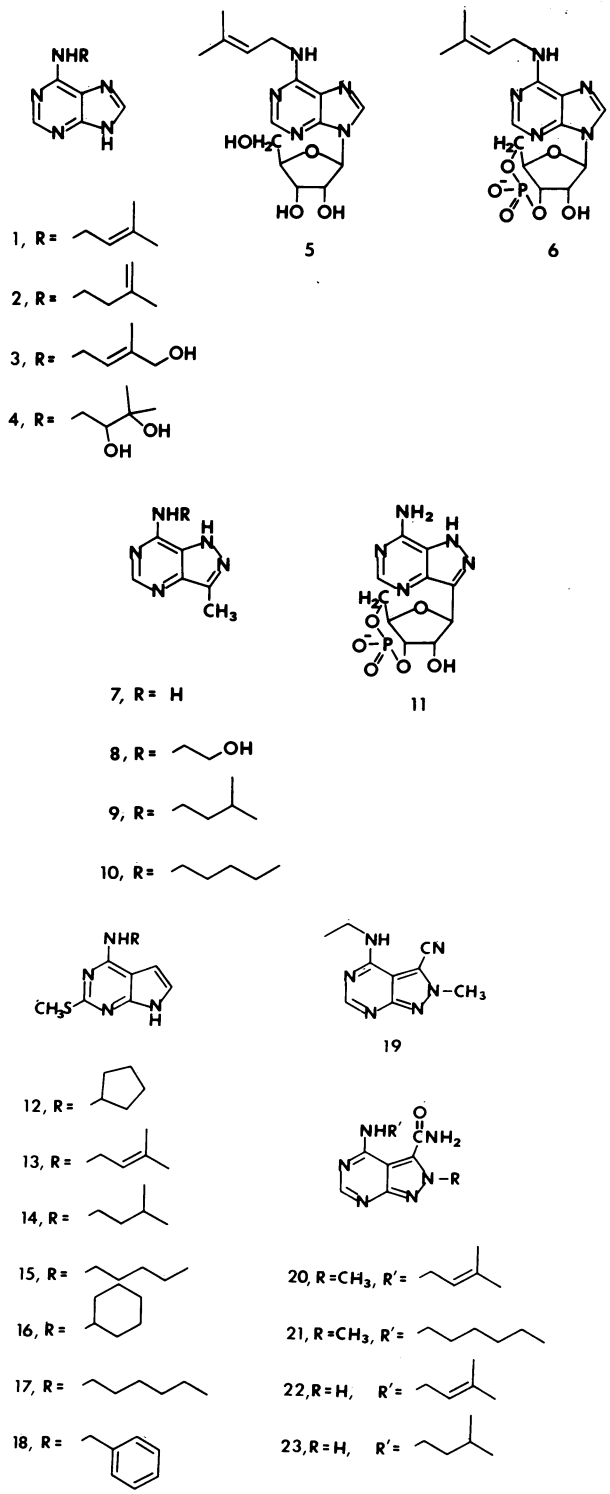

9400) and 233 (7000), λ_{min} 265 (4100) and 222 (5800); λ_{max} (pH 7) 304 (sh), 293 (9400), 285 (sh) and 228 (5500), λ_{min} 252 (3200) and 224 (5400); λ_{max} (pH 12) 302 (7100), 258 (sh) and 232 (14,000), λ_{min} 268 (3100) and 222 (10,700). Chromatography on a polyethyleneimine thin-layer chromatography plate in 0.2 M KCl revealed a single fluorescent spot, R_F 0.06 [R_F (5′-AMP) = 0.07]. Treatment of a portion of the monophosphate with crude snake venom (*Crotalus adamanteus*) reafforded formycin in quantitative yield.

To 36 mg (0.1 mmol) of formycin 5′-monophosphate, converted to the pyridinium salt, was added 29 mg (0.1 mmol) of 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine, and the mixture was dissolved in aqueous pyridine and evaporated to dryness under diminished pressure. Portions of dry pyridine were evaporated from the residue several times to remove traces of water. The ribonucleotide was then dissolved in 15 ml of anhydrous pyridine and added dropwise to a boiling solution of 41 mg (0.2 mmol) of *N*,*N*′-dicyclohexylcarbodiimide in 10 ml of dry pyridine (33). The combined solution was heated at reflux for an additional 2 hr, concentrated to dryness under diminished pressure, and treated with water. The insoluble urea was filtered and the solution was extracted with ether to remove traces of dicyclohexylcarbodiimide. Purification was effected by chromatography on a DEAE-cellulose column (1.8 × 20 cm), elution with a linear gradient of ammonium bicarbonate (2 liters total volume; 0 → 0.3 M) at a flow rate of 150 ml/hr. The fractions containing formycin cyclic 3′:5′-monophosphate (*11*) were combined and desalted by repeated evaporation of portions of water to afford *11* as a white solid, yield 6 mg (18%); λ_{max} (pH 1) 304 nm (sh), 294 (ϵ 9000) and 234 (8800), λ_{min} 270 (7400) and 222 (6400); λ_{max} (pH 7) 304 (sh), 294 (9000), 286 (sh) and 228 (sh), λ_{min} 249 (3700); λ_{max} (pH 12) 302 (5500), 258 (sh) and 232 (11,400), λ_{min} 268 (2800) and 224 (9800). Chromatography on a polyethyleneimine thin-layer chromatography plate in 0.2 M KCl revealed a single fluorescent spot, R_F 0.22 [R_F (cyclic AMP) = 0.27]. Treatment of *11* with beef heart cyclic AMP phosphodiesterase resulted in its complete degradation to a material identical with formycin 5′-monophosphate at a rate 45% of that at which cyclic AMP was hydrolyzed. Further treatment of the hydrolyzed product with crude snake venom (*Crotalus adamanteus*) resulted in its quantitative conversion to formycin.

Enzymatic Assays. Single point assays were carried out in a total volume of 1 ml of 0.05 M Tris·HCl, pH 8.0, containing 3 mM $MgCl_2$, 1 mM EDTA, 0.2 mM cyclic AMP (specific activity 20 Ci/mol) and 2 mM inhibitor. Each reaction was initiated by the addition of 45.9 μg of enzyme, dissolved in 30 μl of buffer, and was maintained at room temperature for 20 min. The reactions were terminated by heating in a boiling-water bath for 3 min. The product (5′-AMP) was separated from cyclic AMP in each case by chromatography on a 12-ml DEAE-cellulose column, elution with a linear gradient of ammonium formate (200 ml total volume; 0 → 0.8 M). Two-milliliter fractions were collected and the radioactivity of the entire fraction was determined in 10 ml of xylene-based scintillation fluid (34). The percent reaction was determined from the sum of the radioactivity in the 5′-AMP fractions, corrected for a boiled enzyme blank. Under these conditions, approximately 50% of the cyclic AMP was converted to 5′-AMP in the absence of any inhibitor. The extent of inhibition of this conversion by a number of heterocyclic species is given in Table 1.

Six representative inhibitors were chosen for kinetic studies with the high and low K_m cyclic AMP phosphodiesterase activities. For the high K_m studies, [^{3}H]adenosine cyclic 3′:5′-monophosphate (2 μCi) was added to each sample of unlabeled cyclic AMP, which was utilized at concentrations of 200, 100, 50, and 20 μM with inhibitor concentrations of 0, 200, 400, and 600 μM. The compounds were dissolved in 0.05 M Tris·HCl, pH 8.0, containing 3 mM $MgCl_2$ and 1 mM EDTA. The assay solutions were equilibrated at 37° and the reactions were initiated by the addition of a portion of the en-

TABLE 1. *Inhibition of cyclic AMP conversion to 5'-AMP*

Compound	% Inhibition*
N^6-(Δ^2-isopentenyl)adenine (*1*)	50
N^6-(Δ^3-isopentenyl)adenine (*2*)	46
N^6-(4-hydroxy-3-methyl-*trans*-2-butenyl)adenine (*3*)	29
N^6-(2,3-dihydroxy-3-methylbutyl)adenine (*4*)	34
N^6-(Δ^2-isopentenyl)adenosine (*5*)	44
Adenosine	0
N^6-(Δ^2-isopentenyl)adenosine cyclic 3':5'-monophosphate (*6*)	35
7-Amino-3-methylpyrazolo[4,3-*d*]pyrimidine (*7*)	0
7-(2-Hydroxyethylamino)-3-methylpyrazolo[4,3-*d*]-pyrimidine (*8*)	43
3-Methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]-pyrimidine (*9*)	74
3-Methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]-pyrimidine (*10*)	80
Formycin cyclic 3':5'-monophosphate (*11*)	0
4-Cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]-pyrimidine (*12*)	74
4-(3-Methyl-2-butenylamino)-2-methylthiopyrrolo-[2,3-*d*]pyrimidine (*13*)	44
4-(3-Methylbutylamino)-2-methylthiopyrrolo-[2,3-*d*]pyrimidine (*14*)	48
2-Methylthio-4-*n*-pentylaminopyrrolo[2,3-*d*]-pyrimidine (*15*)	63
4-Cyclohexylamino-2-methylthiopyrrolo[2,3-*d*]-pyrimidine (*16*)	74
4-*n*-Hexylamino-2-methylthiopyrrolo[2,3-*d*]-pyrimidine (*17*)	50
4-Benzylamino-2-methylthiopyrrolo[2,3-*d*]-pyrimidine (*18*)	59
4-Ethylamino-2-methylpyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (*19*)	20
2-Methyl-4-(3-methyl-2-butenylamino)-pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (*20*)	77
4-*n*-Hexylamino-2-methylpyrazolo[3,4-*d*]-pyrimidine-3-carboxamide (*21*)	65
4-(3-Methyl-2-butenylamino)pyrazolo[3,4-*d*]-pyrimidine-3-carboxamide (*22*)	89
4-(3-Methylbutylamino)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (*23*)	94

* Percent inhibition of cyclic AMP conversion to 5'-AMP by 45.9 μg of enzyme, utilizing substrate and inhibitor concentrations of 0.2 mM and 2 mM, respectively. The experimental conditions are given in *Materials and Methods*.

zyme (2.92 μg of protein) dissolved in 10 μl of the same buffer. The final reaction volume was 2 ml. The incubation time was varied (10–60 min) to permit 1–2% conversion of cyclic AMP to adenosine 5'-monophosphate and the reactions were terminated by immersion in a boiling water bath for 3 min. The extent of conversion of cyclic AMP to 5'-AMP was determined by chromatography of each sample on a DEAE cellulose column, followed by measurement of the radioactivity of the individual fractions as above. The data were plotted as 1/v versus 1/S (Fig. 1) and gave straight lines from which values of K_m and K_I were determined (Table 2). The low K_m studies were carried out in the same fashion, except that cyclic[^{3}H]AMP was utilized at concentrations of 0.2, 0.1, 0.05, and 0.02 μM with inhibitor concentrations of 0–0.6 μM and 0.23 μg of protein per assay. The apparent absence of

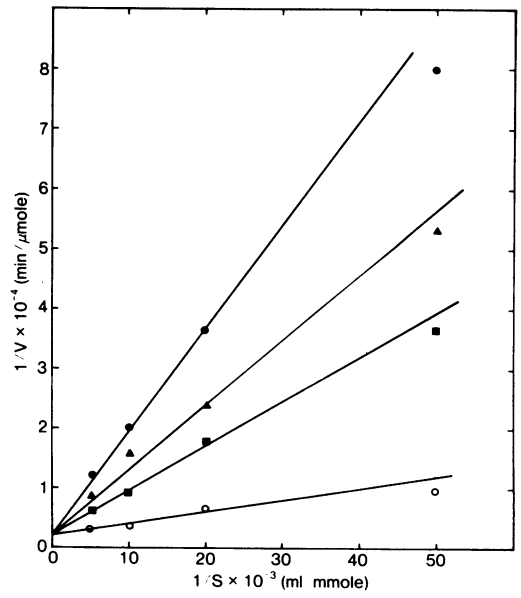

FIG. 1. Initial rate measurements of the conversion of cyclic AMP to 5'-AMP by the high K_m cyclic AMP phosphodiesterase activity in the presence of 20–200 μM cyclic AMP and 0 (○), 200 (■), 400 (▲), or 600 (●) μM 3-methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]pyrimidine (*10*). Experimental conditions are given in *Materials and Methods* and calculated values of K_m and K_I are given in Table 2.

potent inhibition of the low K_m phosphodiesterase activity in these initial studies prompted an examination of the effect of much higher concentrations of inhibitor on initial velocity. Compounds *1*, *5*, *9*, and *10* did not inhibit the phosphodiesterase when utilized at a concentration of 6 μM, in the presence of 0.2 μM cyclic AMP. However, an additional 5-fold increase in the concentration of *9* (to 30 μM) did cause some inhibition of cyclic AMP breakdown and the apparent K_I for *9* (corresponding to an apparent K_m of 0.2 μM) was approximately 33 μM.

RESULTS AND DISCUSSION

Initial experiments with a number of cytokinins and structurally related compounds in four different heterocyclic series, utilizing enzyme and substrate levels of 45.9 μg and 0.2 mM, respectively, and inhibitor concentrations of 2 mM, indicated all of the compounds to be inhibitory to the high K_m phosphodiesterase activity. The heterocycles adenosine, 7-amino-3-methylpyrazolo[4,3-*d*]pyrimidine (*7*), and formycin cyclic 3':5'-monophosphate (*11*), however, were not, suggesting that the *N*-substituent was required for activity of the potential inhibitors in this assay, as is also the case for the expression of activity of these compounds in the tobacco bioassay and assays involving mammalian cells. Six representative compounds were, therefore, chosen for kinetic studies, including the cytokinins N^6-(Δ^2-isopentenyl)adenine (*1*) and N^6-(Δ^2-isopentenyl)adenosine (*5*), and the related compounds 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (*9*), 3-methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]pyrimidine (*10*), 4-cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (*12*) and 4-(3-methyl-2-butenylamino)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (*22*). The six tested compounds were found to be competitive inhibitors of the high K_m cyclic AMP phosphodiesterase activity. As shown in Table 2, cytokinins *1*

TABLE 2. *K_I values for cyclic AMP phosphodiesterase inhibitors**

Compound	K_I (μM)
N^6-(Δ^2-isopentenyl)adenine (*1*)	129
N^6-(Δ^2-isopentenyl)adenosine (*5*)	109
3-Methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]-pyrimidine (*9*)	35
3-Methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]-pyrimidine (*10*)	48
4-Cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]-pyrimidine (*12*)	83
4-(3-Methyl-2-butenylamino)-pyrazolo[3,4-*d*]-pyrimidine-3-carboxamide (*22*)	19

* K_m = 70 μM.

and *5* were found to have K_I's of 129 and 109 μM, relative to a measured K_m of 70 μM. Compounds *9*, *10*, *12*, and *22* were better competitive inhibitors with K_I's of 35, 48, 83, and 19 μM, respectively, consistent with the results observed in the initial experiments at single concentrations of substrate and inhibitor (Table 1).

The establishment of cytokinins as competitive inhibitors of the high K_m cyclic AMP phosphodiesterase activity from beef heart supports prior observations implicating exogenously added cytokinins in cyclic AMP metabolism (13, 18). The substantial activity in this assay of many of the heterocycles structurally related to cytokinins is consistent with their behavior in phytohemagglutinin-transformed human lymphocytes and mouse fibroblasts (S. M. Hecht and R. B. Frye, in preparation), in which they promote the same effects as the cytokinins.† It is important to note that the cytokinins and related heterocycles differ from many other reported inhibitors of this high K_m phosphodiesterase activity (35–37) in that they are neither very potent inhibitors of the low K_m phosphodiesterase activity nor cyclic nucleotide derivatives themselves.

The fate of the low K_m phosphodiesterase activity in the presence of the inhibitors is of interest because this type of activity may be associated with basal metabolism (38–40). Although none of the six compounds (*1*, *5*, *9*, *10*, *12*, or *22*) was at all inhibitory to the low K_m phosphodiesterase activity (apparent K_m measured as approximately 0.2 μM) at concentrations comparable to the K_m, at least one compound (*9*) was found to be an inhibitor at very high concentrations and had similar apparent K_I's for both high and low K_m phosphodiesterase activities. If this were a general phenomenon for these compounds, the simultaneous inhibition of both phosphodiesterase activities might provide a mechanism for a substantial increase in the intracellular cyclic AMP concentration in response to added cytokinin without necessitating a change in the rate of synthesis of the cyclic nucleotide or producing any physiological effect at lower concentration of added inhibitor.

It should be noted that while the inhibition of cyclic AMP phosphodiesterase activity from beef heart with cytokinins and related compounds may be effected at concentration levels similar to those at which physiological responses can be elicited from certain intact cells‡ (about 10 μM) (ref. 13, S. M. Hecht and R. B. Frye, in preparation), other phenomena, such as the growth of cytokinin-dependent tobacco tissue, occur in response to much lower concentrations of exogenously added compound (about $10^{-2} - 10^{-3}$ μM) and may be unrelated to the effects described here.

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† Many of these structural analogs of the cytokinins (e.g., *9* and *10*), which bind competitively to the cyclic AMP phosphodiesterase and mimic the effects of the cytokinins in human lymphocytes and mouse fibroblasts (S. M. Hecht and R. B. Frye, in preparation), are also anticytokinins in the tobacco bioassay. Although their mode of action in relation to the cytokinins cannot be exactly the same in the plant and mammalian assays, it is gratifying to note that the anticytokinins, which were postulated to function in the tobacco bioassay by binding to the same cellular "receptors" as the cytokinins (15, 16), actually can act as structural analogs of the cytokinins in certain mammalian systems.

‡ It is thought that a rise in the intracellular cyclic AMP levels in certain cells in response to external stimuli may be accompanied by an influx of Ca^{2+}, the latter of which may serve to regulate accumulation of the cyclic nucleotide by stimulating a Ca^{2+}-dependent cyclic AMP phosphodiesterase activity (see, e.g., refs. 41 and 42). Although the generality of the cyclic AMP phosphodiesterase inhibition noted here is uncertain, in the sense that the observed inhibition would also increase intracellular cyclic AMP concentrations if operative at the cellular level in species containing Ca^{2+}-dependent cyclic AMP phosphodiesterase activities, the results presented here may also bear relevance to the reported regulation of Ca^{2+} binding and transport by cytokinins (43, 44).

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