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Inhibition of Dopamine-β-Hydroxylase by Cytokinins

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Abstract. The activity of cell-free preparations of dopamine-β-hydroxylase from a mammalian source was inhibited by a number of N^6-substituted adenine derivatives that are hormonally active as cytokinins in plant systems. The synthetic cytokinin N^6-cyclohexylmethyladenine exhibited inhibitory activity equivalent to that of 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTTU), a compound known to be a potent inhibitor of dopamine-β-hydroxylase activity. PTTU itself was found to exhibit cytokinin activity in the tobacco callus bioassay and to inhibit the activity of the plant enzyme, cytokinin oxidase. The possible significance of these observations is discussed in relation to known effects of cytokinins on phenethylamine metabolism.

Dopamine-β-hydroxylase plays an important role in the metabolism of catecholamines in the animal systems where these compounds function as hormones and neurotransmitters (Ljones and Skotland 1984). The enzyme catalyzes a mixed function oxidase reaction in which dopamine (3,4-dihydroxyphenylethylamine) is oxidized to norepinephrine (3,4-dihydroxyphenylethanolamine) with ascorbic acid serving as an electron donor. Other phenethylamines may serve as alternative substrates, and the oxidation of tyramine (4-hydroxyphenylethylamine) to octopamine (4-hydroxyphenylethanolamine) provides the basis of a convenient assay for the enzyme (Wallace et al. 1973). Dopamine-β-hydroxylase has yet to be purified from a plant source, but homogenates of banana tissues have been reported to catalyze the conversion of dopamine to norepinephrine via a reaction that is stimulated by ascorbic acid (Smith and Kirshner 1960). Dopamine and norepinephrine are known to be widely distributed in plant systems (Smith 1977), and the in vivo synthesis of these compounds by a pathway from tyrosine has been observed in *Portulaca* callus tissue (Endress et al. 1984).

The dopamine-β-hydroxylase purified from mammalian systems is sensitive to inhibition by low concentrations of the compound 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTTU) (Johnson et al. 1969, 1970). The structure of PTTU is very similar to that of certain synthetic phenylurea and phenylthiourea derivatives that exhibit biological activity equivalent to that of cytokinins (Bruce and Zwar 1966, Mok et al. 1982). These cytokinin agonists also inhibit degradation of the naturally occurring cytokinins N^6-(Δ^2-isopentenyl)adenine (i^6Ade) and zeatin by the enzyme cytokinin oxidase (Chatfield and Armstrong 1986, Laloue and Fox 1989). The latter enzyme is widely distributed in plant tissues and catalyzes the oxidative cleavage of cytokinins bearing an unsaturated isoprenoid sidechain, forming adenine (McGaw and Horgan 1983) and an aldehyde corresponding to the N^6-sidechain of the cytokinin (Brownlee et al. 1975).

The effects of cytokinins on the activity of dopamine-β-hydroxylase purified from a mammalian source have been investigated in the present study. N^6-substituted adenine derivatives that are active as cytokinins were found to resemble PTTU in serving as effective inhibitors of dopamine-β-hydroxylase activity in cell-free preparations of the enzyme. PTTU itself exhibited cytokinin activity in the tobacco callus bioassay and inhibited the activity of cell-free preparations of cytokinin oxidase. The possible implications of these results for the regulation of phenethylamine metabolism by cytokinins in plant systems are discussed.

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Materials and Methods

Chemicals

The cytokinins N^6-cyclohexylmethyladenine and N^6-isopentyladenine were synthesized as described by Kim and Armstrong (1986) and by Leonard et al. (1968), respectively. N^6-(Δ^2-isopentenyl)adenine-2,3-[^{3}H]) (i^6Ade-[^{3}H] was synthesized as described by Chatfield and Armstrong (1987). Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) was a gift from NOR-AM Chemical Co, Wilmington, DE, USA. All other organic compounds used were purchased from Sigma, St. Louis, MO, USA.

Enzymes

Dopamine-β-hydroxylase (from bovine adrenals, 2.7 U/mg protein) and catalase (from bovine liver, 14,000 U/mg protein) were purchased from Sigma. Crude preparations of cytokinin oxidase were obtained from callus tissues of *Phaseolus vulgaris* cv Great Northern as described by Chatfield and Armstrong (1986).

Assay of Dopamine-β-Hydroxylase Activity

Dopamine-β-hydroxylase was assayed by the procedure of Wallace et al. (1973), which is based upon the use of tyramine as an alternative substrate for the enzyme. In this procedure, the octopamine formed by the enzymatic oxidation of tyramine is converted to *p*-hydroxybenzaldehyde by chemical oxidation with periodate, and the latter compound is measured spectrophotometrically.

The enzyme assay mixtures contained 200 mM sodium acetate (pH 5), 4 mM tyramine-HCl, 10 mM sodium fumarate, 10 mM ascorbic acid, 8000 U of catalase, and 0.1 U (vendor's estimates) of dopamine-β-hydroxylase in a reaction volume of 2 ml. Compounds tested as inhibitors of dopamine-β-hydroxylase were dissolved in 50% (vol/vol) dimethylformamide and added to the reaction volumes in 10-μl aliquots. (The final dimethylformamide concentration in all reaction mixtures was 0.5%.) The reaction mixtures were incubated for 20 min at 37°C in a shaking water bath. The reactions were stopped by the addition of 0.4 ml of 4 M ammonium hydroxide, followed by 0.8 ml of 2% (wt/vol) sodium periodate. After 4 min at room temperature, excess periodate was reduced by the addition of 0.8 ml of 10% (wt/vol) sodium bisulfite. Absorbance was measured at 330 nm against a blank prepared from reaction mixtures that did not contain dopamine-β-hydroxylase. The octopamine produced in the reaction was estimated from standard curves prepared by measuring the absorbance at 330 nm obtained from known amounts of octopamine carried through the same reaction sequence.

Tobacco Callus Bioassay

The cytokinin-dependent callus cultures of *Nicotiana tabacum* cv Wisconsin 38 were grown on media containing the inorganic nutrients defined by Murashige and Skoog (1962) and the organic substances specified by Linsmaier and Skoog (1965). The latter compounds included: thiamine-HCl (400 μg/L), *myo*-inositol (100 mg/L), sucrose (30 g/L), and indole-3-acetic acid (2 mg/L). Kinetin (0.15 μM) was included in the media used for growth of stock cultures. The pH of the medium was adjusted to 5.6–5.8, and Difco Bacto-agar (10 g/L) was added prior to autoclaving. The medium was dispensed into Erlenmeyer flasks (50 ml medium/125 ml flask), and three pieces of stock callus (weighing approximately 25 mg each, derived from 3-week old cultures) were planted per flask. All callus cultures were grown in the dark at 28°C.

For tests of cytokinin activity, PTTU and kinetin were cold sterilized by dissolving these compounds in dimethylsulfoxide (DMSO) (Schmitz and Skoog 1970). Aliquots (50 μl/50 ml medium) of the resulting DMSO stock solutions were added to autoclaved culture flasks prior to solidification of the cytokinin-free medium. The flasks were inoculated with cytokinin-dependent tobacco callus tissue as described above. The yield of callus tissue (fresh weight per flask) was determined after a 5-week culture period. All fresh weight values reported here are the average of two experiments, using four replicate flasks for each treatment of each experiment.

Assay of Cytokinin Oxidase Activity

Cytokinin oxidase activity was assayed as described by Kaminek and Armstrong (1990) using i^6Ade-[^{3}H] as a substrate. The enzyme incubation mixtures contained 100 mM imidazole buffer (pH 7.0), 0.01 mM i^6Ade-[^{3}H] (0.05 μCi, specific activity 100 μCi/μmol), and enzyme equivalent to 100 mg (fresh weight) of *Phaseolus vulgaris* cv Great Northern callus tissue in a total assay volume of 50 μl. PTTU and other test compounds were dissolved in DMSO and incorporated into the standard incubation mixture to give a final DMSO concentration of 1% (vol/vol). The assay volumes were incubated at 37°C for 30 min. The reactions were terminated by the addition of 100 μl of cold 95% (vol/vol) ethanol containing unlabeled adenine (Ade) and i^6Ade (0.75 mM each). The precipitated proteins were removed by centrifugation (minifuge), and 100-μl samples of the assay supernatants were chromatographed on SiC_{18} TLC plates (5-cm wide) over a distance of 5 cm using 40% (vol/vol) ethanol containing 100 mM Na_4EDTA as the developing solvent. The locations of the i^6Ade and Ade standards were determined by inspection under UV light, and the corresponding TLC zones were removed and counted in 5 ml of Beckman Ready-Protein scintillation fluid in a Beckman Model 1801 Scintillation Counter.

Results

The dopamine-β-hydroxylase inhibitor PTTU exhibited weak cytokinin activity when tested in the tobacco callus bioassay (Fig. 1). The PTTU concentration (10 μM) required to obtain optimal growth of the cytokinin-dependent tobacco callus tissue was roughly 100-fold greater than the concentration of kinetin required for the same response. PTTU also inhibited the degradation of i^6Ade-[^{3}H] by cell-free preparations of cytokinin oxidase, although less effectively than the cytokinin-active phenylurea derivative thidiazuron (Table 1). The activity of cytokinin oxidase, as measured using 10 μM i^6Ade-[^{3}H] as a substrate, was inhibited 50% by a PTTU concentration of 100 μM.

The effects of cytokinin-active adenine derivatives on the activity of cell-free preparations of dopamine-β-hydroxylase were examined and compared with the effects of PTTU using a commercial enzyme preparation purified from bovine adrenals.

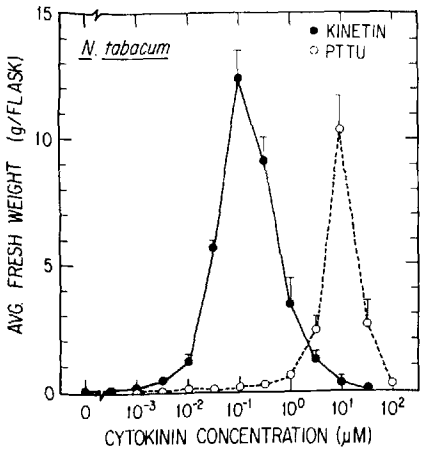

Fig. 1. Cytokinin activity of 1-phenyl-3-(2-thiazolyl)-1-thiourea (PTTU) in the tobacco callus bioassay. The callus tissue was harvested and weighed after a 5-week culture period in the dark at 28°C. All fresh weight values reported are the average of two experiments using four replicate 125-ml flasks (each containing 50 ml medium) for each treatment of each experiment.

Table 1. Comparison of the effects of PTTU and thidiazuron on the degradation of i⁶Ade-[³H] by a cell-free preparation of cytokinin oxidase from *Phaseolus vulgaris* cv Great Northern callus tissue.[a]

Test compound	Test compound concentration 10 μM	100 μM
	Cytokinin oxidase activity (% control)[b]	
PTTU (1-phenyl-3-(2-thiazolyl)-2-thiourea)	80 ± 6	49 ± 5
Thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea)	59 ± 3	25 ± 2

[a] Cytokinin oxidase activity was assayed as described in Materials and Methods.
[b] The activity of the controls incubated in the absence of any of the test compounds was equal to 0.1 nmol i⁶Ade degraded per assay tube per 30-min incubation period. All values are the average of two experiments.

Table 2. Effects of cytokinins on the activity of a cell-free preparation of dopamine-β-hydroxylase.[a]

Cytokinin	Cytokinin concentration 30 μM	100 μM
	Enzyme activity (% control)[b]	
Adenine control	102 ± 3	94 ± 7
N^6-Benzyladenine	42 ± 2	22 ± 2
N^6-Cyclohexylmethyladenine	24 ± 1	9 ± 1
N^6-Furfuryladenine (kinetin)	85 ± 1	65 ± 3
N^6-Hexyladenine	43 ± 4	29 ± 2
N^6-(4-hydroxy-3-methyl-2-butenyl)adenine (zeatin)	96 ± 6	103 ± 10
N^6-(Δ^2-Isopentenyl)adenine	54 ± 1	35 ± 3
N^6-Isopentyladenine	52 ± 2	30 ± 2
N^6-Benzyladenosine	98 ± 2	102 ± 2
N^6-(Δ^2-Isopentenyl)adenosine	107 ± 1	100 ± 2

[a] Dopamine-β-hydroxylase activity was assayed as described in Materials and Methods.
[b] The activity of controls incubated in the absence of any inhibitor was equal to 131 nmol of octopamine formed per hour per 2-ml assay volume. All values are the average of three experiments.

The results of these tests are shown in Table 2. As expected, PTTU strongly inhibited the activity of dopamine-β-hydroxylase. Adenine itself did not affect the activity of the enzyme, but most of the cytokinin-active adenine derivatives tested inhibited the activity of dopamine-β-hydroxylase. Zeatin and the two ribonucleosides, N^6-benzyladenosine and N^6-(Δ^2-isopentenyl)adenosine, were the only cytokinin-active compounds tested that failed to inhibit the enzyme. The cytokinin that was most active in inhibiting dopamine-β-hydroxylase was N^6-cyclohexylmethyladenine (the saturated analog of N^6-benzyladenine). As shown in Table 3, N^6-cyclohexylmethyladenine was as effective as PTTU in inhibiting the activity of the enzyme. Dopamine-β-hydroxylase activity was inhibited approximately 50% by a 10 μM concentration of either compound. The cytokinin activity of N^6-cyclohexylmethyladenine has not been widely tested, but it was only slightly less active than N^6-benzyladenine in promoting the growth of cytokinin-dependent *Phaseolus lunatus* callus cultures (Kim and Armstrong 1986).

The structural specificity of the inhibition of dopamine-β-hydroxylase by cytokinin-active compounds was further examined by comparing the cytokinin i⁶Ade with the corresponding N^3-substituted adenine derivative. N^3-(Δ^2-isopentenyl)adenine (also known as triacanthine) is not active as a cytokinin (Rogozinska et al. 1964). As shown in Table 4, i⁶Ade at a concentration of 30 μM inhibited dopamine-β-hydroxylase activity by about 50%, but triacanthine at concentrations up to 100 μM had no effect on the activity of the enzyme.

To test the possibility that dopamine-β-hydroxylase might exhibit cytokinin oxidase activity, the enzyme was incubated with i⁶Ade-[³H] using reaction conditions similar to those normally employed in assays for cytokinin oxidase activity, as as well as the conditions employed in dopamine-β-hydroxylase assays. No reaction products derived from i⁶Ade-[³H] could be detected using either set

Table 3. Comparison of the inhibitory effects of PTTU and N^6-cyclohexylmethyladenine on the activity of cell-free preparations of dopamine-β-hydroxylase.[a]

	Inhibitor concentration		
	3 μM	10 μM	30 μM
Inhibitor	Enzyme activity (% control)[b]		
PTTU (1-Phenyl-3-(2-thiazolyl)-1-thiourea)	85 ± 7	54 ± 1	26 ± 3
N^6-Cyclohexylmethyladenine	86 ± 2	52 ± 1	27 ± 4

[a] Dopamine-β-hydroxylase activity was assayed as described in Materials and Methods.
[b] The activity of controls incubated in the absence of any inhibitor was equal to 98 nmol of octopamine formed per hour per 2-ml assay volume. All values are the average of three experiments.

Table 4. Comparison of the inhibitory effects of i^6Ade and N^3-(Δ^2-isopentenyl)adenine on the activity of cell-free preparations of dopamine-β-hydroxylase.[a]

	Inhibitor concentration		
	10 μM	30 μM	100 μM
Inhibitor	Enzyme activity (% control)[b]		
N^6-(Δ^2-Isopentenyl)-adenine	80 ± 3	56 ± 2	32 ± 2
N^3-(Δ^2-Isopentenyl)-adenine (triacanthine)	102 ± 2	98 ± 2	99 ± 2

[a] Dopamine-β-hydroxylase activity was assayed as described in Materials and Methods.
[b] The activity of controls incubated in the absence of any inhibitor was equal to 68 nmol of octopamine formed per hour per 2-ml assay volume. All values are the average of three experiments.

of reaction conditions. In an analogous experiment with the cytokinin oxidase from *Phaseolus* callus tissues, the addition of tyramine (at concentrations up to 1 mM) to reaction mixtures containing cytokinin oxidase did not inhibit the degradation of i^6Ade-[^{3}H] by the plant enzyme (data not shown). Tyramine at 10 mM did inhibit the degradation of i^6Ade-[^{3}H] by about 20%, but it is difficult to be certain of the specificity of the effect of such a high concentration (1000-fold greater than the cytokinin substrate).

Discussion

The ability of a number of cytokinin-active adenine derivatives to inhibit dopamine-β-hydroxylase activity in cell-free preparations of the enzyme from a mammalian source has been demonstrated in the present study. Among the compounds tested, the most effective inhibitor of dopamine-β-hydroxylase was N^6-cyclohexylmethyladenine, which exhibited inhibitory activity equivalent to that of PTTU (a compound known to be a potent inhibitor of the enzyme and shown here to be weakly active as a cytokinin in the tobacco bioassay). Zeatin and two cytokinin nucleosides (compounds that contain hydrophillic groups) were the only cytokinins that failed to inhibit dopamine-β-hydroxylase activity, suggesting that a hydrophobic site is involved in the inhibition of dopamine-β-hydroxylase activity by cytokinins. Inhibitors of dopamine-β-hydroxylase include a number of compounds that interact with copper residues at the active site of the enzyme (Ljones and Skotland 1984), and the ability of cytokinin-active adenine derivatives to form complexes with copper (Miller 1985) may be important in this regard. Although it appears unlikely that the particular pharmacological property of cytokinins reported here constitutes a hazard, it may be worth noting that these compounds are not always physiologically inert in animal systems (Visscher 1982).

To our knowledge, there has been only one previous report of an inhibitory effect of adenine derivatives on dopamine-β-hydroxylase. Fujii et al. (1979) reported that a number of N^3-substituted adenine derivatives inhibited the activity of cell-free preparations of dopamine-β-hydroxylase. The only N^3-derivative that was readily available for testing in the present study was triacanthine, N^3-(Δ^2-isopentenyl)adenine. This compound failed to inhibit dopamine-β-hydroxylase activity at concentrations threefold higher than the concentration at which i^6Ade (the corresponding cytokinin) gave 50% inhibition of enzyme activity. Triacanthine was not among the compounds tested by Fujii et al. (1979), but this result would not have been anticipated on the basis of the data from their earlier study. Although N^3-substituted adenine derivatives are known to be susceptible to rearrangement to the corresponding N^6-substituted compounds (Skoog and Leonard 1968), the reason for the apparent discrepancy between the results reported here and those obtained in the earlier work is not certain.

The ability of cytokinins to inhibit dopamine-β-hydroxylase activity in a cell-free system raises the possibility that in vivo interactions of cytokinins with dopamine-β-hydroxylase or a related enzyme may contribute to the biological properties of these hormones. Regulatory effects of cytokinins on phenethylamine metabolism have been reported in a number of plant systems. The ability of cytokinins to stimulate betacyanin synthesis in *Amaranthus*

seedlings is a frequently studied example of an effect of cytokinins on phenethylamine metabolism (Biddington and Thomas 1973, Elliott 1983). Synthesis of the alkaloid hordenine (N,N-dimethyltyramine) has been reported to be promoted by cytokinin treatment of germinating barley embryos (Steinhart et al. 1964), and the production of another phenethylamine-derived alkaloid, sanguinarine, has been observed to be influenced by cytokinin levels in cell suspension cultures of *Papaver bracteatum* (Cline and Coscia 1988). More recently, Christou and Barton (1989) have reported that increased cytokinin levels counteract the growth inhibition and dark pigmentation that results from exposure of tobacco callus tissues to phenethylamine compounds. Although these investigators have suggested that the phenethylamine compounds may be acting as cytokinin antagonists in the tobacco callus system, it appears more likely that cytokinins are affecting some aspect of phenethylamine metabolism in the callus tissue.

Clearly, any hypotheses concerning in vivo effects of cytokinins on dopamine-β-hydroxylase activity in plant tissues that are based upon results with a mammalian enzyme are only speculative. Nonetheless, the observations reported here may be useful in suggesting directions for further investigations of the role of cytokinins in regulating phenethylamine metabolism in plant systems.

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