Effects of Dicyclohexylamine on the Changes in Spermine Levels in Hiproly Barley Callus after Auxin Withdrawal

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Changes of the contents and biosynthetic rates of polyamines and enzyme activities involved in polyamine synthesis as affected by dicyclohexylamine (DCHA) were examined in Hiproly barley callus. The incorporation of [1,4-¹⁴C]putrescine into spermidine increased rapidly after auxin withdrawal, while the incorporation of [1,4-¹⁴C]putrescine or [diaminobutane 1,4-¹⁴C]spermidine into spermine increased gently. The addition of DCHA suppressed the incorporation of [3,4-¹⁴C]methionine or [1,4-¹⁴C]putrescine into spermidine. In contrast, the incorporation of [3,4-¹⁴C]methionine or [diaminobutane 1,4-¹⁴C]spermidine into spermine increased in the callus by treatment of DCHA. The contents of spermine in the callus increased about 2-fold after treatment with 10 mM DCHA. The activity of spermidine synthase in the callus after auxin withdrawal increased rapidly. Inhibition of spermidine synthase by DCHA stimulated spermine synthesis and the activity of spermine synthase was elevated about 10-fold after treatment with 10 mM DCHA. However, DCHA had significant effects on the activity of *S*-adenosylmethionine decarboxylase and the levels of *S*-methyladenosylhomocysteamine. *S*-Methyladenosylhomocysteamine contents increased about 20-fold. The changes in the spermine levels paralleled the changes in the *S*-methyladenosylhomocysteamine contents. These results suggested that in Hiproly barley callus the biosynthesis of spermine were regulated by the levels of aminopropyl donor and that the availability of *S*-methyladenosylhomocysteamine may be rate-limiting for the synthesis of spermine.

In higher plants polyamines are involved in important physiological phenomena such as cell division, growth, and senescence.^{1,2)} The mechanism of polyamine functions may be due to interaction with proteins and nucleic acids.³⁾

In cultured plant cells and tissues, recent work also indicates a role for polyamines in organ differentiation. For example, elongation growth is correlated with high putrescine, and dividing cells are rich in spermidine and spermine.⁴⁾ Katoh *et al.*⁵⁾ found that when Hiproly barley callus was placed on Murashige and Skoog's medium without 2,4-D, adventitious roots formed in the callus. And they showed that the levels of spermidine and spermine in the callus increased during differentiation.⁵⁾ Moreover, the addition of spermidine or spermine to the medium without 2,4-D stimulated the root formation on the callus.⁵⁾

Organ formation of Hiproly barley callus was correlated with high spermidine and spermine.

Spermidine and spermine are synthesized from putrescine by the addition of successive aminopropyl moieties derived from *S*-methyladenosylhomocysteamine.⁶⁾ *S*-Methyladenosylhomocysteamine as aminopropyl donor is a key intermediate in the biosynthesis of spermidine and spermine. Thus, changes in spermidine and spermine biosynthesis may be closely related to the levels of *S*-methyladenosylhomocysteamine or the activities of both polyamine synthases.

In recent work, dicyclohexylamine (DCHA) has been shown to inhibit spermidine accumulation in bacteria, animal, and plant cells.^{7~11)} DCHA is a competitive inhibitor of spermidine synthase. Moreover, DCHA inhibits growth in *Helianthus* tuber explants¹²⁾ and

carrot embryogenesis.¹³⁾

The results in the literature cited above indicated that high spermidine or spermine biosynthesis affected the organ formation in cultured plant cells and tissues. We investigated how spermidine and spermine biosynthesis were regulated in the callus after auxin withdrawal, and DCHA was used to regulate the spermidine and spermine biosynthesis on root formation of the callus. This paper describes the effects of DCHA on the levels of spermidine and spermine in Hiproly barley callus after auxin withdrawal.

Materials and Methods

Materials and chemicals. Hiproly barley (*Hordeum disticum* L. var. Hiproly) was used in the experiments. Spermidine and spermine were obtained from Aldrich Chemical Co., *S*-adenosylmethionine (SAM) and dicyclohexylamine sulfate (DCHA) from Sigma Chemicals, and 2,4-dichlorophenoxyacetic acid (2,4-D) from the Tokyo Kasei Co. Synthetic *S*-methyladenosylhomocysteamine (dSAM) was a generous gift from Dr. K. Samejima, Fac. Pharm., Johsai Univ., Saitama, Japan. Aquazol-2 and L-[¹⁴C-carboxyl]-*S*-adenosylmethionine (59.8 mCi/mmol) were obtained from New England Nuclear, and L-[3,4-¹⁴C]methionine (45~60 mCi/mmol), [1,4-¹⁴C]putrescine (90~120 mCi/mmol), and [diaminobutane 1,4-¹⁴C] spermidine (90~120 mCi/mmol) from Commissariat a l'Energie Atomique. Murashige and Skoog's medium was obtained from Flow Laboratories.

Induction and proliferation of callus from Hiproly barley. Hiproly barley callus was induced and grown on Murashige and Skoog's medium containing 2 mg/l of 2,4-D. Calli were subcultured every 8 weeks. Calli were kept for at least two passages on Murashige and Skoog's medium containing 2 mg/l of 2,4-D before experiments, and transferred to Murashige and Skoog's medium containing various concentrations of DCHA. Hiproly barley calli at different stages during cultivation were placed in Erlenmyer flasks containing a basal medium with L-[3,4-¹⁴C]methionine (1 μ Ci), [1,4-¹⁴C]putrescine (0.5 μ Ci), or [diaminobutane 1,4-¹⁴C]spermidine (0.5 μ Ci). After incubation for 24 hr, the calli were taken out, rinsed twice with distilled water, and stored at -20°C.

Polyamine extractions. Calli (100 mg) were homogenized in 1 ml of chilled 5% perchloric acid with a glass homogenizer. This homogenate was centrifuged at 20,000 $\times g$ for 20 min, and the supernatant containing the free polyamines was stored at -20°C.

Benzoylation and high performance liquid chromatographic analysis of polyamines. The polyamine concentration in the extract was measured by a method modified from that of Redmond and Tseng.¹⁴⁾ The acid-extracted free polyamine solution (1.0 ml) was mixed with 2 ml of 2 N sodium hydroxide in a 50-ml glass centrifuge tube to which was added 10 μ l of benzoyl chloride. The mixture was shaken with a Vortex mixer for 45 sec, and left for 20 min. Two ml of saturated sodium chloride solution was added and the solution was extracted with 4 ml diethyl ether. After centrifugation at 1,500 $\times g$ for 15 min, two ml of the upper ether phase was taken out and evaporated under a stream of nitrogen. The residue was dissolved in 100 μ l of methanol, and 10 μ l of the solution was used for high performance liquid chromatography (HPLC).

HPLC of benzoyl polyamines was run by a Waters model 510 with an ultraviolet detector on a 8NVC₁₈ column (Radial Pak, Waters Associates) at 254 nm. A linear gradient of acetonitrile from 36.5% to 100% in 1% acetic acid over a period of 20 min was used as a mobile phase at a flow rate of 3 ml per min.

Labeled polyamines were extracted with 5% perchloric acid, benzoylated with benzoyl chloride, and fractionated by HPLC as described above. One-ml fractions eluted from HPLC were collected in scintillation vials. Ten ml of Aquazol-2 was added to each vial and the radioactivity was measured with a liquid scintillation counter. Specific incorporation of ¹⁴C activity into polyamines was defined as dpm per one nmol of polyamines per 24 hr.

Extraction and high performance liquid chromatographic analysis of *S*-methyladenosylhomocysteamine. *S*-Methyladenosylhomocysteamine (decarboxylated *S*-adenosylmethionine, dSAM) were measured by a method modified from that of Greenberg and Cohen.¹⁵⁾ Calli (1.0 g) were homogenized and centrifuged by the same method described in polyamine extractions. The supernatants were combined, the pH was adjusted to about 3 with potassium hydroxide, chilled on ice for 30 min, and centrifuged to remove KClO₄. Precipitates were washed with ice-cold 10 mM hydrochloric acid by centrifugation, and supernatants were combined and put on a column of SP-Sephadex 25 (1 $\times$ 5 cm). The column was prepared in 10 mM hydrochloric acid. After washing with 35 ml of 50 mM hydrochloric acid, dSAM was eluted with 40 ml of 500 mM hydrochloric acid. The eluate was dried *in vacuo* at 32°C and the residue was dissolved in 100 μ l of 10 mM hydrochloric acid, and 20 μ l of the solution was used for HPLC.

HPLC of dSAM was run by the method described in the polyamine assay. Gradient elution with mobile phase A (131 ml of 0.1 M NaH₂PO₄ (pH 2.50) containing 8 mM 1-heptanesulfonic acid and 0.1 mM Na₂EDTA and 1 ml of acetonitrile) and mobile phase B (70 ml of 0.2 M NaH₂PO₄ (pH 3.10) containing 8 mM 1-heptanesulfonic acid and 30 ml of acetonitrile) were used. A linear gradient starting from 20% mobile phase B in mobile phase A, and ending

with 60% B over a period of 20 min at a flow rate of 3 ml per min.

Preparation and assay of enzyme. For preparation of SAMDCase, a callus was homogenized in a glass homogenizer with 1 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 0.1 mM dithiothreitol and 1 mM pyridoxal-5'-phosphate at 4°C. The homogenate was centrifuged at $12,000 \times g$ for 20 min at 0°C. The supernatant fraction was used for the SAMDCase assay.

The SAMDCase was measured by a method modified from that of Montague *et al.*¹⁶⁾ The reaction was started by adding 100 μ l of the crude enzyme to 10 μ l of L-[14 C-carboxyl]-S-adenosylmethionine and 10 μ l of 5 mM pyridoxal-5'-phosphate. The reaction mixture was placed in a Warburg flask. A 7-mm diameter filter paper disc (Whatman No. 1) impregnated with 100 μ l of 2 N potassium hydroxide was used to trap the $^{14}\text{CO}_2$ liberated. After incubation at 37°C for 60 min, the reaction was stopped with 0.5 ml of 1 N sulfuric acid, and incubated for an additional 60 min. The paper discs were taken out, dried, and placed in vials containing 1 ml of distilled water and 10 ml of Aquazol-2. ^{14}C activity was measured with a scintillation counter. The enzyme activity is expressed as $^{14}\text{CO}_2$ nmol per mg of protein per hour.

For the preparation of spermidine synthase and spermine synthase, a callus was homogenized in a glass homogenizer with 1 ml of 1 M triethanolamine sulfate buffer (pH 8.2) at 4°C. The homogenate was centrifuged $12,000 \times g$ for 20 min at 0°C. The supernatant fraction was used for the assay of spermidine synthase and spermine synthase.

The spermidine synthase was measured by the method of Cohen *et al.*⁶⁾ The reaction was started by adding 250 μ l of the crude enzyme to 250 μ l of 0.5 M triethanolamine sulfate buffer (pH 8.2) containing 25 μ M dSAM and 10 μ l of [14 C]putrescine (1 μ Ci). After incubation at 37°C for 1 hr, the reaction was stopped by adding 1 ml of 5% perchloric acid, and centrifuged at $1,500 \times g$ for 5 min. After adding 30 nmol of spermidine to one ml of the supernatant, the [14 C]spermidine produced was benzoylated with benzoyl chloride and fractionated by HPLC as described above. One-ml fractions eluted from HPLC were collected in scintillation vials. Ten ml of Aquazol-2 was added to each vial and the radioactivity was measured by a liquid scintillation counter. The enzyme activity is expressed as dpm of ^{14}C per mg of protein per hour.

Spermine synthase was measured by the method described for the assay of spermidine synthase except [diaminobutane 1,4- ^{14}C]spermidine was used in place of [^{14}C]putrescine.

Enzyme protein was measured by the method of Lowry *et al.*¹⁷⁾

Results

Changes in the content of dSAM and the incorporation of [1,4- ^{14}C] putrescine or [diaminobutane 1,4- ^{14}C]spermidine into spermine and/or spermine in Hiproly barley callus after auxin withdrawal

In Hiproly barley callus after auxin withdrawal, the change in the incorporation of [1,4- ^{14}C]putrescine into polyamines is shown in Fig. 1. When 2,4-D was removed from the callus, specific incorporation of ^{14}C into spermidine in the callus increased greatly, while ^{14}C activity of spermine increased gently.

Figure 2 shows changes in the incorporation of [diaminobutane 1,4- ^{14}C] spermidine into spermine in the callus after auxin withdrawal. The specific incorporation of ^{14}C into spermine increased gently. And the content of dSAM increased gently in the callus after auxin withdrawal (Fig. 2).

Hiproly barley callus placed on Murashige and Skoog's medium without 2,4-D formed

Fig. 1. Incorporation of [1,4- ^{14}C]Putrescine into Spermidine and Spermine in Hiproly Barley Callus after Auxin Withdrawal.

Calli at various stages were incubated for 24 hr in the presence of [1,4- ^{14}C]putrescine (0.5 μ Ci), and ^{14}C activities of polyamines were measured. O—O, spermidine; ●—●, spermine.

Fig. 2. Incorporation of [Diaminobutane 1,4-¹⁴C]Spermidine into Spermine and Change in the Content of Decarboxylated S-Adenosylmethionine (dSAM) in Hiproly Barley Callus after Auxin Withdrawal.

Calli at various stages were incubated for 24 hr in the presence of [diaminobutane 1,4-¹⁴C]spermidine (0.5 μ Ci), and ¹⁴C activity of spermine was measured. ○—○, spermine; ●—●, dSAM.

adventitious roots by the 30th day of cultivation. Specific incorporation of [1,4-¹⁴C]putrescine or [diaminobutane 1,4-¹⁴C]-spermidine into spermidine and/or spermine increased, along with development of root formation. The dSAM production also increased in the callus with root formation.

Effects of DCHA on changes in the levels of polyamines in Hiproly barley callus

This experiment was designed to observe the effects of DCHA on changes in the levels of polyamines in Hiproly barley callus after 2,4-D withdrawal.

Figure 3 indicates a strong inhibition of the spermidine synthesis at 10 mM DCHA. On the 30th day after cultivation, levels of spermidine were reduced to about 30% of the control. On the other hand, spermine synthesis was greatly enhanced by DCHA. Levels of spermine in the callus increased about 2-fold after treatment with 10 mM DCHA, as compared with the control, on the 30th day after cultivation.

Fig. 3. Effects of Dicyclohexylamine (DCHA) on Spermidine and Spermine Levels of Hiproly Barley Callus. ○—○, control; △—△, 1 mM DCHA; ▲—▲, 5 mM DCHA; ■—■, 10 mM DCHA.

Effects of DCHA on the incorporation of L-[3,4-¹⁴C]methionine into spermidine and spermine

The effects of DCHA on the incorporation of L-[3,4-¹⁴C]methionine into polyamines in the callus are shown in Table I. The rates of the incorporation of L-[3,4-¹⁴C]methionine into spermine increased greatly when the callus was transferred to the medium containing DCHA. On the 30th day after cultivation, specific incorporation of ¹⁴C activity into spermine had increased about 6-fold in the presence of 10 mM DCHA, compared with the control, but DCHA strongly inhibited the incorporation of L-[3,4-¹⁴C]methionine into spermidine. On the 30th day after cultivation specific incorporation of ¹⁴C activity into spermidine in the callus treated with 10 mM DCHA had decreased to about 14% of the control.

Table I. EFFECTS OF DICYCLOHEXYLAMINE (DCHA) ON THE INCORPORATION OF L-[3,4- 14 C]METHIONINE INTO SPERMIDINE (Spd) AND SPERMINE (Spm) OF HIPROLY BARLEY CALLUS

Chemical	Concentration (mM)	14 C Activity			
		14th day		30th day	
		Spd (dpm/nmol)	Spm (dpm/nmol polyamine/24 hr)	Spd (dpm/nmol)	Spm (dpm/nmol polyamine/24 hr)
Control	—	57.8	30.0	79.3	32.1
DCHA	1.0	36.5	52.8	16.4	69.9
	5.0	24.0	67.0	15.8	74.0
	10.0	19.3	110.1	11.0	176.8

Calli were incubated for 24 hr in the presence of the indicated concentration of DCHA and L-[3,4- 14 C]-methionine (1.0 μ Ci), and 14 C activities of spermidine and spermine were measured.

Table II. EFFECTS OF DICYCLOHEXYLAMINE (DCHA) ON THE INCORPORATION OF [1,4- 14 C]PUTRESCINE INTO SPERMIDINE (Spd) AND THE ACTIVITY OF SPERMIDINE SYNTHASE IN HIPROLY BARLEY CALLUS

Sample	14 C Activity (dpm/nmol spd/24 hr)	Spd synthase (dpm/mg protein/hr)
14th day		
Control	2349.0	166.5
1.0 mM DCHA	1352.5	138.5
5.0 mM DCHA	962.5	108.5
10.0 mM DCHA	959.0	103.5
30th day		
Control	3580.5	325.9
1.0 mM DCHA	596.6	111.0
5.0 mM DCHA	581.3	102.8
10.0 mM DCHA	512.5	94.7

Calli were incubated for 24 hr in the presence of the indicated concentration of DCHA and [1,4- 14 C]putrescine (0.5 μ Ci) and 14 C activity of spermidine was measured. Enzyme activity was measured as described in **Materials and Methods**.

Effects of DCHA on the incorporation of [1,4- 14 C]putrescine into spermidine and the activity of spermidine synthase

The rates of the incorporation of [1,4- 14 C]putrescine into spermidine were decreased greatly by the addition of DCHA. On the 14th and 30th day after cultivation, specific incorporation of 14 C activity into spermidine

Table III. EFFECTS OF DICYCLOHEXYLAMINE (DCHA) ON THE INCORPORATION OF [DIAMINOBUTANE 1,4- 14 C]-SPERMIDINE INTO SPERMINE (Spm) AND THE ACTIVITY OF SPERMINE SYNTHASE IN HIPROLY BARLEY CALLUS

Sample	14 C Activity (dpm/nmol spm/24 hr)	Spm synthase (dpm/mg protein/hr)
14th day		
Control	134.4	23.3
1.0 mM DCHA	1120.0	60.8
5.0 mM DCHA	3353.1	138.0
10.0 mM DCHA	4053.9	156.8
30th day		
Control	217.0	31.0
1.0 mM DCHA	3530.5	120.0
5.0 mM DCHA	4147.5	140.5
10.0 mM DCHA	4837.8	394.5

Calli were incubated for 24 hr in the presence of the indicated concentration of DCHA and [diaminobutane 1,4- 14 C]spermidine (0.5 μ Ci) and 14 C activity of spermidine was measured. Enzyme activity was measured as described in **Materials and Methods**.

was reduced about 66 and 86%, respectively, by 10 mM DCHA as compared with the control (Table II).

The activity of spermidine synthase in the callus was inhibited by treatment of DCHA. Compared with the control, the activity of spermidine synthase was reduced by 10 mM DCHA to about 65 and 30% respectively, on the 14th and 30th day after cultivation (Table II).

Effects of DCHA on the incorporation of [diaminobutane 1,4- 14 C]spermidine into spermine and the activity of spermine synthase

DCHA stimulated spermine biosynthesis. On the 14th and 30th day after cultivation, specific incorporation of 14 C activity into spermine was increased about 30 and 20-fold, respectively, by 10 mM DCHA, as compared with the control (Table III). The results in Table III show that the activity of spermine synthase in the callus treated with 10 mM DCHA increase by about 7 and 13-fold, respectively, of the control on the 14th and 30th day after cultivation.

Table IV. EFFECTS OF DICYCLOHEXYLAMINE (DCHA) ON THE DECARBOXYLATED *S*-ADENOSYLMETHIONINE (dSAM) AND THE ACTIVITY OF *S*-ADENOSYLMETHIONINE DECARBOXYLASE (SAMDCase) IN HIPOPLY BARLEY CALLUS

Sample	dSAM (nmol/g callus)	SAMDCase activity (¹⁴ CO ₂ nmol/mg protein/hr)
14th day		
Control	0.29	9.5
1.0 mM DCHA	1.13	17.3
5.0 mM DCHA	4.01	18.8
10.0 mM DCHA	5.20	24.3
30th day		
Control	1.08	21.6
1.0 mM DCHA	9.61	35.9
5.0 mM DCHA	12.22	39.7
10.0 mM DCHA	23.71	43.8

dSAM and SAMDCase activity were measured as described in **Materials and Methods**.

Effects of DCHA on the content of dSAM and the activity of SAMDCase in Hiproly barley callus

Table IV shows the effects of DCHA on the activity of SAMDCase, which is the rate-limiting enzyme in the pathway of polyamine biosynthesis. DCHA had a significant effect on the activity of SAMDCase. The activity of SAMDCase was increased up to 2-fold by 10 mM DCHA as compared with the controls after cultivation.

Moreover, the contents of dSAM in the callus were greatly increased by the addition of DCHA. As compared with the control, the contents of dSAM were increased about 18 and 22-fold, respectively, by 10 mM DCHA on the 14th and 30th day after cultivation.

Discussion

A decrease in spermidine contents induced by DCHA was already confirmed in pine cotyledons¹⁰⁾ and germinating seeds of *Acer*.¹¹⁾ Sindhu and Cohen¹⁸⁾ have recently reported that DCHA was a specific inhibitor of Chinese cabbage spermidine synthase. DCHA effectively inhibits the synthesis of spermidine.

DCHA may be useful in studies of the regulatory mechanisms of biosynthesis of spermidine and spermine.

In Hiproly barley callus after auxin withdrawal, the rates of incorporation of [¹⁴C]putrescine or [diaminobutane 1,4-¹⁴C]-spermidine into spermidine and/or spermine were increased gradually. However, the addition of DCHA suppressed the incorporation of [¹⁴C]putrescine into spermidine and stimulated the incorporation of [diaminobutane 1,4-¹⁴C]-spermidine into spermine. Specific incorporation of ¹⁴C-spermidine into spermine was increased up to 20-fold, and the spermine contents in the callus treated with DCHA increased about 2-fold, compared with that of the control. Our results demonstrate that DCHA inhibition induced a shift from synthesis of spermidine to that of spermine.

In the presence of DCHA, the activity of spermine synthase in Hiproly barley callus was greatly elevated. dSAM serves as a source of an aminopropyl moiety for the biosynthesis of spermidine and spermine.⁶⁾ The activity of spermine synthase will be closely related to the levels of dSAM. Elevated levels of dSAM have been thought to stimulate the spermine biosynthesis in Hiproly barley callus after auxin withdrawal.

Athman-Hongisto¹⁹⁾ reported that SAMDCase was strongly inhibited by spermidine, and that inhibition of spermidine biosynthesis induced an increase in the SAMDCase activity. Spermidine functions as an effective repressor of SAMDCase. On Hiproly barley callus, the addition of DCHA also leads to increases in the activity of SAMDCase.

During inhibition of spermidine synthase in Hiproly barley callus by DCHA, the incorporation of L-[3,4-¹⁴C]methionine into spermine increased about 6-fold, and the activity of SAMDCase was increased up to 2-fold, compared with the control. Moreover, our results show that the levels of dSAM were increased about 20-fold by the treatment with DCHA. These findings obviously indicate that increases in available size of dSAM pool appeared to increase the synthesis of spermine.

Thus, upon inhibition of biosynthesis of spermidine, the increase in dSAM levels occurs rapidly, and in turn, results in an increased synthesis of spermine. The availability of dSAM may be rate-limiting for the synthesis of spermine in the callus of Hiproly barley after auxin withdrawal.

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