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The Stimulation of Arginine Decarboxylase Activity by α-Difluoromethyl Ornithine in Tobacco Suspension Cultured Cells

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To study the compensatory aspect of putrescine biosynthetic enzyme n tobacco suspension cultured cells, we examined the contents of the cellular polyamines and the activities of arginine decarboxylase (ADC, EC 4.1.1.19) and ornithine decarboxylase (ODC, EC 4.1.1.17) in the tobacco suspension cells treated with α-difluoromethyl arginine (DFMA) or α-difluoromethyl ornithine (DFMO). In the untreated cells, the content of the cellular putrescine was decreased during the first 3 hours and then subsequently increased. However, the content of the cellular spermidine and spermine remained constant during the incubation time. While ADC activity increased after 6 hours, ODC activity decreased following the rapid increase until 6 hours. DFMA induced the decrease in the contents of putrescine and spermidine, and the increase in that of spermine. It also caused the inhibition of ADC and ODC activities throughout the incubation time. DFMO produced the stimulation of ADC activity about 2 times of untreated cells and the decrease in the content of putrescine about 50% of them at 12 hour. The application of putrescine or cycloheximide prevented the increase of ADC activity by DFMO but that of actinomycin-D did not show any detectable effect. The stimulation of ADC activity by DFMO in tobacco suspension cultured cells was probably due to the enhancement of *de novo* synthesis for ADC protein, which might be regulated in the translation step by the content of the cellular putrescine.

Keywords: Arginine decarboxylase, DFMA, DFMO, Ornithine decarboxylase, Polyamines

In higher plant, the biosynthesis of putrescine can be catalyzed by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) (Smith, 1985; Tiburcio *et al.*, 1990). The inhibition of ODC activity blocked cell division but did not affect the cell expansion in tobacco suspension cultured cells (Tiburcio *et al.*, 1990). The ADC activity may be stimulated under environmental stresses such as oxygen deficiency (Reggiani *et al.*, 1989), chilling (Guye, *et al.*, 1986), salt stress, low pH, and the deficiencies of K^+ and Mg^{2+} (Smith, 1985). These results implied that ADC and ODC may have different physiological roles although the function of each pathways is not still clear (Smith, 1990).

It has been observed that the contents of the cellular polyamines were not greatly changed by the addition of the inhibitors of polyamine biosynthesis in all cases (Smith, 1990). This is probably explained with the compensatory aspects between ADC and ODC activity (Smith, 1990). In animals, the application of α-difluoromethyl ornithine (DFMO) causes the increase of SAMDC (S-adenosylmethionine decarboxylase) activity (Pegg, 1984). It was reported that spermidine content was regulated by the compensatory aspects of the in tobacco suspension cultured cells (Lee and Park, 1991). Tiburcio and coworkers (1989) reported that the tobacco cell line resistant to DFMO had the extremely high ADC activity which was increased by 20 times compared to the control cell line. They suggested that the compensatory mechanisms regulating putrescine biosynthesis in inhibitor-treated system may also occur in tobacco cells.

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Therefore, to study the compensatory aspects between ADC and ODC, we examined ADC and ODC activity and the cellular contents of polyamines when α-difluoromethyl arginine (DFMA) or DFMO, the suicide inhibitor of ADC and ODC, respectively was treated in tobacco suspension cultured cells.

MATERIALS AND METHODS

Plant material and cell culture

Callus cells induced from tobacco leaf disc were cultured in Murashige and Skoog (MS) liquid medium with 0.3 μg/mL NAA and 1 μg/mL kinetin. The callus cells were subcultured every 2 weeks in liquid media at 27°C with shaking at 140 rpm. The 4-day-cultured cells were harvested by the filtration and incubated in 50 mL Erlenmeyer flask with 10 mL of the incubation medium containing DFMO, DFMA, or putrescine, which was added either alone or in combination. The filtrated media were used as the incubation media.

Extraction and assay of ADC and ODC

The activities of ADC and ODC were essentially determined as described by Lee and Park (1990). The treated cells were homogenized (by pestle and mortar) with 1 mL extraction buffer that contained 10 mM potassium phosphate (pH 8.0), 1 mM pyridoxal phosphate, 0.1 mM dithiothreitol, and 20 mM Na-EDTA. After centrifugation at 18,000×g, the supernatant was used as enzyme source. The reaction mixtures consisted of 200 μL enzyme source, 125 μL extraction buffer, 123 μL H_2O, 50 μL cold substrate, and 0.1 μCi labeled substrate which was either DL-[U-^{14}C] arginine hydrochloride (309 mCi/mmol) for ADC or DL-[1-^{14}C] ornithine hydrochloride (314 mCi/mmol) for ODC. The added substrate corresponded to 0.1 mM final concentration. The reaction mixture was incubated in flask with center well which contained 50 μL of hyamine (methylbenzethonium hydroxide) on a wick of Whatman No. 1 paper for 1 hour at 37°C. The reaction was stopped by injection of 0.1 mL 5% (v/v) perchloric acid. The flasks were incubated for an additional 1 hour after the termination of reaction, and radioactivity of the wick was measured by a liquid scintillation counter.

Determination of polyamines

Polyamines were determined with the methods as described by Goren *et al.* (1982). The cells were extracted with 5% (v/v) perchloric acid, and then each extract was centrifuged at 15,000 rpm. 0.2 mL saturated sodium carbonate and 0.4 mL dansyl (dimethylaminonaphthalene-1-sulfonyl) chloride (1 mg/mL) were added to 0.2 mL the supernatant, and the mixture was incubated in water bath at 60°C for one and half hours. The mixture was fractionated by TLC and the products were quantified using a spectrophotofluorimeter, in which the emission at 495 nm was recorded after excitation at 350 nm.

RESULTS

Changes in the contents of the cellular polyamines during the growth periods of tobacco suspension cultured cells

The changes in the contents of the cellular polyamines were examined during the growth periods (Fig. 1). The content of the cellular putrescine peaked at day 4 and then decreased by day 7. After the content of the cellular spermidine was slightly increased by day 2, it was decreased by day 5 and then remained constant. But the content of the cellular spermine was not changed during the growth periods. Therefore, in this experiment, the 4-day-cultured cells were used to focus the content of the cellular putrescine.

Effects of DFMA and DFMO on the contents of the cellular polyamines and the activities of ADC and ODC

After the cells were transferred to the incubation medium containing 1 mM DFMA or 1 mM DFMO, both changes in the contents of the cellular polyamines and the activities of ADC and ODC on the each incubation time (Figs. 2, 3, 4) were examined. While the contents of the cellular spermidine and spermine unchanged to some extent in control cells, that of the cellular putrescine was increased continuously after the rapid decrease from 750 to 275 nmol/g fresh weight occurred in first 3 hours. The application of DFMA caused the continuous decrease in the contents of the cellular putrescine and spermidine but the increase in that of spermine after the incubation for 6 hours. In the cells treated with DFMO, the content of the cellular putrescine decreased by 50% of control at 12 hour, but recovered by 85% of control at 24 hour (Fig. 2). Although

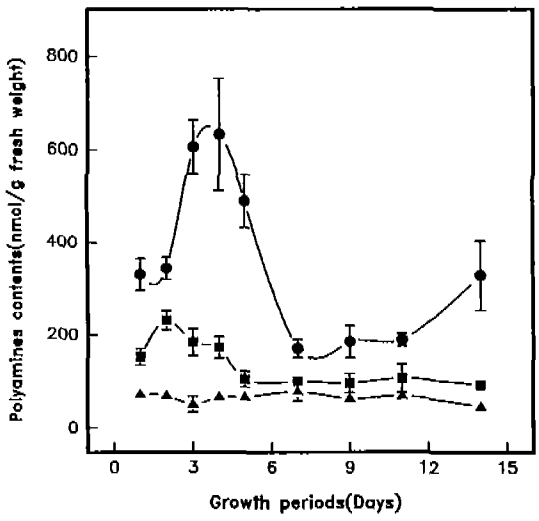

Fig. 1. Changes in the cellular contents of polyamines during the growth periods. -●-, Putrescine; -■-, Spermidine; -▲-, Spermine.

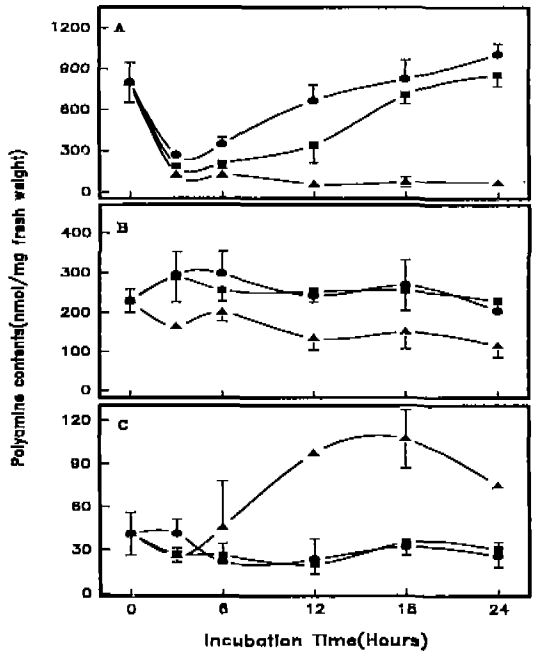

Fig. 2. Changes in the cellular contents of polyamines during the incubation times. After the cells were transferred into 50 mL flask, they were treated with 1 mM DFMA or 1 mM DFMO. A, Putrescine; B, Spermidine; C, Spermine. -●-, Control; -■-, DFMO; -▲-, DFMA.

DFMO completely inhibited ODC activity during the incubation time, it caused the stimulation of ADC activity for 18 hours, which then disappeared (Fig. 3). These results suggest that the increase of ADC activity and the recovery of the content of the

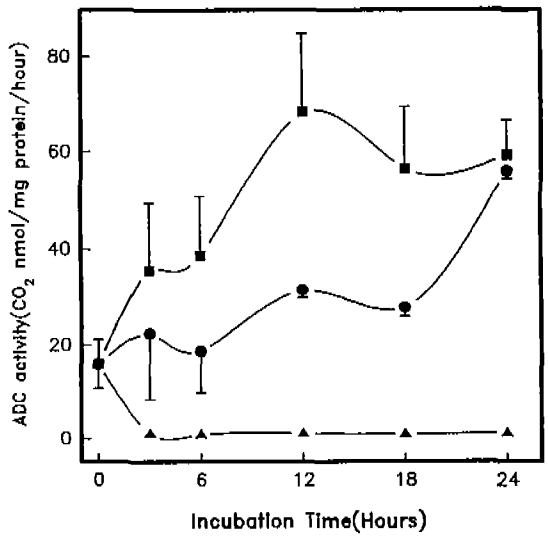

Fig. 3. The effects of DFMA on ADC activity during the incubation times. After the cells were transferred into 50 mL flask, they were treated with 1 mM DFMA, or None. -●-, Control; -■-, DFMO; -▲-, DFMA.

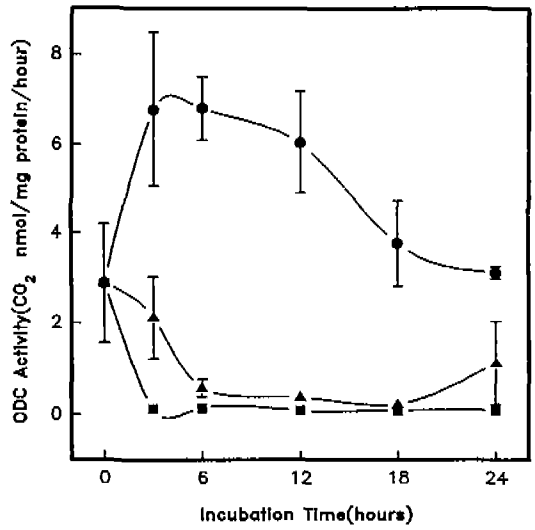

Fig. 4. The effect of DFMA or DFMO on ODC activity during the incubation periods. After the cells were transferred into 50 mL flask, they were treated with 1 mM DFMA, 1 mM DFMO or None. -●-, Control; -■-, DFMO; -▲-, DFMA.

cellular putrescine in DFMO-treated-cells result from the compensatory aspect of the alternative enzyme, ADC.

Effect of putrescine on ADC activity

To investigate that the stimulation of ADC ac-

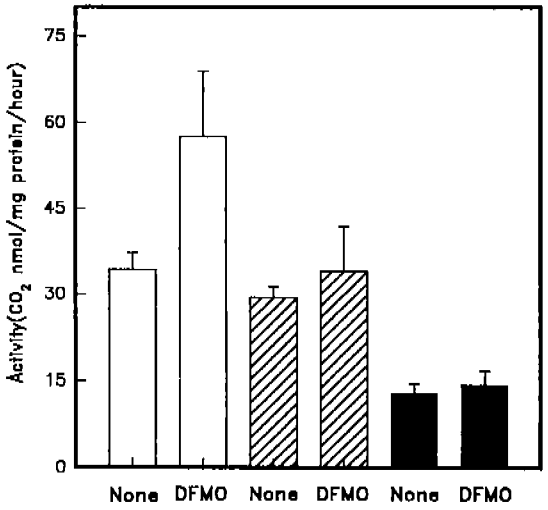

Fig. 5. The effect of putrescine on enhancement of ADC induced by DFMO. After the cells were transferred into 50 mL flask, they were treated with 1 mM DFMO and putrescine. □, Control; ▨, 1 mM Putrescine; ■, 5 mM Putrescine.

tivity results from the low content of the cellular putrescine by DFMO, we examined the ADC activity after the application of putrescine with DFMO. Putrescine (5 mM) declined the activity of ADC by 50% of control cells (Fig. 5) and blocked the DFMO-induced stimulation of ADC activity. Therefore, ADC activity is probably regulated by the content of the cellular putrescine in our system.

Effect of actinomycin-D or cycloheximide on ADC activity

DFMO may have induced the ADC activity stimulation by the change in half-life of enzyme, translation or transcription rate or the post-translational modification of ADC. To find the regulation point of the stimulation of ADC activity by DFMO, the change in the ADC activity was examined when cycloheximide or actinomycin-D was added along with DFMO. When the cells were treated with cycloheximide (10 μg/mL) after being incubated with or without DFMO for 12 hours, the rate of the decrease in ADC activity (ADC half-life) was similar between DFMO-untreated cells and treated cells (Table 1). When DFMO and cycloheximide were treated together, the stimulation of ADC activity by DFMO almost disappeared (Table 2). However, when the cells were incubated with DFMO and actinomycin-D, the stimulation of ADC activity by DFMO was still observed (Table 3).

Table 1. Effect of cycloheximide on the increase of ADC activity induced by DFMO after the incubation for 12 hours

Incubation Time	ADC activity (CO_2 nmol/mg protein/hour)			b/a ratio
	Control(a)	DFMA	DFMO(b)	
0 Hour	16.74	0.33	23.93	1.43
2 Hour	9.65	0.20	13.27	1.38
4 Hour		0.26	7.05	1.61

*After the cells were incubated with DFMA or DFMO for 12 hours, they were treated with cycloheximide.
*The concentration of cycloheximide was 10 μg/mL.
*The values are the mean of three experiments.

Table 2. Effect of cycloheximide on the increase of ADC activity induced by DFMO during incubation time

Incubation Time	ADC activity (CO_2 nmol/mg protein/hour)		
	Control	DFMA	DFMO
0 Hour	5.71	-	-
3 Hour	1.98	0.31	1.84
6 Hour	0.52	0.24	0.54

*The concentration of cycloheximide was 10 μg/mL.
*The cells were simultaneously treated with cycloheximide and DFMA or DFMO during the each incubation.
*The values are the mean of three experiments.
* -: not determined.

Table 3. Effect of actinomycin-D on the increase of ADC activity induced by DFMO during incubation time

Incubation Time	ADC activity (CO_2 nmol/mg protein/hour)		
	Control	DFMA	DFMO
6 Hours	14.75(100)	0.46(3)	17.92(122)
12 Hours	11.62(100)	0.40(3)	18.92(163)

*The concentration of actinomycin-D was 5 μg/mL.
*The cells were simultaneously treated with actinomycin-D and DFMA or DFMO during the each incubation.
*The values are the mean of three experiments.
*The percentage of control values is shown in parentheses.

DISCUSSION

If the compensatory mechanisms regulating putrescine biosynthesis in inhibitor-treated system, which Tiburcio and coworkers (1990) suggested, is probably present, the activity of the alternative enzyme should increase when the specific inhibitor for ADC or ODC is respectively treated. To clarify the compensatory regulation of putrescine biosynthesis

in inhibitor-treated system, we examined the activities of ADC and ODC and the change in the contents of the cellular polyamines after the treatment of DFMA or DFMO, respectively. DFMO induced the decrease in the content of putrescine about 50% of control cells (Fig. 2) and the stimulation of ADC activity about 2 times of untreated cells (Fig. 3) at 12 hours. The treatment of putrescine or cycloheximide prevented the increase of ADC activity which was induced by DFMO (Fig. 5, Table 2) but that of actinomycin-D did not show any detectable effect (Table 3). The treatment of MGBG, a competitive inhibitor of SAMDC, caused the increase in the content of the cellular putrescine and the decrease of ADC activity in tobacco suspension cells (Hiatt, 1986). Although Marmberg (1992) suggested that ADC activity is regulated in post-translational step in oat, it was suggested that ADC activity is regulated in the translation step by the content of putrescine (Hiatt, 1986). Since the above results imply that the DFMO-induced stimulation of ADC activity resulted from the increase in the de novo synthesis for ADC protein which is probably induced by the decrease in the content of the cellular putrescine, we thought that the compensatory overproduction, which was suggested by Smith (1990), maybe occurred between ADC and ODC in tobacco suspension cells.

If the DFMO-induced stimulation of ADC activity results from the low content of the cellular putrescine, ADC activity should be increased as the content of the cellular putrescine is decreased. Nevertheless, although the rapid decrease in the content of the cellular putrescine occurred in the control cells at 3 hour, the ADC activity did not change (Figs. 2, 3, 4). Although these results did not coincide with the fact that 5 mM putrescine blocked the DFMO-induced stimulation of ADC activity, we thought that ADC activity was probably regulated by putrescine because putrescine was declined the activity of ADC in DFMO-treated and untreated cells (Fig. 5).

The content of the cellular putrescine was decreased although the activity of enzyme related to the biosynthesis of putrescine increased or unchanged (Figs. 2, 3, 4). It was previously reported that pea shoots and oat leaves metabolized putrescine and spermidine to GABA (γ-aminobutylic acid) (Flores and Filner, 1985) and one roles of polyamine catabolism may be in the regulation of the contents of the cellular polyamines (Rastogi and Davies, 1989). Therefore, it was speculated that the decline of the putrescine probably resulted from the stimulation of polyamine catabolism. However, since polyamines are present not only as a free form but also as a conjugated or protein-bound form and the transition between them is possible (Tiburcio *et al.*, 1990), it can not be ruled out the probability that the decrease in the content of the cellular putrescine results from the increase in the synthesis of conjugated or protein-bound putrescine.

When the labeled spermidine was fed to thin-layer tobacco cultures, a portion of it was attached to a specific protein with a molecular size of about 18 kD (Apelbaum *et al.*, 1988). Studies with animal tissues have shown that polyamines are substrates for transglutaminase-mediated binding of primary amines to proteins (Willims-Ashman and Canellakis, 1980). The activities of transglutaminase and other polyamine-binding enzyme have recently been reported in plant tissue (Icekson and Apelbaum, 1987; Serafini-Fracassini *et al.*, 1988). These results suggest that the activity of ADC be regulated by the change in the level of protein-bound putrescine. Also, the change in the conjugated putrescine be probably involved in the regulation of the DFMO-induced stimulation of ADC activity because the cultured cells contained a large amount of it (Tiburcio *et al.*, 1990).

Since animal cells only have ODC but not ADC, ODC inhibitors such as DFMO caused the depletion of the content of the cellular putrescine and spermidine but the slight change in that spermine when they were treated in animal tissues or cultured cells (Pegg, 1984; 1986). These include the continued synthesis of a small amount of putrescine which is completely converted to spermine because of the excess decarboxylated SAM (dcSAM) (Pegg, 1986). The content of dcSAM in cells treated with DFMO was reported to increase many times (Pegg, 1984; 1986). The accumulation of dcSAM probably results from substrate limitation for the spermidine and spermine synthase reaction (Manont, 1982). Treatment of DFMO caused the increase of SAMDC activity (Pegg, 1984) which result from a rise in the number of SAM decarboxylase protein (Shirahata *et al.*, 1985; Shirahata and Pegg, 1985). Although DFMA is the inhibitor of ADC, it simultaneously inhibited the activities of ADC and ODC in our system. This may result from the conversion from DFMA to DFMO because DFMA could be converted to DFMO in the tissue exhibiting the high activity of arginase (Slocum and Galston, 1985; Slocum *et al.*, 1988). The inhibition of ADC and ODC activities by DFMA may

be explained by the continued decline of the contents of the cellular putrescine and spermidine during the incubation time (Fig. 2). However, the application of DFMA caused the increase in the content of the cellular spermine. These changes in the content of the cellular polyamines coincide with that in animal tissues treated with ODC inhibitors. Therefore, we concluded that the increase in the content of the cellular spermine by DFMA was probably occurred by the accumulation of the cellular dcSAM which was resulted from the depletion of the content of the cellular putrescine such as animal tissues treated DFMO. Also, we suggest the possibility that the inhibition of putrescine biosynthesis induces the increase of SAMDC activity in tobacco suspension cultured cells.

In conclusion, this study shows that DFMO induced the temporary depletion of putrescine and the increase of ADC activity in tobacco suspension cultured cells. In addition, the application of putrescine or cycloheximide prevents ADC activity from the stimulation by DFMO. Therefore, we suggest that the compensatory aspect of ADC be probably present in the tobacco suspension cultured cells when the ODC activity is inhibited by DFMO, which functions on the step of the translation and is regulated by putrescine.

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