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Regulation of DNA synthesis and cell division by polyamines in *Catharanthus roseus* suspension cultures

R. Minocha [1], S. C. Minocha [2], A. Komamine [3], and W. C. Shortle [1]

[1] USDA Forest Service, NEFES, P.O. Box 640, Durham, NH 03824, USA
[2] Department of Plant Biology, University of New Hampshire, Durham, NH 03824, USA
[3] Biological Institute, Faculty of Science, Tohoku University, Aoba-yama, Sendai 980, Japan

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Summary. Various inhibitors of polyamine biosynthesis were used to study the role of polyamines in DNA synthesis and cell division in suspension cultures of *Catharanthus roseus* (L.) G. Don. Arginine decarboxylase (ADC; EC 4.1.1.19) was the major enzyme responsible for putrescine production. DL α-difluoromethylarginine inhibited ADC activity, cellular putrescine content, DNA synthesis, and cell division. The effect was reversible by exogenous putrescine. Ornithine decarboxylase (ODC; EC 4.1.1.17) activity was always less than 10% of the ADC activity. Addition of DL α-difluoromethylornithine had no effect on ODC activity, cellular polyamine levels, DNA synthesis, and cell division within the first 24 h but by 48 to 72 h it did inhibit these activities. Methylglyoxal bis(guanylhydrazone) inhibited S-adenosylmethionine decarboxylase (EC 4.1.1.50) activity without affecting DNA synthesis and cell division.

Abbreviations: ADC: arginine decarboxylase, ODC: ornithine decarboxylase, SAMDC: S-adenosylmethionine decarboxylase, DFMA: DL α-difluoromethylarginine, DFMO: DL α-difluoromethylornithine, MGBG: methylglyoxal bis(guanylhydrazone).

Introduction

The regulation of cell division is among the variety of growth and developmental phenomena in which a critical role for polyamines (putrescine, spermidine, and spermine) has been implicated (Pegg and McCann 1982; Tabor and Tabor 1984; Smith 1985; Bachrach and Heimer 1989a,b). Serafini-Fracassini et al. (1980) showed a marked increase in polyamine synthesis early during the G1 phase, concomitant with the synthesis of RNA, following the break of dormancy in tuber explants of *Helianthus tuberosus*. A second phase of polyamine accumulation was seen during the progression of the S phase. Torrigiani et al. (1987) and Phillips et al. (1987) further confirmed these observations in the same tissue by showing that the increase in polyamines during the G1 phase was preceded by increases in ODC and ADC activities, the two enzymes regulating the biosynthesis of putrescine in plants (Slocum et al. 1984). Recently, Pfosser et al. (1990) have reported the highest levels of free putrescine at the end of G1 and at the S/G2 boundary in synchronized cells of *Nicotiana tabacum*. No further information on the involvement of a specific polyamine or on the biochemical function of polyamines in cell division in these tissues has been published.

In this paper, we report the effects of different polyamine biosynthesis inhibitors on the DNA synthesis and the activities of ODC, ADC, and SAMDC in batch cultures as well as cellular polyamine levels in synchronously dividing cells of *Catharanthus roseus*.

Materials and Methods

Culture Conditions. Suspension cultures of *C. roseus* were maintained and synchronized in MS medium (Murashige and Skoog 1962) containing 3% sucrose and 2.2 uM 2,4-D as described by Kodama et al. (1989). The medium was adjusted to pH 6.2 before autoclaving. Whereas 2,4-D was added before autoclaving, the inhibitors and the polyamines were filter sterilized. Cells were subcultured at 7 d intervals by transferring 7 ml of cell suspension into 43 ml of fresh medium in 300 ml Erlenmeyer flasks. The flasks were kept in the dark at 27±2°C on a reciprocal shaker at 90 strokes/min. The concentration of each inhibitor was selected on the basis of preliminary studies on their effects on cell division in this tissue (unpublished data).

Offprint requests to: S. C. Minocha

For synchronization, 7 d old cultures were transferred to the phosphate-free MS medium at a low population density of $3x10^5$ cells/ml (Kodama et al. 1989). After 3 d, KH_2PO_4 was added at a final concentration of 0.18 mM. Following 18 h of incubation, the cells were centrifuged at 300 x g for 2 min and washed once with the phosphate-free medium and transferred to phosphate-free medium at a density of $2x10^5$ cells/ml for 2 d. At this time, KH_2PO_4 was added again at a final concentration of 0.675 mM. The time of second phosphate addition is called time zero for all experiments reported here.

For experimental treatments, 10 ml aliquots of cells were transferred from either batch cultures (3 or 4 d old) or synchronously dividing cultures at time zero (as described above) to 50 ml flasks. Inhibitors and/or polyamines were added at this stage and the flasks kept on a shaker until the time of analysis. For determination of cell numbers, protoplasts were prepared from aliquots of cell suspensions and counted in a hemocytometer as described by Amino et al. (1983).

Enzyme Assays. For preparation of extracts, cells were collected on Miracloth by vacuum filtration, washed with deionized distilled water, and homogenized in the extraction buffer (2 ml/g FW) for 90 s at 27000 rpm using a Brinkmann Polytron homogenizer. The extraction buffer contained 0.05 M Tris-HCl, pH 8.4 adjusted at 4°C, 0.05 mM pyridoxal-5-phosphate, 0.1 mM Na_2-ethylenediamine-tetraacetate (EDTA), and 5.0 mM dithiothreitol (DTT). For SAMDC, the extraction buffer contained 0.1 M potassium phosphate, pH 7.5, 3 mM putrescine and 1 mM DTT. The homogenates were centrifuged at 18000 x g for 20 min at 4°C and the supernatants used for enzyme assays. ODC and ADC were assayed according to the procedure of Robie and Minocha (1989) and SAMDC according to Minocha et al. (1991).

Incorporation of ^{3}H-Thymidine. At appropriate intervals, 1.0 uCi of [methyl-^{3}H]-thymidine (sp. act. 43 Ci mmol $^{-1}$, New England Nuclear) was added to 0.5 ml cell suspension in a 12 ml centrifuge tube. Following 30 min incubation at 27°C with constant shaking, 3 ml of ice-cold 4% perchloric acid (PCA) was added to each tube and the tubes were vortexed for 10 s. After 15 min incubation on ice, the cells were filtered through a premoistened Whatman GFC glass fiber filter (24 mm) using a Millipore multifilter manifold under vacuum. The filters were washed with another 3 ml of 4% PCA, 15 ml of cold 80% ethanol and 15 ml of cold 100% ethanol. The filters were dried at 45°C for 2 h and counted for radioactivity in 5 ml scintillation fluid.

Polyamine Analysis. Polyamines were extracted and analyzed according to the procedure of Minocha et al. (1990). Cells were collected on Miracloth and washed with deionized distilled water. After taking fresh weights, the cells were homogenized for 90 s at 21000 rpm in 5% cold PCA with a Brinkmann Polytron homogenizer. Extracts were kept on ice for 1 h and then centrifuged at 18000 x g for 30 min at 4°C. The supernatant was used for dansylation and quantification of polyamines by HPLC (Minocha et al. 1990). Each treatment was run in triplicate and each experiment was repeated at least twice.

Results

Effects of DFMA, DFMO, and MGBG on DNA Synthesis

DNA synthesis was studied using 3 d old cells grown in batch cultures. As shown in Figures 1 and 2, the rate of incorporation of ^{3}H-thymidine into the PCA-insoluble fraction varied considerably in the control cultures at different times. Since the batch cultures were asynchronous and the cell numbers changed between 24 to 72 h, the effects of inhibitors were statistically analyzed only for given times. The results presented in Figure 1 show that the incorporation of thymidine was inhibited in the presence of DFMA (0.1 mM) by about 30% during the first 3.5 h and by 80% at 72 h of incubation. This inhibition was fully reversed by the addition of 0.2 mM putrescine. Increasing the concentration of DFMA (0.5 mM) did not increase the extent of inhibition.

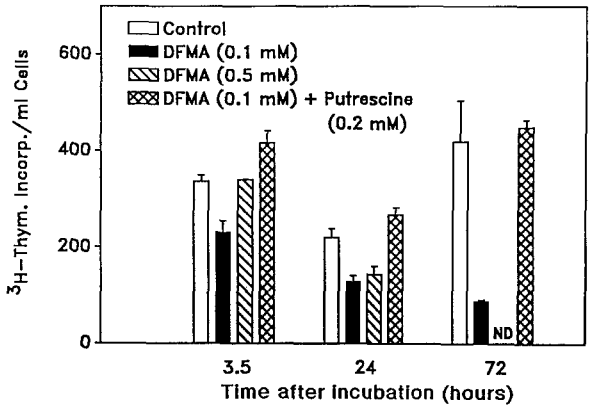

Fig. 1. Effects of DFMA, alone and in combination with putrescine, on the incorporation of ^{3}H-thymidine into DNA in 3 d old cultures of *C. roseus*. Bars represent the mean (in pmol/ml cells) ± SE of three replicates. Data for 72 h come from a separate experiment. ND = Not Determined.

The rate of ^{3}H-thymidine incorporation into DNA was not affected by DFMO (2 mM) during the first 24 h of treatment (Fig. 2A). However, after 72 h there was significant inhibition of DNA synthesis that was not reversed by the addition of 0.2 mM putrescine. Addition of exogenous putrescine alone had no effect on the rate of thymidine incorporation at most of the times tested.

Whereas MGBG caused some inhibition of DNA synthesis at 6 h in these cells, by 24 h the cells seemed to have recovered from the inhibitory effects of this compound (Fig. 2B). Addition of spermidine (0.2 mM) did not reverse the temporary effect of MGBG on thymidine incorporation.

Effects of Inhibitors on ADC, ODC, and SAMDC Activities

The effects of DFMA, DFMO, and MGBG on the activities of ADC, ODC, and SAMDC were studied using 3, 4, and 5 d old cultures. In each case, the enzyme activities were analyzed after 24 h of incubation with various inhibitors unless otherwise stated. Arginine decarboxylase was the predominant enzyme for putrescine biosynthesis at all times. The activity of ODC never exceeded 10% that of ADC.

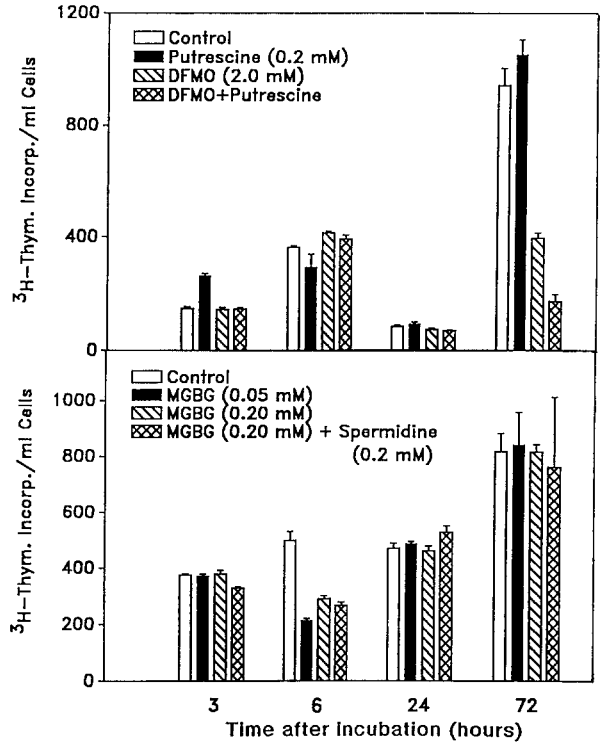

Fig. 2A-B. Effects of DFMO and MGBG alone or in combination with either putrescine or spermidine, respectively, on the incorporation of ^{3}H-thymidine into DNA in 3 d old cultures of *C. roseus*. Bars represent mean ± SE of three replicates. Data for 72 h come from a separate experiment.

Addition of DFMA and DFMO had no effect on ODC activity within the first 24 h but DFMO did inhibit ODC at 48 h. This inhibition was not reversed by exogenous putrescine (data not presented). However, DFMO promoted the activity of ADC by 200 to 400% depending upon the age of the batch culture (Fig. 3). DFMA inhibited more than 90% of the ADC activity in these cells. A time course was also run where the effects of 0.1 mM DFMA and 2 or 5 mM DFMO on ADC activity of 3 day old batch cultures were analyzed at 6, 24, and 48 h intervals. Inhibition of ADC by DFMA was always greater than 85% at each time tested. While neither concentration of DFMO (2 or 5 mM) had an appreciable effect on ADC within the first 6 h, even the lower concentration of DFMO promoted ADC by about 250% at 24 h and 160% at 48 h (data not presented).

The activity of SAMDC was inhibited by approximately 75% in the presence of 0.1 mM MGBG (Fig. 4A). The MGBG effect was further accentuated by the presence of 0.2 mM spermidine. However, both DFMA and DFMO promoted SAMDC activity (Fig. 4B) but to different degrees. Addition of 0.2

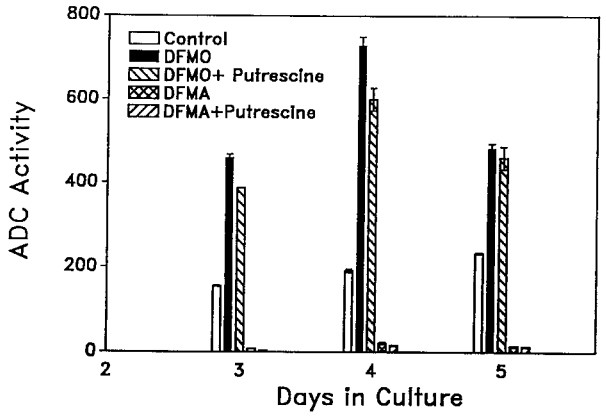

Fig. 3. Effects of DFMO (2 mM) and DFMA (0.1mM) alone and in combination with putrescine (0.2 mM) on ADC activity in 3, 4, and 5 d old cultures of *C. roseus*. Inhibitors were added 24 h prior to enzyme assay on each day. Enzyme activity = nmol CO_2 released h $^{-1}$g $^{-1}$ FW. Bars represent mean ± SE of two assays.

mM putrescine along with DFMA and DFMO did not affect the extent of this promotion.

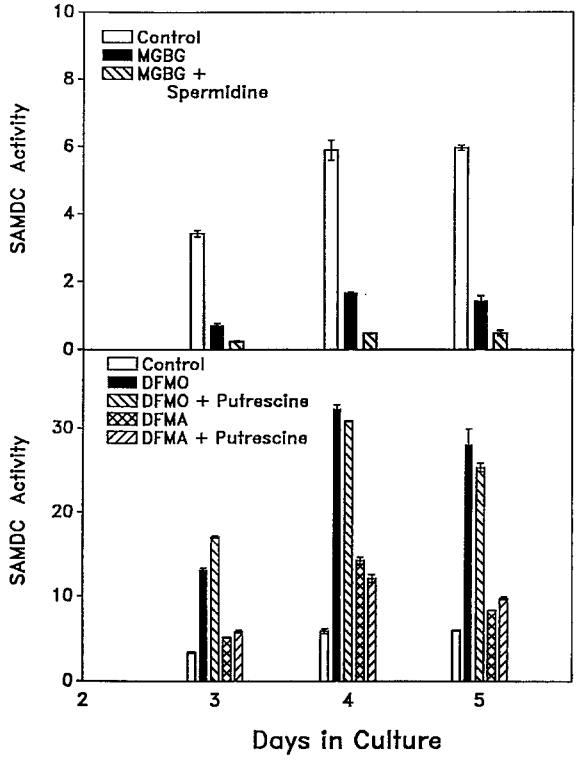

Fig. 4A-B. Effects of MGBG (0.1 mM), DFMO (2.0), and DFMA (0.1 mM) alone and in combination with either spermidine (0.2 mM) or putrescine (0.2 mM) on SAMDC activity in 3, 4, and 5 d old cultures of *C. roseus*. Inhibitors were added 24 h prior to enzyme assay on each day. Enzyme activity = nmol CO_2 released h $^{-1}$g $^{-1}$ FW. Bars represent mean ± SE of two assays.

Synchronized Cell Division and Cellular Polyamines

The results on the synchronization of cell division were similar to those of Kodama et al. (1989). The total duration of the cell cycle was about 32 h. Synchrony was maintained through at least two cell division cycles. The G1 phase was about 12 h in length and the S phase occurred at 13 to 25 h (first cell cycle) and 44 to 57 h (second cycle). The total time for the G2 + M phases was about 7 h. Sixty-five to eighty percent of the cells divided synchronously.

The effects of DFMA and DFMO on cellular polyamine concentrations during the progression of the cell cycle were examined in synchronized cell cultures. Samples were collected at 4, 7, 21, and 50 h from time zero, i. e., from the time of the second addition of phosphate to the medium (see Materials and Methods). The samples at 4 and 7 h constituted two different intervals within the G1 phase, while 21 and 50 h periods represented cells in the middle of DNA synthesis of the first and the second cell cycle, respectively. DFMA significantly reduced the cellular levels of putrescine during the G1 as well as the S phases. Cellular spermidine and spermine were only slightly affected by DFMA (Table 1). DFMA also inhibited cell division by about 20-30%. The inhibitory effects of DFMA on cellular polyamine levels and cell division were reversed by the addition of 0.2 mM putrescine. In the presence of DFMO (2 or 5 mM), cellular putrescine levels were lower by 40% only at 50 h with relatively little effect on spermidine and spermine levels (Table 2). In all these experiments the highest polyamine concentration was seen at 50 h in control cultures.

Table 1. Effect of 0.1 mM DFMA alone and in combination with 0.2 putrescine on cellular polyamines in synchronized cells of *C. roseus*. Each number is a mean ± SE of three replicates.

Time(h)	Treatment	Polyamine Content (nmol/g FW)		
		Putrescine	Spermidine	Spermine
4	Control	104±5	122±3	22±1
	DFMA	46±8	117±1	29±8
	DFMA+Putrescine	116±7	118±6	19±0
7	Control	118±9	116±5	21±1
	DFMA	30±9	112±13	28±6
	DFMA+Putrescine	153±12	135±4	25±3
21	Control	74±12	177±14	28±1
	DFMA	45±3	194±8	47±2
	DFMA+Putrescine	136±20	184±6	33±3
50	Control	150±44	176±16	33±3
	DFMA	20±7	177±7	35±3
	DFMA+Putrescine	17±7	222±11	31±2

Table 2. Effect of 2.0 mM and 5.0 mM DFMO on cellular polyamines in synchronized cells of *C. roseus*. Each number is a mean ± SE of three replicates.

Time(h)	Treatment	Polyamine Content (nmol/g FW)		
		Putrescine	Spermidine	Spermine
4	Control	109±5	157±1	22±2
	2 mM DFMO	112±6	152±8	22±0
	5 mM DFMO	109±7	150±13	20±1
7	Control	152±10	151±10	27±3
	2mM DFMO	137±11	154±4	28±3
	5 mM DFMO	123±4	142±12	24±0
21	Control	131±4	164±13	28±2
	2 mM DFMO	135±8	157±3	30±2
	5 mM DFMO	176±9	167±9	36±3
50	Control	192±5	235±9	33±0
	2 mM DFMO	110±13	191±8	59±3
	5 mM DFMO	116±8	191±4	64±1

Discussion

Whereas ODC is the only enzyme for putrescine biosynthesis in animals, plants generally have an additional pathway using ADC (Slocum et al. 1984; Smith 1985; Pegg 1986). The distribution of these two enzymes and their relative contribution to putrescine biosynthesis in different species and in different tissues of the same plant is not well known. Developmental regulation of these two enzymes has been suggested by several workers (Slocum et al. 1984; Minocha 1988). In some cases both enzymes are simultaneously active in the same tissues (Torrigiani et al. 1987). It has been stated that polyamine biosynthesis during active cell division appears to be regulated by changes in ODC activity, and that ADC activity generally regulates polyamine biosynthesis during the growth of non-dividing but rapidly growing cells (Smith 1981; Altman et al. 1982; Bagni et al. 1983). Data presented here, however, indicate that *C. roseus* cells grown in culture possess ADC as the predominant enzyme for putrescine biosynthesis. Ornithine decarboxylase is barely detectable and does not change appreciably with the age of culture. The observation that DFMO neither affects ODC activity nor the rate of DNA synthesis (Fig. 2A) and cell division (within 24 h of its addition) further supports this conclusion. This situation is similar to that in actively dividing carrot cell cultures, where ODC is completely absent except in fully developed, green somatic embryos (Montague et al. 1979; Robie and Minocha 1989).

The promotion of ADC activity in the presence of DFMO has been observed in suspension

cultures of wild carrot (Fallon and Phillips 1988; Robie and Minocha 1989). The mechanism by which DFMO causes this effect on ADC activity is not understood at present. The inhibition by DFMO of cellular polyamine levels (Table 2) and of DNA synthesis after 72 h of its addition to the medium (Fig. 1) is intriguing and may indicate some effects of DFMO other than interference with ODC, especially since the addition of putrescine did not reverse these effects.

Reversal of the inhibitory effects of DFMA on DNA synthesis (Fig. 1) and cell division by the addition of exogenous putrescine points to a critical role for putrescine in cell division in *C. roseus*. Recently, Torrigiani et al. (1989) and Pfosser et al. (1990) have reported a rapid decline in putrescine content by 40-50% during the S phase DNA synthesis. This reduction was related to high diamine oxidase activity during the S phase. In *C. roseus* also, putrescine levels drop during the S phase of the first synchronous cell cycle. Spermidine content was highest during the S phase and was not inhibited by DFMA or DFMO (Tables 1 and 2). This contrasts with cells of *Nicotiana tabacum* in which spermidine content was reduced following the addition of these inhibitors (Pfosser et al., 1990). Spermine was present only in very low amounts at all times in these cells.

A marked reduction in SAMDC activity was seen in the presence of MGBG (Fig. 4A) without any long term effects on DNA synthesis (Fig. 2B) or cell division. Using *Helianthus tuberosus* tuber explants, which show a highly synchronous cell division, Torrigiani et al. (1987) also observed that the inhibition of spermidine synthesis by dicyclohexylamine did not affect cell division. A peak in the activity of ODC and ADC as well as putrescine was seen in this tissue prior to the beginning of the S phase, and both DFMO and DFMA caused a significant inhibition of cell division. It has been reported earlier from their laboratory that MGBG, which completely inhibited SAMDC activity in *H. tuberosus* explants, also had no effect on cell division (Bagni et al. 1982, 1983). Although MGBG has been widely used as an inhibitor of SAMDC to deplete the cells of spermidine and spermine, it is not as specific as DFMO and DFMA are for ODC and ADC, respectively (Pegg 1986). It has been suggested that while inhibiting SAMDC activity in vitro, the effects of MGBG in vivo may be quite transient. This drug brings about a large increase in the amount of the enzyme protein by stabilizing the enzyme against breakdown and increasing its half-life (Pegg 1986, 1989; Malmberg and Rose 1987).

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