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Interaction of spermine with a signal transduction pathway involving phospholipase C, during the growth of *Catharanthus roseus* transformed roots

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In *Cantharanthus roseus* transformed roots, the application of methylglyoxal bis(guanylhydrazone) (MGBG), an inhibitor of *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50), inhibited the root growth in a dose-dependent manner with a DL_{50} of about 300 μm. Spermidine and spermine (Spm) levels and SAMDC and phospholipase C (PLC; EC 3.1.4.3) activities were reduced in the presence of the inhibitor. The inhibition was reversed by the addition of Spm. Radioactivity from [^{14}C]Spm was detected in an immunoprecipitated fraction with an antibody anti-PLC-δ. To our knowledge, this is the first direct evidence that demonstrates an interaction of Spm with the signal transduction cascade phosphoinositide-Ca^{2+}.

Introduction

The polyamines (Pas) putrescine (Put), spermidine (Spd) and spermine (Spm) are low-molecular-weight cationic molecules present in microorganisms, and in animal and plant cells. They have been implicated in several important cellular processes such as cell proliferation, membrane stability, protein synthesis, protein–DNA interaction and abiotic stress plant responses (Tabor and Tabor 1984, Broeck et al. 1994, Kumar et al. 1997). There is evidence that these compounds are essential for growth and development in prokaryotes and eukaryotes (Tabor and Tabor 1984, Heby and Persson 1990). However, their mode of action remains a matter of speculation, although it is generally accepted that the interaction of Pas with subcellular components constitutes the main mechanism by which these compounds influence cellular function (Tiburcio et al. 1993).

In plants, it has been suggested that most of these functions and interactions of Pas with macromolecules and cellular structures may also exist. The covalent interactions between polyamines and proteins, catalysed by transglutaminases, have been studied (Apelbaum et al. 1988, Mizrahi et al. 1989, Mehta et al. 1991). However, there is little information on the non-covalent binding of polyamines to proteins. Recently, polyamine-binding membrane proteins have been identified in plants (Tassoni et al. 1996, 1998, 2002). An 18-kDa polypeptide and another co-purifying 60-kDa polypeptide, that seems to be directly involved in specific Spd binding, were purified from maize microsome membranes (Tassoni et al. 2002).

Due to the enormous variety of possible biological functions it is difficult to evaluate which biochemical system is mainly affected during specific developmental events induced by Pas treatments. For this reason, to be able to catalogue these compounds as plant growth regulators, the interaction of Pas with membrane proteins and the possible use of a signal transduction mechanism are very important.

It has recently been proposed that Pas may be linked to the phosphoinositide-Ca^{2+} signal transduction pathway (Dureja-Munjal et al. 1992, McDonald and

Abbreviations – [^{14}C]Spm, ^{14}C spermine; DAG, diacylglycerol; IP_3, inositol 1,4,5 trisphosphate; MGBG, methylglyoxal bis(guanylhydrazone); Pas, polyamines; PIP2, phosphatidylinositol 4,5 bisphosphate; PLC, phospholipase C; SAMDC, *S*-adenosylmethionine; SDS-PAGE, electrophoresis in polyacrylamide gels with sodium dodecyl phosphate; Spm, spermine.

Mamrack 1995, Singh et al. 1995). The hydrolysis of inositol phospholipids by the enzyme phospholipase C (PLC) produces two potential intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). This enzyme has a key role in the signal transduction pathway and it has been suggested that it may be implicated in a variety of physiological processes in plants (Coté and Crain 1993, Chapman 1998, Munnik et al. 1998, Kashem et al. 2000).

Using *Catharanthus roseus* transformed roots as a model, our laboratory has focused on the role of Pas and their possible interaction with the signal transduction pathway that involves PLC during the root growth process. These studies have shown that: (1) A high cellular content of Pas during root growth correlates with the increased activity of PLC (De Los Santos-Briones et al. 1997, Echevarría-Machado et al. 2002); (2) Spm stimulates membrane PLC activity at physiological concentrations; while the others Pas (Spd and Put) did not affect the enzymatic activity (Echevarría-Machado et al. 2002); (3) Exogenous Spm (50 μ*M*) stimulates root growth and this dose significantly increases PLC activity (Echevarría-Machado et al. 2002).

These results suggest that Spm interacts with PLC and this interaction may have an important biological action during root growth. The aims of this study were: (1) to evaluate the physiological significance of this event, using methylglyoxal bis(guanylhydrazone) (MGBG), a competitive inhibitor of SAMDC, to change the internal Pas concentrations, and (2) to demonstrate in vivo a possible direct interaction between Spm and PLC.

Materials and methods

Tissue culture

Hairy root line J1 of *C. roseus* was obtained by infecting leaves with *Agrobacterium rhizogenes* (Ciau-Uitz et al. 1994) and was maintained in half-strength B5 medium (Gamborg et al. 1968), supplemented with 30 g l^{-1} sucrose. The pH was adjusted to 5.7 with 0.1 *M* KOH/HCl prior to autoclaving. Medium (100 ml) was placed in a 250-ml Erlenmeyer flask and autoclaved for 20 min. Flasks were inoculated with 0.5 g (fresh mass) of hairy roots. Roots were subcultured every 14 days. Cultures were grown in darkness at 25°C on a rotary shaker at 100 r.p.m.

Application of Pas and/or Pas biosynthetic inhibitors

Freshly prepared stock solutions of MGBG and Pas were filter-sterilized before being added to the autoclaved B5 medium (Millipore filter, 0.45 μm). In some experiments MGBG was incorporated in the medium on subculture day (day 0), 4 or 10 days after subculture (day 4 or 10, respectively); the doses used are described in each case. Pas were always added in the medium on subculture day and the doses are also described in each figure.

Feulgen stain

Roots from days 0, 2, 4 and 6 after subculture were fixed in acetic-ethanol mixture (1:3) overnight and then washed in running water for 5 min. The roots were submitted to hydrolysis in 1 *M* HCl at 62°C for 8 min 30 s. Staining was performed by incubating the roots with basic fuchsine solution (prepared as reported by Sharma and Sharma 1980) for 30–45 min. Finally, roots were transferred to acetic acid solution (45%, v/v) and observed in a stereoscope (Nikon).

The program Rootedge version 2.26 (Root Length Measurement software) developed by T. Ewing and T. Kaspar was used for measurement of root length and width.

Protein extraction and phospholipase C (PLC) activity

Protein extraction and PLC assay were performed as described by Hernández-Sotomayor et al. (1999). Roots were quickly frozen with liquid nitrogen and homogenized in buffer A: 50 m*M* NaCl, 1 m*M* EGTA, 50 m*M* Tris.HCl pH 7.4, 250 m*M* sucrose, 10% glycerol, 1 m*M* phenylmethylsulfonyl fluoride, 10 m*M* sodium pyrophosphate, 0.2 m*M* orthovanadate and 1 m*M* β-mercaptoethanol (approximately 2.5 ml per 1 g root). Extracts were passed through gauze, and tissue debris was removed by centrifugation at 14 000 *g* for 30 min. The supernatant was further centrifuged at 100 000 *g* for 45 min, the pellet was resuspended in buffer A (protein: 0.5–0.9 mg ml^{-1}), and was used as a crude membrane fraction. All steps during the extraction were performed at 4°C.

The hydrolysis of [^{3}H]PIP_2 was measured in a reaction mixture (50 μl) that contained 35 m*M* NaH_2PO_4 (pH 6.8), 70 m*M* KCl, 0.8 m*M* EGTA, 0.8 m*M* $CaCl_2$ (25 μ*M* final Ca^{2+} concentration), 200 μ*M* PIP_2 (approximately 333 Bq), and 0.08% deoxycholate. After incubation at 30°C for 10 min, the reaction was stopped with 100 μl of 1% (w/v) BSA and 250 μl of 10% (w/v) TCA. Precipitates were removed by centrifugation (13 000 *g* for 10 min) and the supernatants were collected for quantification of the [^{3}H]IP_3 released by liquid scintillation counting Aquasol.

S-adenosylmethionine decarboxylase (SAMDC) assay

Roots were quickly frozen with liquid nitrogen and homogenized in buffer B: 50 μ*M* EDTA, 100 m*M* Tris.HCl pH 7.6, and 25 μ*M* pyridoxal phosphate (approximately 3 ml per 1 g root). Extracts were centrifuged at 26 000 *g* for 30 min and the supernatant was used for the assay. The $^{14}CO_2$ released from labelled *S*-adenosyl-l-methionine was measured in a reaction mixture that contained 100 μl of enzymatic extract and 15 μ*M* of *S*-adenosyl-L-[carboxyl-^{14}C]methionine (3700 Bq). Samples were incubated for 2 h at 37°C and the reaction was stopped with 20 μl of 25 (%) (w/v) TCA. The $^{14}CO_2$ released was absorbed on a piece of filter paper

(4×25 mm, folded) impregnated with 20 µl of NaOH (1 *M*). After 1 h incubation at room temperature, the filter paper was removed and placed in a scintillation fluid and the radioactivity was measured in a counter.

Pas extraction and quantification

Roots were homogenized in 5% (v/v) cold PCA (200 mg ml^{-1}), according to Tiburcio et al. (1986). The homogenate was kept on ice for 30–60 min before centrifugation at 27 000 *g* for 20 min. The supernatant obtained contains the free Pas, while the pellet containing conjugated Pas was resuspended in the original volume with 1 *M* NaOH by vortexing. The aliquot (250 µl) of the pellet suspension was mixed 1:1 (v/v) with 12 *M* HCl and hydrolysed at 110°C for 18 h. The filtered hydrolysates were resuspended in 250 µl PCA. The hydrolysed pellet contained Pas liberated from the conjugate. These fractions were dansylated using the method of Flores and Galston (1982) with modifications. Two hundred and fifty µl of each fraction or a standard were mixed with 400 µl of dansylchloride (5 mg ml^{-1} in acetone) and 200 µl of saturated Na_2CO_3. The mixtures were incubated in darkness overnight at 25°C. Excess dansyl reagent was removed by reaction with 100 µl of proline (100 mg ml^{-1}) and dansylpolyamines were extracted in 250 µl of benzene. Dansyl-Pas were separated on high-resolution silica gel TLC plates (Whatman LK6D), using choroform: triethylamine (25:2, v/v) solvent. The fluorescence of dansyl-Pas was measured by excitation at 365 nm in a spectrofluorometer.

Labelling and immunoprecipitation experiments

For [^{14}C]Spm uptake, roots at day 6 (1 g) were incubated in 20 ml of culture medium, previously filtered, containing 74 mBq [^{14}C]Spm (4.07 GBq mmol^{-1}, Amersham Biosciences, Piscataway, NJ, USA) and maintained in darkness at 25°C on a rotary shaker at 100 r.p.m. During incubation, roots were harvested at specified times and protein extraction was performed as previously described for PLC activity. Solubilized membrane extract (SME) was obtained from the crude membrane fraction by using 1% (w/v) n-octyl-1β-D glucopyranoside. The supernatant was recovered after sonication and centrifugation at 14 000 *g* for 30 min.

For immunoprecipitation, 500 µl of SME were incubated with 5 µg of antibovine PLC-δ antibody at 4°C overnight and gently stirred. The immunocomplex was captured after incubation with 100 µl of antimouse IgG-agarose for 2 h at 4°C. The agarose beads, collected by centrifugation (5 s at 14 000 *g*), were washed three times with a buffer containing 1% (v/v) Triton, 24 m*M* deoxycolic acid, 10% (v/v) SDS, 0.5 m*M* Tris. HCl and 150 m*M* NaCl and resuspended into Laemmli and subjected to 10% polyacrylamide gel electrophoresis (PAGE), according to the Laemmli method (Laemmli 1970) at 100V and subsequent Western-blot analysis. The radioactivity was also measured in the supernatant after immunoprecipitation.

For Western-blot analysis, immunoprecipitated proteins separated by electrophoresis were transferred to nitrocellulose membranes for 1 h at 100 V. After blocking for 1 h with blocking agent in Tris buffer saline Tween (TBST) and washing three times with TBST, the blot was incubated with a primary antibody (monoclonal antibody antibovine PLC-δ1, Upstate Biotechnology, Lake Placid, NY, USA) for 2 h at room temperature. After washing three times with TBST, the blot was incubated with a secondary antibody (peroxidase labelled antimouse antibody) and washed under the same conditions as described above. The chemiluminescence detection system (ECL) was used for visualization (Amersham Pharmacia Biotech).

Results

Effect of MGBG on growth of *C. roseus* transformed roots

To understand the physiological importance of the in vitro stimulatory effect of Spm on PLC activity (Echevarría-Machado et al. 2002) MGBG, a competitive inhibitor of SAMDC, was used in this work to vary the physiological concentration of the polyamines.

MGBG was added to the culture medium at three different times: days 0, 4 and 10 after the subculture day, and fresh root weight was measured at day 20 of culture. MGBG inhibited root growth independently of the day it was added to the roots (Fig. 1A–C). The extent of inhibition observed when the inhibitor was added at days 0 and 4 was the same (Fig. 1A,B). When MGBG was added at day 10, and the higher doses were used (500 and 1000 µ*M*) (Fig. 1C), the root growth was affected, although the inhibition was less when compared to day 0 and day 4. The effect of the inhibitor on root growth was dose-dependent in all cases (Fig. 1). Although MGBG affected root growth, a drastic effect on root morphology was observed when it was added at day 0 (Fig. 1, bottom panels). Roots growing in the presence of MGBG (500 and 1000 µ*M*) from day 0, showed a light yellow coloration and the characteristic cluster aggregation did not occur. This aggregation was completely recovered when MGBG was added at day 10 (Fig. 1, bottom panels).

To further investigate the temporal effect on root growth, MGBG was added at the subculture day and root FW was measured every 2 days until days 10 and 20. Three different concentrations of MGBG were used (250, 500 and 1000 µ*M*). After 6 days in the presence of the inhibitor, a significant reduction in fresh root weight was observed and this effect was also dose-dependent (Fig. 2). The larger inhibitory effect was observed at day 10 and did not change when the effect was evaluated after 20 days (Fig. 2).

A dose-response curve for the effect of MGBG was performed. In this experiment, increasing concentrations of the inhibitor were added to the culture medium at subculture day, and root growth was evaluated at day 20. A highly dependent inhibition was observed with a DL_{50} of 300 µ*M* (Fig. 3).

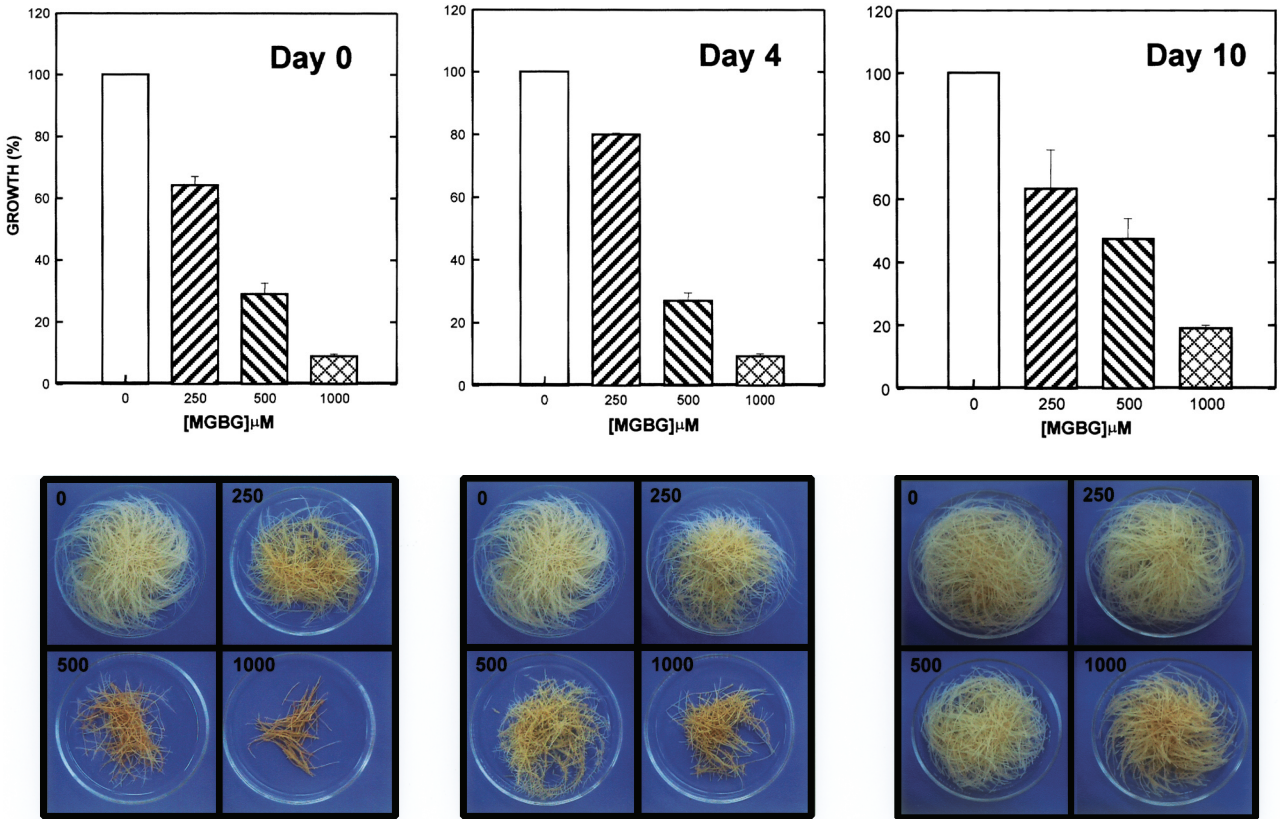

Fig. 1. Effect of MGBG on *C. roseus* transformed root growth. Increasing concentrations of MGBG (250, 500 and 1000 μ*M*) were added to the culture medium at 0, 4 and 10 days after subculture as indicated in Materials and methods. FW (upper panels) was evaluated at day 20 and the data presented as percentage of growth with respect to control (roots growing without inhibitor, which was 12 g). The morphology of the roots is presented in the lower panels.

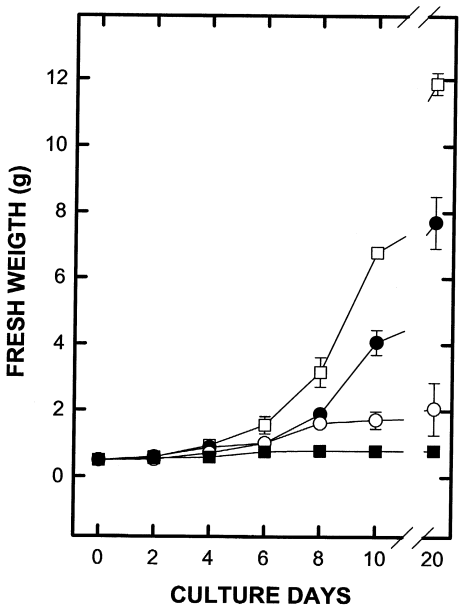

Fig. 2. Time-course for MGBG on *C. roseus*-transformed roots growth. Increasing concentrations of MGBG [(□) 0 (●) 250 (○) 500 and (■) 1000 μ*M* MGBG] were added to the culture medium at subculture day, and FW was evaluated every 2 days after the addition of the inhibitor.

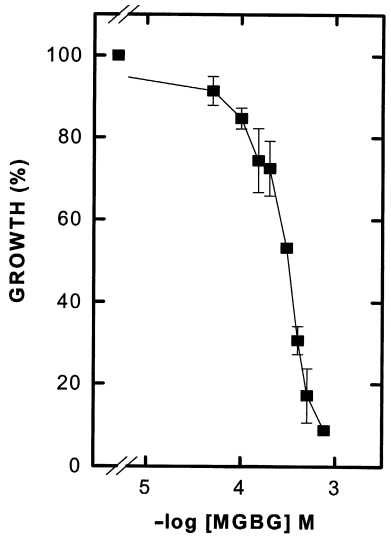

Fig. 3. Dose–response curve for MGBG on *C. roseus* transformed root growth. Increasing concentrations of MGBG were added to the culture medium at subculture day and FW was evaluated on day 20. Data are presented as percentage of growth in control roots (roots growing without inhibitor, which was 12 g).

MGBG may inhibit root growth because it is affecting either cellular division and/or differentiation. With the aim of achieving further knowledge of the effect of MGBG on root growth, a root stain was performed with basic fuchsine (Feulgen stain) to localize the meristematic centres. Figure 4 shows the results obtained when roots were incubated with 250 μ*M* MGBG for different periods (0, 2, 4 and 6 days). The reduction in the number and length of lateral roots was observed in the presence of the inhibitor. These changes were visible from day 2 (Fig. 4B,F,I). Control roots (roots growing without MGBG) formed lateral roots at this time while treated roots developed only sporadically (Fig. 4B,F,I). The difference in lateral root growth was more evident when the roots were incubated for longer periods (Fig. 4G,H,J,K).

Roots used at the subculture day had between 10 and 15 meristematic centres. Lateral roots originated from these centres and continued to proliferate throughout the culture period (Fig. 4A–D). Primary roots also grew, and new meristematic centres developed. When roots were treated with the inhibitor, not all the meristematic centres were able to develop lateral roots, affecting the process of proliferation that originates these roots (Fig. 4I–K).

The number of lateral roots was counted and the length and width of lateral roots and primary roots were measured, to confirm the inhibitory effect of MGBG on root growth. Six-day-old roots growing either with MGBG or without MGBG (control) were used and the evaluations were performed with the Rootedge program. An inhibition of primary root length by MGBG was also observed (Table 1). The greatest inhibition was obtained in the presence of 500 μ*M* MGBG, where a 70% reduction occurred; with this concentration the root width was also significantly affected. The primary root

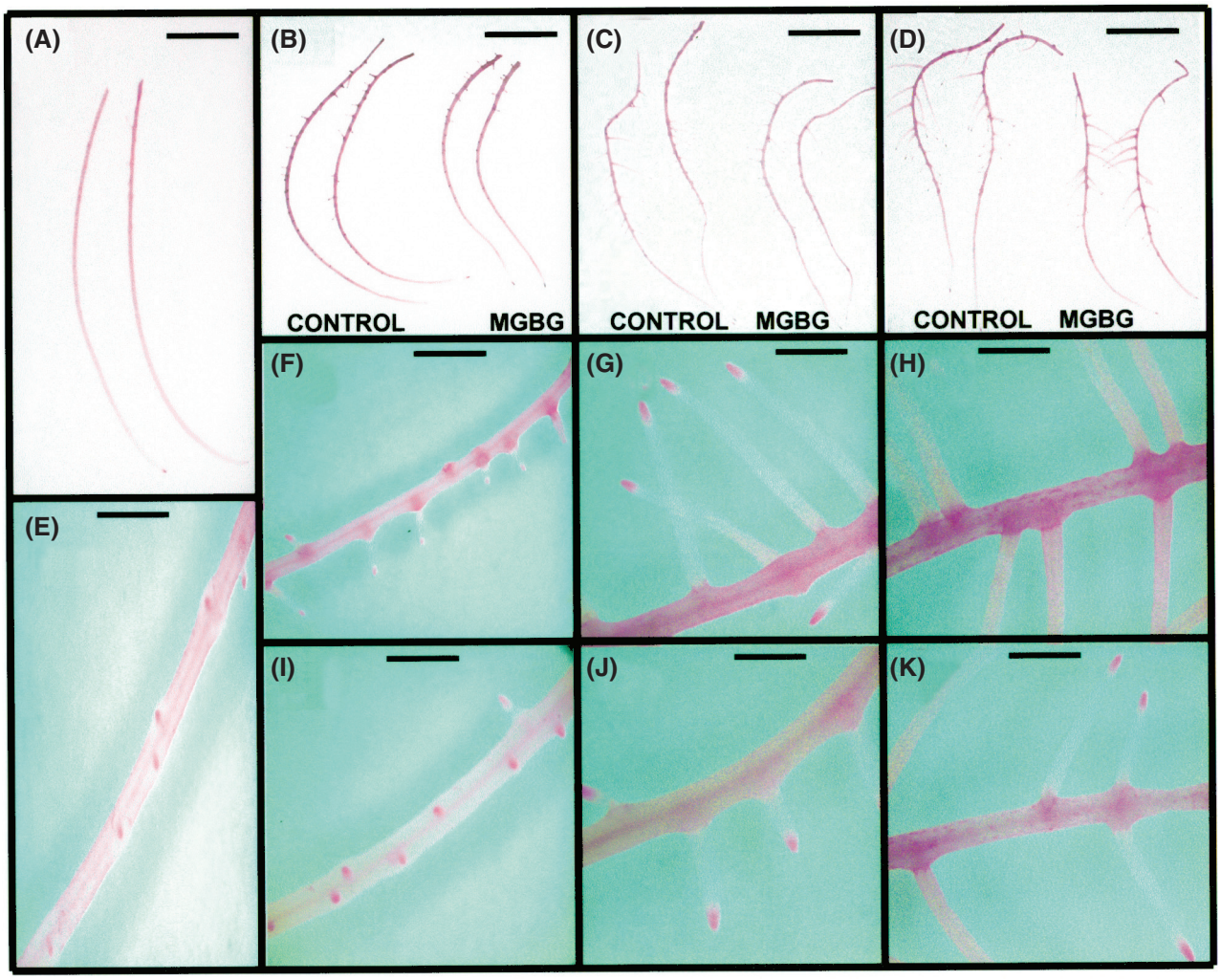

Fig. 4. MGBG effect on lateral root growth. Meristematic centres from roots growing without (control) or with 250 μ*M* of MGBG for 0, 2, 4 and 6 days were stained using Feulgen stain as indicated in Materials and methods. Roots from day zero (A), 2 (B), 4 (C) and 6 (D). (E–H) close-up at stereoscope from roots growing without inhibitor at same days cited above, respectively. (I–K) close-up at stereoscope from roots growing with inhibitor during 2, 4 and 6 days, respectively. Bars = 1 cm in (A), 0.7 cm in (B), (C), (D), 0.4 cm in (E), (F) and 0.3 cm in (G–K).

Table 1. Effect of MGBG on the number of lateral roots, length and width of both primary and lateral roots. Six-day-old *C. roseus* transformed roots growing with or without MGBG were used and the evaluation was performed with the Rootedge program. The results shown are means of five separate experiments ± SE.

[MGBG] μ*M*	0	250	500	1000
Principal Root				
Length (cm)	6.86 (1.37)	4.42 (0.81)	2.08 (0.18)	4.36 (0.24)
Width (mm)	0.53 (0.07)	0.42 (0.03)	0.23 (0.01)	0.58 (0.03)
Lateral Roots (LR)				
Total Number of LR	13.8 (3.12)	10.6 (2.24)	5.2 (0.98)	0.25 (0.43)
No. LR > 1 cm	3.8 (2.4)	0	0	0
No. LR 0.5–1 cm	3.6 (1.02)	2 (1.26)	0	0
No. LR < 0.5 cm	6.44 (1.62)	8.6 (1.36)	5.2 (0.98)	0.25 (0.43)
LR < 0.5 cm				
Length (cm)	0.26 (0.06)	0.30 (0.04)	0.06 (0.02)	0.03
Width (mm)	0.31 (0.06)	0.29 (0.03)	0.13 (0.01)	0.3
LR 0.5–1 cm				
Length (cm)	0.76 (0.07)	0.60 (0.04)	0	0
Width (mm)	0.36 (0.07)	0.30 (0.06)	0	0
LR > 1 cm				
Length (cm)	1.28 (0.15)	0	0	0
Width (mm)	0.36 (0.08)	0	0	0

length in the treatment with 1000 μ*M* MGBG was only slightly lower when compared to the control.

Lateral root formation was severely affected with MGBG; this effect was dependent on the inhibitory dose. Although no significant effect was observed in the primary roots with 1000 μ*M* MGBG, only one lateral root was formed under this condition (Table 1). Lateral roots were grouped by length for the analysis. Lateral roots with a length of > 1 cm were only produced in the control, while lateral roots of between 0.5 and 1 cm were observed in the control and in the roots treated with 250 μ*M* MGBG. In the presence of MGBG these roots had a less that average length when compared to the control (Table 1). Shortened lateral roots (<0.5 cm) were produced in all treatments; however, their average length diminished with higher doses of the inhibitor. The width of the roots was only significantly affected in the presence of 500 μ*M* MGBG (Table 1). These results confirmed that MGBG inhibited root growth, as well as affecting the lateral root number.

Reversion by polyamines of MGBG root growth inhibition

Roots were incubated or not (control) with 300 μ*M* MGBG on the subculture day and increasing concentrations of polyamines were added to MGBG-treated *C. roseus*-transformed root culture. The effect on root growth was evaluated as FW. In this experiment, specific concentration ranges for each polyamine were used: Spd (50–200 μ*M*) and Spm (10–150 μ*M*). These ranges were selected according to the physiological concentration range of these polyamines found in the roots (Echevarría-Machado et al. 2002).

The addition of Spd to the roots treated with 300 μ*M* MGBG resulted in a partial recovery of root growth (Fig. 5A). The greatest recovery was obtained when 150

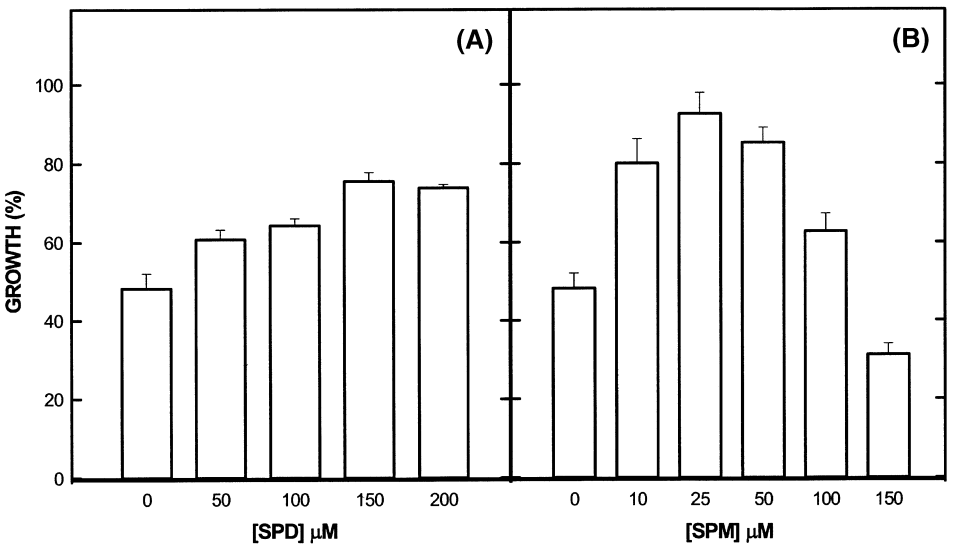

Fig. 5. Pas reversed the effect of MGBG on root growth. Increasing concentrations of Spd (A) or Spm (B) were added to roots growing in the presence of 300 μ*M* MGBG and FW was measured at day 20. Data are expressed as percentage of root growth respect to control roots (roots growing without either inhibitor or Pas, where 100% was 12 g).

and 200 μ*M* Spd was used in which the root growth was approximately 75% of the control; which represented a recuperation of >25% compared to MGBG-treated roots. However, the addition of Spm to the inhibitor-treated roots resulted in a total recovery of root growth (Fig. 5B). The reversion was almost complete in the culture supplemented with 25–50 μ*M* Spm. A higher dose of Spm (150 μ*M*) added to the MGBG-treated roots inhibited the root growth more drastically (70%) than only MGBG (50%) (Fig. 5B).

Effect of MGBG on SAMDC activity and Pas content

The finding that the growth of MGBG-treated roots was restored to essentially the same level of the control roots when Spm was added, implied that the inhibition of the root growth by MGBG was caused by the inhibition of polyamine metabolism. To confirm this, an experiment was performed to analyse the effect of MGBG on SAMDC activity and polyamine content. Increasing concentrations of the inhibitor (250, 500 and 1000 μ*M*) were added to the culture medium on subculture day, and SAMDC activity and Pas levels were evaluated at day 6, since we already knew that the roots had higher Pas content on this day (Echevarría-Machado et al. 2002).

SAMDC activity was drastically diminished when roots were treated with MGBG when compared with untreated roots; the inhibition of the activity was dose-dependent (Fig. 6A). A decrease in SAMDC activity of >90% was obtained with MGBG treatments, indicating a specific inhibition of MGBG on SAMDC in vivo.

To confirm that Pas levels were inhibited in the presence of MGBG, they were quantified in MGBG-treated roots at day 6. Conjugated Pas were not modified by MGBG treatment (data not shown); while free Pas were affected by the inhibitor (Fig. 6B). Put levels increased in the MGBG-treated roots, the increase was marked when 1000 μ*M* MGBG was used. A significant reduction in Spd and Spm levels was observed with MGBG. The greatest inhibition on Pas contents was induced with the higher dose of the inhibitor. These results strongly suggest that the effect of MGBG on root growth is due to regulation of Pas metabolism via an inhibition of SAMDC activity.

PLC activity

In the present work we present evidence of the physiological role of PLC regulation by Pas. Roots were cultured in the presence of increasing doses of MGBG (250, 500 and 1000 μ*M*) and after 6 days PLC activity was evaluated in the MGBG-treated roots, where the Spm levels diminished. Membrane PLC activity decreased in roots treated with 500 and 1000 μ*M* of MGBG (Fig. 7A). Surprisingly, the activity was reduced by approximately 20 and 50%, with 500 and 1000 μ*M*, respectively. Under these conditions Spm levels were reduced to 60 and 80% (Fig. 6B). It is possible that PLC activity is not activated because Spm levels were reduced in the MGBG treated-roots, but also because the protein levels could be reduced in the presence of the inhibitor.

To discard the possibility that the effect of MGBG on PLC activity was due to a reduction in the protein levels, a Western-blot analysis was performed using an anti-bovine PLC-δ antibody. MGBG at 500 and 1000 μ*M* did not modify the detected signal (Fig. 7B). This band was quantified in a Phosphorimager (Bio-Rad Laboratories, Hercules, CA, USA). This analysis confirmed that there

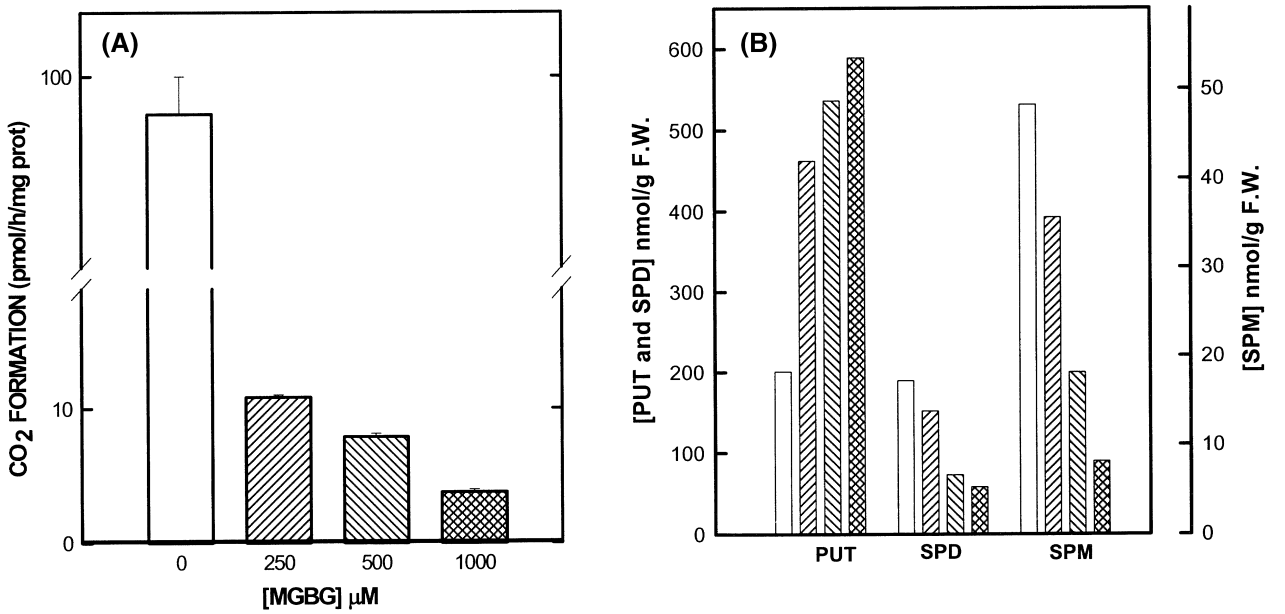

Fig. 6. Effect of MGBG on SAMDC activity and free polyamine levels. Different doses of MGBG were added to the culture medium and after the 6th day, SAMDC activity (A) and free Pas levels (B) were measured. [(□) 0, (▨) 250, (▧) 500, and (▩) 1000 μ*M* MGBG].

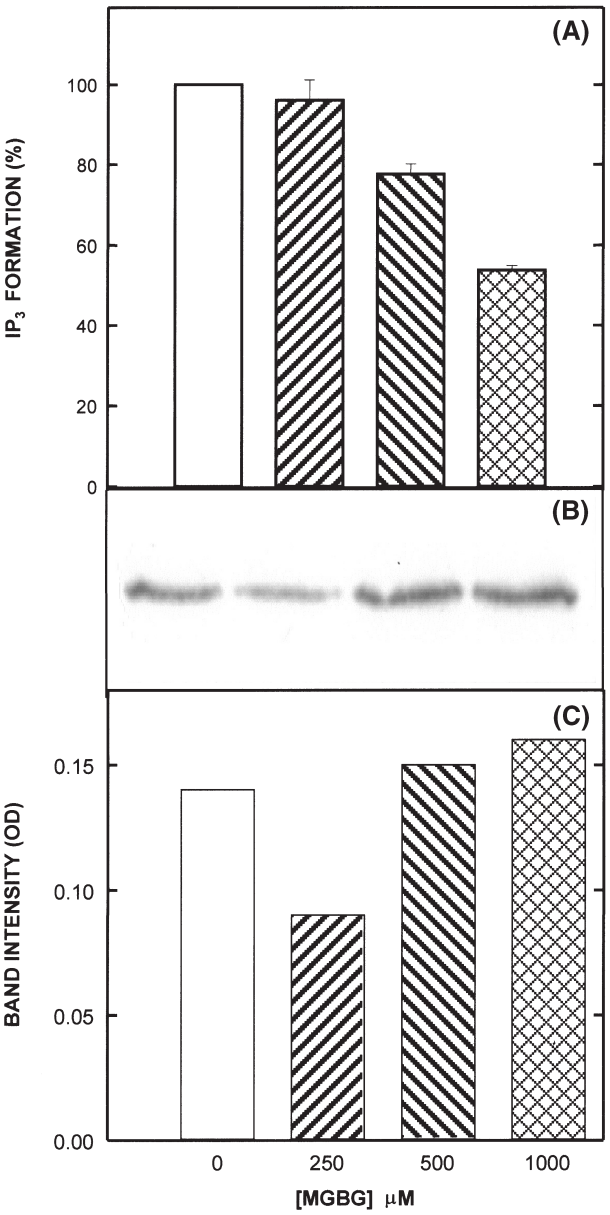

Fig. 7. Effect of MGBG on PLC protein levels and activity. Roots were cultured in the presence of MGBG and after 6 days, PLC protein levels and activity was evaluated. PLC activity (A) as IP_3 formation percentage of basal which was 49.7 nmol min^{-1} mg^{-1} prot); PLC protein levels were determined by immunodetection using antibovine PLC-δ antibody (B) and the band was quantified in a Phosphorimager (C).

is not a significative change in the levels of this polypeptide in these concentrations, only a polypeptide change at 250 μ*M* was observed (Fig. 7C). MGBG did decrease PLC activity without altering the protein levels.

[^{14}C]Spm uptake by *C. roseus* transformed roots

The data presented here suggest that Spm could regulate PLC activity by acting directly on the enzyme. For this reason, a labelling-immunoprecipitation experiment was performed.

Six-day-old roots were incubated with 74 mBq [^{14}C]Spm for different periods. After the exposure time, the roots were harvested and the distinct cellular fractions were obtained, as indicated in Materials and methods. Aliquots of these fractions were collected and the radioactivity was measured. The radioactivity was also measured from the culture medium after the roots were harvested. [^{14}C]Spm was rapidly incorporated by *C. roseus*-transformed roots (Fig. 8). More than 80% of the initial radioactivity in the culture medium was taken up by the roots within 2 h of exposure (Fig. 8).

The radioactivity incorporated into the roots was approximately equally distributed between cellular debris and the total protein extract (Table 2). The radioactivity present in cellular debris increased with the exposure time. The greatest amount of radioactivity incorporated into the total protein extract was found in the soluble fraction (approximately 90%); the remainder was associated with the membrane fraction. Membrane-associated radioactivity was predominantly extracted with the detergent glucopiranoside (Table 2).

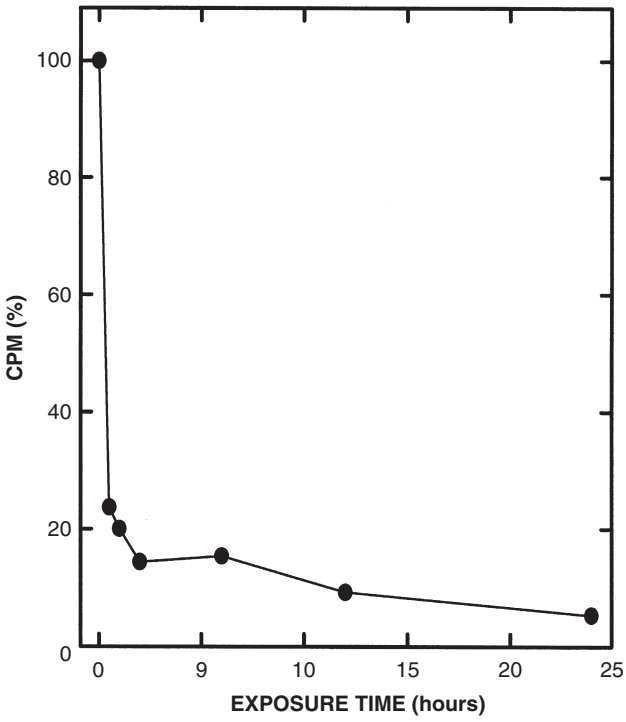

Fig. 8. [^{14}C]Spm uptake for *C. roseus* transformed roots. Six-day-old roots were incubate with 74 mBq [^{14}C]Spm for different periods of time as indicated in Materials and methods. Figure shows radioactivity amount from culture medium after roots were harvested and the data presented as percentage where 100% corresponded to the total radioactivity amount added to the media (approximately 15 000 kcpm).

Table 2. Distribution of radioactivity from [^{14}C]Spm incorporated into *C. roseus* transformed roots. Six-day-old roots were incubated with 74 mBq [^{14}C]Spm during different periods of time and the radioactivity was measured in different cellular fractions.

Exposure Time (hours)	Cellular Debris	Total cpm (x 1000) Total Extract	Soluble Extract	Total Membrane Fraction	Solubilized Membrane Fraction
0.5	1168.85	1317.30	1061.23	75.84	73.80
1	1343.88	1368.84	1268.79	118.73	82.67
2	1085.25	1122.55	818.29	121.76	119.09
6	1444.89	998.21	252.89	117.17	103.77
12	1517.17	1127.36	1097.60	116.21	107.71
24	1688.62	921.71	830.04	120.28	105.42

Spm–PLC interaction

The total membrane extract solubilized with detergent was obtained from 6-day-old roots incubated with [^{14}C]Spm for different periods, as indicated in Materials and methods. PLC from this fraction was immunoprecipitated using an antibovine PLC-δ antibody. The immunoprecipitated fraction was washed three times with a high astringent buffer to eliminate non-specific binding, and an aliquot was used to quantify radioactivity. Radioactivity in the immunoprecipitate was detected only when extracts from roots incubated for short periods with [^{14}C]Spm were used (Fig. 9A). After 30 min of incubation of the roots with [^{14}C]Spm, the radioactivity found in the PLC immunoprecipitated was approximately 12 000 cpm, representing 10% of the total radioactivity present in the solubilized membrane fraction. When the roots were exposed for >2 h, the radioactivity was similar to negative control (immunoprecipitate without antibody).

An immunodetection experiment from the proteins present in the immunoprecipitate was performed using an antibovine PLC-δ antibody (Fig. 9B). Two proteins of molecular weights 57 and 67 kDa were detected.

Discussion

There have been reports of several methods for perturbing the polyamine synthesis pathway, in order to study it's function and other properties, including the use of inhibitors, transgenic organisms and mutants or somaclonal variants (Kumar et al. 1997, Walden et al. 1997, Watson et al. 1998). MGBG, a competitive inhibitor of SAMDC, was used in the present work to determine its effect on polyamine content, PLC activity and root growth. *C. roseus*-transformed roots are an excellent model system for this investigation; the high branching capacity and the presence of many meristematic points in transformed roots account for their fast growth rate.

In this work, MGBG caused a reduction in root growth. This effect was the same when MGBG was added on the first days of the culture cycle (day 0 and day 4) (Fig. 1). By day 4, the roots doubled their initial mass; after day 6, the roots began the growth exponential phase, and on day 10, 50% of the total growth was obtained. We have previously reported that polyamines accumulated between days 2 and 6 of the root culture cycle (Echevarría-Machado et al. 2002). The effect of MGBG on root growth in the first days of culture indicate that polyamines could be required as a signal to activate the root growth process (Figs 1A,B and 2);

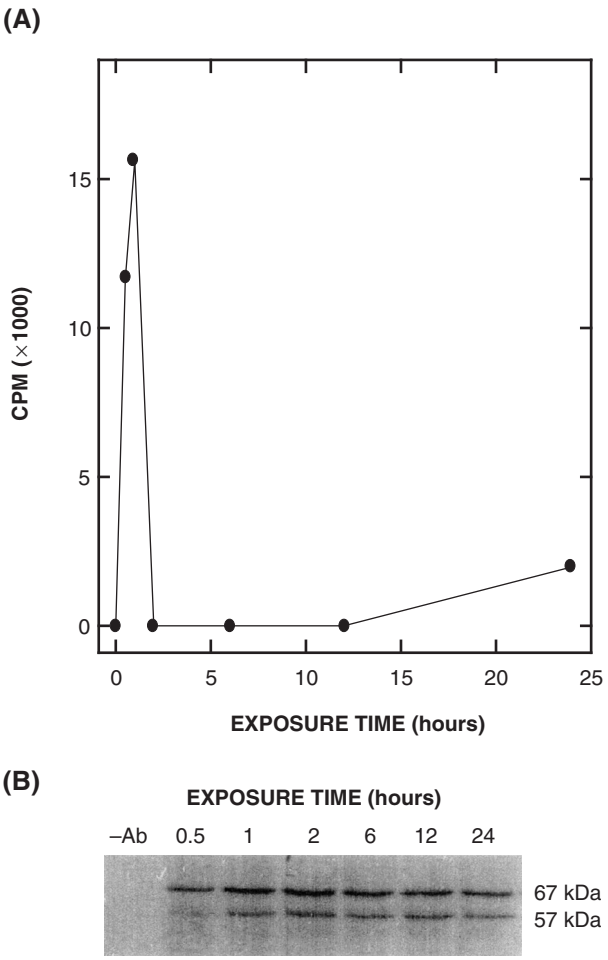

Fig. 9. Spm co-immunoprecipitate with PLC in *C. roseus* transformed roots. PLC was immunoprecipitated using an antibovine PLC-δ antibody from roots incubated with 74 mBq [^{14}C]Spm for different periods of time and radioactivity present in the immunoprecipitate was measured (A). Western-blot analysis from immunoprecipited samples (B). (–Ab) Immunoprecipitation without the antibovine PLC-δ antibody.

when the increase in Pas levels does not occur in this period, growth does not proceed (Fig. 2).

Meristematic points were observed in the roots growing in the presence of all the MGBG doses used, but the formation of lateral roots was delayed or inhibited, depending on the doses used (Fig. 4; Table 1), suggesting that MGBG affects the proliferative process that originates lateral roots. However, further histological analysis is necessary to understand the effect of this inhibitor on the developmental sequence of lateral root formation. MGBG has been reported to modify Pas metabolism affecting plant growth and development in other species (Minocha et al. 1991, Kurosaki et al. 1992).

In the present work, polyamines Spd and Spm were independently effective in restoring root growth. The recovery with Spd was partial, while Spm was able to restore growing activity of the roots to control levels (Fig. 5). It is unknown if Spd was metabolized to Spm or if this amine partially restored the growth per se; more work is needed to identify the role of Spd as a growth regulator in transformed roots. The results in Fig. 5B indicate the important role of Spm on *C. roseus*-transformed roots growth, fundamentally on lateral root growth and formation. A dose–response curve exploring the effect of both of the Pas on root growth was previously performed. Spd did not have a significant effect on root growth in the dose range used (50–200 μM, data not shown); while Spm had only a stimulatory effect at 50 μM, and higher doses drastically inhibited root growth (Echevarría-Machado et al. 2002).

We have observed that *C. roseus*-transformed roots are more sensitive to high concentrations of Spm than of the other Pas (Put or Spd) when these Pas were added exogenously. The endogenous levels of Put and Spd were approximately 3-fold higher in the tissues than Spm levels in the roots (Echevarría-Machado et al. 2002). The negative effect of Spm on root growth can be produced by an excess of positive charges in the tissues; this situation may affect complex processes such as membrane potential and uptake of nutrients, membrane stability and integrity, enzymatic activities, and other events (Bograh et al. 1997, Fromm et al. 1997).

Put levels increased in the presence of MGBG (Fig. 6B), but these levels probably do not affect root growth because with the exogenous addition of Put, the endogenous concentration increased up to 1 mM without any effect on root growth (data not shown). However, the Put/Spd ratio is important for root growth (Schwartz et al. 1986). A low ratio characterized the meristematic apical segment of maize root, while the reverse was true in the more differentiated subapical zone (Schwartz et al. 1986). This phenomenon could be studied in *C. roseus* roots. However, we did not detect a gradient in the Pas levels from the roots (data not shown). The reduction of Put content in roots when DFMO was used inhibited growth, also indicating a possible role of this polyamine (Echevarría-Machado et al. 2002).

It was recently suggested that Pas could interact with the phosphoinositide-Ca^{2+} signal transduction pathway (Dureja-Munjal et al. 1992, McDonald and Mamrack 1995, Singh et al. 1995). We reported that PLC activity from *C. roseus* transformed roots could be regulated during the culture cycle of the roots (De Los Santos-Briones et al. 1997) and in in vitro experiments only Spm stimulated membrane PLC activity (Echevarría-Machado et al. 2002). These results suggest that the effect of Spm on root growth may be due to a modification of a signal transduction pathway linked to PLC.

In the present work, membrane PLC activity was inhibited in roots treated with 500 and 1000 μM of MGBG (Fig. 7A). Under these conditions, free Spm levels were maintained below 20 μM (Fig. 6B). No effect on PLC activity was observed when a similar concentration of Spm was used in an in vitro assay (Echevarría-Machado et al. 2002). Since the effect of MGBG on PLC activity was not due to a reduction in protein levels (Fig. 7B,C), we suggested that when Spm levels were reduced by MGBG treatments, PLC activity was regulated and that this may be the reason for a decrease in PLC activity in the roots growing in the presence of MGBG. We observed a direct relationship between Spm levels-PLC activity-root growth by an increase or decrease on Spm levels (Echevarría-Machado et al. 2002). This behaviour could be supported by the existence of a direct interaction between polyamine and the enzyme in vivo.

When PLC was immunoprecipitated from roots incubated with [^{14}C]Spm, the radioactivity was detected in the immunoprecipitate, after only 30 or 60 min of incubation with [^{14}C]Spm (Fig. 9A). This result strongly indicates that a transient interaction between Spm and PLC enzyme may occur in *C. roseus*-transformed roots. The detection of radioactivity into the solubilized membrane fraction reached a peak after 2 h, differing with the behaviour for the radioactivity detected in the immunoprecipitate, suggesting that the interaction between Spm and PLC is very specific and time-dependent. However, we do not know if Spm is being metabolized in the tissue.

A high amount of radioactivity was presented in the cellular debris (Table 2). The interaction of Pas with the cell wall components has been extensively studied (Mariani et al. 1989, Messiaen et al. 1997, Laurenzi et al. 1999, Messiaen and Van Cutsem 1999), but the function of Pas in this compartment has not been explained. However, it was suggested that pectin-associated Pas is one of many factors that might regulate cell wall expansion by changing the cell wall pH (D'Orazi and Bagni 1987); this association could also result in pectic signal modulation in pathogenesis and in differentiation (Messiaen et al. 1997, Messiaen and Van Cutsem 1999). The degradation of Pas by diamine and polyamine oxidase in the cell wall might be involved in the release of hydrogen peroxide and in the lignification of the cell wall (Laurenzi et al. 1999).

A significant amount of radioactivity was found in soluble extracts (Table 2). In this case, the effect of Pas on enzymatic activities, fundamentally nuclear and

cytosolic protein kinases, has been reported (Polya and Micucci 1985, Datta et al. 1986, Bothma and Dubery 1991, Ye et al. 1994, Chang and Kang 1999). However, it is very important to point out that in the events mentioned above, the effect was independent of the polyamine being used, indicating that the polyamines are acting as general charged agents.

In summary, at day 6 of the culture cycle Spm could be an intracellular signal to start the root growth process. We suggest that this function is mediated by stimulation of PLC activity, performed by this polyamine. The events succeeding downstream of PLC activation by Spm are unknown: variations in the inositol phospholipids levels, increase in IP_3, Ca^{2+} or hyperphosphorylated inositols levels could be the signal that active a cascade bearing at the started of root growth.

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