Polyamines as Physiological Regulators of 14-3-3 Interaction with the Plant Plasma Membrane H^+-ATPase

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Polyamines are abundant polycationic compounds involved in many plant physiological processes such as cell division, dormancy breaking, plant morphogenesis and response to environmental stresses. In this study, we investigated the possible role of these polycations in modulating the association of 14-3-3 proteins with the H^+-ATPase. In vivo experiments demonstrate that, among the different polyamines, spermine brings about 2-fold stimulation of the H^+-ATPase activity and this effect is due to an increase in 14-3-3 levels associated with the enzyme. In vivo administration of polyamine synthesis inhibitors causes a small but statistically significant decrease of the H^+-ATPase activity, demonstrating a physiological role for the polyamines in regulating the enzyme activity. Spermine stimulates the activity of the H^+-ATPase AHA1 expressed in yeast, in the presence of exogenous 14-3-3 proteins, with a calculated $K_{d}$ of 70 $\mu$M. Moreover, spermine enhances the in vitro interaction of 14-3-3 proteins with the H^+-ATPase and notably induces 14-3-3 association with the unphosphorylated C-terminal domain of the proton pump. Comparison of spermine with Mg^{2+}, necessary for binding of 14-3-3 proteins to different target proteins, shows that the polyamine effect is stronger than and additive to that of the divalent cation.

Keywords: H^+-ATPase — Polyamines — 14-3-3 proteins — Zea mays.

Abbreviations: AHA1 Arabidopsis thaliana H^+-ATPase isoform 1; bis-ANS, 4',4'-bis(1-anilinonaphthalene 8-sulfonate); CHA, cyclohexylamine; DFMO, $\alpha$-difluoromethyloctathione; GST, glutathione S-transferase; MGBG, methylglyoxal bis-guanylhydrazone; MHA2 Maize H^+-ATPase isoform 2; PI, polyamine inhibitor.

Introduction

Polyamines are low molecular weight organic cations having two or more primary amino groups. Widespread in eukaryotes, they are involved in various physiological and developmental processes. In plants, the di-amine putrescine, the tri-amine spermidine and the tetra-amine spermine are frequently present in amounts varying from micromolar to more than millimolar, and they are implicated in processes such as stimulation of cell division, response to environmental stresses, regulation of rhizogenesis, embryogenesis, senescence, floral development and fruit ripening (Evans and Malmberg 1989, Kakkar and Rai 1993, Galston et al. 1997, Bouchereau et al. 1999, Kakkar et al. 2000, Kakkar and Sawhney 2002).

The properties and functions of polyamines can be explained by their polycationic character. Interactions of polyamines with phosphate groups of DNA, with anionic components of phospholipids and with cell wall components, such as pectic polysaccharides, have been reported (Tabor and Tabor 1984, Edreva 1996, Walden et al. 1997). Recently it has been demonstrated that polyamines can substitute for divalent cations in modulating the interaction of 14-3-3 proteins with nitrate reductase (Provan et al. 2000, Athwal and Huber 2002, Shen and Huber 2006).

14-3-3 proteins belong to a highly conserved protein family with regulatory roles in all eukaryotes (Aitken 1996, Fu et al. 2000, van Hemert 2001, Aducci et al. 2002). They regulate the cell cycle, differentiation and apoptosis, and coordinate multiple signal transduction pathways in animals (Fu et al. 2000, van Hemert et al. 2001, Tzivion and Avruch 2002). They interact with a broad range of target proteins, and the consequences of binding are diverse: stabilization of the active or inactive proteins, conformational change, different subcellular localization, etc. (Muslin et al. 1996, Yaffe et al. 1997, van Heusden and Steensma, 2006). 14-3-3 proteins bind their targets in a phosphorylation-dependent manner (Muslin et al. 1996, Yaffe et al. 1997), even though proteins interacting in a phosphorylation-independent manner have also been identified (Fu et al. 2000).

In plants, a number of peculiar functions have been ascertained, such as regulation of primary metabolism, ion transport, cellular trafficking and gene transcription. The plasma membrane H^+-ATPase is one of the 14-3-3 targets (Aducci et al. 2002). It is the pivotal enzyme for generation of the electrochemical gradient across the plasma membrane that provides the driving force for a number of key physiological processes such as stomata opening, phloem loading and root ion uptake (Palmgren 1998). Regulatory 14-3-3 proteins have been demonstrated to associate with the C-terminal autoinhibitory domain of
the proton pump (Jahn et al. 1997, Fullone et al. 1998), thereby leading to its displacement and consequently to H\(^{+}\)-ATPase activation (Baunsgaard et al. 1998). The H\(^{+}\)-ATPase-binding site for 14-3-3 proteins is generated upon phosphorylation of a conserved threonine residue within the sequence YTV, located at the very end of the C-terminus (Fuglsang et al. 1999, Svennelid et al. 1999).

The fungal toxin fusicoccin promotes the irreversible association of 14-3-3 proteins with the H\(^{+}\)-ATPase, thus producing a strong activation of the proton pump (Jahn et al. 1997, Oecking et al. 1997, Baunsgaard et al. 1998, Fullone et al. 1998, Fuglsang et al. 1999).

Besides phosphorylation–dephosphorylation of target proteins, other mechanisms can play a general role in the regulation of the interaction. These include phosphorylation of 14-3-3 proteins themselves (Yaffe and Smerdon 2004), endogenous effectors such as AMP (Kaiser et al. 1992, Athwal et al. 2000, Camoni et al. 2001), as well as 14-3-3 isoform specificity or their compartmentalization.

Furthermore, it has been demonstrated that divalent cations or polyamines are required to stabilize 14-3-3 binding to their targets. It is known that Mg\(^{2+}\) can enhance 14-3-3 binding to the nitrate reductase (Athwal et al. 2000) and that the H\(^{+}\)-ATPase–14-3-3 complex as well as the sucrose phosphate synthase–14-3-3 complex are Mg\(^{2+}\) dependent (Fullone et al. 1998, Moorhead et al. 1999).

The present study shows that in vivo and in vitro 14-3-3 binding to the H\(^{+}\)-ATPase is increased by the presence of polyamines, and, among the different polyamines tested, the tetra-amine spermine is the most active.

Moreover, it is demonstrated that spermine treatment increases the interaction of 14-3-3 with H\(^{+}\)-ATPase or with its C-terminal domain. The effect induced by spermine was stronger than that of the cation Mg\(^{2+}\).

**Results**

*Spermine in vivo treatment increases the activity of the proton pump H\(^{+}\)-ATPase*

The effect of the exogenous polyamines putrescine, spermidine, cadaverine and spermine was assayed on the phosphohydrolytic activity of plasmalemma H\(^{+}\)-ATPase from maize roots. Short segments of maize roots after 5 d of growth were incubated for 1 h with 0.2 mM polyamines. Then, the plasma membranes were purified and the phosphohydrolytic activity measured. The results, reported in Fig. 1, show that spermine treatment induces significant stimulation of H\(^{+}\)-ATPase activity (about 200%). The other polyamines, putrescine, spermidine and cadaverine, however, are ineffective.

Some inhibitors of polyamine synthesis were used to lower the endogenous concentration of free polyamines. Maize root segments were incubated for 1 h with the polyamines inhibitors: α-difluoromethylornithine (DFMO), cyclohexylamine (CHA) and methylglyoxal bis-guanylhydrazone (MGBG). The resulting phosphohydrolytic activity, measured on plasma membranes obtained after treatment with PI (polyamine inhibitor) membranes or spermine membranes, is reported in Fig. 2a. The activity in PI membranes is slightly reduced, suggesting a possible effect of endogenous polyamines.

Western blotting with anti-H\(^{+}\)-ATPase antibody demonstrates that the protein level of H\(^{+}\)-ATPase in membranes from spermine- and PI-treated roots is comparable with that of the control (Fig. 2b1); thus the effect of the polyamines and their inhibitors on the proton pump activity may be ascribed to H\(^{+}\)-ATPase regulation following 14-3-3 binding. This was verified by analyzing the level of 14-3-3 proteins associated with the plasma membranes. Western blotting with anti-14-3-3 antibody (Fig. 2b2), in fact, shows a higher amount of 14-3-3 proteins in spermine membranes compared with control and with PI membranes.

The effect of spermine on the association of 14-3-3 proteins with the H\(^{+}\)-ATPase was investigated by means of an overlay assay. In this system, the \(^{32}\)P-labeled GF14-6, a 14-3-3 maize isoform, was used as a probe, and the H\(^{+}\)-ATPase from maize roots was used as a bait. The results are reported in Fig. 2c. The 14-3-3 association is strongly increased in spermine membranes compared with the control, whereas it appears significantly reduced in PI membranes. The higher interaction can be explained by a higher phosphorylation level of the proton pump which is preserved by 14-3-3 binding, in turn induced by the in vivo spermine treatment. These results suggest that the stimulating effect of spermine on the H\(^{+}\)-ATPase

![Fig. 1 Effect of polyamines on H\(^{+}\)-ATPase activity. Plasma membranes (50 µg of total protein) from control (C) roots and from roots treated with cadaverine (Cd), putrescine (Put), spermidine (Spd) and spermine (Spm) were incubated with 2 mM ATP in 0.5 ml of 50 mM Tris–HCl (pH 7.2) buffer containing 5 mM MgCl\(_2\), 1 mM DTT, 50 mM KNO\(_3\), 2 mM NaN\(_3\) and 0.2 mM (NH\(_4\))\(_6\)Mo\(_7\)O\(_{24}\). The illustrated data represent activity means ± SE for two independent experiments run in duplicate.](https://academic.oup.com/pcp/article-abstract/48/3/434/2329608/573x603)
activity is due to an increased binding of 14-3-3 proteins to the proton pump.

**Spermine in vitro treatment increases the interaction of 14-3-3 proteins with \( H^+\)-ATPase and its C-terminal domain**

The effect of polyamines on the 14-3-3-induced \( H^+\)-ATPase activation was tested in vitro by using the AHA1 (Arabidopsis thaliana \( H^+\)-ATPase isoform 1) \( H^+\)-ATPase of \( A. \ thaliana \) expressed in yeast (Regenberg et al. 1995). Since AHA1 localizes mainly in yeast endoplasmic reticulum, this cellular compartment has been purified by sucrose gradient and used in the ATP phosphohydrolytic assay. In this system, a specific and reproducible \( H^+\)-ATPase stimulation is detectable after addition of exogenous 14-3-3 proteins (Baunsgaard et al. 1998).

The administration of different (0.005–1 mM) concentrations of polyamines to a mixture containing endoplasmic reticulum vesicles and the 14-3-3 isoform GF14-6 enhances the AHA1 activity (Fig. 3a). Spermine is effective at micromolar concentrations, the saturation being at 0.2 mM and the half-maximal stimulation (\( S_{50} \)) at approximately 70 \( \mu \)M. Spermidine, cadaverine and putrescine, however, are inactive.

The effect of spermine on the direct interaction of 14-3-3 proteins with the \( H^+\)-ATPase was tested by an overlay assay. Fig. 4a shows that spermine is able to stimulate the association of 14-3-3 proteins with the enzyme. The spermine effect is comparable with that of the fungal toxin fusicoccin, known to stabilize the 14-3-3–\( H^+\)-ATPase interaction (Baunsgaard et al. 1998, Fullone et al. 1998).

The C-terminal domain of the MHA2 isoform of maize \( H^+\)-ATPase, corresponding to the last 103 amino acids of the sequence (residues 845–947), is known to interact with 14-3-3 proteins but only in the presence of physiological concentrations of fusicoccin. The spermine effect on the interaction between 14-3-3 and the \( H^+\)-ATPase C-terminus was tested by overlay assay (Fig. 4b). Spermine allows a strong interaction, as does fusicoccin, while the 14-3-3 probe does not interact with the C-terminus in the presence of spermidine, putrescine and cadaverine (data not shown).

**Comparison of spermine with \( Mg^{2+}\) activation**

The role of spermine in inhibition of nitrate reductase by 14-3-3 proteins is similar to that achieved with excess \( Mg^{2+}\) (Athwal and Huber 2002). This evidence suggests that spermine could mimic divalent cations in promoting the 14-3-3-dependent activation of \( H^+\)-ATPase activity. As it is known that the binding of 14-3-3 to the proton pump is abolished by omitting \( Mg^{2+}\) from the incubation medium (Fullone et al. 1998), we conducted an overlay assay to examine the differences between \( Mg^{2+}\) and
spermine. As shown in Fig. 5, addition of 0.2 mM spermine increases the 14-3-3–H\textsuperscript{+}-ATPase interaction more than does a saturating concentration of Mg\textsuperscript{2+} (5 mM). The addition of both spermine and Mg\textsuperscript{2+} further enhances the interaction.

These data were confirmed by testing the capability of GF14-6 to bind a biotinyl-peptide reproducing the last 15 amino acids of the H\textsuperscript{+}-ATPase isoform MHA2, which contains the phosphorylated 14-3-3-binding site. The peptide was immobilized on a streptavidin-agarose resin and incubated with \textsuperscript{32}P-labeled GF14-6 in the absence or presence of spermine and/or Mg\textsuperscript{2+}. The results (Table 1) show that 14-3-3 binding to the phosphorylated peptide is significantly increased by spermine (40\%). The spermine effect is higher than that achieved by Mg\textsuperscript{2+}. As observed with the full-length enzyme, the addition of both spermine and Mg\textsuperscript{2+} further enhances the interaction.

Binding of divalent cation or spermine to the recombinant Arabidopsis GF14o has been shown to induce a conformational change followed by an increase of surface hydrophobicity (Athwal et al. 1998, Athwal and Huber 2002). We tested the direct interaction between spermine and the recombinant maize GF14-6 by using the fluorescence assay with the environmentally sensitive probe, bis-ANS [4,4'-bis(1-anilinonaphthalene 8-sulfonate)]. As shown in Fig. 6, addition of spermine to GF14-6 results in an increase in bis-ANS fluorescence, indicating that spermine does in fact bind to GF14-6 and causes a conformational change similar to that induced by Mg\textsuperscript{2+}.

**Discussion**

In this study, the role of polyamines in the interaction between 14-3-3 proteins and H\textsuperscript{+}-ATPase has been analyzed. By using different in vitro and in vivo experimental approaches, it is shown that spermine can regulate the 14-3-3 activation of the proton pump.
In vivo incubation of maize roots with 0.2 mM spermine induces an increase of H\(^{+}\)-ATPase activity. This effect is obtained, even though to a much lower extent, with spermidine and putrescine, while cadaverine is ineffective. This differential activity can be ascribed to the structural features of polyamines. In fact, spermine is characterized by the presence of four positive charges, carrying two primary and two secondary amine groups, while the other polyamines are shorter and with fewer positive charges.

Although a differential polyamine activity has been reported in other systems (Tabor and Tabor 1984, Galston and Sawhney 1990), a similar relationship between the number of positive charges and activity has been reported only for polyamine regulation of the interaction between 14-3-3 proteins and nitrate reductase (Athwal and Huber 2002). The role of endogenous polyamines in the regulation of H\(^{+}\)-ATPase is corroborated by the ability of PIs to hamper the phosphohydrolytic activity of plasma membrane preparations. The spermine effect is also detectable in vitro where polyamines were administered to membrane preparations. Spermine is effective at micromolar concentrations, suggesting that the effect occurs at concentrations that have physiological relevance (Evans and Malmberg 1989, Kumar et al. 1997).

The interaction of 14-3-3 with H\(^{+}\)-ATPase requires the phosphorylation of a specific threonine residue located at the C terminus of the proton pump. The recombinant C-terminal domain of the MHA2 isoform of maize H\(^{+}\)-ATPase, which is not phosphorylated, can, however, interact with the 14-3-3 protein but only in the presence of fusicoccin (Fullone et al. 1998). Interestingly, the results show that the 14-3-3 interaction with the C terminus can also occur in the presence of spermine.

This new evidence suggests that spermine can also induce the interaction between 14-3-3 and its target in the absence of C terminus phosphorylation and that, besides the positive charge, its chemical structure can allow the binding between 14-3-3 and its target. In fact, Mg\(^{2+}\), necessary for the interaction of 14-3-3 with the H\(^{+}\)-ATPase, is not effective in the binding of 14-3-3 to the unphosphorylated C-terminal domain.

In this respect, comparison of the effect of spermine with that of a saturating Mg\(^{2+}\) concentration indicates that spermine stimulates, more than Mg\(^{2+}\), the interaction of GF14-6 with the H\(^{+}\)-ATPase or with a peptide reproducing the 14-3-3-binding site of the H\(^{+}\)-ATPase. The interaction is even stronger with the simultaneous addition of spermine and Mg\(^{2+}\).

The effect of Mg\(^{2+}\) and spermine is the possible consequence of their binding to 14-3-3 proteins. It has been shown that both Mg\(^{2+}\) and spermine can bind to the recombinant Arabidopsis GF14o and induce a
conformational change followed by an increase of surface hydrophobicity (Athwal et al. 1998, Athwal and Huber 2002). Also, the recombinant maize GF14-6 used in the present work can bind spermine, as shown in Fig. 6, where either spermine or Mg$^{2+}$ addition to GF14-6 results in an increase in bis-ANS fluorescence.

In conclusion, the present data show that a physiological concentration of spermine can stimulate the interaction between 14-3-3 and H$^+$-ATPase. The effect of spermine appears to have a significant physiological relevance; spermine acts differently from Mg$^{2+}$ as it can induce the interaction of 14-3-3 proteins with an unphosphorylated target.

## Materials and Methods

### Chemicals

[$\gamma$-$^{32}$P]ATP (specific activity 110 TBq mmol$^{-1}$) and thrombin were from Amersham Biosciences (Uppsala, Sweden). Fusicoccin was prepared according to Balio et al. (1998). Protein kinase A was from Sigma (St Louis, MO, USA), bL15Vpeptide biotinyl-LKGLDIDTIQQNYTpV (Tp, phosphothreonine) and bL15V peptide biotinyl-LKGLDIDTIQQNYTV were synthesized by NeoSystem (Strasbourg, France). Chemicals for gel electrophoresis were from Bio-Rad (Hercules, CA, USA).

### Plant material

Maize caryopses (Zea mays L. cv. Santos) from Dekalb (Mestre, Italy) were germinated and seedlings were grown in the dark for 5 d, as already described (Marra et al. 1996).

### In vivo incubation of maize roots with polyamines

Maize roots (20 g) were cut into small pieces (approximately 5 mm) and incubated in 50 ml of 20 mM Tris-Mes (pH 7.0), containing 300 mM sucrose, for 1 h at room temperature. When indicated, polyamines at a concentration of 0.2 mM were added.

### Purification of plasma membrane from maize roots

Two-phase partitioned plasma membrane were obtained from 20 g of maize roots as previously described (Marra et al. 1996).

### Purification of endoplasmic reticulum from yeast expressing AHA1

Plasma membrane H$^+$-ATPase AHA1 isoform was expressed in Saccharomyces cerevisiae as previously described (Regenberg et al. 1995). After cell homogenization, membranes were purified by differential centrifugation, and endoplasmic reticulum, containing most of the AHA1, was isolated by sucrose gradient centrifugation (Marra et al. 2000).

### Expression of recombinant proteins in Escherichia coli

Recombinant GF14-6 and the C-terminal domain of MHA2 H$^+$-ATPase were expressed in E. coli as fusion proteins with glutathione-S-transferase (GST) using pGEX-2TK vector and pGEX-2T vector, respectively, by following the procedure described by Fullone et al. (1998).

### SDS–PAGE and overlay assay

SDS–PAGE was performed as described by Laemmli (1970), in a Mini Protein apparatus (Bio-Rad). The overlay assay was carried out according to Fullone et al. (1998), with minor modifications. The GST-fused 14-3-3 was labeled with [$\gamma$-$^{32}$P]ATP on the cAMP-dependent protein kinase phosphorylation site present at the junction between GST and the cloned protein. $^{32}$P-labeled GF14-6 was used as the probe in the overlay experiments. A 20 µg aliquot of plasma membrane proteins was separated by SDS-PAGE and blotted onto a nitrocellulose membrane by semi-dry electroblothing. The membrane was blocked in buffer HT [25 mM HEPES-OH, 75 mM KCl, 5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.05% Tween-20, pH 7.5] containing 5% no-fat dried milk and then incubated overnight at 4°C in buffer HT with 2% no-fat dried milk and 3 µg of $^{32}$P-labeled GF14-6 (8.3 kBq ml$^{-1}$). The nitrocellulose membrane was extensively washed with buffer HT and radioactivity detected by autoradiography at 80°C.

### Immunoblotting

For immunoblotting analysis, proteins were separated by SDS-PAGE using a mini-gel apparatus (Bio-Rad), then electroblotted onto a PVDF membrane with 39 mM glycine, 48 mM Tris, 0.1% SDS and 10% methanol. After blocking for 1 h in TTBS (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.05% Tween-20) with 5% no-fat dried milk at room temperature, the membrane was incubated with anti-14-3-3 antibodies (1:700) or anti-H$^+$-ATPase antibodies (1:700). Following three washes with TTBS, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2,000; Bio-Rad).

### Binding of GF14-6 to resin-bound phosphopeptides

bL15Vpeptide or bL15V peptides (0.4 nmol) were immobilized onto 40 µl of streptavidin–agarose resin (Sigma) and incubated in 50 µl of buffer H (with or without Mg$^{2+}$) with 3 µg of $^{32}$P-labeled GF14-6 (1.4 kBq µg$^{-1}$) for 60 min at room temperature in the absence or presence of 0.2 mM spermine. The resin was then centrifuged at 2,000 $\times$ g for 5 min and washed three times with 1 ml of buffer H. Resin-bound radioactivity was measured in a liquid scintillation β-counter (LKB Wallac 1410).

### Phosphohydrolytic activity

The phosphohydrolytic activity of the plasma membrane preparation from maize roots was assayed according to Marra et al. (1996) using 50 µg of protein. The AHA1 ATPase activity of yeast endoplasmic reticulum membranes was assayed according to Marra et al. (2000) using 10 µg of sucrose gradient-purified yeast endoplasmic reticulum previously pre-incubated with different spermine concentrations in the presence of 3 µg ml$^{-1}$ GF14-6. ATP hydrolysis was measured according to Serrano (1983).

### Analytical methods

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

### Fluorescence measurements

Fluorescence measurements were performed using a Perkin Elmer LS50B Luminescence Spectrometer. A stock solution of 100 mM bis-ANS (Molecular Probes, Eugene, OR, USA) was prepared and diluted to a final concentration of 1.0 µM during measurements. The final concentrations of GF14-6 used was 0.1 µM. Excitation was at 385 nm and emission was measured at 480 nm.
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References


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