Polyamines cause plasma membrane depolarization, activate Ca\(^{2+}\)-, and modulate H\(^{+}\)-ATPase pump activity in pea roots

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Abstract
Polyamines regulate a variety of cation and K\(^{+}\) channels, but their potential effects on cation-transporting ATPases are underexplored. In this work, noninvasive microelectrode ion flux estimation and conventional microelectrode techniques were applied to study the effects of polyamines on Ca\(^{2+}\) and H\(^{+}\) transport and membrane potential in pea roots. Externally applied spermine or putrescine (1 mM) equally activated eosin yellow (EY)-sensitive Ca\(^{2+}\) pumping across the root epidermis and caused net H\(^{+}\) influx or efflux. Proton influx induced by spermine was suppressed by EY, supporting the mechanism in which Ca\(^{2+}\) pump imports 2 H\(^{+}\) per each exported Ca\(^{2+}\). Suppression of the Ca\(^{2+}\) pump by EY diminished putrescine-induced net H\(^{+}\) efflux instead of increasing it. Thus, activities of Ca\(^{2+}\) and H\(^{+}\) pumps were coupled, likely due to the H\(^{+}\)-pump inhibition by intracellular Ca\(^{2+}\). Additionally, spermine but not putrescine caused a direct inhibition of H\(^{+}\) pumping in isolated plasma membrane vesicles. Spermine, spermidine, and putrescine (1 mM) induced membrane depolarization by 70, 50, and 35 mV, respectively. Spermine-induced depolarization was abolished by cation transport blocker Gd\(^{3+}\), was insensitive to anion channels’ blocker niflumate, and was dependent on external Ca\(^{2+}\). Further analysis showed that uptake of polyamines but not polyamine-induced cationic (K\(^{+}\)+Ca\(^{2+}\)+H\(^{+}\)) fluxes were a main cause of membrane depolarization. Polyamine increase is a common component of plant stress responses. Activation of Ca\(^{2+}\) efflux by polyamines and contrasting effects of polyamines on net H\(^{+}\) fluxes and membrane potential can contribute to Ca\(^{2+}\) signalling and modulate a variety of transport processes across the plasma membrane under stress.

Key words: Ca\(^{2+}\)-ATPase, H\(^{+}\)-ATPase, ion transport, membrane potential, plasma membrane, polyamine, stress.

Introduction
Polyamines (PAs) are polycationic metabolites, which regulate growth, development, senescence, programmed cell death, and stress responses in plants (Kusano et al., 2008; Alcázar et al., 2010; Gill and Tuteja, 2010). Several molecular targets for PAs have been suggested. Polyamines are unique polyvalent cationic metabolites and, as such, can bind to negative surfaces and act as molecular chaperons, protecting membrane, proteins, and nucleic acids and/or otherwise affecting their conformation/assembly (Rhee et al., 2007; Igarashi and Kashiwagi, 2010). Polyamines can also form ternary complexes with Mg-ATP, affecting catalytic activity of protein kinases (Meksuriyen et al., 1998). In addition, PAs can act as reactive oxygen species (ROS) and free radical scavengers (Ha et al., 1998) and stimulate antioxidant system (Tang and Newton, 2005; Kubiś, 2008). However, catabolization of PAs yields H\(_2\)O\(_2\) and may in fact be a source of ROS production, which in turn can induce Ca\(^{2+}\) influx and activation of a signalling cascade in response to biotic and abiotic stresses or in developmental processes (Yoda et al., 2006; Rodriguez et al., 2009; Tisi et al., 2011).

PAs can specifically interact with 14-3-3 proteins, decreasing the activity of the nitrate reductase and stimulating...
Pea seedlings were placed in the induction of nonselective cation and Kir channels (Zhao et al., 2007), whereas in guard cells, PAs inhibited Kir channels from the intracellular side only (Liu et al., 2000; Shabala et al., 2007). In roots, only externally applied PAs were efficient in the inhibition of nonselective cation and Kir channels (Zhao et al., 2007), whereas in guard cells, PAs inhibited Kir channels from the intracellular side only (Liu et al., 2000). To add to the apparent complexity, as a source of ROS, PAs can stimulate cation conductance across the PM (Rodriguez et al., 2009; Wu et al., 2010). In addition, PAs can act as cofactors for hydroxyl radicals (OH•) in the induction of nonselective weakly voltage-dependent conductance (ROS-induced conductance) across root epidermis PM (Zepeda-Jazo et al., 2011; Velarde-Buendía et al., 2012). Apart from this, PAs induced Ca2+ efflux across the PM of pea roots (Bose et al., 2011; Zepeda-Jazo et al., 2011). PAs depolarized membrane potential in roots and leaves, but the underlying mechanisms are not well understood, despite PA-induced membrane potential changes in roots were associated with the increase of membrane conductance (Di Tomaso et al., 1989; Fromm et al., 1997; Ozawa et al., 2010).

In this work, the effects of different PAs on Ca2+ and H+ fluxes and membrane potential in intact pea roots have been analysed. PAs activated PM Ca2+ pumping, and, depending on PA species/concentration, activated or suppressed H+ pumping. Contrasting effects of different PAs on the H+ pumping correlated with a magnitude of the PA-induced depolarization. Overall, this study provides a clue for the regulation of Ca2+ and H+ homeostasis and membrane potential difference in plant roots by PAs and discusses it in the context of plant adaptive response to environment.

Materials and methods

Plant material and growth conditions

Pea (Pisum sativum L. cv Greenfeast) seeds were surface sterilized with commercial bleach and then washed thoroughly with distilled water. The seeds were germinated in a dark growth cabinet at 24°C on the surface of Petri dishes for 3 d. Uniformly germinated seedlings were selected and transferred to a bubbled hydroponic culture unit comprising a plastic container over which seedlings were suspended on a plastic grid so that their roots were almost completely immersed in 3 l growth solution (0.5 mM KCl and 0.1 mM CaCl2). Air was provided by an aquarium air pump via flexible plastic tubing. Seedlings were grown under a 16/8 light/dark cycle (24°C) in an illuminated growth cabinet until 5 d old. Roots 8–10 cm long were used for measurements.

Ion flux measurement

Net K+, Ca2+, and H+ fluxes were measured using the noninvasive microelectrode ion flux estimation (MIFE) technique (UTas Innovation, Hobart, Tasmania). The principles of MIFE measurements, microelectrode fabrication, and calibration and the theory of MIFE measurements are available in previous publications (Newman, 2001; Shabala et al., 2006). Pea seedlings were placed into a 30 ml measuring chamber, containing 0.5 mM KCl, 0.2 mM CaCl2, 5 mM MES, 2 mM Tris base; pH 6.0. H+ flux measurements were made in unbuffered solution. Roots were immobilized in a horizontal position as described elsewhere (Bose et al., 2013) and preincubated in a basic salt medium for at least 30 min. Electrodes were positioned near to the root surface in the mature zone (beyond 2 mm). At the beginning, steady-state ion fluxes were recorded over a period of 5 min, and then individual PAs to a final concentration of 0.1–1 mM were applied and net ion fluxes were measured for up to 40 min. For the pharmacology experiment, the seedlings were pretreated with the given drug for 1 h before the polyanine treatment.

Plasma membrane isolation and H+ transport activity

Plasma membranes were purified by two-phase partitioning as described by Lund and Fuglsang (2012) only with the following modification: 5.7% of dextran and PEG were used in the two-phase system. H+ transport is measured using the fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA). ACMA accumulates inside the vesicles upon protonation; therefore, quenching of ACMA in the solution is correlated to the transport of protons into the vesicles. To obtain inside-out vesicles, PM (protein 20 μg) was pretreated with Brij-58 for 10 min at room temperature in assay buffer (20 mM MOPS-KOH pH 7.0, 2 mM ATP-KOH, 40 mM K2SO4, 25 mM KNO3, 1 μM ACMA, 60 mM valinomycin, 0.05% Brij58) in a total volume of 1.5 ml. When indicated, PAs (1 mM) and/or 14-3-3 protein (10 μg recombinant GF14–14) were added. The assay was initiated by addition of MgSO4 (2.5 mM). Vanadate (100 μM), inhibitor of P-type ATPases, was added in control experiments. Data are presented relatively to the fluorescence level before initiation of the assay. Samples were assayed on a Horiba Jobin Yvon FluoroMax-4 spectrophotometer, with excitation at 412 nm and emission at 480 nm (slit width 1 nm). All chemicals were analytical grade (Sigma).

Membrane potential measurement

The roots of an intact 5-day-old pea seedling were immobilized and preconditioned as for MIFE. Membrane potential (E m) measurements were made using conventional Ag/AgCl microelectrodes (Bose et al., 2013). In brief, borosilicate glass microelectrodes with a tip diameter ~0.5 μm were filled with 1 M KCl, connected to the MIFE electrometer via an Ag/AgCl half-cell, and impaled into the cortical cell of the mature zone root with a manually operated micromanipulator (MFT-5, Narishige). E m was monitored continually using CHART software (for details, see Newman, 2001). Once a stable E m measurement was obtained for 5 min, PA treatment (0.1–10 μM) was imposed. E m measurement was continued for 30–60 min after treatment. In a separate experiment, the effects of increasing external KCl concentration and 1 mM spermine were compared. Spermine-induced E m changes were also tested in...
the roots preincubated over 1 h with 0.3% DMSO, 0.1 mM GdCl₃, 0.2 mM niflumate or 1 mM EGTA.

**Results**

*Polyamines induced Ca²⁺ efflux and altered net H⁺ flux across the root plasma membrane*

Pea roots contain highest concentrations of PAs observed in plants, ranging from hundreds μM for spermine and spermidine to 4 mM for putrescine (Shen and Galston, 1985). In plants, up to two-thirds of tissue PAs are apoplastic (Pistocchi et al., 1988 and references therein). In addition, intracellular PAs can be rapidly mobilized and exported to the apoplastic both in leaves (Yoda et al., 2006) and roots (Kuiper et al., 2001) to combat a pathogen attack and in response to other environmental stimuli (see Pottosin et al., 2014 for a recent review). Consequently, to test the effects of individual PAs, this study chosen the external concentration of 1 mM. Application of 1 mM of putrescine or spermine to intact pea roots provoked a transient Ca²⁺ efflux of a similar magnitude, peaked at 150 nmol m⁻² s⁻¹ (~3 pA/pF; Fig. 1). This efflux was insensitive to amiloride, a nonspecific inhibitor of cation exchangers in animal and plant cells (Frelin et al., 1988; Amalou et al., 1992; Darley et al., 2000; Guo et al., 2009) but was completely abolished by 0.5 μM eosin yellow (EY), a specific inhibitor of P-type Ca²⁺ pumps (De Michelis et al., 1993). EY also abolished the increase of Ca²⁺ in the external medium at the root surface, induced by PAs (Supplementary Fig. S1 available at JXB online). Ca²⁺ efflux induced by spermine was insensitive to 0.1 mM Gd³⁺ (n=3, data not shown), a nonspecific Ca²⁺-transport blocker. PM Ca²⁺-ATPases in animal and plants import several (most likely, two) H⁺ per each exported Ca²⁺ (Beffagna et al., 2000; Niggli and Sigel, 2008). Therefore, H⁺ fluxes in response to PAs were assayed (Fig. 2). Indeed, 1 mM spermine induced a net steady-state H⁺ influx, with a roughly 2:1 stoichiometry between H⁺ and Ca²⁺ (Fig. 1A and 2A). When Ca²⁺-pump activity was inhibited by EY, spermine treatment resulted in a rapid transient H⁺ efflux (Fig. 2A). At the same time putrescine induced a significantly sustained H⁺ efflux (150 nmol m⁻² s⁻¹, ~1.5 pA/pF; Fig. 2B). Neither spermine- nor putrescine-induced H⁺ fluxes were sensitive to a pretreatment with 0.1 mM Gd³⁺ (Supplementary Fig. S2).

As activity of the CLC-type anion/H⁺ antiporter is thermodynamically unfavourable under these experimental conditions (Teakle and Tyerman, 2010), the only transport system at the PM which can be responsible for a net H⁺ efflux is the P-type H⁺-ATPase. Both P-type Ca²⁺ and H⁺ PM ATPases are sensitive to vanadate, and the pretreatment with vanadate caused a substantial suppression of both spermine-induced H⁺-influx and putrescine-induced H⁺-efflux (Fig. 2) as well as of the respective alkalinization and acidification of the external medium (Supplementary Fig. S3) and substantially decreased Ca²⁺ efflux induced by PAs (Supplementary Fig. S4). The simplest explanation for this data may be that spermine and putrescine induced Ca²⁺ pumping and that putrescine in addition caused an activation of the H⁺-ATPase pump, overriding the Ca²⁺ pump-mediated H⁺ influx. However, one result did not fit this simple model: EY suppressed, not increased, the net H⁺ efflux induced by putrescine (Fig. 2B), while such increase was expected in the case when Ca²⁺- and H⁺-pump activities and putrescine effects on them were independent.

In the light of this controversy, the effects of spermine and putrescine on the PM H⁺-pumping activity were addressed in direct experiments using purified PM vesicles. Again, the outcome may come as a surprise, because putrescine did not affect the PM H⁺-ATPase-mediated vanadate-sensitive H⁺ pumping at all, and spermine suppressed the H⁺-ATPase-mediated acidification by a factor of two (Fig. 3). Thus, the stimulatory effect of putrescine on the H⁺ pumping is mediated by an additional component, lacking in the isolated PM fraction. Further, the H⁺-ATPase activity was stimulated by the 14-3-3 protein, but spermine again caused a 2-fold decrease in H⁺ pumping, without any sign for a positive interaction between spermine and 14-3-3 protein, previously reported for maize roots by Garufi et al. (2007).

**Fig. 1.** Polyamine-induced Ca²⁺ efflux in the mature zone of pea roots is mediated by the plasma membrane Ca²⁺-ATPase. Ca²⁺ fluxes were generated by application of 1 mM spermine (A) or putrescine (B) in the bath as indicated by arrows. Polyamine-induced Ca²⁺ fluxes were strongly suppressed by eosin yellow, a specific Ca²⁺-pump inhibitor but were insensitive to a cationic exchanger inhibitor amiloride. Roots were preincubiated for 1 h with 0.5 μM EY or 1 mM amiloride before the addition of a poliamine. The sign convention is ‘efflux negative’ for all MIFE measurements. Fluxes were measured in the mature zone, about 20 mm from root tip. Data are means±SE. EY, eosin yellow; Put, putrescine; Spm, spermine.

**Polyamines-induced changes in membrane potential are correlated with effect on net H⁺ fluxes**

At 1 mM, all PAs caused a substantial membrane depolarization, spermine>spermidine>putrescine (Fig. 4B). As already shown, spermine induced a net H⁺ influx at a steady state, whereas putrescine caused a net H⁺ efflux. The effect of
spermidine, which is an intermediate in its chemical structure between spermine and putrescine, was also an intermediate in terms of magnitude and direction of the response (Fig. 4A). At a lower concentration (0.1 mM), spermine was unable to activate any significant Ca\(^{2+}\) efflux \((n=4, \text{ data not shown})\) but induced a transient H\(^{+}\) efflux \((\text{Fig. 5A})\). Moreover, instead of a depolarization, 0.1 mM spermine caused a small but significant \((P<0.05)\) membrane hyperpolarization \((\text{Fig. 5B})\). Both effects were consistent with a transient activation of the PM H\(^{+}\)-ATPase. The effect of 0.1 mM spermidine \((n=3, \text{ data not shown})\) on the membrane potential was similar to that of 0.3 mM spermine \((\text{Fig. 5B})\). Thus, only spermine displayed dual effects on the membrane potential: a hyperpolarization at low concentration (0.1 mM) and depolarization at higher concentrations; other PAs apparently caused only depolarization.

Because 1 mM spermine caused the largest depolarization as compared to other PAs, this treatment was selected for further pharmacological analysis. First, this work verified that the depolarization was not produced by ROS as a result of the spermine catabolization in the apoplast. H\(_{2}\)O\(_{2}\) is neither efficient in the induction of cation fluxes nor in modulation of membrane potential in the pea mature root zone \((\text{Zepeda-Jazo et al., 2011; Bose et al., 2013)}\). On the contrary, OH\(^{•}\) was efficient in this model system. However, 0.3% DMSO (a potent OH\(^{•}\) scavenger; \text{Demidchik et al., 2010}) did not significantly affect spermine-induced depolarization \((n=4, \text{ data not shown})\). The anion-channel-blocker niflumate \((0.2 \text{ mM})\) did not affect spermine-induced depolarization either, but 0.1 mM Gd\(^{3+}\) (an efficient blocker of nonselective cation channels) completely abolished the spermine-induced depolarization and unmasked the spermine-induced hyperpolarization \((\text{Fig. 6A})\). This residual Gd\(^{3+}\)-insensitive hyperpolarization was very similar to that induced by 0.1 mM spermine \((\text{Fig. 5B})\).

To test whether the external Ca\(^{2+}\) contributed to the spermine-induced depolarization, 0.2 mM Ca\(^{2+}\) was chelated by 1 mM EGTA. Under these conditions, spermine-induced depolarization was not abolished but it was dramatically slowed down (>50-times, based on the comparison of signal slopes; \text{Fig. 6B}). A much slower time course of the spermine-induced depolarization in the absence of Ca\(^{2+}\) again unmasked the fast spermine-induced hyperpolarization.

**Discussion**

**Effects of polyamines on membrane potential**

Membrane potential in plants is defined by a balance of passive ion fluxes and active transports; the latter mainly contributed
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by the electrogenic PM H\(^{+}\) pump (Spanswick, 1981; Lew, 1991). At increased concentration of external K\(^{+}\) (millimolar range), the balance may be switched to the K\(^{+}\)-state and membrane potential value is approaching the equilibrium potential for K\(^{+}\) (Hirsch et al., 1998). Pea root, however, displays an unusual capacity to rapidly revert a depolarization induced by high external concentrations of NaCl (Bose et al., 2013). The same is also true for high (10 mM) external KCl, whereas 1 mM spermine caused a large and stable depolarization (Supplementary Fig. S5). Efficient control of the resting membrane potential against depolarization challenge induced by high external salt implies a dominance of the PM H\(^{+}\)-pump activity over the background passive ion conductance. Spermine-induced depolarization was completely abolished by 0.1 mM Gd\(^{3+}\) (Fig. 6A), but Gd\(^{3+}\) affected neither Ca\(^{2+}\) nor H\(^{+}\) fluxes induced by spermine (Supplementary Fig. S2). As depolarization is likely associated with a spermine uptake (as will be discussed), spermine seems to activate the PM Ca\(^{2+}\) pump from the extracellular side.

A possible explanation may be found in a fact that spermine might induce some novel passive conductance. Additional measurements were performed to exclude a contribution of the anion efflux to the spermine-induced depolarization. Indeed, application of spermine (as tetrachloride) caused an abrupt increase of external Cl\(^{–}\) concentration but did not affect the net Cl\(^{–}\) flux (Supplementary Fig. S6). Based on this and the pharmacology data presented in Fig. 6, such a conductance can be a nonselective cation one, dependent on, or directly contributed by, external Ca\(^{2+}\). This hypothesis, however, does not match the existing experimental evidence. If spermine induced a nonselective cation conductance, it has to be K\(^{+}\)-permeable. As spermine at 1 mM induced a depolarization well above E\(_K\), this has to evoke a K\(^{+}\) efflux via this nonselective pathway. However, no significant K\(^{+}\) efflux is induced by 1 mM spermine in pea roots (Zepeda-Jazo et al., 2011).

The MIFE technique allows quantification of net fluxes of all physiologically important cations present in the bath solution (K\(^{+}\), H\(^{+}\), and Ca\(^{2+}\)). Here, Ca\(^{2+}\) and H\(^{+}\) fluxes induced by 1 mM spermine or putrescine were combined with data for K\(^{+}\) flux previously reported by Zepeda-Jazo et al. (2011) for the same species and identical experimental conditions. As shown in Fig. 7A, spermine induced a net steady-state cation
influx (due to a dominant spermine-induced H⁺ influx), qualitatively consistent with the observed membrane depolarization. However, putrescine caused a net efflux of Ca²⁺, H⁺, and K⁺ (hence, an overall net cation efflux) but, at the same time, induced a significant membrane depolarization (Fig. 7B). The only possible explanation to this controversy is that this depolarization was caused by uptake of PAs themselves across the PM and that the extent of this depolarization overcame the otherwise hyperpolarizing effect of fluxes of all external cations measured.

Most of information about the transport of PAs across the PM in plants has come from early studies undertaken in 1980s and 1990s (reviewed by Kakkar et al., 1997/1998; see also Igarashi and Kashiwagi, 2010). Briefly, PA uptake can be relatively rapid, causing their intracellular concentration rise of 0.1–1 mM min⁻¹ (Pistocchi et al., 1987, 1988; DiTomaso et al., 1992a). This process may be channel-mediated (Colombo et al., 1992), although carrier-mediated or energy-dependent uptake have been suggested by other studies (Kakkar et al., 1997/1998). There is also evidence that PA uptake is Ca²⁺ stimulated (Pistocchi et al., 1987; Antognoni et al., 1993), although it is suppressed by a high concentration (mM) of external Ca²⁺ (DiTomaso et al., 1992a). Uptake of PAs is inhibited by lantanides (Pistocchi et al., 1988; DiTomaso et al., 1992a). All these results are compatible with the data presented in Fig. 6.

It remains to be elucidated whether the uptake of PAs by pea roots is rapid enough to account for the kinetics of membrane potential changes in Fig. 4B. Because spermine-induced depolarization was not accompanied by K⁺ efflux, the PA uptake route needs to be relatively selective for PAs against inorganic cations. Previously, other authors (e.g. DiTomaso et al., 1992a) also came to a conclusion that PAs do not share their transport routes in the PM with other cations. Finally, the difference in depolarization induced by different PAs (spermine>spermidine>putrescine) is probably related to the direction of the net H⁺ fluxes induced by them (Fig. 4A). The difference in PA effects on the net H⁺ flux in turn is likely linked to the difference in the direct effects of a higher PA (spermine) and diamine (putrescine) on H⁺ pumping (inhibition and no effect, respectively; Fig. 3).
Effects of polyamines on the plasma membrane Ca\(^{2+}\) and H\(^{+}\) pumps

The results shown in Fig. 1 provide evidence that spermine or putrescine are equally efficient in activation of the PM Ca\(^{2+}\) pump. However, as evidenced by Fig. 4A, PAs caused contrasting effects on H\(^{+}\) fluxes, ranging from a net steady-state influx (spermine) to a net efflux (putrescine); this sequence correlated with a degree of depolarization (spermine>spermidine>putrescine) caused by different PAs (Fig. 4B). There is only one known PM transporter that is able to mediate a net H\(^{+}\) efflux under these experimental conditions, namely the primary H\(^{+}\) pump. If the effect of the putrescine on the H\(^{+}\)-ATPase was independent on its stimulatory effect on the Ca\(^{2+}\) pump, a selective inhibition of the PM Ca\(^{2+}\) pump by EY should enhance the putrescine-induced H\(^{+}\) efflux. Instead, in this work almost complete suppression of the putrescine-induced H\(^{+}\) pumping was observed (Fig. 2B). This raises the question of how the activity of the two PM cationic pumps, H\(^{+}\) and Ca\(^{2+}\), may be coupled? One of the possible links may be through the H\(^{+}\) gradient generated by the PM H\(^{+}\)-ATPase, which can fuel Ca\(^{2+}\)-pump activity. The stoichiometry of Ca\(^{2+}\)/H\(^{+}\) exchange by the plant PM Ca\(^{2+}\)-ATPase may be about two H\(^{+}\) to one Ca\(^{2+}\) (Beffagna et al., 2004), same as for its counterpart in animal cells (Thomas, 2009). This is in a good agreement with the ratio between steady state H\(^{+}\) and Ca\(^{2+}\) fluxes induced by spermine (Figs 1A and 2A). However, the exact stoichiometry between Ca\(^{2+}\) and H\(^{+}\) could not be determined from the data because spermine caused additional complex effects the H\(^{+}\) pumping—a direct inhibition at high concentration (1 mM) and a transient stimulation, apparent already at lower concentration (0.1 mM; Fig. 5A)—which was similar to one induced by 1 mM spermidine (Fig. 4A). Transient activation of H\(^{+}\) pumping likely underlies a transient hyperpolarization, observed at 0.1 mM spermine (Fig. 5B). At 1 mM of spermine, such hyperpolarization was unmasked by block or deceleration of the spermine-induced depolarization by Gd\(^{3+}\) and Ca\(^{2+}\), respectively (Fig. 6). Based on the H\(^{+}\) flux data in Fig. 4A, one may suggest that such transient hyperpolarization may be also induced by putrescine and spermidine. Yet this anticipated hyperpolarization, if any, was apparently masked by a massive depolarization, as observed in the membrane potential data.

On the other hand, an increase of intracellular Ca\(^{2+}\) caused an indirect inhibition of the PM H\(^{+}\)-ATPase (Kinoshita et al., 1995; Lino et al., 1998; Brault et al., 2004). It can be proposed, therefore, that the activation of the PM Ca\(^{2+}\) pump by PAs can have the opposite effect, resulting in an increase of PM H\(^{+}\)-pump activity. This may explain why the inhibition of the Ca\(^{2+}\) pump by EY abolished the putrescine-induced H\(^{+}\)-pump activation (Fig. 2B).

The working model for the effects of PAs on Ca\(^{2+}\) and H\(^{+}\) pumping and the membrane potential is summarized in Fig. 8. This model involves differential effects of putrescine and spermine on the H\(^{+}\) pump, intracellular Ca\(^{2+}\)-mediated crosstalk between Ca\(^{2+}\) and H\(^{+}\) pumps, and direct effects of PAs on the membrane potential. It assumes that both putrescine and spermine can traverse the membrane, depolarizing it to a different extent. PA (spermine) uptake is inhibited by Gd\(^{3+}\). Both putrescine and spermine can activate the PM Ca\(^{2+}\) pump, which exchanges Ca\(^{2+}\) for H\(^{+}\) with an approximated stoichiometry of 1:2. Activation of the Ca\(^{2+}\) efflux can partly relieve PM H\(^{+}\)-pump inactivation by intracellular Ca\(^{2+}\) (Brault et al., 2004). A coupled mechanism of H\(^{+}\)-pump activation follows from the fact that EY, a specific Ca\(^{2+}\)-pump inhibitor, suppresses the putrescine-induced net H\(^{+}\) efflux instead of increasing it. Spermine (0.1–1 mM) transiently stimulates the H\(^{+}\) pump, but at 1 mM is able to directly inhibit H\(^{+}\) pumping in isolated PM vesicles. Due to the induction of a net H\(^{+}\) influx instead of a net H\(^{+}\) efflux, spermine causes a larger depolarization than putrescine.

**Physiological implications**

The results suggest that PAs can modulate three fundamental parameters, namely PM potential, cytosolic pH, and cytosolic free Ca\(^{2+}\) levels. Each of these parameters, in turn, controls a huge variety of transport processes across the PM. Stress-induced elevation in PA level is widely reported in response to salinity, drought, chilling, heat, hypoxia, ozone, UV radiation, heavy metal toxicity, and herbicide treatment (reviewed in Alcázar et al., 2010; Gill and Tuteja, 2010). All these stresses result in a significant disturbance to intracellular ionic homeostasis and, specifically, in a transient elevation in the cytosolic free Ca\(^{2+}\) (Sanders et al., 1999). From this point of view, the reported PA-induced activation of the PM-based Ca\(^{2+}\)-ATPase may be an important component of adaptive mechanism allowing restoration of the basal...
cytosolic Ca\(^{2+}\) level once the signalling was completed (Bose et al., 2011; Shabala et al., 2011). On the other hand, PAs export to the apoplasm, and catabolization therein leads to ROS generation (Yoda et al., 2006; Moschou et al., 2008; Rodriguez et al., 2009). ROS can then evoke Ca\(^{2+}\) influx via diverse conductance pathways (Pei et al., 2000; Foreman et al., 2003; Demidchik et al., 2007; Zepeda-Jazo et al., 2011) in a tissue-, species- (e.g. H\(_2\)O\(_2\) or OH\(^-\)), and concentration-dependent manner. Taken together, these two factors, Ca\(^{2+}\) efflux and uptake, will shape a specific Ca\(^{2+}\) ‘signature’ (Bose et al., 2011), activating signalling cascade and triggering gene expression required for adaptation (Kudla et al., 2010; Reddy et al., 2011).

Cytosolic pH is another important second messenger known to affect activity of a large number of membrane transporters. Cytosolic alkalinization leads to the inhibition and activation of inward and outward K\(^+\) currents in guard cells, respectively (Grabov and Blatt, 1997). In Arabidopsis, drought stress causes increase in the putrescine level, without changes in spermine or spermidine, whereas salt stress causes increases both in spermine and putrescine levels (Alcázar et al., 2010). In this context, drought stress-induced putrescine elevation may be a factor contributing to stomata closure via putrescine-induced activation of the PM H\(^{+}\)-ATPase (Fig. 2B), resulting in alkalinization of the cytosol and, hence, inhibition of K\(^+\) uptake and stimulation of K\(^{+}\) efflux. Another example is oxygen deprivation caused by anoxia or hypoxia, which result in a substantial cytosolic pH acidification (Felle, 2005). At the same time, hypoxia causes a massive increase in putrescine and decrease in spermine content in rice roots and shoots (Reggiani et al., 1993), and elevated putrescine levels stimulate PM H\(^{+}\)-ATPase activity (Reggiani et al., 1992). It is plausible to suggest that the above phenomena may be of adaptive significance and used as a mean to reduce intracellular acidification.

As putrescine is a precursor of higher PAs (e.g. spermine and spermidine), it is plausible to think that not only individual PAs concentrations but also the ratio of putrescine to spermine+spermidine may determine plant’s physiological status. For instance, this ratio increases upon aging and K\(^+\) deficiency but decreases upon adaptation to salt stress (Shen and Galston, 1985; Watson and Malmberg, 1996; Gill and Tuteja, 2010). Opposite effects of lower and higher PAs on the H\(^{+}\)-ATPase activity (i.e. its activation by putrescine but inhibition by spermine) may be considered as a biochemical ‘switch’ for the differential activity of membrane transporters under stress conditions. Thus, the balance between higher PAs and putrescine may be a critical factor determining plant’s adaptive strategy, switching from an energy-consuming to an energy-saving (H\(^{+}\)-ATPase activity kept at minimum) mode or vice versa. The fine print of this regulation remains to be revealed in the future studies.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Fig. S1. Changes in Ca\(^{2+}\) concentration induced by polyamines in the vicinity of a pea root.

Supplementary Fig. S2. Gadolinium had no effect on H\(^{+}\) flux induced by polyamines.

Supplementary Fig. S3. Changes in pH induced by polyamines in the vicinity of a pea root.

Supplementary Fig. S4. Vanadate suppresses Ca\(^{2+}\) efflux induced by polyamines.

Supplementary Fig. S5. Kinetics of membrane potential change in pea roots in response to different stimuli.

Supplementary Fig. S6. Application of spermine raises the external Cl\(^-\) concentration without a significant effect on the net Cl\(^-\) flux.

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