Polyamines prevent NaCl-induced K⁺ efflux from pea mesophyll by blocking non-selective cation channels

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Abstract Despite numerous reports implicating polyamines in plant salinity responses, the specific ionic mechanisms of polyamine-mediated adaptation to salt-stress remain elusive. In this work, we show that micromolar concentrations of polyamines are efficient in preventing NaCl-induced K⁺ efflux from the leaf mesophyll, and that this effect can be attributed to the inhibition of non-selective cation channels in mesophyll. The inhibition by externally applied polyamines developed slowly over time, suggesting a cytosolic mode of action. Overall, we suggest that elevated levels of cellular polyamine may modulate the activity of plasma membrane ion channels, improving ionic relations and assisting in a plant’s adaptation to salinity.

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1. Introduction

Polyamines (putrescine, spermidine, and spermine) are plant growth regulators, critical for a number of developmental processes, including cell division, somatic embryogenesis, root growth, floral initiation, and flower and fruit development [1,2]. Despite some significant variations between species, the reported estimated levels of free concentrations of polyamines (PA) in plant cells are as follows: Spm⁺⁺, from a few, up to tens of micromolar; Spd⁺⁺, tens to hundreds of micromolar; and Put⁺⁺, up to several millimolar [1–4]. In addition to their role in plant development, PA may also play an important role in plant stress responses. Several fold increases in the level of free PA in plant cells in response to a variety of abiotic stresses, including salinity, have been reported [1,5,6]. Furthermore, since the early 1990s, it has been demonstrated that salt-resistant plant varieties contain higher polyamine levels under stress conditions [7,8] and more recent work has shown that the application of exogenous PA can improve the adaptation of plants to salt-stress [9,10]. Overexpression of genes for ADC or ODC, two key enzymes involved in putrescine biosynthesis, improves plant salt-tolerance in tobacco [11] and oat [12]. In addition, increased salt-tolerance was reported in plants over-expressing SAMDC (S-adenosylmethionine decarboxylase; [13]) and SPDS (spermidine synthase; [14]), while loss-of-function Arabidopsis mutants deficient in polyamine biosynthesis, showed a higher sensitivity to salt-stress [15,16]. At the same time, salt-induced injury in the adc2-1 mutant was partly reverted by putrescine addition [17]. All this points towards the importance of PA in adaptive mechanisms against salt-stress. However, the specific ionic mechanisms of PA-mediated adaptation to salinity remain elusive.

One plausible hypothesis is that elevated polyamine levels in the cell cytosol modulate the activity of plasma membrane ion channels in plant tissues, thus improving ionic relations and assisting plants in their adaptation to salinity. Some evidence supporting this hypothesis comes from the animal literature. Polyamines are organic polycations, protonated at a physiological pH, which can potentially interact with a variety of cellular targets including nucleic acids and proteins. In recent years, specific interactions of polyamines with a number of different types of ion channels, including KIR inward-rectifying K⁺ channels in animal tissues, have been reported [18–21]. In plant systems, cytosolic polyamines have been shown to be efficient inhibitors of both slow (SV) and fast (FV) vacuolar cation channels [22,23] as well as potent blockers of inward K⁺ currents across the plasma membrane of guard cells [5]. However, none of these channels are considered to be major players in mediating plant adaptive responses to salinity [24,25].

One of the “hallmark” cellular responses to salt-stress is a massive K⁺ efflux, observed in both root [26–30] and leaf [27,31–33] tissues. Such K⁺ efflux is initiated within seconds of the acute NaCl stress and may last for several hours [28,29], reducing the intracellular K⁺ pool [34,35], and significantly impairing cell metabolism. Consistent with the key role of K⁺ homeostasis in salt-tolerance mechanisms [24], a reduction of K⁺ efflux correlates with increased salt-tolerance [28,29,34]. Electrophysiological and pharmacological studies have suggested that NaCl-induced K⁺ efflux across the plasma membrane is mediated by at least two transport systems, namely outward-rectifying K⁺ permeable channels (KOR) and non-selective cation (NSCC) channels [27,36]. It has also been shown that various substances known to ameliorate the detrimental effects of salinity on plants (such as supplementary Ca²⁺ or compatible solutes), are efficient in preventing NaCl-induced K⁺ efflux [26,30,31,36,37]. Is it possible that polyamines play the same role?

The above question was addressed by a comprehensive electrophysiological study of the effects of polyamine on

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Abbreviations: KOR, potassium outward-rectifying channel; NSCC, non-selective cation channel; Vᵳ, reversal potential

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NaCl-induced K⁺ fluxes and currents from pea leaf mesophyll tissues (using the non-invasive ion flux measuring (the MIFE) technique) and protoplasts isolated from these tissues (using the patch-clamp technique). We report the presence of NSCC in pea mesophyll tissue and show that polyamines are efficient in preventing K⁺ efflux from mesophyll cells, most likely by blocking these NSCC. Both MIFE and patch-clamp experiments show a clear time-dependency of the polyamine effect on K⁺ transport across the cell membrane, suggesting a cytotoxic mode of action.

2. Materials and methods

2.1. Plant material

Seeds of the Argenteum mutant of Pisum sativum L. were grown in a standard potting mix [38] in a glasshouse (16 h/8 h light/darkness) for 20–30 days. Fully unfolded, but still growing leaves were used for experiments.

2.2. Potassium flux measurements

The leaf epidermis was gently removed, and mesophyll segments of about 5 × 7 mm were cut and left floating in a shallow Petri dish filled with nutrient solution (0.1 mM CaCl₂ + 0.2 mM KCl) for several hours, essentially as described elsewhere [32,38]. Various concentrations of polyamines (as chloride salts) were added at various times during pre-incubation to certain samples. One hour prior to measurement, mesophyll segments were immobilized in a Perspex holder, then placed into a measuring chamber filled with the appropriate solution. Net fluxes of K⁺ were measured using the non-invasive ion-selective vibrating microelectrode (the MIFE) technique (University of Tasmania, Hobart), generally as described by in our previous publications [33,36,39], and calculated from the measured differences in electrochemical potential for each ion between these two positions as described earlier [39,40].

2.3. Protoplast isolation

Protoplasts from leaf mesophyll cells were isolated from plasmolyzed leaves using a procedure adapted from [27]. Briefly, the adaxial leaf epidermis was removed with forceps. Peeled leaves were floated in a “wash” solution (as above, minus enzymes) for 3–5 min before the above conditions. Protoplasts were then released by gently shaking the plasmolysed leaf tissue in 1.5 mL of “release” solution (1 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7 adjusted with Tris base, 760 mOsm adjusted with à-sorbitol) at 28–30 °C and 90 rpm rotation (an orbital shaker). After 12–15 min incubation, leaves were washed in a “wash” solution (as above, minus enzymes) for 3–5 min under the above conditions. Protoplasts were then released by gently shaking the plasmolysed leaf tissue in 1.5 mL of “release” solution (1 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.6, 380 mOsm adjusted with à-sorbitol) directly in the measuring chamber. After 10 mL of the “scaling” solution (as above, but osmolality adjusted to 480 mOsm) was then pumped through the chamber to remove cell debris and all unattached protoplasts.

2.4. Patch-clamp electrophysiology

Pea mesophyll protoplasts of 20–25 µm diameter were patch-clamped in the whole-cell mode. GΩ resistance seals were obtained in the “sealing” solution (1 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.6, 480 mOsm adjusted with à-sorbitol). Basic “pipette solution” (PS) contained (in mM): 100 KCl, 2 MgCl₂, 0.8 CaCl₂ (100 nM free Ca²⁺), 2 EGTA, 10 HEPES, pH 7.2 adjusted with Tris base. Osmolality of the PS was 540 mOsm. In some experiments, 50 mM NaCl was used in the bath solution instead of 5 mM KCl. Polyamines were added as a 50 mM stock made in the appropriate solution, with both pH and osmolality adjusted to the required level.

Measurements were made using an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA) in the conventional whole-cell configuration as described in our previous publications [27,41]. Membrane potentials were clamped at −80 mV throughout experiments, and volt- 

age pulses applied in steps (−160 to +100 mV range, with 20 mV increments). Typical access resistance was 18–23 MΩ, and whole cell capacitance; 15–20 pF.

2.5. Membrane potential measurements

Conventional KCl-filled Ag/AgCl microelectrodes [36] with tip diameter ~0.5 µm were used. Measurements were taken from at least five individual leaf segments for each treatment, and recorded for 1.5–2 min after the initial cell penetration.

3. Results

Leaf pre-incubation in 1 mM concentrations of polyamines significantly (P < 0.001) prevented NaCl-induced K⁺ efflux from pea mesophyll (Fig. 1a), with a 85–90% inhibition observed for Spm⁴⁺ and Spd³⁺, and 90–95% inhibition for Put²⁺ (measured for steady-state conditions, 30 min after NaCl treatment). When analyzed for the first 5 min of stress, the efficiency of polyamine block followed the sequence: Put²⁺ > Spd³⁺ > Spm⁴⁺. The effect of polyamines was time-dependent, with increasing duration of pre-treatment resulting in a stronger inhibition of the NaCl-induced K⁺ leak (illustrated for Put²⁺ in Fig. 1b). A clear dose-dependency of polyamine effect

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Fig. 1. (a) Transient K⁺ fluxes measured from pea mesophyll tissue in response to 100 mM NaCl treatment from control samples and from leaf segments pre-incubated in 1 mM of either Put²⁺ or Spm⁴⁺ for 2–3 h. Results for Spd³⁺ were in between those for Spm⁴⁺ and Put²⁺ (not shown). Means ± S.E. (n = 6–8 leaf segments isolated from 4 to 5 different leaves for each treatment). (b) The peak magnitude of NaCl-induced K⁺ efflux from pea mesophyll segments pre-incubated in 1 mM Put²⁺ for various amounts of time before adding 50 mM NaCl. Mean ± S.E. (n = 4–7). The sign convention is “efflux negative” for all MIFE measurements.
on NaCl-induced K⁺ efflux was found (Fig. 2), with \( K_{\text{m}} \) values being 40 and 50 \( \mu \text{M} \) for Put²⁺ and Spm⁴⁺, respectively (the dose-dependence of Spd³⁺ was not studied).

Membrane potential measurements suggested that cell pre-treatment with polyamines reduced the magnitude of NaCl-induced depolarization of the membrane potential (Fig. 3). In general, Spm⁴⁺ was more efficient than Put²⁺ in preventing membrane depolarization. The efficiency of the polyamine effect also increased with increasing concentrations of polyamines (Fig. 3).

Further characterization of the effects of polyamines on the electrophysiological characteristics of mesophyll cells was conducted using the patch-clamp technique. Several types of ion channels were observed in pea mesophyll in the whole-cell configuration; the most abundant of which (observed in >80% of protoplasts) were the instantaneously activated currents (Fig. 4a). The reversal potential (\( V_r \)) for all currents was about −60 mV under standard conditions (5 mM K⁺ in the bath; 100 mM K⁺ in the pipette) (Fig. 4e). This is close to the \( E_K \) (−72 mV), implying their high cationic selectivity. However, as the instantaneously activated currents also mediated Na⁺ fluxes (Fig. 4b), as well as Ca²⁺ fluxes (data not shown); we term the respective channels as NSCC (for non-selective cation channels). These channels were our focus for further investigation.

Both Put²⁺ and Spm⁴⁺ (1 mM concentration) were effective in blocking ~70% of NSCC inward currents (as illustrated for Put²⁺ in Fig. 4c). Also reduced was the outward current (~30% reduction 3 min after Spm⁴⁺ application, significant at \( P < 0.05; \) ~20% reduction 3 min after Put²⁺ application, +60 to +100 mV range). The above blocking effect of polyamines on NSCC currents showed a clear time-dependency (illustrated for Put²⁺ in Fig. 4f). The characteristic time of this process was 4–5 min as estimated from the single exponential fit of the normalized channel-mediated current at −160 mV (Fig. 4e). It should be mentioned that in each experiment, polyamines were added only after the stabilization of the current amplitude in the control (7–10 min after obtaining the whole-cell configuration), after the absence of run-downs was confirmed.

Adding 50 mM NaCl to the bath solution dramatically (2.5-fold, significant at \( P < 0.01 \)) increased the magnitude of inward NSCC currents, without any substantial effect on the chord conductance of the outward current (Fig. 4b and e). There was also an obvious shift in \( V_r \) values (from −60 mV in 5 mM KCl bath, to −20 mV in 5 mM KCl + 50 mM NaCl bath), suggesting that NSCC was indeed conducting Na⁺. Adding 1 mM Put²⁺ significantly (\( P < 0.05 \)) inhibited the inward (Na⁺) current (Fig. 4c). The protoplast washout with 50 mM NaCl solution showed only partial reversibility of polyamine effects on NSCC currents (Fig. 4d).

Another type of the channel observed in some patches was KOR channels (Fig. 5). Accordingly, we undertook a study on the effect of polyamines on KOR currents in whole-cell patch-clamp experiments. This was not a trivial task, as KOR channels were present only in ~10% of successful patches, and the overall success rate of seal formation on pea mesophyll was rather low (~5%) in our experiments. Nonetheless, we were able to analyze PA effects on two samples with KOR activity (Fig. 5). In both cases, no effect of PA on the time-dependent K⁺ outward current was found (illustrated for 1 mM Spm⁴⁺ in Fig. 5), although instantaneous inward currents (through NSCC) were substantially suppressed.

### 4. Discussion

Elevated Na⁺ levels in the leaf apoplast cause a substantial K⁺ efflux from mesophyll cells [27,31–33]. With K⁺ being an activator of a large number of enzymes, the consequences of such a K⁺ loss to cell metabolism are rather drastic.

Reports on the effect of polyamines on plasma membrane K⁺ transport date back two decades [42]. In this work, we show that micromolar concentrations of externally applied polyamines (\( K_{\text{m}} = 40–50 \mu \text{M} \); Fig. 2) substantially reduced
The inhibitory effect of externally applied polyamines on both K⁺ efflux (Fig. 1b) and on NSCC-mediated currents (Fig. 4f) developed slowly over time, thus ruling out a direct inhibition of membrane cation conductance from outside the cell. More likely, polyamines are transported across the plasma membrane [43,44] and exert their inhibitory effects from the cytosolic side. Therefore, the potency of polyamine block will depend on how fast a threshold concentration is built up within the cytosol. Interestingly, in situ effects of exogenous polyamines (measured in MIFE experiments) are much slower than those observed in patch-clamp measurements, suggesting that removing the cell wall may cause severe perturbation to

Fig. 4. Non-selective cation channels (NSCC) in pea mesophyll cells and the effect of polyamines on their currents. (a)–(d) Typical examples of recordings of NSCC currents. (a) Control (5 mM KCl + 1 mM CaCl₂). (b) −50 mM NaCl in the bath; (c) −50 mM NaCl + 1 mM Put²⁺ (after 15 min); (d) Washout (>30 min in 50 mM NaCl). (e) I–V relationships for instantaneous currents (mean ± S.E.; n = 4–7). (f) Normalized protoplast currents as a function of time for Put²⁺ effects. Mean ± S.E. (n = 4). Solid line is a best fit to monoexponential function with an offset.

Fig. 5. Outward-rectifying K⁺ currents (mediated by KOR channel) in pea mesophyll cells are not blocked by exogenously applied polyamines. A typical example of KOR recording from a small outside-out vesicle (~0.4 pF) isolated from pea protoplast is shown. From a holding potential of −80 mV voltage steps from −160 mV up to +100 mV were applied in 20 mV increments. Adding 1 mM Spm⁴⁺ to the bath did not substantially inhibit outward K⁺ current, while instantaneous inward currents (mediated by NSCC) was strongly suppressed. The bath contained (in mM): 1 CaCl₂, 5 KCl, 2 MES-KOH (pH 5.6), and pipette solution: 100 KCl, 2 MgCl₂, 0.1 µM free Ca²⁺, 10 HEPES–Tris (pH 7.2).
cell metabolism (and, possibly, transport properties). More likely, however, is that in MIFE experiments, a substantial amount of applied polyamines is strongly bound to cell walls. It has been reported that polyamines adsorb selectively on plant cell walls according to their valency (up to 70% of the total wall CEC for Put\(^{2+}\); up to 90% for Spd\(^{3+}\) and Spm\(^{4+}\) [45]). Thus, it is reasonable to assume that it should take far longer for the same amount of polyamine to be accumulated in the cytosol of intact cells (compared with isolated protoplasts) to reach the same threshold concentration. Furthermore, stronger Spm\(^{4+}\) binding to the cell wall [45] may be an explanation of why Put\(^{2+}\) was more efficient in preventing NaCl-induced K\(^{+}\) efflux during the first moments of acute salt-stress (Fig. 1a). The only report of effect of polyamines on plasma membrane ion channels in plant cells so far is from a study of K\(^{+}\) inward-rectifying (KIR) channels in *Vicia* guard cell [5]. Polyamines in that case indirectly inhibited KIR current from the cytosolic side. Similar to our study, no large specificity of polyamine effect was observed: at 1 mM concentration Put\(^{2+}\), Spd\(^{3+}\) and Spm\(^{4+}\) inhibited 40%, 60%, and 55% of KIR-mediated currents, respectively [5]. In contrast to our data for NSCC, up to 15 min incubation with externally applied polyamines did not affect the KIR current amplitude. One possible explanation is that different cell types, such as guard and mesophyll cells, possess different capacities for polyamine transport across the plasma membrane.

To the best of our knowledge, no direct information about the free cytosolic polyamine levels is available in the literature. However, experiments on protoplasts with radiolabelled [\(^{14}\)C]-polyamines suggested that only 42% of Put\(^{2+}\) and 28% Spd\(^{3+}\) were compartmentalized in the vacuole, with less than 2% of free polyamines found in either the chloroplasts or mitochondria [46]. Thus, it might be reasonable to assume that a substantial portion of free polyamines, not bound to the cell wall, may reside in the cytosol under our conditions. Keeping in mind the \(K_m\) values for Put\(^{2+}\) and Spm\(^{4+}\) effect on NaCl-induced K\(^{+}\) efflux being 40–50 \(\mu\)M, even resting PA levels may be sufficient to (at least partially) block K\(^{+}\) efflux from the cell. The efficiency of this block will increase if the total polyamine pool is increased under stress conditions. Polyamine levels increase in response to a variety of abiotic stresses, showing a stress-specific pattern. When it comes to salt-stress, most researchers are unanimous in their reports of a significant elevation in Spd\(^{3+}\) or Spm\(^{4+}\). Data for Put\(^{2+}\) is rather controversial, with both elevation [17,47–49] and decline [50–52] reported. The impact of salt-stress on free Put\(^{2+}\) content also appears to be tissue- and cell-type specific [48,51]. However, polyamine control of NaCl-induced K\(^{+}\) efflux showed little specificity, with Put\(^{2+}\) being as efficient as Spd\(^{3+}\) or Spm\(^{4+}\) (Figs. 1 and 2). Therefore, it is the size of the total pool of free polyamines, rather than specific polyamine content, that is important to control intracellular K\(^{+}\) homeostasis, at least in pea mesophyll.

Polyamines are ideally suited as physiological channel blockers. While being efficient channel blockers, most inorganic polycations (e.g. Al\(^{3+}\); Gd\(^{3+}\); La\(^{3+}\)) are, at the same time, highly toxic to cell metabolism, so cannot be accumulated in the cytosol at the concentrations needed for “safe” control of intracellular homeostasis. Polyamines are the only organic polycations present in sufficient quantities to perform the role of channel blockage, without compromising cell metabolism.

The simplest explanation for the observed reduction of NaCl-induced K\(^{+}\) efflux by polyamines is a blockage of KOR channels. However, in the case of pea mesophyll cells, KOR currents were insensitive to polyamines (Fig. 5). This observation is consistent with reports on polyamine effects on the KOR currents in guard cells [5]. Moreover, KOR channels were expressed in less than 10% of successfully patched protoplasts in our experiments. Thus, the involvement of some other K\(^{+}\)-permeable channel should be considered.

It is generally agreed that NaCl-induced depolarization of the membrane potential is caused by excessive Na\(^{+}\) entry into the cytosol. NSCC are often cited as a major route for this Na\(^{+}\) uptake, at least in root tissue [53,54]. Evidence for leaves is scarce, although Na\(^{+}\)-permeable cation channels were reported earlier for Arabidopsis mesophyll cells [27], and Elzenia and Van Volkenburgh [56] have reported TEA-insensitive cation channels in the plasma membrane of pea epidermal cells that poorly discriminate between potassium, sodium and lithium. In this work, non-selective, K\(^{+}\)- and Na\(^{+}\)-permeable cation channels was reported in pea mesophyll protoplasts (Fig. 3). Although its detailed characterization is outside the scope of this paper, the general features of this channel (current rectification, instantaneous activation, selectivity) are very similar to previously reported NSCC in plant root [53], guard [55] or leaf epidermal [56] cells. Importantly, both inward- and outward-currents were effectively blocked by polyamines (illustrated for Put\(^{2+}\) in Fig. 4 and Spm\(^{4+}\) in Fig. 5).

Taken together with MIFE flux measurements and membrane pore potential determinations, these results suggest the following scenario. Under saline conditions, Na\(^{+}\) enters the cytosol through NSCC, inducing plasma membrane depolarization (Fig. 3). This results in increased K\(^{+}\) efflux (Fig. 1) through depolarization-activated outward-rectifying KOR channels [27] and NSCC channels. Both Put\(^{2+}\) and Spm\(^{4+}\) block NSCC (from the cytosolic side, in a time-dependent manner), reducing the magnitude of NaCl-induced plasma membrane depolarization and, consequently the depolarization-induced K\(^{+}\) efflux through KOR. In addition to this mechanism, polyamines may directly block outward K\(^{+}\) currents through NSCC (Figs. 3 and 4) or activate the plasma membrane H\(^{+}\)-ATPase [4], so restoring the membrane potential. One way or another, polyamine regulation of membrane transport activity would enable the maintenance of an optimal cytosolic K\(^{+}\)/Na\(^{+}\) ratio for cell metabolism. This scenario may explain both the reported observations of reduced Na\(^{+}\) accumulation in leaves [10] and higher K\(^{+}\)/Na\(^{+}\) ratios [9] in the shoot of polyamine-treated plants grown in the presence of NaCl.

A direct confirmation for this model could come from measurements of polyamine effects on net Na\(^{+}\) flux from salinized mesophyll tissue. Unfortunately, for the moment, these measurements are completely out of question due to the poor selectivity of all available Na\(^{+}\) LIX (see [28] for details). The acute NaCl stress causes significant K\(^{+}\) and Ca\(^{2+}\) efflux (due to plasma membrane depolarization and resulting from Na\(^{+}\)/Ca\(^{2+}\)-Donnan exchange in the cell wall, respectively). This efflux is measured by the Na\(^{+}\) LIX (sensitive to both K\(^{+}\) and Ca\(^{2+}\); [28]) and interpreted as an apparent Na\(^{+}\) efflux, thus completely masking any Na\(^{+}\) uptake.

In summary, our results suggest that the observed stress-induced elevation in the level of endogenous polyamines under saline conditions, may represent an important adaptive mechanism: reducing the uptake of Na\(^{+}\) and leakage of K\(^{+}\) from mesophyll cells, thus assisting plants in their adaptation to salinity.
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