

Phosphatidylinositol (4,5)Bisphosphate Inhibits K⁺-Efflux Channel Activity in NT1 Tobacco Cultured Cells^{1[W][OA]}

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In the animal world, the regulation of ion channels by phosphoinositides (PIs) has been investigated extensively, demonstrating a wide range of channels controlled by phosphatidylinositol (4,5)bisphosphate (PtdInsP₂). To understand PI regulation of plant ion channels, we examined the in planta effect of PtdInsP₂ on the K⁺-efflux channel of tobacco (*Nicotiana tabacum*), NtORK (outward-rectifying K channel). We applied a patch clamp in the whole-cell configuration (with fixed "cytosolic" Ca²⁺ concentration and pH) to protoplasts isolated from cultured tobacco cells with genetically manipulated plasma membrane levels of PtdInsP₂ and cellular inositol (1,4,5)trisphosphate: "Low PIs" had depressed levels of these PIs, and "High PIs" had elevated levels relative to controls. In all of these cells, K channel activity, reflected in the net, steady-state outward K⁺ currents (I_K), was inversely related to the plasma membrane PtdInsP₂ level. Consistent with this, short-term manipulations decreasing PtdInsP₂ levels in the High PIs, such as pretreatment with the phytohormone abscisic acid (25 μM) or neutralizing the bath solution from pH 5.6 to pH 7, increased I_K (i.e. NtORK activity). Moreover, increasing PtdInsP₂ levels in controls or in abscisic acid-treated high-PI cells, using the specific PI-phospholipase C inhibitor U73122 (2.5–4 μM), decreased NtORK activity. In all cases, I_K decreases stemmed largely from decreased maximum attainable NtORK channel conductance and partly from shifted voltage dependence of channel gating to more positive potentials, making it more difficult to activate the channels. These results are consistent with NtORK inhibition by the negatively charged PtdInsP₂ in the internal plasma membrane leaflet. Such effects are likely to underlie PI signaling in intact plant cells.

Many environmental and internal signals induce increased metabolism of phosphoinositides (PIs) in both animal and plant cells. PIs are involved in numerous cellular processes important for cell develop-

ment and growth, including secretion of metabolites, vesicular transport, organization of the cytoskeleton, and regulation of ion channels and transporters (for review, see Hilgemann, 2003; Meijer and Munnik, 2003; Suh and Hille, 2005; Huang, 2007). In many signaling cascades, phosphatidylinositol (4,5)bisphosphate (PtdInsP₂) undergoes cleavage by phospholipase C (PLC), producing diacylglycerol and inositol (1,4,5)trisphosphate (InsP₃). While diacylglycerol operates within the plane of the membrane, production of InsP₃ is practically synonymous with further signaling through mobilization of Ca²⁺ from internal stores (Berridge and Irvine, 1984; Berridge et al., 1998).

In the last decade, signaling via PtdInsP₂ itself, rather than via its cleavage products, has become the focus of intense study in animal cells. Detailed descriptions have accumulated about PtdInsP₂ interactions with membrane ion channels and other ion transporters, highlighting its important roles in conveying information about physical and chemical stimuli and in maintaining cellular ionic homeostasis (for review, see Suh and Hille, 2005; Huang, 2007).

In the plant world, this topic has been evolving at a much slower pace. Over two decades of study focusing on plant signaling via the PI pathway and Ca²⁺ mobilization have linked signals that cause cell shrink-

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ing with the cleavage of PtdInsP₂, for example, in the case of the phytohormone abscisic acid (ABA) in *Vicia faba* guard cells (Lee et al., 1996) or blue light in the leaf motor cells, such as “flexors” of *Samanea saman* (Kim et al., 1996) and “extensors” of *Phaseolus coccineus* (Mayer et al., 1997). Moreover, a strong positive correlation has been revealed between these “shrinking signals” and the activation of anion- and K⁺-release channels in the plasma membrane of the shrinking cells (Blatt, 2000; Suh et al., 2000; Hetherington, 2001; Schroeder et al., 2001; Moran, 2007a, 2007b; Pandey et al., 2007). Accumulating evidence dissociated the activation of these channels from obligatory Ca²⁺ mobilization, suggesting alternative signaling pathways: for example, Marten et al. (2007), using fluorescent Ca²⁺-reporter dye, demonstrated ABA activation of anion channels in *V. faba* and tobacco (*Nicotiana tabacum*) guard cells in the absence of or prior to an observable rise in cytosolic [Ca²⁺]. Similarly, Lemtiri-Chlieh and MacRobbie (1994) found in patch-clamp experiments with Ca²⁺-buffered cytosolic milieu that ABA activation of K⁺-release channels in *V. faba* guard cells was independent of cytosolic Ca²⁺. Importantly, and consistent with the latter report, direct examination of K⁺-release channels in excised membrane patches from *V. faba* guard cells (Hosoi et al., 1988) or from *S. saman* motor cells (Moshelion and Moran, 2000) revealed that their activity did not require Ca²⁺ at their cytosolic surface. Cytosolic alkalinization or membrane depolarization resulting from H⁺ pump inhibition and anion channel activation have been proposed as alternative mediators of ABA activation of guard cell K⁺-release channels (Irving et al., 1992; MacRobbie, 1992; Blatt and Armstrong, 1993; for a recent review, see Pandey et al., 2007). However, depolarization accounted only partially for the stimulation of K⁺-release channel activity by a “shrinking” blue light in the case of *S. saman* leaf motor cells (Suh et al., 2000).

Could PtdInsP₂ itself modulate K⁺-release channels? To address this question, Liu et al. (2005) examined three plant K channels, two K⁺ influx channels, LKT1, a tomato (*Solanum lycopersicum*) homolog of the Arabidopsis (*Arabidopsis thaliana*) AKT1, the Arabidopsis KAT1, and the Arabidopsis K⁺-efflux channel, SKOR. Applying a patch clamp to frog oocytes expressing these channels, they demonstrated Ca²⁺-independent regulation by PI lipids. Isomers of PtdInsP, PtdInsP₂, or PtdInsP₃ applied to the cytosolic side of excised membrane patches prevented the rundown and enhanced the activity of these channels (Liu et al., 2005). These results, resembling direct activation of various types of animal ion channels by PtdIns(4,5)P₂ (for review, see Hilgemann, 2004; Suh and Hille, 2005; Huang, 2007), suggested a direct correlation between PtdInsP₂ level and SKOR activity in oocytes (Liu et al., 2005).

In contrast, interpretation of in planta experiments leads to a direct relation between the up-regulation of the K⁺-release channel and PtdInsP₂ cleavage (i.e. to an inverse correlation between PtdInsP₂ level in the

membrane and channel activity). This can be seen in the case of SKOR-like channels in guard cells (Blatt, 1990; Blatt et al., 1990; Lemtiri-Chlieh and MacRobbie, 1994; Lemtiri-Chlieh, 1996) or in *S. saman* flexors (Kim et al., 1996; Suh et al., 2000).

To reconcile this apparent discrepancy, we undertook to examine this relationship more explicitly in planta. We investigated NtORK in tobacco cultured cells (NT1), the previously described representative of the SKOR-related K⁺-release channel family (Kasukabe et al., 2006; Sano et al., 2007, 2008). We used NT1 with genetically manipulated basal levels of PtdInsP₂ and InsP₃ (Perera et al., 2002; Im et al., 2007). Three types of cell lines were studied: “Low PIs,” cells with lowered (relative to controls) levels of PtdInsP₂ and InsP₃; “High PIs,” cells with elevated levels of both PIs; and control cell lines transformed with “empty” vectors and the wild type. Using a patch clamp in a whole-cell configuration, with the cytosolic concentrations of Ca²⁺ and protons tightly controlled by appropriate buffers, we established in these cells a negative correlation between the basal level of PtdInsP₂ and NtORK activity, as expected from the reported plant motor cell findings. Further manipulations of the PtdInsP₂ membrane levels, causing either its cleavage by the plant hormone ABA and by elevation of bath pH or its accumulation by PLC inhibition, strengthened this conclusion. Taken together, our results strongly suggest the inhibition of NtORK by PtdInsP₂. In addition, the initiation of a signaling cascade by ABA in the NT1 cells, which is characteristic of guard cells, should further encourage the use of this model system in studies of signal transduction.

RESULTS

K⁺-Release Channels in Tobacco Cultured Cells with Modified Membrane Lipids

Channel Identification through Comparison with Previous Work

In whole-cell patch-clamp configuration, increasingly depolarizing voltage pulses evoked ion channel activity. This is evident in all protoplasts of the three types of NT1 cell lines, the Low PIs, the High PIs, and the various controls (Fig. 1A; see “Materials and Methods”), as time-dependent outward currents. The sigmoidal time and voltage dependencies of these currents resembled those of outward K⁺ currents, flowing through K channels, described already in the similar tobacco cell line BY2 (Stoeckel and Takeda, 2002; Sano et al., 2007).

Channel Identification by Blockers

We identified the channels as K⁺-conducting channels by following treatments with the classical K channel blockers: (1) internal Cs⁺ to block the outward current (Supplemental Fig. S1A), (2) external Cs⁺ to

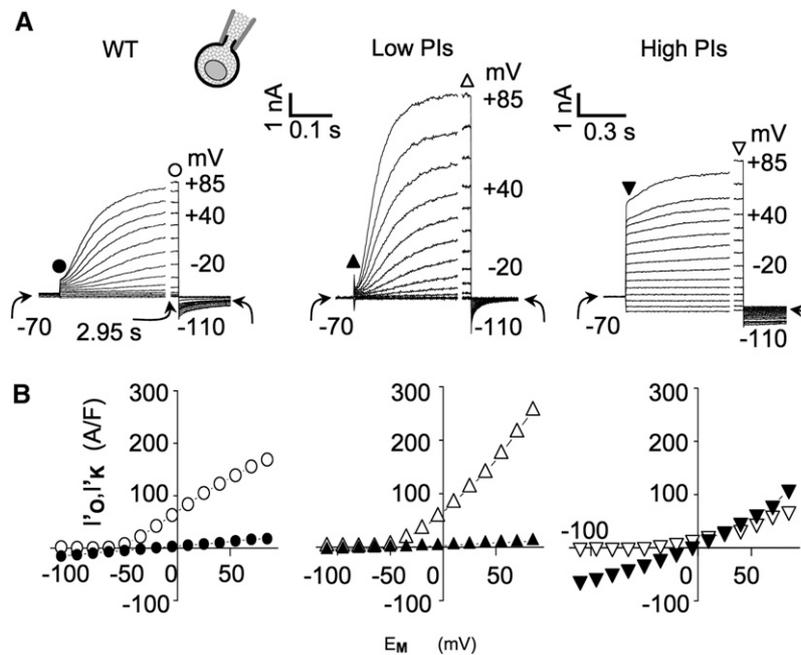


Figure 1. Comparison of steady-state and instantaneous currents in NT1 cells. A, Whole-cell currents (superimposed traces) evoked by a series of increasingly depolarizing voltage pulses in tobacco protoplasts with different membrane PI levels. WT (Wild type), one of the control cell lines with intermediate PI levels; Low PIs, cell line (I2-8) with diminished level of PIs; High PIs, cell line (HsPIP1 α -2) with elevated PI levels. Numbers to the right of traces or near leftward- or rightward-pointing arrows indicate the values of E_M , membrane potential (in mV), during the recording. Black symbols indicate instantaneous currents, I_O (leak), and white symbols indicate steady-state current values, which, after subtraction of corresponding I_O , yield the net steady-state current, I_K . Note the 2.7-s-long break in the time scale; the 0.05-s scale bar relates to the first part, and the 0.2-s scale bar relates to the second part, restarting about 2.9 s after beginning of the pulse (upward arrow). The inset shows the recording configuration. B, Membrane potential dependence of whole-cell current (so-called current-voltage, I-V or I- E_M relationship) obtained from the records, in A, of I_K or I_O , as indicated by the symbols. Prime (') indicates normalization of the currents to the cell's capacitance (see "Materials and Methods"). Note the constant slope of I'_O , indicating voltage-independent leak conductance, and its close-to-null reversal potential, indicating poor ion selectivity.

block the inward "tail" current (Supplemental Fig. S1B), and (3) external Ba²⁺ to block both the outward and the inward currents (Supplemental Fig. S1C). Furthermore, in all of the cell lines, the reversal potentials of these currents were only slightly more depolarized than the calculated K⁺ equilibrium (Nernst) potential of -81 mV (Supplemental Fig. S2), providing additional support for identifying these channels as K⁺-selective K⁺-release channels, NtORK channels (Sano et al., 2007). Actually, although two genes, *NtORK1* and *NtORK2*, have been mentioned in the literature, only *NtORK1* has been characterized to some extent so far (Langer et al., 2002; Kasukabe et al., 2006; Sano et al., 2007). Because our measurements do not indicate clearly two separate types of channels, we use the inclusive name, NtORK.

Correlating Channel Activity with PtdInsP₂ Level

At external pH 5.6, the NtORK channels were most active in the Low PIs, least active in the High PIs, and they displayed intermediate activity in the control protoplasts, as evident from the net, steady-state K⁺ currents normalized to cell capacitance, I'_K (Fig. 1;

Supplemental Fig. S3, A and B; see "Materials and Methods"). The effect of the InsP₃ 5-phosphatase with which the Low PIs were generated was evident in the cells where the 5-phosphatase was targeted to the plasma membrane (I2-8 and I4-2; Perera et al., 2002; Supplemental Fig. S4). When the 5-phosphatase was inactive (C348S) or missing the C terminus and therefore no longer bound to the plasma membrane (Δ C), the channels behaved similarly to the wild-type or "empty-vector" (C5, GFP) controls (Supplemental Fig. S3A). These data indicate that plasma membrane targeting, not just the ability to hydrolyze InsP₃ (Supplemental Fig. S5), is essential. It is most likely that the InsP₃ 5-phosphatase needs to be in close proximity to the channel to have an effect.

In contrast to I'_K , the baseline "leaks" (normalized instantaneous currents), I'_O , were larger in the High PIs than in all other cells, but in all cell types, leak conductance was constant over the whole voltage range and its ion selectivity was poor. We base this latter conclusion on the linearity of the I'_O - E_M (current-membrane potential) curves and their nearly null intersection with the abscissa, which was an intermediate value between all equilibrium potentials calcu-

lated for the ions in the solutions on both sides of the membrane (Fig. 1; Supplemental Fig. S3, C and D).

Components of Channel Inhibition

In an attempt to resolve the possible effects of PtdInsP₂ on NtORK into effects on properties of the open channel versus effects on channel gating (i.e. opening and closing), we examined the voltage dependence of its chord conductance, $G'_{K'}$, extracted from $I'_{K'}$ (Eq. 1 in “Materials and Methods”). Three characteristic Boltzmann parameters served to compare the cell lines: G'_{max} , the maximum attainable conductance (normalized to cell capacitance); $E_{1/2}$, the voltage at which half of G'_{max} is attained; and z , the effective charge of the gating subunits (Eq. 2 in “Materials and Methods”). As could be deduced from the current measurements, cells with higher PtdInsP₂ levels had lower G'_{max} , and vice versa (Fig. 2, A and B). This analysis revealed also a mean difference of about 23 mV between $E_{1/2}$ values of the High PIs and the rest of the cell lines. This difference is concealed in the $I'_{K'}$ - E_M plots (Supplemental Fig. S3, A and B) and

even in the $G'_{K'}$ - E_M plots (Fig. 2A) and is clearly visible only in the plots of the probability of opening, P_O , versus membrane potential (Eq. 3 in “Materials and Methods”). Thus, the P_O -voltage relationships (P_O - E_M s) of the High PIs, reflecting the voltage dependence of the channel-gating subunits, were shifted significantly to more depolarizing membrane potentials (rightward) relative to Low PIs and controls (Fig. 2, B and C). z values, reflected in the “slopes” of the $G'_{K'}$ - E_M curves, did not vary among the three cell types (High PIs, Low PIs, and controls; Fig. 2B).

Effects of ABA

Effects of ABA on Plasma Membrane PtdInsP₂ Content

If indeed NtORK activity depends inversely on the membrane PtdInsP₂ content, lowering the PtdInsP₂ level should increase NtORK activity. Therefore, we examined whether we can lower the PtdInsP₂ in the high-PI NT1 cells utilizing the plant hormone ABA, which is known to activate PLC in guard cells (Lee et al., 1996). Indeed, a 15-min incubation of protoplasts

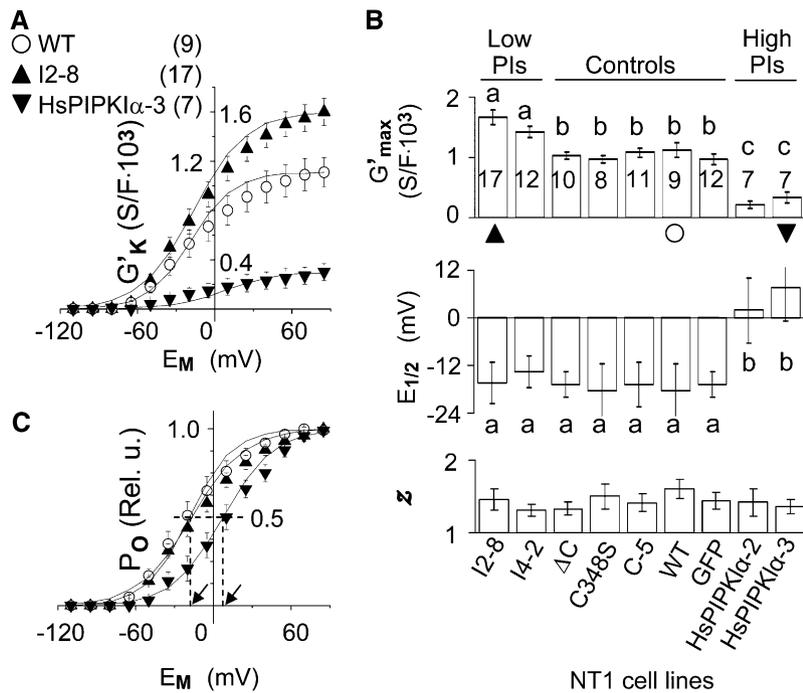


Figure 2. The effect of PtdInsP₂ on the K channel chord conductance. A, Symbols show mean ± SE conductance-voltage relationships normalized to cell capacitance ($G'_{K'}$ - E_M s), obtained by averaging individual $G'_{K'}$ - E_M s ($G'_{K'}$ units: Siemens/Farad) extracted from each cell's $I'_{K'}$ - E_M relationships (see “Materials and Methods”). The chosen cell lines represent the Low PIs, High PIs, and controls shown in Supplemental Figure S3 and described in “Results” and “Materials and Methods.” The numbers of assayed cells are indicated in parentheses. Lines were calculated using Equation 2 and the averaged best-fit Boltzmann parameters (G'_{max} , $E_{1/2}$, z ; shown in B) obtained from analyses of individual $G'_{K'}$ - E_M s. WT, Wild type. B, Mean ± SE best-fit Boltzmann parameters resulting from fitting Equation 2 to the individual $G'_{K'}$ - E_M s of cells in A (see “Materials and Methods”): G'_{max} , the maximum (asymptotic) conductance (top panel); $E_{1/2}$, the E_M value at half- G'_{max} (middle panel); and z , the effective charge of the gating subunit (bottom panel). The parameter values differ significantly if they are denoted by different letters (a, b, or c). Other details are as in A. C, Symbols show mean ± SE probability of channel opening versus voltage, P_O - E_M relationships. Lines are as in A, except for using Equation 3 here. Vertical dashed lines and arrows denote the corresponding $E_{1/2}$ values.

isolated from the high-PI cells with 25 μM ABA significantly lowered the PtdInsP₂ levels (Fig. 3). The mean effects of ABA on the already low levels of PtdInsP₂ present in wild-type cells were minute, and in the GFP cells they were insignificant.

Effects of ABA on NtORK

In the patch-clamp experiments, 10- to 20-min preincubation of protoplast with 25 μM ABA increased I'_{K} in the High PIs and had no effect on I'_{K} in the control cell lines (Fig. 4A). This correlated with changes in the PtdInsP₂ levels. Boltzmann analysis of NtORK G'_{K} revealed that in the High PIs, but not in the controls, ABA pretreatment increased G'_{max} considerably and shifted $E_{1/2}$ by about 15 mV in a hyperpolarizing (leftward) direction (Fig. 5). z was not affected by ABA (Fig. 5B).

Effects of pH

Effects of pH on the Content of Plasma Membrane PtdInsP₂

Because a small alkalinization of the apoplast was correlated with stomatal closure in *Vicia faba* and potato (*Solanum tuberosum*) leaves (Hedrich et al., 2001), we hypothesized that pH would have an ABA-mimicking (although ABA-independent) effect. Incubating the wild-type cells for about 15 min in external solution buffered to pH 7.0 (rather than to pH 5.5) decreased the plasma membrane PtdInsP₂ levels only by roughly 10 units ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ plasma membrane protein), and in GFP cells the mean

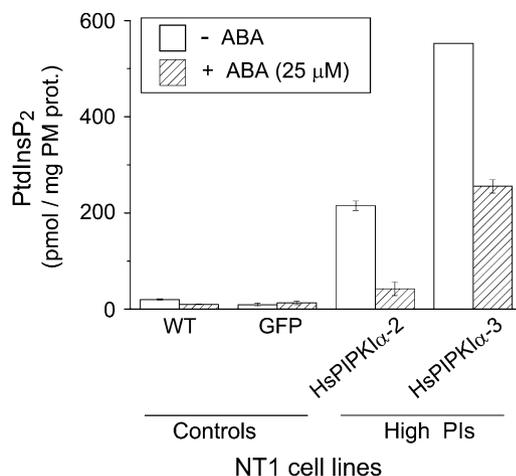


Figure 3. The effect of ABA on PtdInsP₂ levels in the plasma membrane (PM). Mean \pm sd of PtdInsP₂ levels in protoplasts isolated from the indicated cell lines, incubated for 15 min without and with 25 μM ABA at a temperature of 24°C to 25°C. Plasma membrane lipid levels were determined based on an InsP₃ assay of the lipid hydrolysate (Im et al., 2007; see "Materials and Methods"). The results shown are from one of two independent experiments performed with two replicates that gave similar results. WT, Wild type.

PtdInsP₂ level was not affected (Fig. 6). In contrast, in high-PI protoplasts, the pH shift decreased the PtdInsP₂ levels by about 100 to 440 units (Fig. 6), resembling the effect of ABA (Fig. 3).

Effects of pH on NtORK

In correlation to the effect of pH on PtdInsP₂ levels, 10- to 20-min incubation of protoplasts in the external solution buffered to pH 7 prior to attaining a whole-cell patch-clamp configuration increased I'_{K} in the High PIs but did not affect I'_{K} in the control cell line (Fig. 7A). Boltzmann analysis of NtORK $G'_{\text{K}}-E_{\text{M}}$ relationships (Fig. 8) revealed an increase of the maximum conductance, G'_{max} (Fig. 8, A and B) and a hyperpolarizing (leftward) shift of about 36 mV of the $P_{\text{O}}-E_{\text{M}}$ relationship (Fig. 8, B and C). In contrast to enhancing I'_{K} (i.e. NtORK activity), pH 7 significantly decreased the leak currents in both High PIs, down to its levels in the control cells (Fig. 7B).

Effects of PLC Inhibitor

If PtdInsP₂ inhibits NtORK activation, then preventing its cleavage by inhibiting PLC, consequently leading to PtdInsP₂ accumulation, should decrease NtORK conductance. In ABA-pretreated protoplasts from High PIs (to promote their NtORK activity) or in wild-type protoplasts, a bath exposure to U73122 (the commonly used PI-PLC inhibitor; Bleasdale et al., 1990) decreased I'_{K} progressively within a few minutes (Fig. 9). Adding the same volume of the external solution to the bath with the High PIs or adding U73343, the inactive analog of U73122 (Bleasdale et al., 1990), to wild-type protoplasts had no immediate discernible effect on I'_{K} (Fig. 9, B and D). Longer term incubation of control cell protoplasts with U73122 (including several minutes prior to attaining the whole-cell configuration) resulted, on average, in about 65% inhibition of I'_{K} ; however, the same treatment with U73343 inhibited I'_{K} by only about 20% (Fig. 10A). U73122 also decreased I'_{O} , while U73343 did not affect it (Fig. 10B).

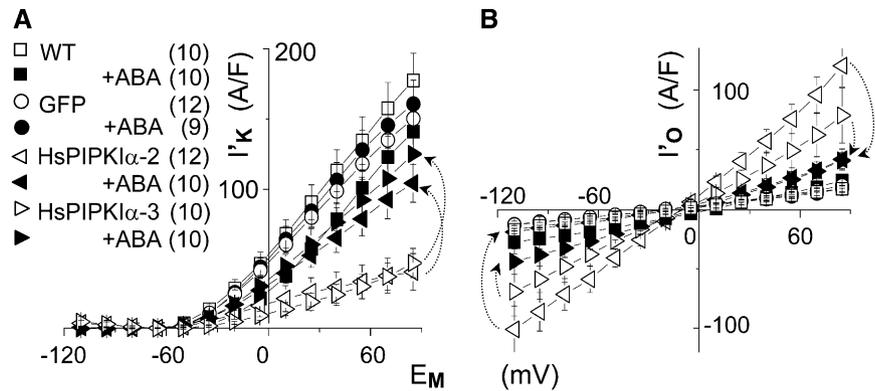
Boltzmann analysis of the $G'_{\text{K}}-E_{\text{M}}$ relationship under these treatments in steady-state conditions revealed a roughly 65% inhibition of G'_{max} by U73122 (Fig. 10, C and D) and, notably, also a shift of the activation curve in the depolarizing (rightward) direction (Fig. 10, D and E).

DISCUSSION

NtORK Activity Is Inversely Related to Genetically Manipulated Levels of PtdInsP₂ by Two Molecular Mechanisms

Two molecular mechanisms can explain the diminished NtORK activity accompanying the increased levels of PtdInsP₂ in the High PIs. A major effect can be attributed to PtdInsP₂ inhibition of the maximum chord conductance, G'_{max} (which is G'_{K} at its satura-

Figure 4. The effect of ABA on I'_K and leak current. A, Mean \pm SE normalized net steady-state K^+ current-voltage (I'_K - E_M) relationships in High PIs (HsPIP1 α -2 and HsPIP1 α -3) and their controls (wild type [WT] and GFP). The numbers of assayed cells are indicated in parentheses. Round arrows indicate the effect of ABA. B, Normalized instantaneous currents, leak voltage (I'_O - E_M) relationships in High PIs and in their controls with and without ABA, obtained from the same current records as those used for A. Round arrows are as in A. ABA decreased I'_O significantly only in one high-PI line.



tion level and, hence, voltage independent). The other can be attributed to a G'_K - E_M shift.

G'_{max}

G'_{max} is a product of the unitary conductance of a single NtORK channel, γ_s ; of the maximum (voltage-independent) fraction of the time a channel dwells in the open state (maximum open probability [P_{max}]); and of the number of NtORK channels in the plasma membrane (actually, in a unit area of it) attainable for voltage activation, N' . Thus, PtdInsP₂ could decrease N' , or P_{max} , or γ_s , or any combination of them. Which of the three factors is the more likely target for PtdInsP₂ effects?

γ_s

In different channels, these effects seem to vary. Binding of an anionic lipid palmitoylphosphatidylglycerol to the two-transmembrane-domain bacterial K channel, KcsA, in a lipid bilayer increased γ_s (Marius et al., 2008). But PtdInsP₂ did not seem to affect γ_s , neither while inhibiting the animal olfactory cyclic nucleotide-gated channels (Zhainazarov et al., 2004) nor while activating the two-transmembrane-domain KIR channels (inward-rectifying K channels; Yang et al., 2000), or the Shaker-like KCNQ channels (voltage-gated KQT-like K channels) in mammalian cells (Li et al., 2005), or the plant Shaker-like SKOR (K^+ -release) channel in frog oocytes (Liu et al., 2005).

P_{max}

Palmitoylphosphatidylglycerol also increased the open probability of KcsA channels (Marius et al., 2008), and increased open probability was also a hallmark of PtdInsP₂ activation of several other different animal channels. For example, dissociation of PtdInsP₂ from the epithelial sodium channel, ENaC, decreased its open probability without affecting its plasma membrane abundance (Mace et al., 2008; for review, see Suh and Hille, 2008, and references therein). PtdInsP₂ also increased the open probability of the

plant SKOR channels in excised oocyte membrane patches (Liu et al., 2005). A word of caution: a change of open probability at a single membrane potential can actually mean a shift of the voltage activation curve rather than a change in the P_{max} ; we have not found this distinction in many of the published reports.

N'

Regulation of the activity of some ion channels, such as TRPV5 and ENaC channels in animal cells, has been

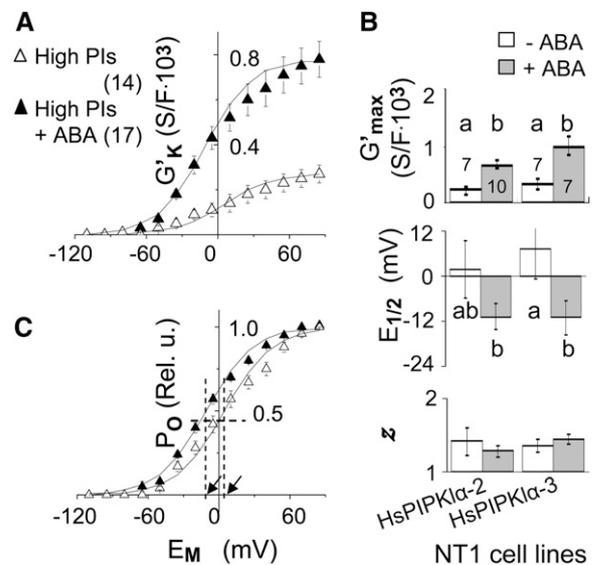


Figure 5. The effects of ABA on the K channel chord conductance. A, Symbols show mean \pm SE G'_K - E_M relations of the cells in Figure 4A (data of both high-PI cell lines were pooled together for the presentation). Lines were calculated using Equation 2 and the averaged best-fit Boltzmann parameters (G'_{max} , $E_{1/2}$, z ; shown in B) obtained from analyses of individual G'_K - E_M s. B, Mean \pm SE best-fit Boltzmann parameters. The parameter values differ significantly if they are denoted by different letters (a or b). C, Symbols show mean \pm SE probability of channel opening, P_O - E_M , relationships. Data of both cell lines were pooled together as in A. Lines are as in A, except for using Equation 3 here. Vertical dashed lines and arrows denote $E_{1/2}$ values without (right) and with (left) ABA. Note the ABA-induced hyperpolarizing shift of P_O - E_M .

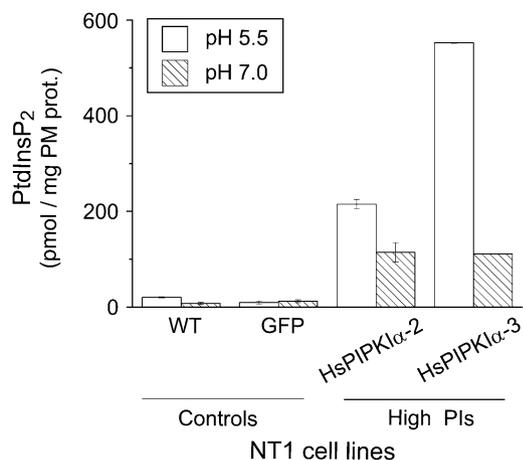


Figure 6. The effect of protons on PtdInsP₂ levels. Mean (±SD) PtdInsP₂ levels in protoplasts isolated from the indicated cell lines, incubated for 15 min at pH 5.5 and pH 7 at a temperature of 24°C to 25°C. Lipid levels were determined as described in the Figure 3 legend. The control values are those of Figure 3. The results shown are from one of two independent experiments performed with two replicates. PM, Plasma membrane; WT, wild type.

shown to involve recycling between the plasma membrane and endomembranes (Lambers et al., 2007; Mace et al., 2008). For example, treatments causing the dissociation of PtdInsP₂ from the ENaC subunits initiated the retrieval of ENaC protein from the plasma membrane (Mace et al., 2008). In tobacco plants, ABA treatment, known to enhance the metabolism of PtdInsP₂, caused the endocytosis of the K⁺-influx channel, KAT1-GFP, in epidermal and guard cells within 10 to 20 min. This effect was selective, since the H⁺-ATPase (PMA2-GFP) in the same cells was not affected even after a 120-min exposure (Sutter et al., 2007). Endocytosis and membrane recycling have already been linked with PtdInsP₂, for example in the growth of a pollen tube (Dowd et al., 2006; Helling et al., 2006). This complex link has been clarified recently in yeast. There, sites of endocytosis needed to be enriched in PtdInsP₂, but for endocytosis to be completed PtdInsP₂ had to be removed (Sun et al.,

2007). This is consistent with enhanced turnover of PtdInsP₂ being capable of promoting endocytotic internalization of ion channels. Based on this, we may expect enhanced endocytosis in the plasma membrane of the High PIs, where PtdInsP₂ is metabolized at an enhanced rate (Im et al., 2007), and consequently NtORK removal from the plasma membrane is reflected in a decreased N'. Thus, we deem it most likely that a combined decrease in P_{max}·N' underlies the inhibitory effect of PtdInsP₂ on NtORK G'_{max}.

G'_K-E_M Shift

The other inhibitory effect of the high PtdInsP₂ levels in the High PIs consists of shifting the voltage dependence of NtORK gating (the P_O-E_M relationship, which is the G'_K-E_M relationship normalized to G'_{max}) by over 25 mV to more positive values. This means that it is more difficult to activate the channel in the High PIs, since in order to activate the same fraction of channels as in controls the High PIs require an additional depolarization of 25 mV (Fig. 3C). Such a shift has been observed also in animal cells under similar conditions of increased PtdInsP₂ levels (Yaradanakul et al., 2007; for review, see Suh and Hille, 2008). The direction of this shift is consistent with the NtORK channel protein sensing an increased density of negative surface charges at the internal surface of the plasma membrane, which is consistent, in turn, with the enrichment of the internal leaflet with PtdInsP₂.

The general importance of electrostatic interactions between the membrane and proteins (Mulgrew-Nesbitt et al., 2006) and, in particular, the importance of the negative surface charges of PtdInsP₂ for channel activity has been highlighted elegantly (in the case of the animal KCNQ potassium channel) by increasing the cytosolic concentration of Mg²⁺ and applying a series of organic polycations with increasing valency. This, in quantitative agreement with neutralization of the internal negative charges, diminished channel activity (Suh and Hille, 2007; Lundbaek, 2008; see discussion by Suh and Hille, 2008). Interestingly, quantitative modeling of PtdInsP₂-scavenging polycations predicted also a lack of the screening effect of polycations

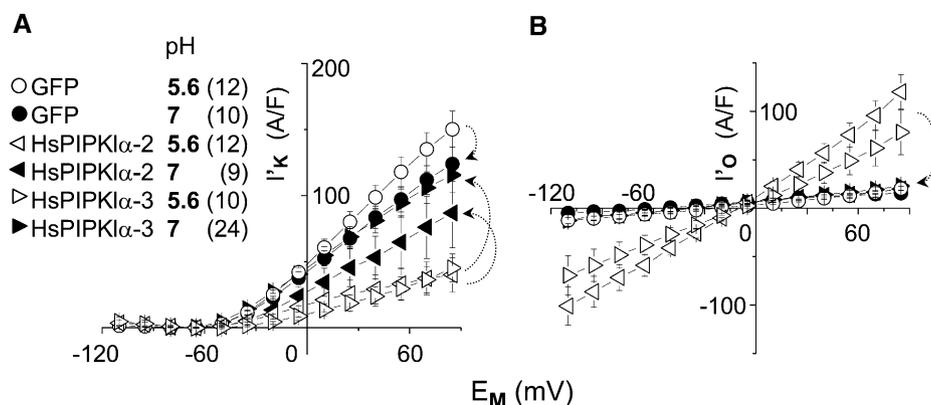
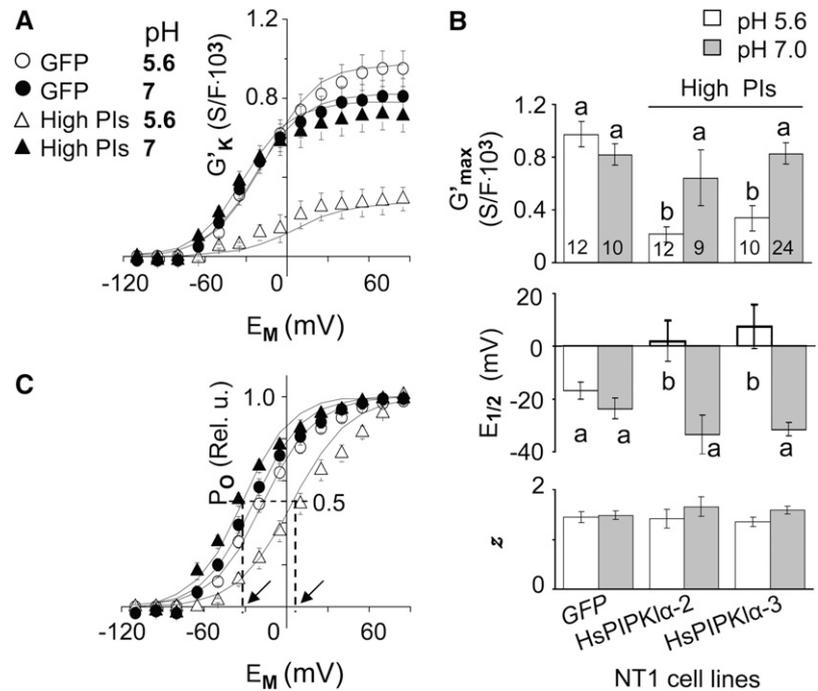


Figure 7. The effects of pH 7 on I'_K and leak current. A, Mean ± SE I'_K-E_M relationships of the High PIs and their control, GFP. Round arrows indicate the effect of pH neutralization. The numbers of assayed cells are indicated in parentheses. B, Mean ± SE I'_O-E_M relationships in High PIs and the GFP control obtained from the same current records as the data of A. Round arrows are as in A.

Figure 8. The effects of pH 7 on the K channel chord conductance. A, Symbols show mean \pm SE G'_{K-E_M} relations of the cells in Figure 7. Data of both high-PI cell lines were pooled together for clarity. Lines were calculated using Equation 2 and the averaged best-fit Boltzmann parameters (G'_{max} , $E_{1/2}$, z , shown in B) obtained from analyses of individual G'_{K-E_M} s. B, Mean \pm SE best-fit Boltzmann parameters. The parameter values differ significantly if they are denoted by different letters (a or b). Numbers inside columns are the numbers of cells contributing to the averages. C, Symbols show mean \pm SE P_{O-E_M} relationships. Data of both high-PI cell lines were pooled together for clarity. Lines are as in A, except for using Equation 3 here. Vertical dashed lines and arrows denote $E_{1/2}$ values at pH 5.6 (right) and at pH 7 (left). Note the pH neutralization-induced hyperpolarizing shift of P_{O-E_M} of the High PIs.



when PtdInsP₂ concentrations in the membrane are increased (Suh and Hille, 2008). Such may be the explanation of a reported insensitivity of a plant K⁺-release channel to cytosolic polycations in *V. faba* guard cells (Liu et al., 2000). The concurrent inhibition by polycations of a K⁺-influx channel in these guard cells, in a whole-cell configuration but not in excised patches, was interpreted as due to a different specific mechanism rather than to PtdInsP₂ charge screening (Liu et al., 2000).

In contrast to the G'_{K-E_M} shift, the sensitivity to voltage of the gating process (i.e. the slope of the P_{O-E_M} relationship, embodied in the parameter z) was not affected by PtdInsP₂ levels. This is consistent with unchanged properties of the channel-gating subunits themselves.

Two Mechanisms of Enhancing NtORK Activity in High PIs by ABA Degradation of PtdInsP₂

Applying ABA as a means to hydrolyze PtdInsP₂ through PLC activation according to the *V. faba* guard cell paradigm indeed resulted in considerable lowering of the PtdInsP₂ level in the High PIs (Fig. 3). Alteration of NtORK activity had the same underlying biophysical components as did genetic manipulation: lowering of the PtdInsP₂ level increased maximum attainable NtORK conductance (per unit area), G'_{max} , i.e. increased $P_{max} \cdot N' \cdot \gamma_s$, or, perhaps, as suggested earlier, $P_{max} \cdot N'$.

Could ABA increase N' by transcriptional activation of NtORK? It is quite unlikely, since in intact Arabidopsis suspension cells a short-term ABA treatment induced GORK mRNA, which was noticeable only

after about 4 h (Becker et al., 2003). Rather, an increase of N' could occur through removal (by PLC-mediated hydrolysis) of the PtdInsP₂ from a direct inhibitory interaction with channels already in the membrane, or by recruitment of channels to the membrane through tipping the balance between endocytosis and exocytosis toward the latter when the PtdInsP₂ levels declined.

Additionally, the ABA-induced decrease in the PtdInsP₂ level led to a reversal of the voltage shift of the High PIs channel gating back to the control range, in line with the presumably diminished negative charge density at the internal plasma membrane surface. Resembling our results, in patch-clamp experiments conducted on protoplasts of *V. faba* guard cells, ABA not only increased the outward K⁺ currents but, under certain conditions, also caused a similar, hyperpolarizing shift of the activation curve of the K⁺-release channels (Lemtiri-Chlieh, 1996). These specific conditions were cytosolic K⁺ concentrations below 150 mM, which we interpret as cytosolic solutions of ionic strength low enough to not mask the negative surface charges on the inside of the plasma membrane.

In the controls, the minute ABA-induced changes in PtdInsP₂ levels (even if significant in the case of the wild type) did not affect the activity of NtORK. This effective lack of modification of the already low PtdInsP₂ level can be attributed to a simultaneous ABA activation of phospholipase D (PLD; Ritchie and Gilroy, 1998; Jacob et al., 1999; Hallouin et al., 2002; Zhang et al., 2004). Phosphatidic acid, released from the abundant phosphatidylcholine by PLD, is expected to activate the endogenous PtdIns(4)P 5-kinase (Jenkins et al., 1994; Jones et al., 2000; Wang, 2000, 2004;

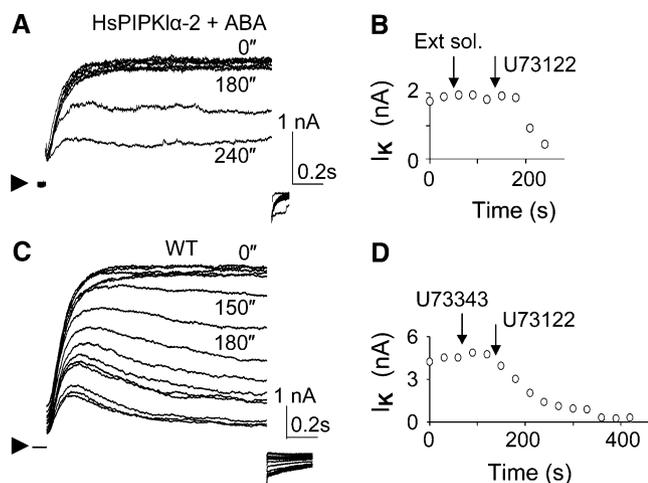


Figure 9. The effect of U73122. Superimposed traces of membrane currents recorded in a whole-cell patch-clamp configuration, elicited by depolarizing pulses to +85 mV applied repeatedly every 30 s from a holding potential of -70 mV. A, High-PI cells (HsPIP2K α -2) preincubated with $25 \mu\text{M}$ ABA prior to beginning the recording, then exposed to $5 \mu\text{L}$ of plain external solution added to the bath and, subsequently, to $5 \mu\text{L}$ of $250 \mu\text{M}$ U73122, to a final concentration of approximately $4 \mu\text{M}$. The arrowhead indicates the holding current at -70 mV. B, Time course of I'_K during the experiment in A. Vertical arrows show timing of treatments. C, An experiment similar to A, using control wild-type (WT) protoplasts without preincubation (the traces were shifted slightly to superimpose at the holding current). U73343, the inactive analog of U73122, and then U73122 were added, as in A, each to a final concentration of $4 \mu\text{M}$. The other details are similar to those in A. D, Time course of I'_K during the experiment in C. Two representatives of four similar independent experiments are shown.

Wang et al., 2006) and thereby enhance the production of PtdInsP₂. PtdInsP₂ promotes the PLD activity, in turn producing more PtdInsP₂ in a positive feedback (Huang, 2007). We are tempted to speculate that it is quite likely that in the control NT1 cell lines the ABA-stimulated opposing effects of the two phospholipases, PLC and PLD, are sufficiently well balanced to keep the PtdInsP₂ level practically constant (as demonstrated in Fig. 3) and, consequently, the NtORK activity unchanged.

The lack of short-term effects of ABA on GORK channel activity in Arabidopsis guard cells (Wang et al., 2001) resembles our results with the control cell lines, but in Arabidopsis it could be due to technical reasons (the application of ABA after attaining a whole-cell configuration, i.e. on the background of altered cytoplasmic milieu and disrupted signaling cascade). In contrast, ABA enhanced within minutes K⁺-release channels (identified as GORK; Becker et al., 2003) in Arabidopsis suspension cells (Jeannette et al., 1999). In this case, the cells were assayed with an impaling electrode, which presumably minimized the perturbation of the internal milieu. These results resemble our own results with the High PIs, and it would be interesting to assay the effect of ABA on the PI levels in the membranes of the Arabidopsis suspension cells.

Two Mechanisms of Enhancing NtORK Activity in High PIs by Degradation of PtdInsP₂ through the Removal of External Protons

In the experiments presented here, in which protoplasts were incubated at pH 7 for several minutes prior to attaining whole-cell configuration (i.e. before introducing the strongly pH-buffered patch-pipette solution to the cytosolic milieu), it is very reasonable to assume that external alkalization of an intact proto-

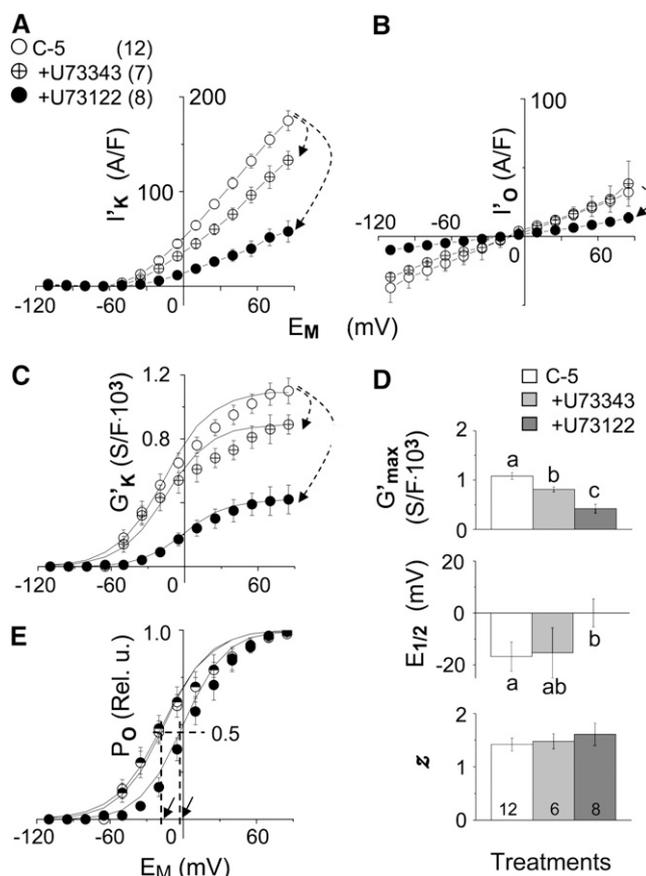


Figure 10. Steady-state effect of U73122 on NtORK conductance. Protoplasts of the control line C-5 (see “Materials and Methods”) were exposed to U73122 or U73343 in the bath ($2.5 \mu\text{M}$) starting about 3 min prior to attaining the whole-cell configuration. A depolarizing pulse of +85 mV was applied repeatedly every 45 s from a holding potential of -70 mV. A, Mean \pm SE I'_K - E_M relations. Round arrows indicate the effect of the treatments: U73122 (long arrow) and U73343 (short arrow). Numbers of assayed cells are indicated in parentheses. B, Mean \pm SE I'_O - E_M relations of the data of A. The only arrow indicates the effect of U73122. C, Symbols show mean \pm SE G'_K - E_M relations of the data of A. Lines were calculated using Equation 2 and the averaged best-fit Boltzmann parameters (G'_{max} , $E_{1/2}$, z ; shown in D) obtained from analyses of individual G'_K - E_M s. Arrows are as in A. D, Mean \pm SE best-fit Boltzmann parameters. The parameter values differ significantly if they are denoted by different letters (a, b, or c). E, Symbols show mean \pm SE P_O - E_M relationships. Lines are as in C, except for using Equation 3 here. Vertical dashed lines and arrows denote $E_{1/2}$ values without (left) and with (right) U73122. Note the U73122-induced depolarizing shift of P_O - E_M of the K channels in the control cell.

plast led immediately to a partial, even if only transient, cytosolic alkalinization (Heppner et al., 2002; Boron, 2004). Thus, the effects of external alkalinization on NtORK could be mediated by cytosolic alkalinization.

In patch-clamp experiments on excised guard cell plasma membrane patches, cytosolic alkalinization turned out to increase the number of K^+ -release channels available for activation. Moreover, this process was membrane delimited (i.e. independent of soluble cytosolic components, and, in particular, independent of cytosolic $[Ca^{2+}]$, at least at 50 nM and 1 μ M; Miedema and Assmann, 1996). This seems to suggest direct pH sensing located on the interior of the membrane. Alternatively, intracellular alkalinization, which is likely to deprotonate Ca^{2+} -binding sites, could increase their availability for Ca^{2+} binding, activating PLC even at a Ca^{2+} concentration as low as 50 nM (Hunt et al., 2004).

Interestingly, external alkalinization enhanced the activity of TRPV5 channels in animal cells, with patch-clamp experiments excluding pH effects through the cell interior. Excluding also a direct effect on the channel protein, these experiments suggested the mediation of an external pH sensor other than the channel itself (Lambers et al., 2007).

Why were control NT1 cells unaffected by the higher pH? The minute changes in the originally low levels of membrane PtdInsP₂ in the controls could be due to a balance in the activities of PLD and PLC in these cells, as argued earlier for ABA stimulation. Consequently, this could result in unchanged NtORK activity. Future experiments should address this hypothesis.

Two Mechanisms of Inhibiting NtORK Activity in High PIs and Controls by Accumulation of PtdInsP₂ through PLC Inhibition

Targeting the PLC for inhibition by U73122 was expected to shift the dynamic balance between PtdInsP₂ production and its cleavage (Staxen et al., 1999; Perera et al., 2001; De Jong et al., 2004; Parre et al., 2007) in both the High PIs and the control cells, wild type and C5, leading to PtdInsP₂ accumulation and, consequently, to NtORK inhibition. Indeed, the inhibition of NtORK by U73122 included two elements, resembling the effect of the genetically elevated PtdInsP₂ levels: depression of G'_{max} (likely via decreasing $P_{max} \cdot N'$) and a shift of the voltage activation range to depolarizing potentials, consistent with PtdInsP₂ accumulation in the internal leaflet of the membrane.

Physiological Significance

Taken together, our data strongly suggest that PtdInsP₂ inhibits NtORK. PtdInsP₂ also inhibited an anion channel in guard cells of *Arabidopsis* and *V. faba* (Lee et al., 2007). While preventing stomatal closure can be achieved, in principle, by inhibiting only one of

these two ion-releasing channels, no osmotically significant loss of ions (K^+ and Cl^-) or stomatal closure would occur without both channels operating simultaneously. We propose that lowering of PtdInsP₂ levels in guard cell plasma membrane is what activates both channels or, at least, predisposes them for activation, eventually leading to stomatal closure. The physiological initiating signal may be ABA. This type of signaling may mediate ABA-induced stomatal closure even in the absence of an observable rise in the concentration of cytosolic Ca^{2+} (Levchenko et al., 2005; Marten et al., 2007). PtdInsP₂ hydrolysis might not elevate cytosolic Ca^{2+} , for example, if the resulting InsP₃ fails to convert to InsP₆, a proposed physiological agent of Ca^{2+} rise in guard cells (Lemtiri-Chlieh et al., 2000, 2003). Lowering of PtdInsP₂ levels may also be the underlying mechanism of the induction of stomatal closure by intracellular or extracellular alkalinization. Notably, transgenic *Arabidopsis* plants with lowered PtdInsP₂ and InsP₃ (expressing the same gene of human 5-phosphatase as in the tobacco I2-8 and I4-2 Low PIs) were more resistant to drought, losing less water through their leaves and exhibiting more efficient stomata closure (Perera et al., 2008), as would be expected from the hypothesized relief of PtdInsP₂ inhibition of the ion-release channels in their guard cells.

Inverse or Direct

Interestingly, a reversible rundown of channel activity in the absence of cytosolic components required for membrane-delimited phosphorylation, hydrolyzable ATP and Mg^{2+} , was documented for the SKOR-related K^+ -release channel of *S. saman* (presumed to be SPORK; Moran, 1996). This resembles the rundown of SKOR activity in the oocytes (Liu et al., 2005). Such rundown has been highlighted as symptomatic of a PtdInsP₂ requirement for channel activity (Suh and Hille, 2008). If, indeed, SPORK requires elevated PtdInsP₂ for its activity, the current models of motor cell signaling in moving leaves (Kim et al., 1996; Moran, 2007a, 2007b) will have to be altered. We posit that NtORK behavior represents the physiological relationship between K^+ release channel activity and PtdInsP₂ in all plant motor cells.

The fact that the PtdInsP₂ level in the High PIs was elevated constitutively could not be the reason for depressed NtORK activity in these cells, since even a short-term PtdInsP₂ accumulation caused by U73122 had the same inhibitory effect on the channel. The different responses of SKOR (Liu et al., 2005) and NtORK to increased PtdInsP₂ level (activation versus inhibition, respectively) may be due, at least partially, to the following reasons: (1) heterologous versus homologous expression of the channels: different interactions with the surrounding milieu (types of membrane lipids, enzymes, cytoskeleton, other proteins) or different protein modifications of the channels themselves; (2) different mode of lipid application: exogenous versus

in vivo; (3) different channel sequences: in spite of close homology, there could be differences, in particular at the termini and the cytosolic loops, dictating different three-dimensional structures, leading to different interactions with PtdInsP₂ or with an intervening protein (Suh and Hille, 2008).

The last possibility seems the most likely. For example, SKOR, the Arabidopsis xylem-loading channel (Gaymard et al., 1998), may indeed be stimulated by elevating PtdInsP₂ even in planta. Thus, a K⁺-release channel of maize (*Zea mays*) root stele, and therefore, a likely SKOR ortholog, was inhibited by pretreating the plants with ABA (Roberts, 1998), as was K⁺ xylem loading in barley (*Hordeum vulgare*; Cram and Pitman, 1972). This was quite opposite from the stimulating effect of ABA on the (unresolved) K⁺-release channels in *V. faba* guard cells (Lemtiri-Chlieh and MacRobbie, 1994; Lemtiri-Chlieh, 1996) or on GORK in Arabidopsis suspension cells (Jeannette et al., 1999).

Is NtORK like SKOR or like GORK? Based on comparison of the amino acid sequences, NtORK1 (the sole gene of the K⁺-release channel in the tobacco suspension cells; Sano et al., 2008) is more similar to SKOR than to GORK (72% versus 66% identical; Sano et al., 2008). However, because NtORK1's dominant distribution in tobacco plant leaves (Sano et al., 2008) resembles that of GORK in Arabidopsis (Becker et al., 2003), NtORK is likely a GORK ortholog (Sano et al., 2008). Our present results support this view.

CONCLUSION

The previously observed correlations between the phospholipid level and GORK (and, very likely, SPORK) activity in plant motor cells were extended here into a causal relationship between the PtdInsP₂ level and the activity of a homologous channel, NtORK. This was achieved by manipulating the endogenous levels of PtdInsP₂ in the plant cell membrane, on a long- or a short-term scale, while monitoring the in situ NtORK activity, as prescribed by a recent critical review: "The more ways you can change the PIP₂ [PtdInsP₂], the stronger the evidence becomes" (Suh and Hille, 2008). Our findings are consistent with NtORK inhibition by the negatively charged PtdInsP₂ in the internal plasma membrane leaflet. Biophysical analysis of NtORK whole-cell outward K⁺ currents provided an insight into a possible mechanism underlying this causality. Thus, NtORK activity was diminished mainly through decreased maximum available conductance via the channels (G_{\max}), and, to a somewhat lesser extent, by altering the voltage dependence of channel activation and making the channels more difficult to open. Such effects are likely to underlie PI signaling in intact plant cells.

We are aware that our observations are only the beginning of a prolonged exploration of the possible

interactions of PIs with plant ion channels and with other proteins (Suh and Hille, 2008). Although PIs are much less abundant in the plant plasma membrane than in the animal plasma membrane, the wealth of reports on plant PI signaling suggests that channel-PtdInsP₂ interactions in the plant cell will not be much rarer. Because ion channels are crucial for signaling and osmotic homeostasis, understanding these interactions will provide a handle for manipulating plant water relations.

MATERIALS AND METHODS

Plant Material

Cell Lines

One wild-type and eight transgenic lines of tobacco (*Nicotiana tabacum*) cultured cells, NT1, were used in this work: two High PIs, cell lines expressing the GFP-fused human phosphatidylinositol (4)phosphate 5-kinase, type 1 α , HsPIPK1 α -2 and HsPIPK1 α -3, and a control GFP line, transformed with the same vector but with GFP alone (Im et al., 2007); two Low PIs, cell lines expressing the human-type InsP 5-phosphatase, I2-8 (Perera et al., 2002) and I4-2, both with membrane-associated phosphatase; three controls for the Low PIs: C-5, an empty vector control, C348S, an inactive mutant phosphatase, and Δ C, a truncated soluble phosphatase localized in the cytosol. The soluble form of the InsP 5-phosphatase Δ C lacks the C-terminal isoprenylation site (the last four amino acids; De Smedt et al., 1997). The inactive mutant C348S has a single amino acid substitution (Cys-348 to Ser) in the catalytic domain (Communi and Erneux, 1996). The three InsP 5-phosphatase constructs (I4-2, C348S, and Δ C) were subcloned into the Gateway binary vector pK2GW7 containing a cauliflower mosaic virus 35S promoter (Functional Genomics Division, Department of Plant Systems Biology, University of Gent, Belgium), and the DNA sequence was verified by sequencing. Tobacco cell transformation and selection of transgenic tobacco lines were as described previously (Perera et al., 2002).

Tissue Culture

Tobacco ('Bright Yellow 2') cultured cells (NT1) were maintained in 16 mL of liquid culture medium (see "Solutions" below). The 100-mL Erlenmeyer flasks with cells were agitated at about 125 rpm in the dark at about 28°C. Cells were subcultured every 7 d at a 1:16 (v/v) dilution with fresh medium at room temperature (Perera et al., 2002).

Protoplast Isolation

Protoplasts were isolated from the tobacco cells at 4 d (4 × 24 h) after subculture. A 0.5-mL suspension was centrifuged at 36g for 5 min, and the upper medium was poured off and quickly replaced with 2.5 mL of cell wall digestion solution (see "Solutions" below). Subsequently, the cells were agitated on a rotary shaker at about 60 rpm for 1.5 h at 30°C. The enzymatic reaction was stopped by washing the cells with 5 mL of isotonic solution (see below) through a nylon filter (50- μ m mesh) into a 12- to 15-mL test tube. The test tube was centrifuged at 36g for 5 min. The supernatant was discarded, and the protoplasts were resuspended in 300 μ L of isotonic solution in the same test tube. The isolation procedure was conducted at room temperature. To prevent regrowth of cell walls and reproduction of bacteria, the protoplasts were placed on ice for the duration of the experiment (up to 9 h).

Patch-Clamp Experiments

Procedure

Patch-clamp experiments were performed at room temperature (20°C–22°C) using a Digidata 3122A interface and the pClamp 9 or 10 program suite from Axon Instruments, which was used both for running the experiment and analysis. Fire-polished borosilicate glass patch-clamp pipettes (Sutter Instru-

ments; catalog no. BF150-86-10) had resistance of 50 to 100 M in the external solution (see "Solutions" below). A drop with protoplasts was added to the external solution in the bath, and the protoplasts were allowed to settle for several minutes or the bath was filled with the external solution after the drop with protoplasts was placed on the chamber bottom (the order did not influence the results). Whole-cell configuration was attained usually spontaneously without obtaining a giga-seal. Recording started several minutes after decrease and stabilization of the baseline current at the holding potential of -70 mV, usually within about 10 min. Outward K^+ currents were recorded from the protoplasts applying 1- to 3-s-long voltage pulses between $+85$ and -110 mV in -15 -mV voltage steps at 45-s intervals. All experiments were performed in voltage-clamp mode. The error in the voltage clamping of the whole-cell membrane, largely due to the series resistance of the patch pipette, was compensated at approximately 80% by analog circuitry of the amplifier. Mean series resistance was 15.8 ± 5.4 M (\pm SD, $n = 106$). Currents were filtered at 500 Hz and sampled usually at 1 to 2 kHz.

Analysis

Prior to averaging, a normalized net steady-state current, $I'_{K'}$, was obtained by subtracting the instantaneous current from the total steady-state current, then dividing by the cell's capacitance, as read off the amplifier dial. The normalized instantaneous current, $I'_{O'}$, was obtained similarly. Mean capacitance was 32.9 ± 9.4 pF ($n = 106$). The mean diameter of protoplasts selected for these experiments was 45.4 ± 6.3 μ m ($n = 106$). The average specific capacitance was 0.51 ± 0.10 μ F cm^{-2} ($n = 106$).

The normalized chord conductance, $G'_{K'}$, was extracted from $I'_{K'}$ according to Equation 1:

$$G'_{K'} = I'_{K'} / (E_M - E_{rev}) \quad (1)$$

where E_M is the membrane potential and E_{rev} is the current reversal potential, determined separately for each cell line (as described in Supplemental Fig. S2). Individual $G'_{K'}$ - E_M relationships were fitted with the Boltzmann equation (Hille, 2001) using Origin (version 7.0220; Origin Lab Co.), according to Equation 2:

$$G'_{K'} = G'_{max} / (1 + e^{-zF(E_M - E_{1/2})/RT}) \quad (2)$$

where G'_{max} is the maximum conductance (normalized to capacitance), $E_{1/2}$ is the voltage at which half of G'_{max} is attained, and z is the effective charge of the gating subunits. The individual best-fit parameter values (G'_{max} , $E_{1/2}$, and z) were then averaged for each cell line.

Channel open probability, $P_{O'}$, was obtained by normalizing $G'_{K'}$ to G'_{max} according to Equation 3:

$$P_{O'} = 1 / (1 + e^{-zF(E_M - E_{1/2})/RT}) \quad (3)$$

Other details were as published previously (Yu et al., 2001). Differences between means were deemed significant if, using a two-sided Student's t test, $P < 0.05$.

Solutions

Bath solution included (in mM): 5 KCl, 1 CaCl₂, and 10 MES. pH was 5.6, and osmolarity was 435 mOsm. Pipette solution included (in mM): 150 KCl, 20 HEPES, 5 MgCl₂, 2 K₄-1,2-bis(*o*-aminophenoxy) ethane-*N,N,N,N*-tetraacetic acid (BAPTA), and 2 K₂ATP. pH was 7.5, and osmolarity was 470 mOsm. pH in both solutions was adjusted by *N*-methyl *D*-glucamine base and osmolarity by *D*-sorbitol. Pipette solution was filtered before use.

Culture solution was 4.3 g L⁻¹ Murashige and Skoog solution (Sigma; catalog no. M-5524) supplemented with 2 g L⁻¹ KH₂PO₄, 1 g L⁻¹ myoinositol, 30 g L⁻¹ Suc, 100 μ L L⁻¹ thiamine-HCl, and 4 μ L L⁻¹ 2,4-dichlorophenoxyacetic acid. pH was adjusted to 5.8 with KOH, and the solution was autoclaved for 20 min.

ABA (Sigma; catalog no. 862169) 10 mM stock solution was prepared by dissolving 13 mg of powder in approximately 100 μ L of 1 M KOH and completing the volume with bath solution to 5 mL. After adjusting pH to 5.6 by MES, the stock solution was stored at -20° C.

U73122 (Calbiochem; catalog no. 662035) and U73343 (Calbiochem; catalog no. 662041) 5 mM stock solutions were prepared in dimethyl sulfoxide (Fluka; catalog no. 41650).

K₄-BAPTA was from Molecular Probes, and other chemicals were from Sigma or Merck and were of analytical grade.

Determination of PI Levels

PtdIns(4,5)P₂ Mass Measurements

Protoplasts were harvested by centrifugation, plasma membranes were isolated by aqueous two-phase partitioning, lipids were extracted, and PtdIns(4,5)P₂ mass measurements were carried out as described (Heilmann et al., 2001).

InsP₃ Determination

Either the membrane lipid hydrolysate (as above) was assayed, or, to measure total InsP₃ level, cells were harvested by filtration and immediately frozen in liquid N₂, ground to a fine powder, and precipitated with cold 10% (*v/v*) perchloric acid. InsP₃ assays were carried out using the TRK1000 InsP₃ assay kit (GE Healthcare Life Sciences) as described previously (Perera et al., 1999, 2002).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Identification of K channel currents using K channel blockers.

Supplemental Figure S2. Determination of the reversal potential, E_{rev} .

Supplemental Figure S3. The effect of PtdInsP₂ on whole-cell currents.

Supplemental Figure S4. Subcellular localization of the 5-phosphatase in the Low PIs.

Supplemental Figure S5. InsP₃ content in the InsP 5-phosphatase-harboring transgenic cells and their controls.

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CORRECTIONS

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Ma X., Shor O., Diminshtein S., Yu L., Im Y.J., Perera I., Lomax A., Boss W.F., and Moran N. Phosphatidylinositol (4,5)Bisphosphate Inhibits K^+ -Efflux Channel Activity in NT1 Tobacco Cultured Cells.

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The first two sentences in the “Solutions” section of “Materials and Methods” should read: Bath (external) solution included (in mM): 5 KCl, 1 CaCl₂, and 10 MES. pH was 5.6 or 7, as indicated in the text, and osmolarity was 435 mOsm.

The following paragraph should be added to the “Solutions” section of “Materials and Methods”:

Isotonic solution: 10 mM KCl, 1 mM CaCl₂, 10 mM MES, pH 5.5, adjusted osmolality to 450 mOsm by D-sorbitol. Cell wall digestion solution: Isotonic solution with the following final concentrations of enzymes: 1% (w/v) Onozuka R10 cellulase (Gallard-Schlesinger Chemical; catalog no. B6144), 0.1% pectolyase Y23 (Seishin Pharmaceutical), and 0.5% bovine serum albumin (Sigma; catalog no. A6003).