Phosphatidylinositol 4,5-bisphosphate regulates plant K⁺ channels

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Abstract
Phosphoinositides play an important role in both abiotic and biotic signalling in plants. The signalling cascade may include the production of second messengers by hydrolysis of PtdIns(4,5)P₂. However, increasingly, PtdIns(4,5)P₂ itself is shown to mediate signalling by regulating target proteins. The present mini-review summarizes the experimentally demonstrated effects of PtdIns(4,5)P₂ on plant K⁺ channels and examines their structure for candidate sites of direct PtdIns(4,5)P₂-protein interaction.

Introduction
Many abiotic and biotic signals enhance the metabolism of PIs (phosphoinositides) in both animal and plant cells. In animals, PIs mediate signalling which regulates numerous cellular processes that are important for cell development and growth, including vesicular transport and membrane recycling, secretion of metabolites, organization of the cytoskeleton, and regulation of ion channels and transporters (reviewed recently in [1]). Much of this signalling has been attributed to cleavage of PtdIns(4,5)P₂ by PLC (phospholipase C) [2], producing DAG (diacylglycerol) and Ins(1,4,5)P₃. Whereas DAG operates within the plane of the membrane, production of the soluble Ins(1,4,5)P₃ is practically synonymous with further signalling through mobilization of Ca²⁺ from internal stores [3,4]. In the last decade, signalling via PtdIns(4,5)P₂, rather than via its cleavage products, became the focus of intense study in animal cells (reviewed in [5–13]).

PIs in plants
In higher plant cells, the ratio of PtdIns(4,5)P₂ to PtdIns(4)P is approx. 10–20-fold lower than in animals (reviewed in [14,15]). In spite of this, PtdIns(4,5)P₂ has been implicated in similar cellular functions affecting plant development and growth ([14,15], reviewed in [16,17], and more recently in [18–20]).

Because PtdIns(4,5)P₂ and Ins(1,4,5)P₃ are affected simultaneously by signals activating PLC, it is frequently not clear which of these molecules is more important in a given signalling cascade. Apart from direct biochemical assays (such as [21]), patch–clamp assays of ion channel activity, in which the cytosolic surface of the cell plasma membrane is exposed to solutions completely devoid of Ins(1,4,5)P₃, appear to be the most convincing experiments establishing the second messenger role of PtdIns(4,5)P₂ itself. So far, in contrast with the abundance of such experiments, ion channels and other transporters in animals (see the Introduction), in plants there are only very few such reports (see also [20,22] for reviews).

The effect of PtdIns(4,5)P₂ on plant ion channels
Three plant K⁺ channels were expressed heterologously in frog oocytes: the K⁺-influx channels, KAT1 from Arabidopsis and LKT1 from tomato (a homologue of the Arabidopsis AKT1), and the Arabidopsis thaliana K⁺-efflux channel, SKOR. Their activities were examined using patch–clamp by applying isomers of PtdInsP, PtdInsP₂ or PtdInsP₃ to the cytosolic side of excised membrane patches. In these experiments, the PI lipids reversed the rundown and restored, or even enhanced, channel activity in a Ca²⁺-independent manner (Figure 1A) [23]. These results suggested a positive correlation between PtdIns(4,5)P₂ levels and channel activity.

In contrast, we reported recently on an inhibitory effect of PtdIns(4,5)P₂ on the activity of NtORK, the SKOR-related K⁺-efflux channel of Nicotiana tabacum cultured cells (NT1) [22]. In our experiments, we monitored the channel in situ using patch–clamp in a whole-cell configuration, with the cytosolic concentrations of Ca²⁺ and protons tightly controlled by appropriate buffers [22] (Figure 1B). In these cells, the levels of PtdIns(4,5)P₂ and Ins(1,4,5)P₃ had been altered genetically, to produce cell lines with lowered or elevated levels of both PIs: ‘Low PIs’ and ‘High PIs’ respectively. Control lines consisted of cells transformed with ‘empty’ vectors, and WT (wild-type) cells [22,24,25]. We quantified NiORK activity as the maximum available K⁺ conductance (G′ max), which represents the product of the conductance of a single open channel, the number of the channels in the membrane and the maximum probability of the channel to be in the open state (for ease of comparison among cells, we used values of G′ max normalized to the membrane surface area as given by cell

Key words: basic residues motif, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4,5-bisphosphate-binding domain, phosphatidylinositol 4,5-bisphosphate-protein interaction, phosphorinositol, plant K⁺ channel.

Abbreviations used: CNG, cyclic nucleotide-binding; DAG, diacylglycerol; PI, pleckstrin homology; PI, phosphoinositide; PLC, phospholipase C; WT, wild-type.

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The effect of PtdIns(4,5)P₂ on K⁺ currents via plant K⁺ channels

(A) Traces of currents recorded in frog oocytes expressing SKOR, KAT1 or LKT1, elicited by the indicated depolarizing or hyperpolarizing voltage pulses (membrane potential is in mV), applied to the membrane patch in the different configurations. Note the current rundown upon patch excision and revival of current upon application of exogenous PtdInsP₂ (PIP₂) to the cytosolic surface of the patch. Inset: on-cell and inside-out patch configurations. Reprinted from [23] with permission. © 2005 Blackwell Publishing Ltd. (B) Outward whole-cell currents (superimposed traces) via NtORK channel evoked by a series of increasingly depolarizing voltage pulses in tobacco protoplasts isolated from the following cell lines with different levels of endogenous membrane PIs: WT, with intermediate PIs levels; Low PIs, with diminished PI levels; High PIs, with elevated PI levels. Numbers on the right of the traces, or near arrows, indicate the values of membrane potential (in mV), during the recording. Note the 2.7-s-long break in the traces; 0.1 s and 0.3 s scale bars relate to the first and second part respectively. Inset: whole-cell recording patch configuration. Reprinted from [22] with permission.

Figure 2 | Percentage inhibition of NtORK activity according to the relative PtdInsP₂ levels in the plasma membrane

Relative PtdIns(4,5)P₂ levels were determined as follows: we designated the PtdIns(4,5)P₂ level in control (WT) cells as 1, and calculated the ratio between PtdIns(4,5)P₂ levels determined in the protoplasts of each of the Low PI and the High PI lines and of WT. Percentage inhibition was determined as follows: the highest G_max value of a Low PI line (G_max') was defined as zero inhibition and all other percentage inhibition values were calculated as 100×(1−G_max/G_max'). The absolute PtdIns(4,5)P₂ level in WT protoplasts was 20.3 pmol/mg of plasma membrane protein [22] and G_max' was 1.66 (nS/pF) [22]. The line describes percentage inhibition=V_max/[1+K_m/(relative PtdInsP₂ level)], where V_max and K_m are the best-fit parameters, 89% and 1.2 respectively.

The mechanism of PtdIns(4,5)P₂ effects on the channels: general considerations

Jänmey and Kinnunen [28] suggested a mechanical view: the insertion of the non-cylindrical molecule of PtdIns(4,5)P₂ (the polar head of which occupies a larger surface area than its tail) into the membrane inner leaflet introduces a mechanical tension in the membrane; the increased tension may affect directly the energy distribution among the conformational states of the channels, consequently affecting gating. On the basis of this, differences between local PtdIns(4,5)P₂ concentrations, and/or the channel structure will determine the rates of transitions between the open and closed states and the final balance among them.

The general importance of electrostatic interactions between the membrane lipids and proteins [29], and, in particular, the importance of the negative surface charges of PtdIns(4,5)P₂ for channel activity has been highlighted elegantly (in the case of the animal KCNQ K⁺ channel) by increasing the cytosolic concentration of Mg²⁺ and applying a series of organic polycations with increasing valency. This, in a quantitative agreement with neutralization of the internal negative charges, diminished channel activity ([30], see also discussion in [31,32]). Electrostatic interactions can exhibit membrane capacitance). NtORK activity was the lowest in the High PIs and the highest in the Low PIs. Moreover, we manipulated the endogenous levels of PtdIns(4,5)P₂ in the plant cell membrane also on a short timescale. Treating the High PIs with the stress hormone ABA (abscisic acid) or changing the external pH from 5.5 to 7 decreased the High PIs with the stress hormone ABA (abscisic acid) or changing the external pH from 5.5 to 7 decreased the rates of transitions between the open and closed states and the final balance among them.
a large range of specificity. They can occur sparsely or in dense clusters, depending on the protein structure and on the abundance of PtdIns(4,5)P₂ in the membrane.

**The effect of PtdIns(4,5)P₂ abundance on channel activity**

Whereas PtdIns(4,5)P₂ is only a very minor phospholipid in plants, being roughly 10–20-fold less abundant than its precursor PtdIns4P (which accounts for approx. 0.5% of total phospholipids), it is by far (99%) the most abundant of all the PtdInsP₂ isoforms [14]. Also, it accumulates in the membrane following various stresses or signals, for example: in Arabidopsis plants grown in liquid medium, PtdIns(4,5)P₂ synthesis increased rapidly in response to the addition of NaCl, KCl or sorbitol to the medium [33]; in rice leaves, PtdIns(4,5)P₂ levels increased rapidly (<30 min) up to 4-fold in response to salt (NaCl) stress [34]; in cultured tobacco BY-2 cells, heat stress induced a rapid <10-fold accumulation of PtdIns(4,5)P₂ [35]. Even non-stressing signals, such as white light irradiation of V. faba guard cells at 170 μmol · m⁻² · s⁻¹, increased PtdIns(4,5)P₂ levels in the membrane, as suggested by a translocation of the classical ‘PtdIns(4,5)P₂ indicator’, the PH (pleckstrin homology) domain of PLCδ1 fused to GFP (green fluorescent protein), expressed in these cells, from the cytosol to the plasma membrane [26].

Is the range of PtdIns(4,5)P₂ effects on NtORK activity representative of the range of the above physiological changes in PtdIns(4,5)P₂ levels? To address this we plotted the percentage inhibition of Gmax (a measure of NtORK activity) against the relative PtdIns(4,5)P₂ levels in the membrane (Figure 2). Indeed, the range between the normal and the elevated PtdIns(4,5)P₂ levels of the High PIs NT1 (BY-2) cells spans the dynamic range of NtORK activation (Figure 2).

**Protein structure and PtdIns(4,5)P₂ effect on channels**

**PI-binding protein domains**

At least ten different protein domain types bind phospholipids at the membrane surface; some interactions are highly specific, others are not, and involve attraction to general physical attributes of the membrane (such as charge and curvature) [36]. In the animal kingdom, some channel proteins seem to interact with PtdInsP₂ through a structured PtdInsP₂-binding pocket, deduced from the apparent affinity, higher for PtdIns(4,5)P₂ than for PtdIns(3,4)P₂ or PtdIns(3,5)P₂. Other channels show little selectivity among polyphosphoinositides, sometimes accompanied by general low affinity for PtdInsP₂ [31].

**Seeking PtdInsP₂-binding domains in SKOR, NtORK, LKT1 and KAT1**

We used SMVProt (17 Jan 2008 update, http://jing.cz3.nus.edu.sg/cgi-bin/smvprot.cgi; the FASTA format of the proteins was retrieved from NCBI http://www.ncbi.nlm.nih.gov/ using the following accession numbers: KAT1, NP_199436.1; SKOR, NP_186934.1; LKT1, CAA65254.1; TORK1, BAD81036.1) to predict whether or not the above four plant PtdIns(4,5)P₂-regulated K⁺ channels are lipid-binding proteins. SMVProt is a prediction software for classification of a protein into functional families from its primary sequence. SMVProt classification is based on the analysis of physicochemical properties of a protein generated from its sequence, such as hydrophobicity, normalized van der Waals volume, polarity, polarizability, charge, surface tension, secondary structure and solvent accessibility [37]. All four channel proteins were predicted to have features of the lipid-binding protein family, at different levels. The P-value (expected classification accuracy—probability of correct classification) was 97.0% for SKOR, 92.9% for NtORK, 89.3% for LKT1 and 73.8% for KAT1.

To identify known domains in the four channels, we used InterProScan (release 24.0, http://www.ebi.ac.uk/Tools/InterProScan/), a tool that combines different protein signature-recognition methods from the InterPro databases into one resource [38]. As expected, the software recognized all the four-channel proteins as ion-transport proteins (IPR05821), and as K⁺ channels (IPR003938), and also identified the following known domains: CNB (cyclic nucleotide-binding) (IPR00595) and CNB-like (IPR018490) domains, and RmlC-like jelly-roll fold (IPR014710). Additionally, in SKOR, LKT1 and NtORK, ankyrin repeat (IPR002110) and ankyrin repeat-containing domains (IPR020683) were identified. However, no domains known to bind phospholipids have been found.

**Seeking clusters of basic residues**

In contrast with the more specific binding sites typical of PI binding to PH domains, ENTH (epsin N-terminal homology) domains [39] or Tubby motifs [40], the binding sites of PtdInsP₂-regulated animal ion channels have been generally only described by clusters of basic residues with positive charged side chains, usually interspersed with hydrophobic and/or aromatic residues [41].

We thus hypothesized that SKOR, NtORK, LKT1 and KAT1 possess similar domains with conserved basic residues. In order to search for such motifs, we used MEME (version 4.2.0) [42] to identify motifs common to all four channels. Again, as expected, the software recognized motifs, which we then identified as known conserved domains, such as the GYGD in the K⁺ selectivity filter in the pore region of K⁺ channels and the positively charged residues of the ‘voltage sensor’ in the S4 transmembrane domain.

In addition, we identified a new, previously unrecognized, domain with basic residues (Figure 3). This 44-amino-acid-long domain has three conserved arginine (R) or lysine (K) residues, interspersed with conserved hydrophobic residues and a conserved histidine (H) residue, therefore we named it the RKH domain. To resolve whether this domain is at the cytosolic surface of the plasma membrane, we resorted to the Uozumi model already established experimentally for KAT1, to identify the six membrane-spanning regions [43] and localized the RKH domain at the cytoplasmic C-terminus.
end of the S6 helix of KAT1. Multiple alignment of the channel proteins using ClustalW (version 2.0.12) helped to localize the RKH domain on SKOR, LKT1 and NtORK.

The RKH region includes other basic residues in addition to the three conserved basic residues: a total of seven in KAT1, eight in LKT1 and 11 in both SKOR and NtORK (Figure 3). Interestingly, among the three channels, SKOR, LKT1 and KAT1, assayed for rundown reversal, SKOR was the most sensitive to PtdIns(4,5)P_2 and KAT1 was the least sensitive [23]. This rank of sensitivity correlates positively with the number of basic residues in the identified domain of each channel protein, and also with the SMVProt-predicted probability of correct classification of these proteins as lipid-binding. It would be interesting to examine directly the PtdInsP_2-binding properties of the RKH domains of each of these channels.

The CNB-related motif

Further down the C-terminus, we identified another candidate PtdIns(4,5)P_2-binding site containing three conserved basic residues overlapping the distal part of the nucleotide-binding domain. This domain has the structure RX_RXX(R/K). Similarly, a polybasic segment of 19 residues exists at the C-terminus of the CNB domain of the human K+ channel HERG (human ether-a-go-go-related gene), the closest animal K+ channel relative of plant shaker K+ channels [44] and the first channel shown to interact with PtdIns(4,5)P_2 [45]. Both CNB-related motifs may participate in the dynamic binding of PtdInsP_2.

Conclusion

Exploration of the possible interactions of PIs with plant ion channels has only just begun. Understanding these interactions will provide a handle for manipulating ion channels, which is crucial for signalling and osmotic homeostasis, and thus enable enhancement of plant growth and stress tolerance.

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References

1 Michell, R.H. (2009) First came the link between phosphoinositides and Ca^{2+} signalling, and then a deluge of other phosphoinositide functions. Cell Calcium 45, 521–526
41 Hernandez, C.C., Zai, O. and Shapiro, M.S. (2008) A carboxy-terminal inter-helix linker as the site of phosphatidylinositol 4,5-bisphosphate action on Kv7 (M-type) K+ channels. J. Gen. Physiol. 132, 361–381
45 Bai, J.S. and McDonald, T.V. (2007) Phosphatidylinositol 4,5-bisphosphate interactions with the HERG K+ channel. Pflugers Arch. 455, 105–113

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