Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells

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myo-Inositol hexakisphosphate (InsP₆) is the most abundant inositol phosphate in cells, yet it remains the most enigmatic of this class of signaling molecule. InsP₆ plays a role in the processes by which the drought stress hormone abscisic acid (ABA) induces stomatal closure, conserving water and ensuring plant survival. Previous work has shown that InsP₆ levels in guard cells are elevated in response to ABA, and InsP₆ inactivates the plasma membrane inward K⁺ conductance (Iₖ,in) in a cytosolic calcium-dependent manner. The use of laser-scanning confocal microscopy in dye-loaded patch-clamped guard cell protoplasts shows that release of InsP₆ from a caged precursor mobilizes calcium. Measurement of calcium (barium) currents Iₖ₈ in patch-clamped protoplasts in whole cell mode shows that InsP₆ activates both the fast and slow conductances of the guard cell vacuole. These data define InsP₆ as an endomembrane-acting calcium-release signal in guard cells; the vacuole may contribute to InsP₆-triggered Ca²⁺ release, but other endomembranes may also be involved.

InsP₆ (myo-inositol hexakisphosphate), a physiological signal generated in guard cells in response to the drought-stress hormone abscisic acid (ABA), is a potent inhibitor of the K⁺-inward rectifier (Iₖ₈,in) (1), one of the fundamental elements of the control of guard cell turgor and hence of stomatal aperture. Whereas much is known of the physiological circumstances and molecular mechanisms by which the second messenger, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃], acts almost ubiquitously as a calcium-mobilizing signal (2, 3), little is known of the circumstances or mode of action of InsP₆. In animal cells, Ins(1,4,5)P₃ functions to mobilize calcium by acting as a ligand at the cytosolic face of ligand-gated endomembrane calcium-release channels. To date, no candidate Ins(1,4,5)P₃ receptor has yet been identified in the Arabidopsis or yeast (Saccharomyces cerevisiae) genomes. Nevertheless, Ins(1,4,5)P₃ elevates [Ca²⁺]ₘ when released from caged precursor in guard cells, with consequent inhibition of Iₖ₈,in and decrease in aperture (4, 5). The calcium reservoir, claimed initially to be the vacuole (6), could also include nonvacuolar stores, because most of the InsP₆-binding capacity in the cell is in nonvacuolar membranes (7). Our ignorance of the role on InsP₆ in signaling chains stems in part from a general lack of responsiveness of InsP₆ to extracellular stimuli in systems other than guard cells (1), fission yeast (8), or yeast (Schizosaccharomyces pombe) (8), and that in plants, treatment with ABA also quickly increases InsP₆ levels in guard cells (1). It is clearly important to establish the role of InsP₆ in ABA-induced signaling chains. The stomatal guard cell has emerged as a uniquely tractable experimental system in which to study the function of InsP₆ in osmotic-stress biology. By applying patch-clamp electrophysiology in whole-cell and -vacuole mode, we have investigated the cellular effectors of InsP₆ signaling. In guard cell protoplasts (GCPs), inhibition of Iₖ₈,in by InsP₆ does not occur in the presence of internal calcium chelators such as EGTA or BAPTA (1), suggesting that InsP₆ is a calcium-mobilizing signal. In the present work, by use of laser-scanning confocal microscopy in dye-loaded patch-clamped GCPs, we show first that laser uncaging of InsP₆ does indeed mobilize calcium and then investigate the source of this increase.

InsP₆-induced calcium transients in guard cells could arise from calcium entry through the plasma membrane or by calcium release from internal stores. In pancreatic β cells, InsP₆ activates an Iₖ₈-type calcium current (10). Recent studies (11–14) have shown that plants harbor calcium-permeable channels [hyperpolarization-activated calcium current (Iₖ₈ᵥ)] that activate, in contrast to those in animal cells on membrane hyperpolarization, and that in guard cells, these channels increase their opening probability in response to ABA (13). We have tested the effect of InsP₆ on Iₖ₈ᵥ and show that the InsP₆-induced increase in cytoplasmic Ca²⁺+ is not a consequence of Ca²⁺ influx from outside but of release of Ca²⁺+ from internal stores. Study of tonoplast ion channels in patch-clamped isolated vacuoles shows activation of both fast-activating [fast vacuolar (FV)] and slowly activating [slow vacuolar (SV)] channels by InsP₆.

Methods

Protoplast Isolation. Vicia faba L. cv. (Bunyan) Bunyan Exhibition was grown on vermiculite and GCPs isolated from abaxial epidermal strips of 3- to 4-week-old leaves as described (15). Epidermal strips were floated on medium containing 1.8–2.5% (w/v) sucrose, 0.26% BSA, and 1 mM CaCl₂ (pH 5.5 and osmolality 360 mOsm kg⁻¹ adjusted with mannitol), and incubated at 28°C, with gentle shaking. After 120–150 min, released protoplasts were passed through a 25-µm mesh and kept on ice for 2–3 min before being centrifuged at 100 × g for 4 min (at room temperature). The pellet of GCPs was resuspended and kept on ice in 1 or 2 ml of fresh medium containing 0.42 M mannitol, 10 mM (Mes), 200 μM CaCl₂, and 2.5 mM KOH (pH 5.55 and osmolality 466 mOsm kg⁻¹).

Vacuole Isolation. Freshly made protoplasts were allowed to settle to the bottom of the recording chamber and stick well to the

Abbreviations: ABA, abscisic acid; Iₖ₈,in, K⁺-inward rectifier; InsP₆, myo-inositol hexakisphosphate; FV, fast vacuolar; SV, slow vacuolar; GCP, guard cell protoplast; cADPR, cyclic adenosine diphosphoribose; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Iₖ₈ᵥ, current-voltage.

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glass, then given a hypotonic shock (200 mOsm kg⁻¹) by a continuous perfusion of a solution containing (in mM): 100 KCl, 10 Hepes, 2 EGTA, pH adjusted to 8 (KOH). Perfusion was stopped when >50% of the protoplasts had released their vacuoles (usually in ~20–30 min). Vacuoles were then perfused with a suitable external solution.

**Solutions.** Protoplasts (or vacuoles) were placed in a 0.5-ml chamber, allowed to settle, then perfused continuously at flow rates of 1–2 ml per min. The bath and pipette solutions were chosen appropriately for the particular membrane conductance measured. Four different conductances were studied: the inward K⁺-rectifier (I₉) and the hyperpolarization-activated calcium current (or Iᵥ) located at the plasma membrane, and the FV vacuolar current and the SV vacuolar current at the vacuolar membrane. For recording of Iᵥ, the solutions contained (in mM): 5 KCl, 2 MgCl₂, 2 KEGTA, 10 Mes (KOH) at pH 5.5, osmolality 460 mOsm kg⁻¹ (sorbitol) in the bath; and 92 K⁺-glutamate, 2 MgATP, 3.4 CaCl₂, 5 KEGTA, 10 Hepes (KOH) at pH 7.5, osmolality 520 mOsm kg⁻¹ (sorbitol) in the pipette. For Iᵥ, the solutions contained 100 BaCl₂, 10 Mes (Tris) at pH 7.5, osmolality 230 mOsm kg⁻¹; and 92 K⁺-glutamate, 2 MgATP, 3.4 CaCl₂, 5 KEGTA, 10 Hepes (KOH) at pH 7.5, osmolality 520 mOsm kg⁻¹ (sorbitol) in the pipette. For recording of both vacuolar channels, the bath contained 200 KCl, 0.1 CaCl₂, 10 Hepes (Tris) at pH 7.5, osmolality 440 mOsm kg⁻¹, with the further addition of 4 mM KEGTA for the FV channel; for both SV and FV, the pipette contained 200 KCl, 10 CaCl₂, 10 Mes (KOH) at pH 5.5, osmolality 460 mOsm kg⁻¹.

**Current-Voltage (I-V) Recording and Analysis.** Patch pipettes (5–10 MΩ) were pulled from Kimax-51 glass capillaries (Kimble/ Kontes, Vineland, NJ) by using a two-stage puller (Narishige PP-83, Tokyo). Experiments were done at room temperature (20–22°C) by using the standard whole-cell patch–clamp techniques, with an Axopatch 200B Integrating Patch Clamp amplifier (Axon, Union City, CA). Voltage commands and simultaneous signal recordings and analysis were assessed by a microcomputer connected to the amplifier via a multipurpose input/output device (Digidata 1200A, Axon) using pCLAMP 8.0 software (Axon). After gigaseal formed were sealed, the whole-cell configuration was achieved by gentle suction, and the membrane was immediately clamped to a holding voltage (hv) set close to the Nernst potential for K⁺ (Ek⁺). For Iᵥ, hv was set close to E₁ (the Nernst potential for Ca²⁺). For recording of both vacuolar channels, the bath contained 200 KCl, 0.1 CaCl₂, 10 Hepes (Tris) at pH 7.5, osmolality 440 mOsm kg⁻¹, with the further addition of 4 mM KEGTA for the FV channel; for both SV and FV, the pipette contained 200 KCl, 10 CaCl₂, 10 Mes (KOH) at pH 5.5, osmolality 460 mOsm kg⁻¹.

**Confocal Microscopy.** Laser-scanning confocal microscopy of dye-loaded patch-clamped GCPs was used to determine the consequences of laser uncaging of InsP₆ on cytosolic calcium. GCPs, isolated from either V. faba or Solanum tuberosum as described above (see also ref. 17), loaded through the patch pipette with P(4,5)-NBZ InsP₆, P(4,5)-NBZ InsP₅, and P(4,5)-NBZ InsP₄ (100–200 μM) (18) and a calcium-sensitive single wavelength emission dye, Calcium Green-1 (100 μM; Molecular Probes Europe, Leiden, The Netherlands). A Leica TCS-NT-UV CLSM was used to uncage InsP₆ and to follow changes in [Ca²⁺]. InsP₆ was uncaged by simultaneous exposure to the 354- and 361-nm lines of an UV laser (10–30 randomly chosen spots of 10-ms duration each). Calcium Green-1 was excited by using the 488-nm line of an ArKr laser with the other lines attenuated to zero. A reflectance short-pass filter of 510 nm was used, and emitted fluorescence was measured between 515 and 545 nm by using a water immersion 1.2 N.A. objective lens. Ten images of the Calcium Green-1 fluorescence were collected at a resolution of 512 × 512 pixels before uncaging and a further 200 after uncaging at 200-ms intervals. Images were analyzed by using the commercial software (Universal Imaging, West Chester, NY). Each image was pseudocolor-coded, and a scale bar was generated to indicate low (blue) to high [Ca²⁺] (red). GCPs were bathed in solutions (in mM): 10 KCl, 0.5 CaCl₂/2 MgCl₂/10 Mes (KOH), pH 5.5 (KOH)/sorbitol to give 480 mOsm kg⁻¹. The patch pipette contained (in mM) 92 K⁺-glutamate, 2 MgCl₂, 2 K₃ATP, 10 Hepes at pH 7.5 (KOH), and sorbitol to give 520 mOsm kg⁻¹ (some experiments contained 3.4 CaCl₂/5 EGTA to give resting [Ca²⁺] = 100 nM).

**Results.** InsP₆, Released from Caged Precursor, Mobilizes Ca²⁺ in Patch-Clamped GCPs. GCPs were loaded with Calcium Green-1 and caged InsP₆ to determine the effects on cytoplasmic Ca²⁺ of uncaging InsP₆. In six of nine dye- and P(4,5)-NBZ InsP₆-loaded GCPs, we observed transient increases in Calcium Green-1 fluorescence on uncaging. One such experiment is shown in Fig. 1A and B, in which [Ca²⁺]₉₉ levels increased to a maximum within 1 s of uncaging, persisting at maximal level for ~5 s before decaying to resting levels within 25–30 s. The time course varied in different experiments, and Fig. 1C shows another time course.
with a faster return to preflash levels within 6 s, together with a nonflashed control. UV irradiation was without effect in dye- or dye-loaded protoplasts and also in protoplasts coloaded with caged ATP (not shown). The nature of transients induced by InsP₆ release in this study is similar to the transients induced by ABA in GCPs from V. faba (19) and are among the most transitory [Ca²⁺]ᵪcyt signals yet recorded in single protoplasts of a higher plant. We have also investigated the consequence of repetitive uncaging events. Successive release events at intervals of 2–5 min elicited immediate and repetitive excursions in [Ca²⁺]ᵪcyt levels but of diminishing amplitude (not shown). It is therefore clear that uncaging of InsP₆ did not exhaust the reserve of calcium from which the excursions in [Ca²⁺]ᵪcyt levels originate. Similarly, ionomycin treatment of a dye- and P(4,5)-NBZ InsP₆ coloaded and UV laser-responsive protoplast resulted in a massive increase in dye fluorescence, assumed to result from entry of calcium from the bath solution (not shown). This indicates clearly that the GCPs used were able to regulate their internal free calcium before and after uncaging of InsP₆. These data identify InsP₆ as a calcium-mobilizing agent and provide an unambiguous explanation of the [Ca²⁺]ᵪcyt dependence of inhibition of Iₖᵢₚᵦ by InsP₆.

**InsP₆ Has No Effect on Ca²⁺ Currents at the Plasmalemma.** To measure Iₖᵢₚᵦ, GCPs isolated from V. faba were bathed in 100 mM BaCl₂, pH 5.5 (Mes/Tris), with 10 mM BaCl₂, pH 7.5 (Heps/Tris) in the pipette. The use of barium instead of calcium has two advantages: that Ba permeates calcium channels more freely than Ca, and that Ba blocks potassium channels more efficiently than Ca. Moreover, in these solutions Eₖᵢₚᵦ (+29 mV) and E_Ca (-58 mV) are far removed from each other, allowing easy identification of the conducting species. GCPs exhibited large whole-cell hyperpolarization-activated barium-permeable channels (Iₖᵢₚᵦ) (Fig. 2). Large Iₖᵢₚᵦ were observed even in the absence of stimuli such as H₂O₂ or ABA, which were essential for channel activity in Arabidopsis GCPs (14). We also confirmed substantial activation by ABA (up to 5-fold) and H₂O₂ (up to 16-fold) of whole-cell currents in Vicia protoplasts (not shown). In Fig. 2A (Lower Inset), Ba-permeable current traces activated instantly without a time-dependent kinetic component, irrespective of voltage (-198 to +42 mV) and up to 4-s square wave test pulses). The I-V relationship of whole-cell Iₖᵢₚᵦ showed a weakly voltage-dependent rectification with current reversing near the predicted Nernstian equilibrium for barium, E_Ba (Fig. 2B). External addition of GdCl₃ (20–100 μM) suppressed these currents (Fig. 2B), whereas in other experiments designed to measure Iₖᵢₚᵦ similar concentrations of GdCl₃ were without effect (not shown). LaCl₃ also inhibited Iₖᵢₚᵦ but less potently than GdCl₃. Verapamil, a calcium-channel blocker, was, at 50 μM concentration, much less effective than GdCl₃ or LaCl₃. Moreover addition of 1 mM CsCl, which blocks Iₖᵢₚᵦ completely, has no or little effect on Iₖᵢₚᵦ (Fig. 2B Inset). More importantly, we show (Fig. 2C) that InsP₆ (in the range 1–20 μM in the patch pipette) was without major effect on Iₖᵢₚᵦ (n = 16, control, n = 14, InsP₆). This contrasts markedly with the effects of ABA on Iₖᵢₚᵦ (13) and also with the strong inhibition of Iₖᵢₚᵦ manifest in Vicia at submicromolar InsP₆ concentrations (1). The present results therefore indicate that InsP₆-mediated [Ca²⁺]ᵪcyt-dependent inhibition of Iₖᵢₚᵦ is not a consequence of InsP₆-induced calcium influx via hyperpolarization-activated calcium current.

Further confirmation of the absence of an apoplast-derived calcium influx component in InsP₆-dependent inhibition of Iₖᵢₚᵦ was afforded by measuring Iₖᵢₚᵦ in the absence of external calcium. Fig. 3 shows one such experiment performed with 2 mM EGTA in the bath solution. Loading the cell with InsP₆ (0.1–1 μM in the patch pipette), we observed an up to 80% inhibition of Iₖᵢₚᵦ in 4–6 min (n = 4). We conclude from the foregoing data (Figs. 1–3) that the InsP₆-induced calcium mobilization and the [Ca²⁺]ᵪcyt dependence of inhibition of Iₖᵢₚᵦ resides with an InsP₆-sensitive endomembrane store of calcium.

**InsP₆ Activates Vacuolar Ion Channels.** By patch-clamping of isolated vacuoles, we tested whether the endomembrane-store-dependent inhibition of Iₖᵢₚᵦ could be explained by mobilization of vacuolar calcium. Three vacuolar conductances have previ-
wards directions. Challenge of vacuoles with cytosolic InsP6 (100 nM) to measure FV or high calcium (100–200 nM) to measure SV. In low external calcium and no added EGTA, all membrane potentials are specified as the potential on the cytosolic side relative to the vacuolar side.

Experiments in high calcium and in the whole-vacuole mode were also carried out to assess the effects of InsP6 on SV conductance. Addition of InsP6 (5 μM) activated SV currents. Fig. 4C shows one such experiment where control, InsP6, and bath contained (in mM): 200 KCl, 2 MgCl2, 0.1 CaCl2, 4 EGTA (∼2 mM Free Ca2+), 10 Hepes at pH 7.5 (KOH); patch pipette composition (in mM): 200 mM KCl, 2 mM MgCl2, 10 mM CaCl2, 10 mM Mes at pH 7.5 (KOH). Solutions in C were as in A and B but with 100 μM external calcium and no added EGTA. All membrane potentials are specified as the potential on the cytosolic side relative to the vacuolar side.

Discussion
The measurements of cytosolic Ca2+ after the flash release of InsP6 from its cage establish InsP6 as an effective Ca2+-mobilizing agent in guard cells, to be added to the list of such agents already identified. We now have four agents capable of mobilizing Ca2+ in guard cells and need to establish their physiological relevance. Both Ins(1,4,5)P3 and InsP6 elicit elevations in [Ca2+]cyt in guard cells and both signal to IK, in inhibiting IK,in in a calcium-dependent manner (1, 5). However, we have shown that InsP6 is 50–100 times more potent as an inhibitor of IK,in than Ins(1,4,5)P3 (1). Two other intracellular signaling agents, cyclic adenosine diphosphoribose (cADPR) (25) and sphingosine-1-phosphate (26), have also been shown to modulate [Ca2+]cyt when introduced into guard cells. Evidence has been presented for the involvement of all four agents in the response of guard cells to ABA (1, 25–29), and an important goal for the future is to establish their relative contributions in different conditions.
The [Ca\textsuperscript{2+}]\textsubscript{cyt} response of guard cells to uncaging of InsP\textsubscript{6} (Fig. 1) is fundamentally different from the response of guard cells to Ins(1,4,5)P\textsubscript{3}, cADPR, and sphingosine-1-phosphate. The instantaneous increase of [Ca\textsuperscript{2+}]\textsubscript{cyt} observed with InsP\textsubscript{6} and the rapid decay to resting level within 30 s or less contrasts markedly with the sustained increases of [Ca\textsuperscript{2+}]\textsubscript{cyt} (5–10 min) observed after flash photolysis of caged-Ins(1,4,5)P\textsubscript{3} (4) or the 3- to 5-min periodic elevations of [Ca\textsuperscript{2+}]\textsubscript{cyt} observed with cADPR (25) and sphingosine-1-phosphate (26). However, it is important to note that the method of application of the agent differs in different studies, and the rapid decay in the response to a pulse of InsP\textsubscript{6} may reflect the diffusion of InsP\textsubscript{6} in the patch pipette or its metabolism; the accurate time course of change in InsP\textsubscript{6} levels after stimulation with ABA in intact cells is not established.

InsP\textsubscript{6}-induced calcium transients in guard cells could result from calcium entry through plasma membrane-permeable channels or calcium release from internal stores. Our findings that InsP\textsubscript{6} has no effect on I\textsubscript{K,in} and that InsP\textsubscript{6} can inhibit I\textsubscript{K,in} in the absence of external Ca\textsuperscript{2+} rule out an effect through activation of calcium influx and point to an endomembrane source for calcium release.

In the context of endomembrane calcium, the vacuole constitutes an important pool of mobilizable calcium in higher plants but may not be the dominant signaling pool. Among the different agents reported to increase guard cell [Ca\textsuperscript{2+}]\textsubscript{cyt}, both Ins(1,4,5)P\textsubscript{3} and cADPR are believed to target endomembranes (25, 30). Thus both agents activate calcium release from the vacuole (30) but also from other endomembranes such as the endoplasmic reticulum (ER) (7, 31). A further Ca\textsuperscript{2+}-mobilizing agent in higher plants, nicotinic acid adenine dinucleotide phosphate, is active exclusively at the ER (32). Our results establish that InsP\textsubscript{6} activates two tonoplast ion channels, the FV and SV channels. The activation of the FV channel is reminiscent of the effects of cADPR (25), which activated a fast tonoplastic channel inhibited by cytoplasmic Ca\textsuperscript{2+} above ~600 nM, but which appeared to be permeable to Ca\textsuperscript{2+}, because the reversal potential was shifted by increase in vacuolar Ca\textsuperscript{2+}. Our results show activation also of the SV channel by InsP\textsubscript{6}, and this channel is permeable to Ca\textsuperscript{2+} (24). Thus our results suggest that the vacuole contributes to the InsP\textsubscript{6}-triggered Ca\textsuperscript{2+} release, but other endomembranes may also be involved, as is the case for both InsP\textsubscript{6} and cADPR.

Our study further defines the mechanism by which InsP\textsubscript{6} mediates physiological (ABA-dependent) inhibition of I\textsubscript{K,in}. The discovery that InsP\textsubscript{6} is a calcium-mobilizing agent and produces inhibition of I\textsubscript{K,in} much more effectively than does InsP\textsubscript{3} may yet explain the absence of InsP\textsubscript{3} receptors in the yeast and Arabidopsis genomes; conversion of InsP\textsubscript{3} introduced into the cytoplasm to InsP\textsubscript{6} would produce the effects observed. The work identifies release from endomembrane stores rather than Ca\textsuperscript{2+} influx as the source of InsP\textsubscript{6}-triggered increase in cytoplasmic Ca\textsuperscript{2+}, and the demonstration that InsP\textsubscript{6} regulates vacuolar release channel(s) provides further evidence that endomembrane channels are cellular targets of InsP\textsubscript{6}. It remains to be established whether this reflects a direct interaction with the channels or action via a regulatory agent. That such channels are involved in osmoregulation adds considerably to our understanding of the physiological and cellular signaling function of this, the most enigmatic of inositol phosphates.

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