Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells

Foud Lemtiri-Chlieh†, Enid A. C. MacRobbie*, Alex A. R. Webb*, Nick F. Manison†, Colin Brownlee‡, Jeremy N. Skepper§, Jian Chen*, Glenn D. Prestwich*, and Charles A. Brearley**

*Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom; †Marine Biological Association, Citadel Hill, Plymouth PL2 5PB, United Kingdom; ‡Multi-Imaging Centre, Department of Anatomy, University of Cambridge, Tennis Court Road, Cambridge CB2 3DY, United Kingdom; §Department of Medicinal Chemistry, University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, UT 84108; and ¶School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom

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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 rapidly increased 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 ICa 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 (IK,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⁺ when released from a caged precursor in guard cells, with consequential inhibition of IK,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), and hippocampal neurons (9). It is striking that the levels of InsP₆ quickly increase in hyperosmotically stressed 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 signal-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 IK,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 I₉-type calcium current (10). Recent studies (11–14) have shown that plants harbor calcium-permeable channels [hyperpolarization-activated calcium current (Iᵥ)] 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 Iᵥ 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% mannitol, 10 mM MES, pH 5.6, and incubated 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, 0 mM (Mes), 200 μM CaCl₂, and 2.5 mM KOH (pH 5.55 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 360 mOsm/kg⁻¹ adjusted with mannitol), and incubated 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 360 mOsm/kg⁻¹).

**Vacuole Isolation.** Freshly made protoplasts were allowed to settle to the bottom of the recording chamber and stick well to the
glass, then given a hypoosmotic 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 (Iₖ) 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 K₂EGTA, 10 Mes (KOH) at pH 5.5, osmolality 480 mOsm·kg⁻¹ (sorbitol) in the bath; and 92 K-glutamate, 2 MgATP, 3.4 CaCl₂, 5 K₂EGTA, 10 Hepes (KOH) at pH 7.5, osmolality 520 mOsm·kg⁻¹ (sorbitol) in the pipette. For Iₖ, the solutions contained 100 BaCl₂, 10 Mes (Tris) at pH 5.5, osmolality 230 mOsm·kg⁻¹ in the bath, and 4 K₂EGTA, 10 BaCl₂, 10 Hepes (Tris) at pH 7.5, osmolality 230 mOsm·kg⁻¹ in the pipette. For recording of both vacuolar channels, the bath contained 200 KCl, 2 MgCl₂, 0.1 CaCl₂, 10 Hepes (Tris) at pH 7.5, osmolality 440 mOsm·kg⁻¹, with the further addition of 4 mM K₂EGTA 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 gigahm seals were formed, the whole-cell configuration was achieved by gentle suction, and the input resistance was confirmed to be >500 MΩ. Nernst potentials were calculated after correction for ionic activities (estimated by GEOCHEM-PC software, public domain freeware). I–V relationships for Iᵥ, FV, and SV were plotted as steady-state currents vs. test potential, but Iᵥ was plotted as the time-dependent current (i.e., the steady-state current minus the instantaneous current) vs. test potential. Results are expressed as mean ± SEM.

**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)-nitrobenzyl) inositol hexakisphosphate [P(4,5)-NBZ InsP₆] (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 monitored 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 METAMORPH 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 at 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. 1 A and B, in which [Ca²⁺]₂C 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. 1 C 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-only loaded protoplasts and also in protoplasts loaded with caged ATP (not shown). The nature of transients induced by InsP$_6$ release in this study is similar to the transients induced by ABA in GCPs from V. faba (19) and are among the most transitory [Ca$^{2+}$]$_{cyt}$ 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 [Ca$^{2+}$]$_{cyt}$ levels but of diminishing amplitude (not shown). It is therefore clear that uncaging of InsP$_6$ did not exhaust the reservoir of calcium from which the excursions in [Ca$^{2+}$]$_{cyt}$ had originated. Similarly, ionomycin treatment of a dye- and P(4,5)-NBD-InsP$_6$ co-loaded 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 InsP$_6$. These data identify InsP$_6$ as a calcium-mobilizing agent and provide an unambiguous explanation of the [Ca$^{2+}$]$_{cyt}$ dependence of inhibition of $I_{K,in}$ by InsP$_6$.

**InsP$_6$ Has No Effect on Ca$^{2+}$ Currents at the Plasmalemma.** To measure $I_{K,in}$, GCPs isolated from V. faba were bathed in 100 mM BaCl$_2$, pH 5.5 (Mes/Tris), with 10 mM BaCl$_2$, pH 7.5 (Hepes/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 $E_{Ba}$ (+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 ($I_{Ba}$) (Fig. 2). Large $I_{Ba}$ were observed even in the absence of stimuli such as H$_2$O$_2$ or ABA, which were essential for channel activity in Arabidopsis GCPs (14). We also confirmed substantial activation by ABA (up to 5-fold) and H$_2$O$_2$ (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 $I_{Ba}$ showed a weakly voltage-dependent rectification with current reversing near the predicted Nernstian equilibrium for barium, $E_{Ba}$ (Fig. 2B). External addition of GdCl$_3$ (20–100 μM) suppressed these currents (Fig. 2B), whereas in other experiments designed to measure $I_{K,in}$ similar concentrations of GdCl$_3$ were without effect (not shown). LaCl$_3$ also inhibited $I_{Ba}$ but less potently than GdCl$_3$. Verapamil, a calcium-channel blocker, was, at 50 μM concentration, much less effective than LaCl$_3$ or GdCl$_3$. Moreover addition of 1 mM CsCl, which blocks $I_{K,in}$ completely, has no or little effect on $I_{Ba}$ (Fig. 2B Inset). More importantly, we show (Fig. 2C) that InsP$_6$ (in the range 1–20 μM) in the patch pipette was without major effect on $I_{Ba}$ ($n = 16$, control; $n = 14$, InsP$_6$). This contrasts markedly with the effects of ABA on $I_{Ba}$ (13) and also with the strong inhibition of $I_{K,in}$ manifest in Vicia at submicromolar InsP$_6$ concentrations (1). The present results therefore indicate that InsP$_6$-mediated $[Ca^{2+}]_{cyt}$-dependent inhibition of $I_{K,in}$ is not a consequence of InsP$_6$-induced calcium influx via hyperpolarization-activated calcium current.

Further confirmation of the absence of an apoplast-derived calcium influx component in InsP$_6$-dependent inhibition of $I_{K,in}$ was afforded by measuring $I_{K,in}$ 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 InsP$_6$ (0.1–1 μM in the patch pipette), we observed an up to 80% inhibition of $I_{K,in}$ in 4–6 min ($n = 4$). We conclude from the foregoing data (Figs. 1–3) that the InsP$_6$-induced calcium mobilization and the $[Ca^{2+}]_{cyt}$ dependency of inhibition of $I_{K,in}$ resides with an InsP$_6$-sensitive endomembrane store of calcium.

**InsP$_6$ Activates Vacuolar Ion Channels.** By patch-clamping of isolated vacuoles, we tested whether the endomembrane-store-dependent inhibition of $I_{K,in}$ could be explained by mobilization of vacuolar calcium. Three vacuolar conductances have previ-
Fig. 3. InsP₆ inactivates Iᵥᵣᵢᵳ in the absence of external calcium. Current recordings from a GCP 1 min (Left) and 5 min (Right) after breaking the patch seal to go whole cell. The patch pipette contained 0.1 μM InsP₆. Time-dependent current values (indicated by arrows) were plotted on the corresponding I–V curve; note the inhibition of Iᵥᵣᵢᵳ by InsP₆ but absence of effect on Iᵥᵯᵢᵠ. The bath contained (mM): 5 KCl, 2 K₂EGTA, 2 MgCl₂, 10 Mes at pH 5.5 (KOH), 0.1 CaCl₂, 4 EGTA (resting [Ca²⁺]ᵥᵣᵢᵳ = 100 nM), 2 MgATP, 10 Hepes at pH 7.5 (KOH), and sorbitol to give 480 mOsmkg⁻¹. The patch pipette contained (mM): 92 K⁺, 1.6 glutamate, 3.4 CaCl₂/5 EGTA (resting [Ca²⁺]ᵥᵣᵢᵳ = 100 nM), 2 MgATP, 10 Hepes at pH 7.5 (KOH), and sorbitol to give 520 mOsmkg⁻¹.

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⁺]ᵥᵣᵢᵳ in guard cells and both signal to Iᵥᵣᵢᵳ, inhibiting Iᵥᵣᵢᵳ in a calcium-dependent manner (1, 5). However, we have shown that InsP₆ is ~100 times more potent as an inhibitor of Iᵥᵣᵢᵳ 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⁺]ᵥᵣᵢᵳ 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 $[Ca^{2+}]_{\text{cyt}}$ response of GCPs to uncaging of InsP$_6$ (Fig. 1) is fundamentally different from the response of guard cells to Ins(1,4,5)P$_3$, cADPR, and sphingosine-1-phosphate. The instantaneous increase of $[Ca^{2+}]_{\text{cyt}}$ observed with InsP$_6$ and the rapid decay to resting level within 30 s or less contrasts markedly with the sustained increases of $[Ca^{2+}]_{\text{cyt}}$ (5–10 min) observed after flash photolysis of caged-Ins(1,4,5)P$_3$ (4) or the 3- to 5-min periodic elevations of $[Ca^{2+}]_{\text{cyt}}$ 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$_6$ may reflect the diffusion of InsP$_6$ in the patch pipette or its metabolism; the accurate time course of change in InsP$_6$ levels after stimulation with ABA in intact cells is not established.

InsP$_6$-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$_6$ has no effect on $I_{Ca}$ and that InsP$_6$ can inhibit $I_{K,in}$ in the absence of external $Ca^{2+}$ 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 $[Ca^{2+}]_{\text{cyt}}$, both Ins(1,4,5)P$_3$ 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 $Ca^{2+}$-mobilizing agent in higher plants, nicotinic acid adenine dinucleotide phosphate, is active exclusively at the ER (32). Our results establish that InsP$_6$ activates two tonoplasmon channels, the FV and SV channels. The activation of the FV channel is reminiscent of the effects of cADPR (25), which activated a fast tonoplasmon channel inhibited by cytoplasmic $Ca^{2+}$ above $\approx 600 \text{ nM}$, but which appeared to be permeable to $Ca^{2+}$, because the reversal potential was shifted by increase in vacuolar $Ca^{2+}$. Our results show activation also of the SV channel by InsP$_6$, and this channel is permeable to $Ca^{2+}$ (24). Thus our results suggest that the vacuole contributes to the InsP$_6$-triggered $Ca^{2+}$ release, but other endomembranes may also be involved, as is the case for both InsP$_6$ and cADPR.

Our study further defines the mechanism by which InsP$_6$ mediates physiological (ABA-dependent) inhibition of $I_{K,in}$. The discovery that InsP$_6$ is a calcium-mobilizing agent and produces inhibition of $I_{K,in}$ much more effectively than does InsP$_6$ may yet explain the absence of InsP$_6$ receptors in the yeast and Arabidopsis genomes; conversion of InsP$_6$ introduced into the cytoplasm to InsP$_6$ would produce the effects observed. The work identifies release from endomembrane stores rather than $Ca^{2+}$ influx as the source of InsP$_6$-triggered increase in cytoplasmic $Ca^{2+}$, and the demonstration that InsP$_6$ regulates vacuolar release channel(s) provides further evidence that endomembrane channels are cellular targets of InsP$_6$. 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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