Inositol hexakisphosphate is a physiological signal regulating the K^+-inward rectifying conductance in guard cells

Fouad Lemtiri-Chlieh*, Enid A. C. MacRobbie, and Charles A. Brearley*†

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom

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(*RS*)-2-*cis*, 4-*trans*-abscisic acid (ABA), a naturally occurring plant stress hormone, elicited rapid agonist-specific changes in *myo*-inositol hexakisphosphate ($InsP_6$) measured in intact guard cells of *Solanum tuberosum* ($n = 5$); these changes were not reproduced by (*RS*)-2-*trans*, 4-*trans*-abscisic acid, an inactive stereoisomer of ABA ($n = 4$). The electrophysiological effects of $InsP_6$ were assessed on both *S. tuberosum* ($n = 14$) and *Vicia faba* ($n = 6$) guard cell protoplasts. In both species, submicromolar concentrations of $InsP_6$, delivered through the patch electrode, mimicked the inhibitory effects of ABA and internal calcium (Ca_i^{2+}) on the inward rectifying K^+ current, $I_{K,in}$, in a dose-dependent manner. Steady state block of $I_{K,in}$ by $InsP_6$ was reached much more quickly in *Vicia* (3 min at $\approx$1 μM) than *Solanum* (20–30 min). The effects of $InsP_6$ on $I_{K,in}$ were specific to the *myo*-inositol isomer and were not elicited by other conformers of $InsP_6$ (e.g., *scyllo*- or *neo*-). Chelation of Ca^{2+} by inclusion of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid or EGTA in the patch pipette together with $InsP_6$ prevented the inhibition of $I_{K,in}$, suggesting that the effect is Ca^{2+} dependent. $InsP_6$ was $\approx$100-fold more potent than $Ins(1,4,5)P_3$ in modulating $I_{K,in}$. Thus ABA increases $InsP_6$ in guard cells, and $InsP_6$ is a potent Ca^{2+}-dependent inhibitor of $I_{K,in}$. Taken together, these results suggest that $InsP_6$ may play a major role in the physiological response of guard cells to ABA.

Despite the fact that *myo*-inositol hexakisphosphate ($InsP_6$) is the most abundant *myo*-inositol phosphate in nature (1) and is the most readily identified of this class of compounds, the cellular functions of $InsP_6$ remain enigmatic. This is in part a consequence of an apparent lack of response of $InsP_6$ to extracellular signals, with a notable exception in the response of *Schizosaccharomyces pombe* to hypertonic osmotic shock, which leads to rapid increases in the levels of $InsP_6$ (2). A role for $InsP_6$ in cell signaling is suggested by recent work in two other systems. In the first, three genes, whose products constitute a path linking Ins $(1,4,5)P_3$ to $InsP_6$, have been identified as members of a group that functionally complement lesions in mRNA export from the nucleus in yeast; the genes respectively encode phospholipase C, an Ins $(1,4,5)P_3$ 3-/Ins $(1,3,4,5)P_4$ 6-kinase and Ins $(1,3,4,5,6)P_5$ 2-kinase (3, 4). Secondly, in insulin-secreting pancreatic β cells (5), it was shown that 10 μM $InsP_6$ in the patch pipette increased an L-type Ca^{2+} current; the effect may reflect the ability of $InsP_6$ to inhibit protein phosphatases, types PP1, PP2A, and PP3, with consequent activation of the L-type Ca^{2+}-channels. In this paper, we define a function for $InsP_6$ in plants, as a signal, generated in guard cells in response to the stress hormone, (*RS*)-2-*cis*, 4-*trans*-abscisic acid (ABA). We have further defined an electrophysiological target for $InsP_6$. In drought conditions, ABA is produced and induces changes in ion channel activity in both plasmalemma and tonoplast of guard cells. The ensuing net loss of K^+ and associated anions from the vacuole and from the cell leads to reduction in turgor and closure of the stomatal pore. The mechanisms by which ABA leads to activation and inactivation of specific ion channels have been the subject of much study, but detailed description of the events and signaling chains remains very incomplete. Ca^{2+} has been identified as a signaling intermediate in some, but not all, of the ABA-induced changes in guard cell ion channels; ABA-induced inactivation of the plasmalemma inward K^+ channel, $I_{K,in}$ (6–8), is Ca^{2+} mediated (8), whereas ABA-induced activation of the plasmalemma outward K^+-channel, $I_{K,out}$ (6–9), is Ca^{2+} independent (8, 10). In neither case is the detailed signal transduction chain established.

Several of the signaling functions of specific inositol phosphates seem to be intimately associated with the second messenger function of internal calcium (Ca_i^{2+}); thus the Ins $(1,4,5)P_3$ receptor is well documented as a Ca^{2+}-release channel in the endoplasmic reticulum in animal cells, and other studies have assigned a specific function to Ins $(3,4,5,6)P_4$ as an uncoupler of Ca^{2+}-activated Cl^- secretion in T_{84} colonic epithelial cells (11). The role of $InsP_3$ as a second messenger in plants is less well established, but it has been suggested to play a role in the signal transduction processes in the guard cells responsible for ABA-induced stomatal closure (12–14). In this paper, evidence is presented for involvement of $InsP_6$ in the ABA response, with the demonstration that $InsP_6$ regulates inward K^+ current, probably the best characterized electrophysiological target of ABA in guard cells (15). We show that ABA increases the level of $InsP_6$ in guard cells, that $InsP_6$ in the patch pipette is a potent inhibitor of guard cell plasmalemma inward K^+ current, and that $InsP_6$-dependent inhibition of inward K^+ current is manifest in a calcium-dependent manner.

Materials and Methods

Biochemistry. Epidermal fragments isolated (16) from 5–6 g of leaves of *Solanum tuberosum* (Desirée) were labeled for 24 h in 10 ml of 10 mM Mes, pH 5.5 (KOH)/0.2 mM $CaCl_2$, osmolality adjusted to 470 mOsm·kg^{-1} with sorbitol, containing 25 μCi·ml^{-1} *myo*-[2-^{3}H]inositol (specific activity 16–18 Ci·$mmol^{-1}$, Amersham Pharmacia). Tissue was split into approximately equal aliquots before challenge for 5 min with 30 μM ABA, (*RS*),-2-*trans*, 4-*trans*-abscisic acid (obtained from Sigma), or water. ABA was stored frozen in the dark and thawed immediately before use. Tissue was rapidly filtered on 200 μm nylon mesh, washed with 50 ml water, and frozen in liquid nitrogen. Inositol phosphates were extracted, mixed with standards obtained from previously defined sources (17, 18) and resolved on Partisphere SAX (Whatman) HPLC columns with gradients of NaH_2PO_4: 0 min 0-M; 5 min, 0-M; 75 min, 2.5-M.

Abbreviations: $InsP_6$, *myo*-inositol hexakisphosphate; ABA, (*RS*)-2-*cis*, 4-*trans*-abscisic acid; GCP, guard cell protoplast; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetate or -tetraacetic acid.

*F.L.-C. and C.A.B. contributed equally to this work.

†To whom reprint requests should be addressed. E-mail: cab1000@cus.cam.ac.uk.

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Electrical Recording. Guard cell protoplasts (GCPs) from *Vicia faba* were obtained as described (9). GCPs from *S. tuberosum* (Desirée) were prepared from epidermal fragments (as obtained in ref. 16). Enzymatic solution and protocol for isolation of GCPs are as described for *V. faba* (9). The bath medium contained 14 mM K^+ glutamate, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Mes, pH 5.5 (KOH), osmolality adjusted to 480–500 mOsm·kg^{-1} with mannitol or sorbitol. Pipette solutions were 100 or 180 mM K^+ glutamate/3.4 mM $CaCl_2$ (or 3.21 mM at 180 mM K^+ glutamate)/5 mM EGTA (giving ≈100 nM free Ca^{2+})/2 mM K_2-ATP/2 mM $MgCl_2$/10 mM Hepes, pH 7.5 (KOH), osmolality adjusted to 520–540 mOsm·kg^{-1} with mannitol or sorbitol. Electrical recordings and analysis were performed as described (9). All chemicals, including $InsP_3$ and $InsP_6$, were from Sigma. *Scyllo-* and *neo*-inositol hexakisphosphate were obtained from the laboratory of the late Dennis Cosgrove, Commonwealth Scientific and Industrial Research Organization, Plant Industry, Canberra, Australia. The structures of these inositol phosphates were confirmed by ^{31}P NMR before use.

Results

Biochemical Studies. Guard cell preparations from *S. tuberosum* were prelabeled with [^{3}H]inositol, and the distribution of label in different inositol phosphates and the effect of treatment of guard cells with 30 μM (*RS*)-2-*cis*, 4-*trans* abscisic acid on distribution of label between different inositol phosphates were determined. For identification of individual inositol phosphates, guard cell extracts were mixed with internal ^{14}C standards (Fig. 1*A*) or ^{32}P standards (Fig. 1*B*) and resolved by HPLC as shown. The extent of labeling of inositol phosphates was variable between different experiments, and the low levels of labeling precluded rigorous identification of stereoisomers. Inositol phosphates were identified as follows: $InsP_2$ (coeluting with [Ins (1,4)P_2]); three [$InsP_3$] peaks, one of which coeluted with Ins (1,4,5)P_3; $InsP_4$; three $InsP_5$ peaks; $InsP_6$ and a peak eluting later than $InsP_6$, which is probably a diphosphoinositol polyphosphate, perhaps a diphosphoinositol pentakisphosphate (colloquially known and referred hitherto in this manuscript as "$InsP_7$").

Marked differences were found in the pattern of labeling between ABA-treated cells and water-treated controls, with a large ABA-induced increase in the fraction of label associated with $InsP_6$. The pattern of change was similar in different experiments even if the extent of labeling differed. Fig. 1*C* shows the pattern of labeling and the response to ABA in one such experiment, in which cells were treated with ABA for 5 min. There was little ABA-induced difference in the fraction of label in $InsP_1$ to $InsP_5$, but the label in $InsP_6$ increased nearly 5-fold from 3% in the control to 15% in the ABA-treated cells, and that in $InsP_7$ rose from 0.02% to 0.4%; the label in $InsP_2$ fell by a much smaller fraction, from 56% to 43%. Thus $InsP_6$, and perhaps $InsP_7$, was the inositol phosphate, the labeling of which was most responsive to ABA. Table 1 summarizes the extent of the ABA-induced increases in labeling in $InsP_6$ in five experiments, with treatments of 30 sec to 15 min. Although we were unable to quantify the chemical level of $InsP_6$ in our system, even with the mass-sensitive metal dye detection HPLC system of Mayr (19) with which we can measure as little as 100 pmol $InsP_6$ on-column, it is clear that ABA induces rapid changes in $InsP_6$ metabolism.

By way of control, we have tested the effect of another naturally occurring isomer of ABA, 30 μM (*RS*)-2-*trans,* 4-*trans*-abscisic acid, which does not induce stomatal closure in stomata opened in the dark by CO_2-free air (20). Treatment with 30 μM (*RS*)-2-*trans,* 4-*trans*-abscisic acid elicited within 5 min a rapid and dramatic reduction in the labeling of $InsP_6$; in three experiments, the label in $InsP_6$ was reduced to 5 ± 3% of that in the control. In one further experiment, after a 5-min treatment with

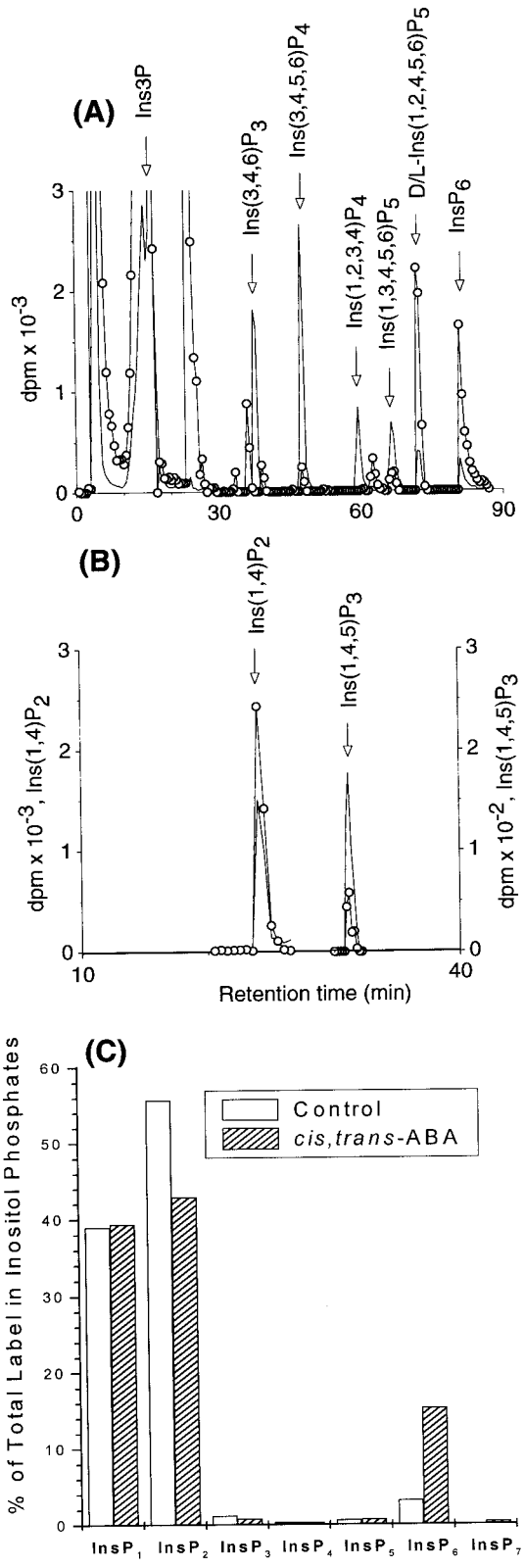

Fig. 1. Activation of inositol phosphate metabolism in guard cells. Inositol phosphates in ^{3}H-labeled guard cells (○), mixed with internal ^{14}C (no symbol) standards (*A*) and ^{32}P (no symbol) standards (*B*). The traces shown in *A* and *B* were from HPLC runs performed on different extracts of ^{3}H-labeled guard cells. The bar graph represents the distribution of label in different inositol phosphates, as a percentage of the total label in $InsP_1$ to $InsP_7$, from control (transparent bars) or (*RS*)-2-*cis*, 4-*trans*-abscisic acid-treated (hashed bars) guard cells (*C*).

Table 1. ABA-induced increases in $InsP_6$ in guard cells

	Period of treatment	dpm in $InsP_6$	
		Control	ABA
Expt. 1	30 sec	2541	3533
Expt. 2	1 min	n.d.	575
	5 min	85	7444
Expt. 3	5 min	2536	10608
Expt. 4	5 min	7100	40491
Expt. 5	5 min	312	4989
	15 min	n.d.	7204

Epidermal fragments were labeled with *myo*-[2-^{3}H]inositol for 24 hours before challenge with 30 μM (*RS*)-2-*cis*, 4-*trans* abscisic acid. Inositol phosphates were extracted and resolved by Partisphere SAX HPLC. Radioactivity in peak fractions was determined by scintillation counting. To account for differences in the amount of tissue extracted for control and ABA treatments, dpm recovered in the $InsP_6$ peaks were normalized to the inositol peak. Expt., experiment.

the *trans, trans* isomer, labeling in $InsP_6$ was reduced more than 3-fold. $InsP_7$ was below the limit of detection in these experiments. These results show that the control by ABA of $InsP_6$ metabolism in guard cells is stereospecific; the massive increase in label in $InsP_6$ is effected only by the isomer that is active in promoting stomatal closure. The most likely explanation of the reduction in the level of $InsP_6$ produced by the *trans, trans* isomer is that this reflects competition for an ABA receptor with endogenous *cis, trans* abscisic acid, which determines the resting level of $InsP_6$ before external ABA is added.

Electrical Recordings. The discovery of rapid stereospecific control of $InsP_6$ metabolism by ABA isomers identifies $InsP_6$ as a candidate signal in ABA-dependent processes in guard cells. It is important, therefore, to determine whether $InsP_6$ regulates the activity of guard cell ion channels. We have used the patch-clamp technique in whole protoplast configuration to manipulate cytosolic composition to identify the cellular targets of $InsP_6$. Specifically, we have studied the control of potassium channels in both *S. tuberosum*, on which the biochemistry described above was performed, and *V. faba*, which has been the subject of much electrophysiological work. Two K^+ conductances have been characterized in *V. faba,* both showing time- and voltage-dependent characteristics. The first is an inward K^+ current, open only at voltages more negative than about -120 mV, which is inhibited by cytoplasmic Ca^{2+} (21–23) and is down-regulated by ABA (6–8); the inhibition by ABA is prevented by the inclusion of Ca^{2+} chelator in the patch pipette (8). The second is an outward K^+ channel, opening at voltages positive of the potassium equilibrium potential (E_K), which is insensitive to Ca^{2+} (21) and is activated by ABA in a Ca^{2+}-independent manner (8, 10). There are fewer studies of *Solanum* guard cell K^+ channels (16, 24), but nevertheless Dietrich *et al.* (24) have shown that the K^+ channels of this species are similar to those of *Vicia* with respect to voltage dependence, selectivity, and single-channel conductance.

K^+ Currents in *Solanum* GCPs. Fig. 2*A* shows typical current traces of potassium inward and outward rectifier ($I_{K,in}$ and $I_{K,out}$, respectively) recorded from a GCP isolated from *Solanum* after going from protoplast-attached to whole-protoplast configuration. This experiment is included here to show that when the standard internal medium was used, with ≈100 nM free Ca^{2+} in the patch pipette, time- and voltage-dependent $I_{K,in}$ and $I_{K,out}$ currents were present and stable for as long as the gigaohm seal resistance between the cell and the pipette was maintained (n = 6). The current-voltage (I/V) plot shows two I/V scans made at

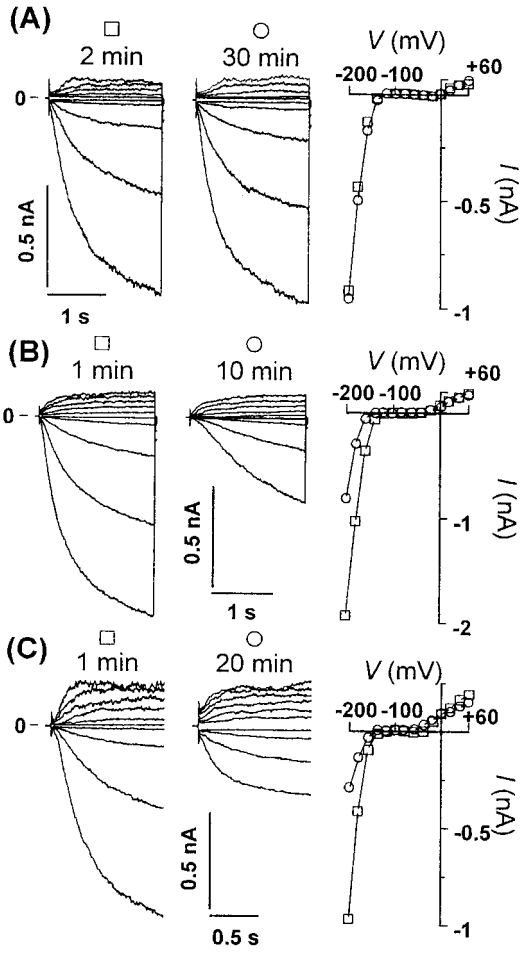

Fig. 2. $InsP_6$ and increased Ca_i^{2+} inactivate $I_{K,in}$ but not $I_{K,out}$ in *Solanum* GCPs. Current traces of $I_{K,in}$ and $I_{K,out}$ (*Left*) and corresponding I/V curves (*Right*) were measured at the indicated times shown above each set of current traces (referring to the time after achieving whole-protoplast configuration). Control experiment carried out with ≈0.1 μM free Ca^{2+} in the patch pipette (*A*). Typical experiments with pipette solutions containing ≈1.3 μM free Ca^{2+} (*B*) and those containing 20 μM $InsP_6$ (with ≈0.1 μM free Ca^{2+}) (*C*).

2 and 30 min with little or no variation between them. Thus, the effects of specific inositol phosphates described in the following are not a consequence of "run down" of currents because of depletion of undefined cytosolic components.

Previous studies have noted that $I_{K,in}$ measured from *Solanum* protoplasts was largely insensitive to external calcium (16, 24), a result in contrast to that obtained with *Vicia* (8, 25). The sensitivity of the *Solanum* channel to internal Ca^{2+} (Ca_i^{2+}) has not been defined, and this represents an issue of some importance to the regulation of $I_{K,in}$. We show here that, as for *Vicia* (21–23), $I_{K,in}$ in *Solanum* is sensitive to Ca_i^{2+}. Thus, in contrast to the temporally stable currents observed with low- (≈100 nM) pipette Ca^{2+} (Fig. 2*A*), inclusion of high Ca^{2+} (1.3 μM) caused a time-dependent reduction in currents consequent on equilibration of the cytoplasm with high-pipette Ca^{2+} (Fig. 2*B*). In all of three separate experiments, elevation of Ca^{2+} reduced the current carried at all tested voltages in the range within which $I_{K,in}$ is activated, with no or little effect on $I_{K,out}$.

$InsP_6$ Modulates $I_{K,in}$ in *Solanum* GCPs. With confidence in the stability of K^+ currents in GCPs from *Solanum* and in our ability to manipulate the cytosolic composition successfully, we tested the effect on both $I_{K,in}$ and $I_{K,out}$ of 20 μM $InsP_6$ in the pipette,

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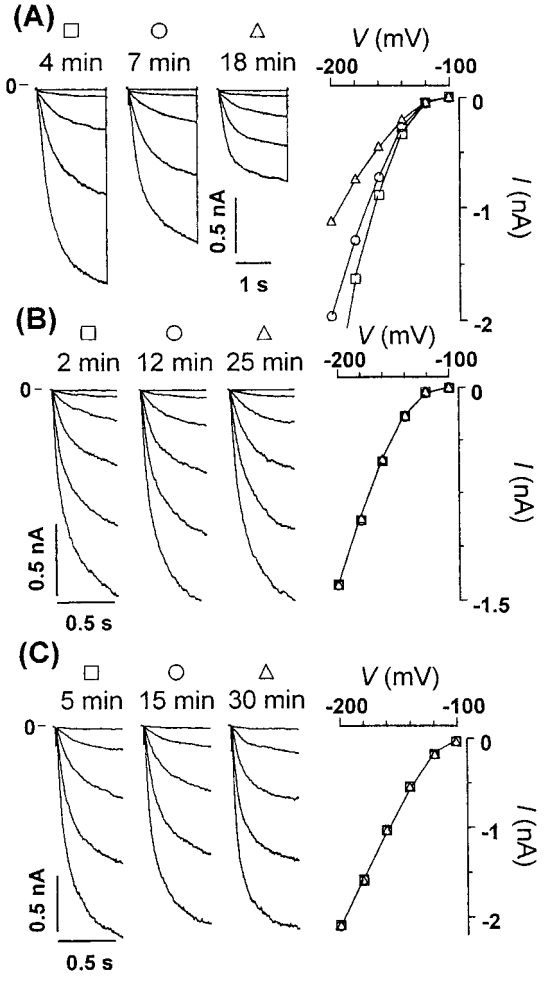

Fig. 3. Stereospecific inactivation of $I_{K,in}$ by $InsP_6$ conformers is relieved by buffering Ca_i^{2+} in *Solanum* GCPs. Current traces of $I_{K,in}$ and corresponding I/V curve obtained with pipette solutions containing 1 μM *myo*-$InsP_6$ (*A*), 1 μM *neo*-$InsP_6$ (*B*), and 1 μM *myo*-$InsP_6$ together with 20 mM BAPTA (*C*).

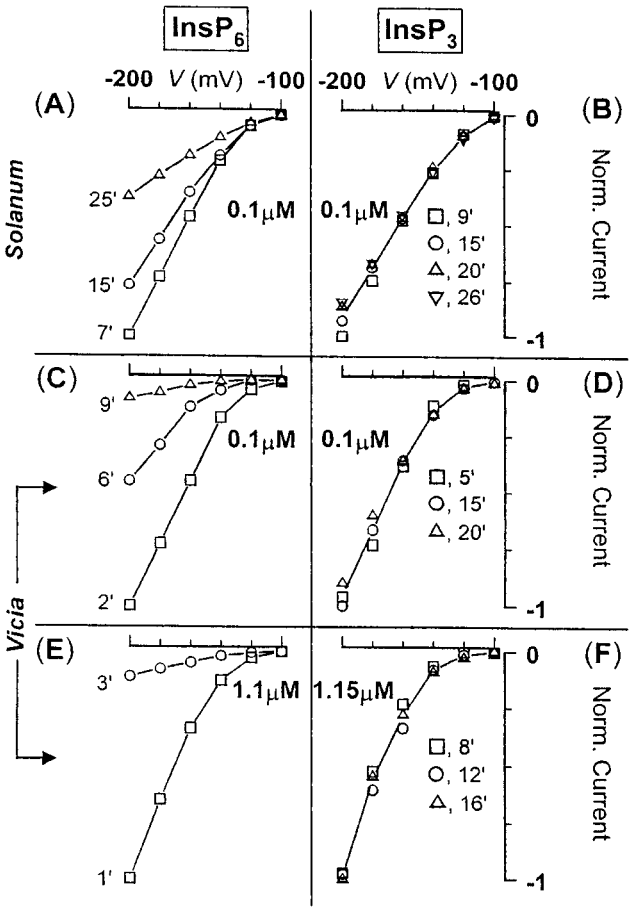

Fig. 4. At the submicromolar range, $InsP_6$ is more potent in the inhibition of $I_{K,in}$ than Ins (1,4,5)P_3 in both *Solanum* (*A* and *B*) and *Vicia* GCPs (*C–F*). In *A–F*, I/V scans were taken at different times, as indicated in the graph; the amount of inositol polyphosphate used is also indicated. *A*, *C*, and *E* are experiments carried out with $InsP_6$ in the patch pipette. *B*, *D*, and *F* are experiments carried out with Ins (1,4,5)P_3 in the patch pipette. Note the remarkable lack of effect of Ins (1,4,5)P_3 compared with $InsP_6$-induced large inhibition of $I_{K,in}$ at either concentration. This figure also highlights the speed at which GCPs from *Vicia* respond to $InsP_6$ [at −200 mV, 90% block in only 9 min (*C*)] when compared with GCPs from *Solanum* (at −200 mV, 60% block in 25 min; *A*).

a concentration within the range quoted for this inositol phosphate in animal cells (26). An example is shown in Fig. 2*C* in which currents were measured 1 and 20 min after achieving whole-protoplast mode. A substantial decrease in the current carried by $I_{K,in}$ at any tested voltage was observed with no change in the amplitude of the current carried by $I_{K,out}$. The sensitivity of $I_{K,in}$ to lower concentrations of $InsP_6$ was also determined in *Solanum* (see Figs. 3*A* and 4*A*, with 1 μM and 0.1 μM $InsP_6$ in the pipette, respectively). At lower concentrations, the inhibition developed more slowly, but up to 90% block was observed when gigaohm seals lasted for 30 to 60 min or more. Pipette concentrations as low as 0.1 μM were sufficient to trigger inactivation of $I_{K,in}$ by more than 70% at the steady state, with no effect on $I_{K,out}$ ($n = 3$).

$InsP_6$ Modulation of $I_{K,in}$ Is Faster in *Vicia* than in *Solanum*. We have further extended our observation of $InsP_6$-dependent inhibition of $I_{K,in}$ to guard cell protoplasts of *Vicia*, which is the only species in which the electrophysiological consequences of release of Ins (1,4,5)P_3 have been determined (13). Our results show that $InsP_6$-mediated inhibition of $I_{K,in}$ is evident in both species. Thus, $InsP_6$ gave a response in 11 of 14 *Solanum* protoplasts tested and in all of 6 *Vicia* protoplasts tested. *Solanum* and *Vicia* differed markedly in the speed of response to $InsP_6$ with the response developing more rapidly in *Vicia*. Measured at −180 mV, inhibition of $I_{K,in}$ by 1 μM $InsP_6$ reached 43% in 18 min for *Solanum* (Fig. 3*A*) compared with greater than 85% inhibition achieved in 3 min for *Vicia* (Fig. 4*E*). Also compare Fig. 4 *A* and *C*, where 0.1 μM $InsP_6$ was used (at −200 mV: 60% inhibition in 25 min for *Solanum* vs. 90% inhibition in just 9 min for *Vicia*).

$InsP_6$-Induced Inhibition of $I_{K,in}$ in *Solanum* GCPs Is Stereo Specific. Having defined $I_{K,in}$ as a specific downstream target of the elevation of $InsP_6$ observed in ABA-stimulated *Solanum* guard cells, we sought to define the stereo specificity of the interaction of $InsP_6$ with its effector(s) or target ion channels. For this purpose, we used two conformers of $InsP_6$, *scyllo*-$InsP_6$ and *neo*-$InsP_6$. These molecules differ from *myo*-$InsP_6$ in the orientation of phosphate ester substituents relative to the plane of the cyclohexane ring (1). In *scyllo*-$InsP_6$, all phosphates are equatorial. In *myo*-$InsP_6$, all phosphates with the exception of that in the 2-position are equatorial (2- is axial), whereas in *neo*-$InsP_6$, the 2- and 5-phosphates are both axial. Fig. 3 *A* and *B* show that when

1 μM *neo*-$InsP_6$ (Fig. 3*B*) was substituted for *myo*-$InsP_6$ (Fig. 3*A*), no effect could be seen 12 or even 25 min after achieving whole-protoplast mode. Likewise, by using 1 μM *scyllo*-$InsP_6$, no inhibition could be detected after even 23 min (data not shown). No effect on potassium currents was seen in seven of all eight protoplasts challenged with these two conformers. These results unequivocally discount one of the potential criticisms of the interpretation of $InsP_6$ action in cell signaling, namely that $InsP_6$ effects may be unspecific and merely a function of the high negative charge density and the potential to chelate internal Mg^{2+}. Our standard patch-pipette medium contains 2 mM $MgCl_2$.

$InsP_6$-Induced Inhibition of $I_{K,in}$ Is Relieved by Ca^{2+} Chelators. Our results show that $InsP_6$ is competent to regulate the Ca^{2+}-dependent inward K^+ channel in guard cells. Because of the precedent of inactivation of $I_{K,in}$ by increasing $[Ca^{2+}]_i$, we have investigated whether $InsP_6$-driven inhibition of the inward K^+ current is similarly a calcium-dependent process. This was tested by the inclusion of 20 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) with 1 μM *myo*-$InsP_6$ in the patch pipette. As shown in Fig. 3*C*, buffering of cytoplasmic Ca^{2+}, by inclusion of BAPTA in the pipette, completely relieved the inactivation of $I_{K,in}$ by $InsP_6$, and this effect was persistent up to 30 min. The effect was confirmed in three further experiments by using 20 mM BAPTA and in one by using 40 mM EGTA.

$InsP_6$ Is Much More Potent than Ins (1,4,5)P_3 in Modulating $I_{K,in}$. Because our results with $InsP_6$ are reminiscent of the inhibition of $I_{K,in}$ observed on photolytic release of 1–10 μM Ins (1,4,5)P_3 into intact guard cells (13), we have compared the effectiveness of $InsP_6$ and Ins (1,4,5)P_3 in modulating I_{Kin}. We now show that $InsP_6$ is considerably more potent in the inactivation of $I_{K,in}$ in GCPs than is Ins (1,4,5)P_3. Fig. 4 *A–D* compares the effects of $InsP_6$ and Ins (1,4,5)P_3, each at 100 nM, in both *Vicia* and *Solanum*, and it is clear that Ins (1,4,5)P_3 is without effect at this concentration. Ins (1,4,5)P_3 was also without effect at 1 μM in *Vicia* (Fig. 4*F*). Only when added at supraphysiological concentrations (10 to 22 μM; $n = 5$) did Ins (1,4,5)P_3 produce a strong inhibition (up to 87% block at 22 μM; data not shown). Thus $InsP_6$ is some 100 times more potent than Ins (1,4,5)P_3 for inactivation of $I_{K,in}$. This observation is important as it demonstrates that the potent effects of $InsP_6$ are not a consequence of its metabolism to Ins (1,4,5)P_3; note also the absence of consistent and detectable change of $InsP_3$ in Fig. 1*C*.

Discussion

The work described above establishes that ABA produces rapid changes in $InsP_6$ in guard cells and that $InsP_6$, delivered at submicromolar concentrations through the patch pipette, mimicked the inhibitory effects of ABA and Ca_i^{2+} on the inward K^+ channel, and that this inhibition was abolished by the inclusion of Ca^{2+} chelator in the pipette. The changes in $InsP_6$ induced by ABA are rapid, detectable within a minute of application, and comparable with the time scale for ABA-induced electrical changes in intact guard cells (6, 7). The response of the inward current to diffusion of $InsP_6$ from the patch pipette is similarly rapid, within a few minutes, especially in *Vicia* GCPs [at 1.1 μM $InsP_6$, maximum inhibition of $I_{K,in}$ was reached in 3 min (Fig. 4*E*)]. $InsP_6$ is also much more efficient than Ins (1,4,5)P_3 in the inhibition of $I_{K,in}$ at submicromolar concentrations. Thus, the results are consistent with a dominant role for $InsP_6$ in a Ca^{2+}-dependent signaling chain by which ABA inhibits the inward K^+ current in guard cells.

Various explanations of the calcium dependency of $InsP_6$ results are possible, and two seem prominent. The first is that $InsP_6$ may elicit Ca^{2+} increases either by entry of Ca^{2+} from the outside, as seen for example in the activation of L-type Ca^{2+} channels by $InsP_6$ in animal cells (5), or may release Ca^{2+} from internal stores, perhaps by analogy to Ins (1,4,5)P_3 induced-calcium release. An alternative possibility is that $InsP_6$ acts downstream of Ca^{2+} to modulate the interactions with Ca^{2+}-sensitive targets. Indeed, Luan and coworkers (27) have shown that Ca^{2+}-dependent inhibition of $I_{K,in}$ is abolished by immunosuppressants but induced by the inclusion of a constitutively active fragment of bovine brain calcineurin in the patch pipette, arguing perhaps that calcineurin-like mediated protein dephosphorylation may be a consequence of elevation of Ca^{2+} in guard cells. Future measurements of the effect of $InsP_6$ on cytoplasmic Ca^{2+} will allow the two possibilities to be distinguished.

The changes in $InsP_6$ metabolism detected here are among the most rapid of the biochemical responses to ABA detected in guard cells. The size and the speed of the changes of $InsP_6$ to a stimulus, in this case ABA, are more dramatic than responses reported in animals (26) and are similar to that reported in yeast (2). The response to ABA observed here is also fundamentally different from the developmentally programmed accumulation of $InsP_6$ in turions (specialized mesophyll cells) of the duckweed, *Spirodela polyrhiza*, which incidentally also occurs in response to ABA (17). The latter study reported a pathway proceeding from Ins $\Rightarrow$ Ins3P $\Rightarrow$ Ins (3,4)P_2 $\Rightarrow$ Ins (3,4,6)P_3 $\Rightarrow$ Ins (3,4,5,6)P_4 $\Rightarrow$ Ins (1,3,4,5,6)P_5 $\Rightarrow$ $InsP_6$. We have further confirmed the presence of Ins (3,4,5,6)P_4 1-kinase activity in mesophyll cells (28). It is not possible to speculate at present about the route of activated $InsP_6$ synthesis in guard cells. The lack of significant changes in the unidentified $InsP_3$ coeluting with [^{32}P]Ins (1,4,5)P_3 might argue that inositol phospholipid turnover does not contribute to the production of $InsP_6$ observed here. However, in contrast, Michell and coworkers (2) have characterized an enzyme activity that converts Ins (1,4,5)P_3 to $InsP_6$ in *S. pombe*, an experimental system in which $InsP_6$ increases in response to hyperosmotic shock. It will be important in future work to establish the pathway for synthesis of $InsP_6$ in guard cells.

The present results raise, but do not answer, the question of the relation between the $InsP_6$-mediated signaling pathway and other pathways previously postulated in guard cells, those involving Ins (1,4,5)P_3 and cyclic adenosine diphosphate-ribose cADPR. There is clear evidence that guard cells are competent to respond to Ins (1,4,5)P_3 by increase in cytoplasmic calcium and inhibition of $I_{K,in}$ (12, 13) and to cADPR by increase in cytoplasmic calcium (29), but the evidence that these are major contributors to the ABA response in intact guard cells is not compelling. There is no evidence for stimulus-dependent changes in cADPR in guard cells, and indeed the case for significant stimulus-dependent change in Ins (1,4,5)P_3 (30) in guard cells is, to our mind, still lacking (31). The relatively small effects of inhibitors of phospholipase C, or of synthesis of cADPR, on ABA-induced changes in aperture does not identify a predominant role for either pathway (29, 32, 33). Thus the relative contributions of the three potential signaling intermediates, Ins (1,4,5)P_3, cADPR, and $InsP_6$, in the physiological response to ABA are not yet established, and this remains an important goal for future work.

It is remarkable that $InsP_6$ and Ins (1,4,5)P_3 share common effects on a common target, $I_{K,in}$, and that inhibition of $I_{K,in}$ by both of these agents is manifest in a calcium-dependent manner. Although it is widely held that the calcium dependence of inactivation of $I_{K,in}$ by Ins (1,4,5)P_3 resides with an Ins (1,4,5)P_3-triggered release of Ca^{2+} from internal stores, the evidence now attributes ABA-dependent inactivation of $I_{K,in}$ rather more potently to $InsP_6$ than to Ins (1,4,5)P_3. An intriguing possibility raised by our experiments is that the reported effects of release of Ins (1,4,5)P_3 in guard cells is a consequence of conversion to $InsP_6$. Although we find no evidence to support this suggestion in the present study, the work of Michell and coworkers (2)

indicates that $InsP_6$ production observed in response to hyperosmotic shock in *S. pombe* may derive from Ins $(1,4,5)P_3$.

Returning to guard cells, the exact nature of the calcium dependence of $InsP_6$ action on $I_{K,in}$ is not defined. Activation of plasma membrane channels that are permeable to calcium, either selectively [witness activation of L-type calcium channels by $InsP_6$ (4)] or nonselectively, could represent a mechanism by which $InsP_6$ might regulate $I_{K,in}$. Indeed, Blatt and coworkers (34, 35) have characterized a hyperpolarization-activated plasma membrane calcium influx channel that is activated by ABA and that contributes significantly to ABA-dependent increase in cytoplasmic Ca^{2+} and inactivation of $I_{K,in}$.

Consequently, in light of the ABA-driven changes in $InsP_6$, the potency of inhibition of inward-K^+ current by $InsP_6$, and the calcium dependency of inhibition, irrespective of the exact mechanism by which $InsP_6$ effects are manifested, the evidence suggests that $InsP_6$ may play a major, if not dominant, role in ABA signaling to $I_{K,in}$. In a much more general sense, our work defines a physiological target of $InsP_6$ action in plants. That this target, the inward K^+ current, has a well-defined function in control of guard cells by ABA further serves to highlight an important and emerging osmoregulatory context (2) for the function of this, the most enigmatic of inositol phosphates.

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1. Cosgrove, D. J. *Inositol Phosphates, Their Chemistry, Biochemistry and Physiology* (Elsevier, Amsterdam, 1980).
2. Ongusaha, P. P., Hughes, P. J., Davey, J. & Michell, RH. (1998) *Biochem J.* **335,** 671–679.
3. York., J. D., Odom, A. R., Murphy, R., Ives, E. B. & Wente, S. R. (1999) *Science* **285,** 96–100.
4. Odom, R. A., Stahlberg, A., Wente, S. R. & York, J. D. (2000) *Science* **287,** 2026–2029.
5. Larsson, O., Barker, C. J, Sj-oholm, A., Carlqvist, H., Michell, R. H., Bertorello, A., Nilsson T., Honkanen, R. E., Mayr, G. W., Zwiller, J., *et al.* (1997) *Science* **278,** 471–474.
6. Blatt, M. R. (1990) *Planta* **180,** 445–455.
7. Thiel, G., MacRobbie, E. A. C. & Blatt, M. R. (1992) *J. Membr. Biol.* **126,** 1–18.
8. Lemtiri-Chlieh, F. & MacRobbie, E. A. C. (1994) *J. Membr. Biol.* **137,** 99–107.
9. Lemtiri-Chlieh, F. (1996) *J. Membr. Biol.* **153,** 105–116.
10. Blatt, M. R. & Armstrong, F. (1993) *Planta* **191,** 330–341.
11. Vajanaphanich, M., Schultz, C., Rudolf, M. T., Wasserman, M., Enyedi, P., Craxton, A., Shears, S. B., Tsien, R. Y., Barrett, K. E. & Traynor-Kaplan, A. (1994) *Nature (London)* **371,** 711–714.
12. Gilroy, S., Read, N. D. & Trewavas, A. J. (1990) *Nature (London)* **346,** 769–771.
13. Blatt, M. R., Thiel, G. & Trentham, D. R. (1990) *Nature (London)* **346,** 766–769.
14. Allen, G. J., Muir, S. R. & Sanders, D. (1995) *Science* **268,** 735–737.
15. Blatt, M. R. & Grabov, A. (1997) *Physiol. Plant.* **100,** 481–490.
16. Muller-Rober, B., Ellenberg, J., Provart, N., Willmitzer, L., Busch, H., Becker, D., Dietrich, P., Hoth, S. & Hedrich, R. (1995) *EMBO J.* **14,** 2409–2416.
17. Brearley, C. A. & Hanke, D. E. (1996) *Biochem. J.* **314,** 227–233.
18. Brearley, C. A. & Hanke, D. E. (1996) *Biochem. J.* **318,** 279–286.
19. Mayr, G. W. (1988) *Biochem. J.* **254,** 585–591.
20. Kriedemann, P. E., Loveys, B. R., Fuller, G. L. & Leopold, A. C. (1972) *Plant Physiol.* **49,** 842–847.
21. Schroeder, J. I. & Hagiwara, S. (1989) *Nature (London)* **338,** 427–430.
22. Grabov, A. & Blatt, M. R. (1997) *Planta* **201,** 84–95.
23. Grabov, A. & Blatt, M. R. (1999) *Plant Physiol.* **119,** 277–287.
24. Dietrich, P., Dreyer, I., Wiesner, P. & Hedrich, R. (1998) *Planta* **205,** 277–287.
25. Fairley-Grenot, K. A. & Assmann, S. M. (1992) *J. Membr. Biol.* **128,** 103–113.
26. Shears, S. B. (1998) *Biochim. Biophys. Acta* **1436,** 49–67.
27. Luan, S., Li, W., Rusnak, F., Assmann, S. M. & Schreiber, S. L. (1993) *Proc. Natl. Acad. Sci. USA* **91,** 2202–2206.
28. Brearley, C. A. & Hanke, D. E. (2000) *Plant Physiol.* **122,** 1209–1216.
29. Leckie, C. P., McAinsh, M. R., Allen, G. J., Sanders, D. & Hetherington, A. M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15837–15842.
30. Lee, Y., Choi, Y. B., Suh, S., Lee, J., Assmann, S. M., Joe, C. O., Kelleher, J. F. & Crain, R. C. (1996) *Plant Physiol.* **110,** 987–996.
31. Brearley, C. A., Parmar, P. N. & Hanke, D. E. (1997) *Biochem J.* **324,** 123–131.
32. Jacob, T., Ritchie, S., Assmann, S. M. & Gilroy, S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 12192–12197.
33. Staxen, I., Pical, C., Mongomery, L. T., Gray, J. E., Hetherington, A. M. & McAinsh, M. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 1779–1784.
34. Grabov, A. & Blatt, M. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4778–4783.
35. Hamilton, D. W. A., Hills, A., Kohler, B. & Blatt, M. R. *Proc. Natl. Acad. Sci. USA* **97,** 4967–4972.