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Role of Calcium in the Modulation of *Vicia* Guard Cell Potassium Channels by Abscisic Acid: A Patch-Clamp Study

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Abstract. There is evidence for a role of increased cytoplasmic Ca^{2+} in the stomatal closure induced by abscisic acid (ABA), but two points of controversy remain the subject of vigorous debate—the universality of Ca^{2+} as a component of the signaling chain, and the source of the increased Ca^{2+}, whether influx across the plasmalemma, or release from internal stores. We have addressed these questions by patch-clamp studies on guard cell protoplasts of *Vicia faba,* assessing the effects of ABA in the presence and absence of external Ca^{2+}, and of internal Ca^{2+} buffers to control levels of cytoplasmic Ca^{2+}. We show that ABA-induced reduction of the K^+ inward rectifier can occur in the absence of external Ca^{2+}, but is abolished when Ca^{2+} buffers are present inside the cell. Thus, some minimum level of cytoplasmic Ca^{2+} is a necessary component of the signaling chain by which ABA decreases the K^+ inward rectifier in stomatal guard cells, thus preventing stomatal opening. Release of Ca^{2+} from internal stores is capable of mediating the response, in the absence of any Ca^{2+} influx from the extracellular medium. The work also shows that enhancement of the K^+ outward rectifier by ABA is Ca^{2+} independent, and that other signaling mechanisms must be involved. A role for internal pH, as suggested by H.R. Irving, C.A. Gehring and R.W. Parish (*Proc. Natl. Acad. Sci. USA* **89:**1790–1794, 1990) and M.R. Blatt (*J. Gen. Physiol.* **99:**615–644, 1992), is an attractive working hypothesis.

Key words: Abscisic acid — Guard cell protoplast — *Vicia faba* — Whole-cell configuration — K^+ channels — Cytoplasmic Ca^{2+}

Introduction

There is evidence for a role of increased cytoplasmic Ca^{2+} in the stomatal closure induced by abscisic acid (ABA), the phytohormone produced in, or imported into, leaves under water deficit conditions, which induces net loss of potassium salt from the guard cell, with consequent reduction in turgor, cell shrinkage and closure of the stomatal pore. However, two points of controversy remain the subject of vigorous debate—the universality of Ca^{2+} as a component of the signaling chain (McAinsh, Brownlee & Hetherington, 1990, 1992; Gilroy et al., 1991), and the source of the increased Ca^{2+}, whether influx across the plasmalemma (Schroeder & Hagiwara, 1990*a*) or release from internal stores (Gilroy et al., 1991). The aim of the present work is to address these questions, by patch-clamp studies on guard cell protoplasts of *Vicia faba,* assessing the effects of ABA in the presence and absence of external Ca^{2+}, and in the presence of internal Ca^{2+} buffers to control levels of cytoplasmic Ca^{2+}.

Measurements using Ca^{2+}-sensitive dyes show that free cytoplasmic Ca^{2+} can increase in response to ABA (McAinsh et al., 1990, 1992; Gilroy et al., 1991; Schroeder & Hagiwara, 1990*a*), but there is controversy over whether stomatal closure can be induced by ABA without any increase in cytoplasmic Ca^{2+}. Gilroy et al. (1991) argue that the increase in response to ABA is not universal, and that other unknown mechanisms can be triggered by ABA to close stomata in the absence of any change in cytoplasmic Ca^{2+}. McAinsh et al. (1992), who see a Ca^{2+} increase more frequently, argue that Ca^{2+} is a central requirement in the signaling chain, but that methodological problems may interfere with the visualization of Ca^{2+} changes. It is therefore important to assess the electrical effects of ABA in conditions where very low concentration of cytoplasmic Ca^{2+} is maintained by the presence of cytoplasmic Ca^{2+} chelators.

The other point of current debate concerns the source of Ca^{2+} for the ABA-induced increase in the cy-

toplasm. The guard cell can respond to release of inositol 1,4,5-trisphosphate (IP_3) in the cytoplasm, by increase in cytoplasmic Ca^{2+} and closure of stomata (Gilroy, Read & Trewavas, 1990). Gilroy et al. (1991) argue that release of internal stored Ca^{2+} is involved in the ABA response, rather than influx at the plasma membrane. However, Schroeder and Hagiwara (1990*a*), combining measurements of membrane currents with photometric measurements of internal Ca^{2+}, by introducing Fura-2 in the patch pipette, found ABA-induced repetitive transient increases in cytoplasmic Ca^{2+}, each associated with an inward current. On the basis of the reversal potential of -11 mV, they argued that this current reflects Ca^{2+} influx through nonselective Ca^{2+}-permeable channels, and that Ca^{2+} influx at the plasmalemma, rather than Ca^{2+} release from internal stores, is primary in the ABA response. We have addressed this question by assessing the ABA-induced electrical effects in the absence of external Ca^{2+}.

The electrical and flux responses to ABA must be understood in terms of the established properties of the various electrogenic ion transport systems which have been identified by different electrophysiological techniques in the guard cell plasma membrane (in intact guard cells or their protoplasts). In addition to the electrogenic proton-translocating ATPase characteristic of all plant and fungal cells, a number of ion channels have been identified. These include: (1) an inward K^+ channel, activating slowly after a voltage step, opening at membrane potentials more negative than about -100 mV, which is responsible for K^+ influx to the cell when the pump generates a sufficiently negative membrane potential at suitable external K^+ and pH. (2) An outward K^+ channel, opening at membrane potentials positive of the potassium equilibrium potential (E_K), and responsible for K^+ efflux from the cell when other changes drive the membrane potential sufficiently positive for this channel to activate. Again, this channel activates only slowly after a voltage step. (3) An ill-defined "leak" conductance, which includes more than one charge-carrying current. The contribution of this current can be distinguished from those of the two K^+ channels by its "instantaneous" response to a voltage step. (4) Two types of anion channel have been identified in patch-clamp studies. A rapidly activating type (R-type) is active over the voltage range -100 to -50 mV, but as it inactivates with a half-time of 10–12 sec, it cannot be responsible for the sustained anion efflux required for stomatal closing. It is Ca^{2+} activated in the presence of nucleotides (Keller, Hedrich & Raschke, 1989; Hedrich, Busch & Raschke, 1990; Schroeder & Hagiwara, 1990*b*). More recently, a slowly activating anion channel has been identified (Linder & Raschke, 1992; Schroeder & Keller, 1992), which does not inactivate; this is active in the voltage range -200 to 60 mV, and in the presence of 5 μM cytoplasmic Ca^{2+}. If activated, this channel would produce cell depolarization, and could give the prolonged anion efflux required. Finally, three different stretch-activated ion channels are also present in the plasma membrane of the guard cell protoplasts, specific for Cl^-, K^+ and Ca^{2+}, respectively (Cosgrove & Hedrich, 1991).

The electrical changes in the guard cell plasma membrane, which follow treatment with ABA, have been established by whole-cell current-voltage analysis of intact guard cells of *Vicia faba* (Blatt, 1990; Thiel, MacRobbie & Blatt, 1992). Three electrical changes are identified: (1) activation of a component of the background conductance, carrying inward current at physiological potentials. The nature of this channel is still to be identified, but either an anion channel or a channel capable of carrying Ca^{2+} influx (whether selective or not) are likely candidates. (2) Reduction of the K^+ inward rectifier, by shift of its activation voltage to more negative potentials. (3) A slower enhancement of the K^+ outward rectifier, seen as an increase in the capacity of this channel to carry current, without any change in its kinetics or voltage for activation.

Two of the three ABA-induced changes, the reduction of the K^+ inward rectifier and the activation of the "leak" current, are also produced by increase of cytoplasmic Ca^{2+} (Schroeder & Hagiwara, 1989), or by release of IP_3 in the cytoplasm (Blatt, Thiel & Trentham, 1990). However, these electrical studies also show that the third change, the enhancement of the K^+ outward rectifier, is not induced by increase in cytoplasmic Ca^{2+}, whether produced by high Ca^{2+} in the patch pipette or by internal release of IP_3 in intact cells. The present work examines the changes in K^+ channels in response to ABA when any change in cytoplasmic Ca^{2+} is prevented by the presence of Ca^{2+} chelator in the cytoplasm, and when Ca^{2+} influx is prevented by the absence of external Ca^{2+}.

Materials and Methods

Protoplast Isolation

Vicia faba L. cv (Bunyan) Bunyan Exhibition was grown on vermiculite under conditions described previously (Blatt, 1987). Guard cell protoplasts were isolated from abaxial epidermal strips of 3- to 4-week-old leaves following a procedure similar to the one described by Schroeder and Hagiwara (1990*a*). Briefly, epidermal strips were floated on 10 ml medium containing 1.8–2.5% of Cellulase Onozuka RS (Yacult Honsha, Japan), 1.7–2% Cellulysin (Calbiochem, Behring Diagnostics, La Jolla, CA), 0.026% Pectolyase Y-23, 0.26% BSA, 1 mM $CaCl_2$ (pH 5.56 and osmolality 360 mOsm · Kg^{-1} adjusted with mannitol). Epidermal strips were incubated in a temperature-controlled chamber at 28°C. The incubation medium was gently stirred with a Variomag shaker (Model R100, Luckham, Sussex, UK) at a frequency of 0.5 Hz. After 120 to 150 min, released protoplasts were purified by passage through a 30 μm mesh, kept on ice for 2 to 3 min before being centrifuged at 100× *g* for 4 min (at room temperature). The pellet consisting of guard cell protoplasts was resuspended and kept on ice in 1 or 2 ml of fresh medium containing

0.42 M mannitol, 10 mM 2-[N-Morpholino]ethanesulfonic acid (MES), 200 μM $CaCl_2$, 2.5 mM KOH (pH 5.55 and osmolality 366 mOsm · Kg^{-1}).

Solutions

Protoplasts were placed in a 0.5 ml chamber. When the protoplasts reached the bottom of the glass, a bath solution containing 10 mM K^+-glutamate, 0.5–1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM MES, pH 5.5 to 6.1 (adjusted with KOH) and osmolality 480–500 mOsm · Kg^{-1} (adjusted with mannitol) was perfused continuously at a rate of 3–4 chamber volumes per minute. In experiments without calcium in the bath, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) was added, at 0.4 or 2 mM. Patch pipettes were filled with 100 mM K^+-glutamate (unless otherwise specified), 2 mM MgATP (or K_2ATP), 5 mM KOH, 2 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), pH adjusted as required between 7.18 and 7.92; osmolality was set to 520 mOsm · Kg^{-1} (adjusted with mannitol). In experiments with no calcium in the pipette, BAPTA was added to a final concentration of 10–20 mM. Ethylene glycol bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) was sometimes used, at final concentrations of 1.8, 5.4 and 44 mM. Abscisic acid was included as required. All chemicals were from Sigma Chemical (Poole, Dorset, UK).

Current-Voltage Recording and Analysis

Patch pipettes (5 MΩ) were pulled from Kimax-51 glass capillaries (Kimble 34500; Kimble Div., Owens-Illinois) using a two-stage puller (Narishige PP-83, Japan). All the experiments were performed at room temperature (20 to 22°C), using the standard whole-cell patch-clamp technique (Hamill et al., 1981). To monitor cell currents, a patch-clamp amplifier L/M EPC7 (List-Medical, Germany) was used. Voltage commands and simultaneous signal recordings and analysis were assessed by a microcomputer (Tandon 386/16) connected to the amplifier via a multipurpose I/O device (LM-125) using Bioclamp software (Biologic, Claix, France). The plasma membrane surrounding the prepared guard cell protoplasts (GCP's) was able to bind to the glass microelectrodes, forming high-resistance seals (usually 1 to 5 GΩ) in more than 50% of the attempts. Resistances greater than 5 GΩ were obtained but less frequently. The whole-cell configuration was achieved by breaking the underlying membrane using gentle suction, after which the membrane was immediately clamped to voltages close to E_K. The GCP's were then left for 3–5 min to allow equilibration between the pipette solution and the cytoplasmic content before starting any current measurements. All the current-voltage relationships (except in Fig. 1) were plotted as the time-dependent current (i.e., the steady-state current minus the instantaneous current) *vs.* test potential.

Results

K^+ Channels of *Vicia* Guard Cells: Effect of Ca^{2+}

Figure 1 (*A* and *C*) shows a series of typical whole-cell current recordings from a GCP bathed in 10 mM K^+ and 0.5 mM Ca^{2+} (pH_o 5.56), with 200 mM K^+ (pH_i 7.71) in the pipette. Time- and voltage-dependent inward currents (IK_i) were elicited by stepping the membrane potential, from a holding potential of −80 mV, to voltages more negative than −100 mV. Depolarizing voltage steps to voltages more positive than −20 mV produced time- and voltage-dependent outward currents (IK_o). The nonohmic current-voltage relationships (lined area, *see* Fig. 1*B* and *D*) are obtained by subtracting from each steady-state current (○) the corresponding instantaneous current (●) (*see* Fig. 1*A* and *C*). The *I-V* relations highlight the presence of two conductances rectifying in opposite directions. These conductances have been shown by different groups to have essentially the characteristics of K^+-conducting channels (Schroeder, Hedrich & Fernandez, 1984; Schroeder, Raschke & Neher, 1987; Blatt, 1988; Hosoi, Iino & Shimazaki, 1988; Schroeder, 1988; Blatt et al., 1990; Fairley-Grenot & Assmann, 1992*a,b*).

Addition of Ba^{2+} in the mM range to the bathing medium results in a dose-dependent block of both conductances (*data not shown*). With 100 mM K^+ and pH_i 7.8 in the pipette, addition of 1.5 mM Ba^{2+} to the bath reduced both IK_i (by 80% at −200 mV), and IK_o (by 62% at 100 mV), while addition of 3.6 mM Ba^{2+} totally suppressed both time-dependent K^+ currents.

External Ca^{2+} seems to have a similar effect since its removal from the bathing medium increases both conductances (Fig. 2). This effect was seen either with no added Ca^{2+} (nominal zero Ca^{2+}) or with BAPTA (0.4 or 2 mM), a specific calcium chelating agent, added to the bath. Figure 2 shows the current-voltage relationships for the K^+ currents from the same GCP in the presence (○) and absence (●) of external calcium (the current traces for the extremes of the voltage range in the voltage protocol are shown in the inset). In the example shown in Fig. 2, with 0.4 mM BAPTA in the bath, the increase of K^+ current was more pronounced for outward than for inward K^+ current; thus IK_i increased by 62% at −160 mV while IK_o was about 13-fold greater at 80 mV. The percentage increase in K^+ currents produced by removal of calcium from the external medium was extremely variable; for the eight cells examined, the mean increase in IK_i was 236 ± 113% (mean ± SEM).

Effect of ABA on K^+ Channels

Effect of ABA in the Presence of Ca^{2+} on Both Sides of the Plasma Membrane

Current-voltage profiles were constructed for the time-dependent currents, attributable to K^+ currents, from current recordings obtained by stepping the membrane voltage from a holding voltage near the calculated E_K to values between −200 and +100 mV (20 mV increments) every 5 sec. The size of the K^+ currents was variable in different experiments, depending on internal pH and K^+ as well as the tightness of the seal (*see* Table 1). It is therefore important that conclusions be drawn only from recordings made in two different conditions on the same GCP, each GCP thus acting as its

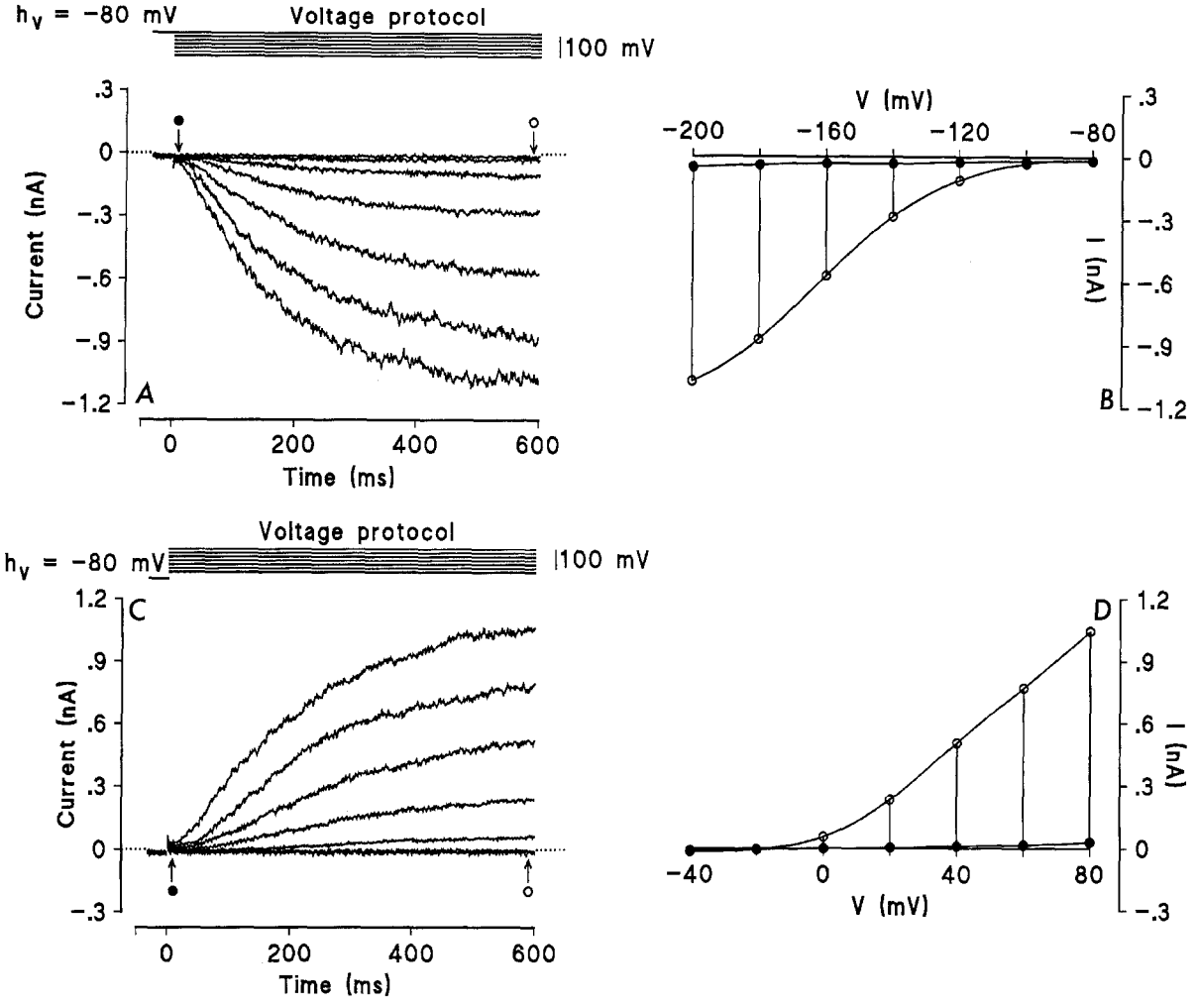

Fig. 1. (*A*) and (*C*) Typical recordings of inward and outward K^+ current in a whole GCP. The external medium contained 10 mM K^+, pH_o 5.56, while the pipette medium contained 200 mM K^+, pH_i 7.71. These currents were obtained by applying 600 msec clamp square pulses with different amplitudes, every 5 sec, from a holding potential of −80 mV to voltages in the range of −200 to 100 mV (20 mV increments). (*B*) and (*D*) Current-voltage relationships for the current traces. Measurement of the current in the first 10 msec of the clamp pulse gave the instantaneous currents (●). Time-dependent currents (lined area) were obtained by subtracting these instantaneous currents from the steady-state currents measured at the end of the clamp pulse (○).

own control. Figure 3 shows an example of the response of a patch-clamped GCP to ABA, showing the current-voltage relationships for K^+ currents before and after treatment with ABA (the effect of ABA was seen in 23 out of the 33 GCP's challenged with ABA in a concentration range between 10 and 35 μM). The effects are similar to that seen in intact guard cells (Blatt, 1990; Thiel et al., 1992), namely a reduction of the K^+ inward rectifier, by shift of the activation voltage to more negative values, and an enhancement of K^+ outward rectifier, by increase in its capacity to carry current at any given membrane voltage, without effect on the voltage for activation. In this example, ABA decreased IK_i measured at −160 mV by about 86% and increased IK_o at 80 mV by 509% (the collected results for ABA-induced changes in IK_i at −160 mV and IK_o at 80 mV are summarized in Table 2). Figure 3 (inset) illustrates time- and voltage-dependent inward and outward K^+ currents obtained by stepping the potential from a holding potential of −60 mV to two voltages, −180 and 80 mV, before (−ABA) and 8 min after (+ABA) adding 10 μM ABA to the bath medium.

Effect of ABA in the Absence of Ca^{2+} Inside the Patch Pipette

It is possible to address the question of whether cytoplasmic Ca^{2+} is necessary in the ABA response by assessing the electrical effects of ABA in the presence of Ca^{2+}-chelating agents, such as BAPTA or EGTA, in the patch pipette, thereby maintaining cytoplasmic Ca^{2+} at very low levels. It is much more difficult to achieve

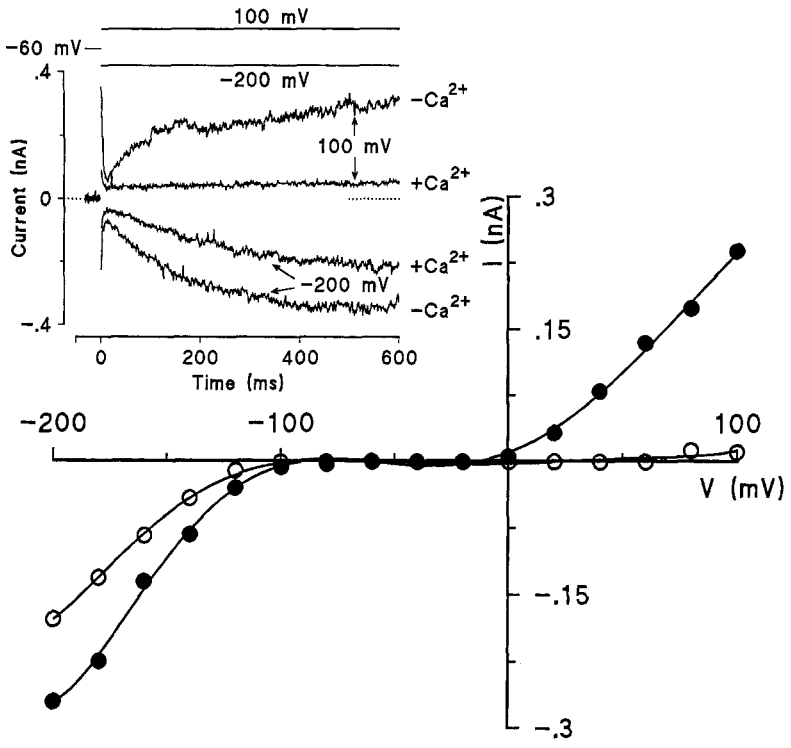

Fig. 2. Effect of external Ca^{2+} removal on K^+ currents. *I/V* relationships for the time-dependent IK_i and IK_o in the presence (○) and absence (●) of Ca^{2+} ($CaCl_2$ was omitted and 0.4 mM K_4 BAPTA was added to the bath medium). Current traces corresponding to the extremes of the voltage range of the *I/V* curves in both conditions ($+Ca^{2+}$ and $-Ca^{2+}$) are shown in the inset (*see* upper panel for voltage protocol). The pipette solution contained 100 mM K^+ with a pH of 7.8.

Table 1. Effect of pH_i and $[K^+]_i$ on IK_i (−160 mV) and IK_o (+80 mV)

Experimental condition	IK_i (pA)	IK_o (pA)	*n*
pH_i			
7.2	−267 ± 28	0.00	15
7.8	−171 ± 31	68 ± 23	18
$[K^+]_i$			
150	−437 ± 52	256 ± 32	12
200	−232 ± 66	498 ± 74	9

Values of IK_i and IK_o are given as mean ± SEM.

seals in the presence of Ca^{2+} chelator, but Fig. 4 shows one of the six successful experiments. This shows the full current-voltage profile for the standard pulse protocol, together with current traces (inset) for the extremes of the voltage range (−200 and +100 mV), before and after application of ABA (20 μM). With no Ca^{2+} and 20 mM K_4BAPTA in the pipette, the ABA-induced reduction of the K^+ inward rectifier is abolished, whereas the enhancement of the K^+ outward rectifier occurs as normal, with a 352% increase in K^+ current at 80 mV. Qualitatively similar results were obtained by using EGTA instead of BAPTA, but high concentrations of EGTA (>40 mM) were required to prevent any decrease of IK_i (*see* Fig. 5). With lower concentration of EGTA (5.4 mM) in the pipette, some reduction in IK_i was observed on adding ABA, but by not more than 17% at −180 mV in two cells, much less than the reduction observed in the absence of added internal Ca^{2+} buffer (*see* Table 2). Thus, it appears that at least physiological levels of Ca^{2+} are required for the ABA-induced reduction of the K^+ inward rectifier, that if Ca^{2+} is buffered to very low levels, the response is prevented. Thus, Ca^{2+} plays an obligatory role in the signaling chain, although it remains to be determined whether this is a direct effect on the channel or indirect through other signaling systems.

The present results show that although some minimum Ca^{2+} level is necessary for the reduction of the K^+ inward rectifier, the K^+ outward rectifier can activate normally in response to ABA, even when cytoplasmic Ca^{2+} is buffered to very low levels. Thus, Ca^{2+}-independent signaling mechanisms must be sought for the enhancement of the K^+ outward rectifier.

Effect of ABA in the Absence of Extracellular Ca^{2+}

To determine whether Ca^{2+} influx from outside the cell is necessary for the ABA response, as is claimed by Schroeder and Hagiwara (1990*a*), we have investigated the effects of ABA on K^+ currents in the absence of external Ca^{2+}. Figure 6 shows current-voltage profiles for the K^+ currents, before and after the addition of ABA, in nominally Ca^{2+}-free solutions. Both effects of

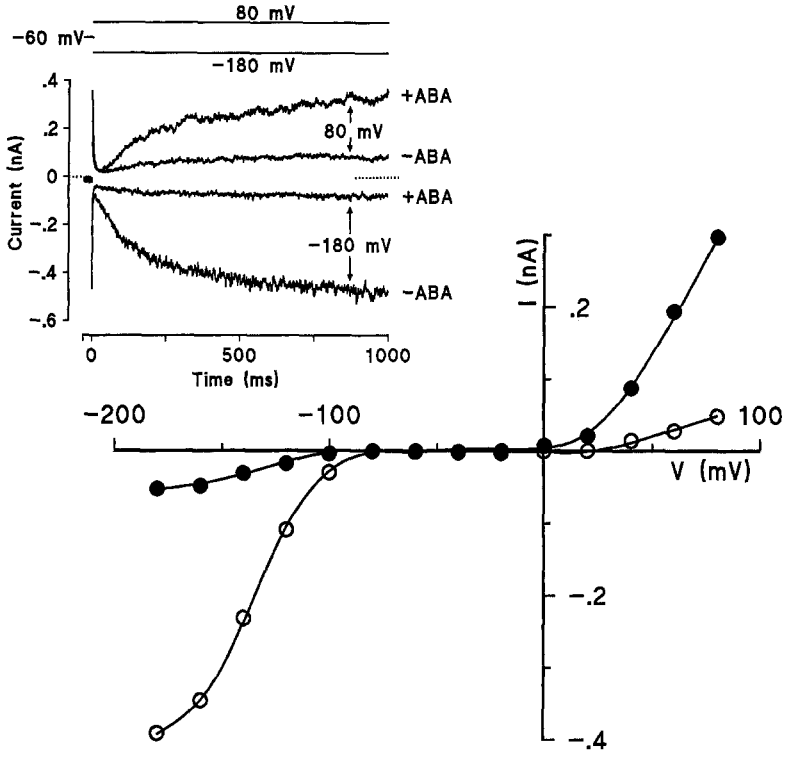

Fig. 3. Effect of ABA on K^+ currents in the presence of Ca^{2+} on both sides of the plasma membrane. *I/V* relationships of the time-dependent IK_i and IK_o before (○) and 8 min after (●) addition of 10 μM ABA to the bath medium. Inset showing current traces of IK_i and IK_o recorded respectively at −180 and 80 mV (*see* upper panel for voltage protocol) in the absence (−ABA) and presence (+ABA) of ABA. The pipette solution contained 100 mM K^+ with a pH of 7.8.

Table 2. Effect of ABA on IK_i (−160 mV) and IK_o (+80 mV)

Experimental condition		IK_i (pA)		Ratio	IK_o (pA)		
		Range	Mean ± SEM	ABA/pre-ABA	Range	Mean ± SEM	
No internal chelator:							
pH_i 7.2	Pre-ABA	294–720	430 ± 75		0		(5)
	ABA	150–440	250 ± 61	0.57 ± 0.08	0		
pH_i 7.4–7.8	Pre-ABA	75–450	250 ± 51		0–180*	37 ± 48	
	ABA	0–220	87 ± 28	0.31 ± 0.08	0–297	146 ± 27	(8)
Internal chelator:							
pH_i 7.6–7.9	Pre-ABA	60–346	194 ± 31	1.09 ± 0.13	70–477	203 ± 44	(10)
	ABA	50–436	225 ± 45		142–1,204	474 ± 121	(10)

* Pre-ABA: 3 cells 0 pA; 5 cells in the range 7–180 pA. ABA: 1 cell 0 pA; 7 cells in the range 50–297 pA.

ABA are still seen, the enhancement of the K^+ outward rectifier, and the reduction of the K^+ inward rectifier, with a shift in their activation voltages to more negative values; ABA reduces the inward K^+ current at −200 mV by about 80%, while increasing the outward K^+ current at +100 mV about 14-fold.

The reduction in the Ca^{2+}-sensitive inward current by ABA was also seen in the complete absence of external Ca^{2+}, in the presence of 2 mM BAPTA outside; an example (one of six such cells) is seen in the current-voltage profiles, before and after ABA, shown in Fig. 7. In this example, ABA decreases IK_i at −160 mV by 58%, a value close to the average decrease of IK_i (*see* Table 2).

Discussion

Taken together, our results show that cytoplasmic Ca^{2+} is necessary for the full K^+ flux changes in response to ABA, but that the presence of external Ca^{2+} is not. Thus, Ca^{2+} is a necessary component of the signaling chain by which ABA decreases the K^+ inward rectifi-

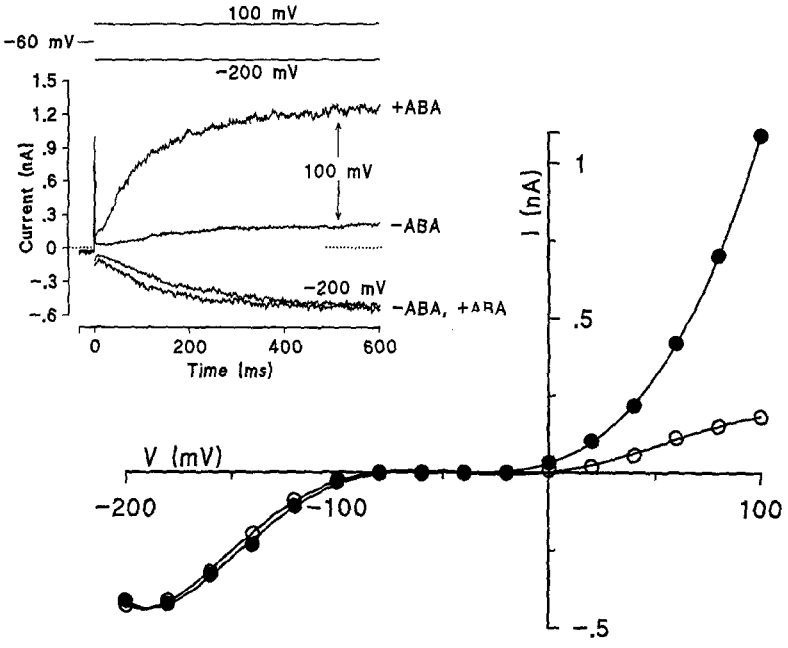

Fig. 4. Effect of ABA on K^+ currents in the absence of Ca^{2+} inside the patch pipette ($CaCl_2$ was omitted and 20 mM K_4BAPTA was added to the patch pipette medium; 150 mM K_i in total and pH_i 7.8). *I/V* relationships of the time-dependent IK_i and IK_o before (○) and 10 min after (●) addition of 20 μM ABA to the bath medium. Inset showing current traces of IK_i and IK_o recorded respectively at −200 and 100 mV (*see* upper panel for voltage protocol) in the absence (−ABA) and presence (+ABA) of ABA.

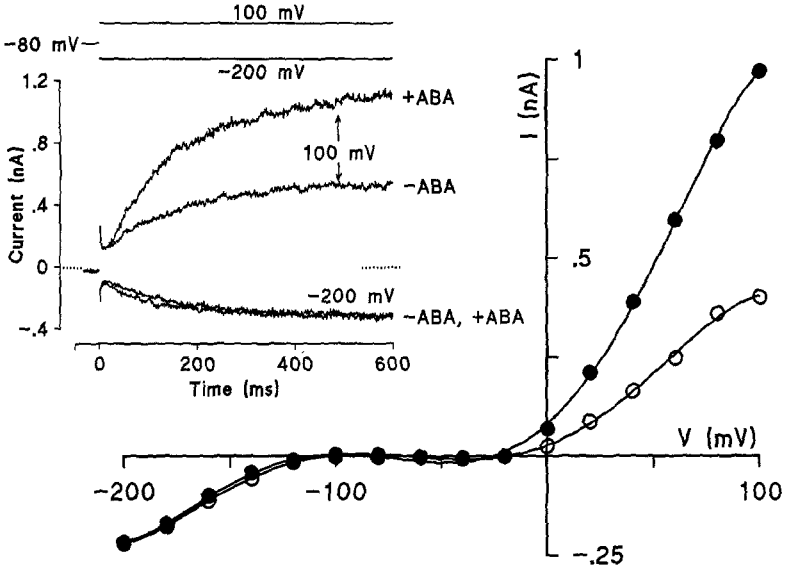

Fig. 5. Effect of ABA on K^+ currents in the absence of Ca^{2+} inside the patch pipette ($CaCl_2$ was omitted and 44 mM K_2EGTA was added to the patch pipette medium; 270 mM K_i in total and pH_i 7.91). *I/V* relationships of the time-dependent IK_i and IK_o before (○) and 11 min after (●) addition of 10 μM ABA to the bath medium. Inset showing current traces of IK_i and IK_o recorded respectively at −200 and 100 mV (*see* upper panel for voltage protocol) in the absence (−ABA) and presence (+ABA) of ABA.

er in stomatal guard cells, preventing stomatal opening, but release of Ca^{2+} from internal stores is capable of mediating this response, in the absence of any Ca^{2+} influx from the medium. This does not rule out an ABA-induced stimulation of Ca^{2+} influx when external Ca^{2+} is present, but such influx is not required for the ABA response, and the results imply that internal release of Ca^{2+} is an integral part of the ABA response. The evidence that guard cells are capable of responding to IP_3, by both Ca^{2+}-mediated electrical changes and by stomatal closure, has already been cited. Parmar and Brearley (1993) have identified the components of the phosphoinositide signaling pathway in guard cells (both inositol phospholipids and inositol phosphates). Parmar (1991, and *unpublished data*) has shown that changes in the labeling patterns of these compounds occur within 20 sec of exposure to ABA; this response is of a type consistent with ABA-induced turnover in this signaling pathway. Such evidence suggests that an increase in cytoplasmic Ca^{2+}, at least locally, is likely, even when measurements of Ca^{2+}-sensitive fluorescence do not indicate a global increase throughout the cell. It may be that in some cells intracellular Ca^{2+} buffering prevents the translation of a local change near the plasmalemma into a global increase throughout the cell, capable of being seen in the imaging experiments. This

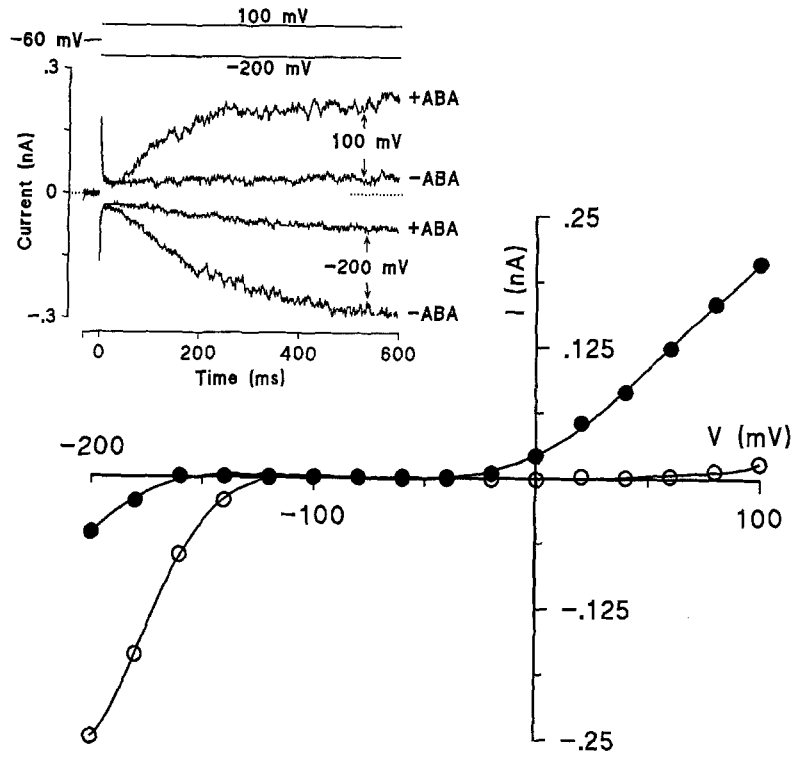

Fig. 6. Effect of ABA on K^+ currents in the absence of extracellular Ca^{2+} (nominal Ca^{2+}-free bathing medium). *I/V* relationships of the time-dependent IK_i and IK_o before (○) and 22 min after (●) addition of 17.5 μM ABA to the bath medium. Inset showing current traces of IK_i and IK_o recorded respectively at −200 and 100 mV (*see* upper panel for voltage protocol) in the absence (−ABA) and presence (+ABA) of ABA. The pipette solution contained 100 mM K^+ with a pH of 7.8.

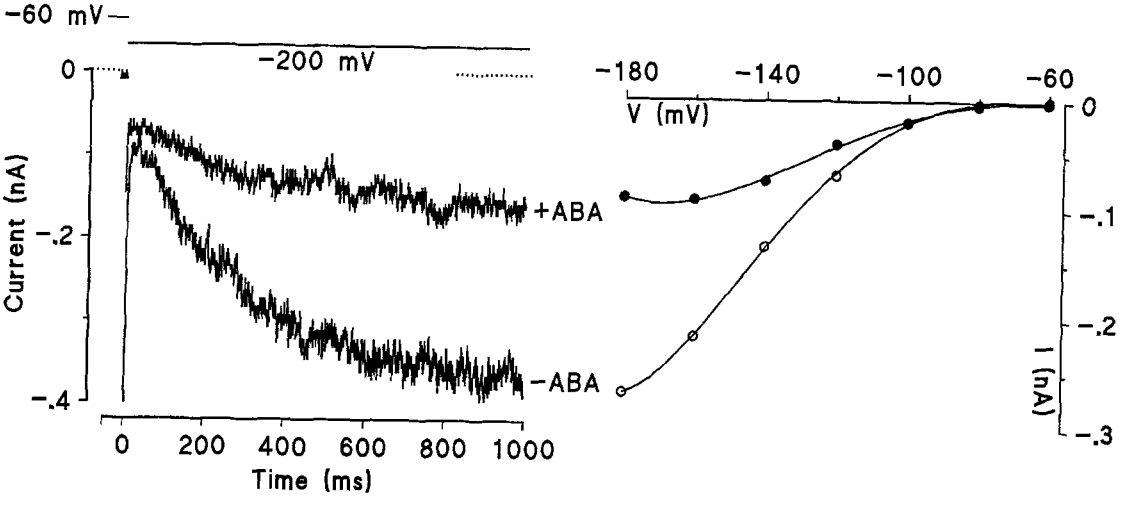

Fig. 7. Effect of ABA on the inward K^+ current in the total absence of extracellular free Ca^{2+} ($CaCl_2$ was omitted and 2 mM K_4BAPTA was added to the bath medium). *I/V* relationships of the time-dependent IK_i before (○) and 11 min after (●) addition of 10 μM ABA to the bath medium. Inset showing current traces of IK_i recorded at −180 mV (*see* upper panel for voltage protocol) in the absence (−ABA) and presence (+ABA) of ABA. The pipette solution contained 100 mM K^+ with a pH of 7.2.

possibility is highlighted by the demonstration by Alexandre and Lassalles (1992) that the level of Ca^{2+} buffering can influence the ability of IP_3-triggered Ca^{2+} flux in patch-clamped vacuoles to modulate Ca^{2+}-sensitive channels in the same membrane.

The results also show that the effect of ABA on the outward K^+ channel is not mediated by a Ca^{2+}-dependent signal pathway, reinforcing previous demonstrations which indicated that the gating of the K^+ outward rectifier is insensitive to cytoplasmic Ca^{2+} concentration (Hosoi et al., 1988; Schroeder & Hagiwara, 1989; Blatt et al., 1990). Thus, other signaling system(s) must be responsible for the ABA-induced enhancement of this current. Blatt (1992) has shown inhibition of the K^+ outward rectifier by acidification of the cytoplasm after addition of butyric acid to the bathing medium, and has suggested a role for cytoplasmic pH in the regulation of this channel. This sensitivity of the K^+ outward

rectifier to pH_i is confirmed in the present study (*see* Table 1), and it should be noted that at pH_i 7.2, there is no outward K^+ current even after treatment with ABA (*see* Table 2). Irving et al. (1990) have shown ABA-induced alkalinization of the guard cell cytoplasm, by a mechanism which remains unknown, but which may be important in mediating Ca^{2+}-independent flux changes.

A recent report by Fairley-Grenot and Assmann (1992*b*) has shown an increase of about 60% in the amplitude of both inward and outward K^+ rectifiers of *Vicia* GCP's by reduction of cytoplasmic Ca^{2+} from 180 to 2 nM; the increase is even greater in GCP's from *Zea mays* (around 80%). This contrasts with the insensitivity of the K^+ outward rectifier to Ca^{2+} in the range 0.1–1.5 μM found by Schroeder and Hagiwara (1989). The rise in Ca^{2+} concentrations reported in cells responding to ABA, or to the injection of IP_3, are in the higher range, from basal levels around 100–200 nM to values up to 1 μM or more (McAinsh et al., 1990; Schroeder & Hagiwara, 1990*a;* Gilroy et al., 1991).

In summary, our results show that ABA must initiate at least two signaling cascades in the guard cell, and Ca^{2+} is an obligatory component of only one of these; in the Ca^{2+}-dependent chain, release of Ca^{2+} from internal stores can support the response, and Ca^{2+} influx at the plasmalemma is not required. The nature of the second, Ca^{2+}-independent chain, responsible for enhancement of the K^+ outward rectifier, remains unknown, but a role for pH changes in this chain is an attractive hypothesis.

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