Increases in cytosolic Ca$^{2+}$ are not required for abscisic acid-inhibition of inward K$^{+}$ currents in guard cells of Vicia faba L.

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Abstract. The inward K$^{+}$ channels (IKin) of guard cells are inhibited upon application of abscisic acid (ABA). It has been postulated that IKin inhibition requires an elevation in cytosolic free Ca$^{2+}$ levels ([Ca$^{2+}$]$_{c}$) because: (i) experimental increases in [Ca$^{2+}$]$_{c}$ can mimic the ABA effect, and; (ii) ABA can trigger an elevation of [Ca$^{2+}$]$_{c}$ in guard cells. However, not all guard cells respond to ABA with a [Ca$^{2+}$]$_{c}$ increase, and the magnitude of the increases that do occur is variable. Therefore, an obligate role for Ca$^{2+}$ in the regulation of downstream effectors of ABA response, such as the IKin channels, remains in question. In this study, we developed a methodology for simultaneous patch clamping and confocal ratiometric Ca$^{2+}$ imaging of Vicia faba L. guard-cell protoplasts. This allowed us to directly assess the relationship between ABA-induced changes in [Ca$^{2+}$]$_{c}$ and IKin inhibition. In the presence of extracellular Ca$^{2+}$, the extent of [Ca$^{2+}$]$_{c}$ elevation correlated with the extent of IKin inhibition. However, upon chelation of either extracellular Ca$^{2+}$, [Ca$^{2+}$]$_{c}$, or both, extracellular Ca$^{2+}$ and [Ca$^{2+}$]$_{c}$ elevation did not occur in response to ABA yet IKin currents were still strongly inhibited. These data illustrate that Ca$^{2+}$-independent regulation is involved in ABA-inhibition of stomatal opening processes.

Key words: Abscisic acid – Cytosolic calcium – Guard cell – K$^{+}$ channels – Vicia (K$^{+}$ channels)

Introduction

Abscisic acid (ABA) regulates stomatal apertures via effects on osmotically-driven changes in guard cell turgor. At the plasma membrane of guard cells, ABA inhibits the H$^{+}$-ATPase (Goh et al. 1996) and activates non-selective Ca$^{2+}$-permeable channels (Schroeder and Hagiwara 1990) and “slow” (S-type) anion channels (Grabov et al. 1997; Pei et al. 1997; Li et al. 2000). Inhibition of H$^{+}$ extrusion, promotion of anion loss, and Ca$^{2+}$ uptake all will serve to depolarize the membrane potential of the guard cell, shifting from a situation in which the transmembrane electrochemical gradient favors K$^{+}$ uptake (as occurs during stomatal opening) to a situation in which the gradient favors K$^{+}$ efflux (as occurs during stomatal closure). This shift in membrane potential also affects voltage-regulated K$^{+}$ channels, favoring deactivation of the channels that mediate K$^{+}$ entry (IKin channels), and activation of the channels that mediate K$^{+}$ loss (IKout channels). In addition to channel regulation via effects on membrane potential, at a fixed membrane potential ABA also inhibits IKin channel activity (Blatt 1990; Lemitiri-Chlieh and MacRobbie 1994) and increases the availability of IKout channels (Blatt and Armstrong 1993), actions that will also retard K$^{+}$ uptake and accelerate K$^{+}$ loss.

There are remarkable parallels in the effects of ABA and Ca$^{2+}$ on guard-cell transport processes. Like ABA, experimental elevation of Ca$^{2+}$ levels inhibits the H$^{+}$-ATPase (Kinoshita et al. 1995), inhibits the IKin channels (Schroeder and Hagiwara 1989; Fairley-Grenot and Assmann 1992), and activates anion channels (Schroeder and Hagiwara 1989; Schroeder and Keller 1992). One exception is the regulation of IKout currents, which appear to be largely Ca$^{2+}$-insensitive (Schroeder and Hagiwara 1989; Lemitiri-Chlieh and MacRobbie 1994; Miedema and Assmann 1996) but are nonetheless activated by ABA (Blatt 1990; Blatt and Armstrong 1993).

Measurements of the cytosolic calcium concentration ([Ca$^{2+}$]$_{c}$) in living guard cells using fluorescent [Ca$^{2+}$]-indicators indeed have shown that an increase in [Ca$^{2+}$]$_{c}$ can be one of the earliest events seen in response to ABA, preceding (e.g. McAinsh et al. 1990), and potentially triggering (Gilroy et al. 1990), ABA-induced
changes in stomatal aperture. In addition to the non-selective Ca\(^{2+}\)-permeable, voltage-gated channels (Schröder and Hagiwara 1990), the guard-cell plasma membrane contains stretch-activated Ca\(^{2+}\)-permeable channels (Cosgrove and Hedrich 1991), and an inward rectifying K\(^+\) channel with some Ca\(^{2+}\) permeability (Fairley-Grenot and Assmann 1992). Abscisic acid-induced increases in \([\text{Ca}^{2+}]_\text{c}\) may also arise by release from intracellular stores possibly mediated by channels modulated by inositol 1,4,5-trisphosphate (Blatt et al. 1990; Gilroy et al. 1990; Lee et al. 1996; Stuxen et al. 1999), cyclic ADP-ribose (McAinsh et al. 1996), voltage (Allen and Sanders 1994), or Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Ward and Schroeder 1994).

Abscisic acid-induced changes in \([\text{Ca}^{2+}]_\text{c}\) represent attractive candidates for signal transduction elements because they could integrate the many processes involved in stomatal aperture regulation (McAinsh et al. 1997). On the other hand, it has been repeatedly observed that ABA-induced changes in \([\text{Ca}^{2+}]_\text{c}\) are variable, ranging from no detectable change to sustained or oscillatory increases of up to 1 \(\mu\text{M}\) (McAinsh et al. 1990, 1992; Schroeder and Hagiwara 1990; Gilroy et al. 1991; Irving et al. 1992; Allan et al. 1994) leading to the idea of an interplay of signaling activities such as phospholipase C,D and cyclic-ADP ribose (Jacob et al. 1999), leading to Ca\(^{2+}\)-dependent and -independent pathways. Downstream effectors of ABA action also show variability. For example, Lemtiri-Chilieh and MacRobbie (1994) reported that the range of \(\text{I}_{\text{Kin}}\) current values before ABA treatment was 294–720 pA, and the range of current values post-ABA was 150–440 pA. Thus, there were some cells for which the pre-ABA current values were smaller than the post-ABA currents of other cells.

Given this variability, along with the known Ca\(^{2+}\)-independence of some ABA events, the question remains as to whether the ABA-induced Ca\(^{2+}\)-rise is truly a causative event in ion transport regulation in general and in \(\text{I}_{\text{Kin}}\) regulation in particular. One way to address this issue would be to measure, in the same cell, both \([\text{Ca}^{2+}]_\text{c}\) and the relevant ionic response(s). We combined confocal ratiometric Ca\(^{2+}\) imaging and patch-clamp measurements of \(\text{I}_{\text{Kin}}\) to address the following questions: (i) In cells where an inhibition of \(\text{I}_{\text{Kin}}\) by ABA is observed, is this effect always correlated with an increase in \([\text{Ca}^{2+}]_\text{c}\)? (ii) Conversely, in cells where no such inhibition is observed, does \([\text{Ca}^{2+}]_\text{c}\) always remain at resting levels? (iii) In cells where an increase in \([\text{Ca}^{2+}]_\text{c}\) is experimentally prevented, can ABA still inhibit \(\text{I}_{\text{Kin}}\)? We focused on \(\text{I}_{\text{Kin}}\) because, while the relationship of ABA, \([\text{Ca}^{2+}]_\text{c}\), and stomatal closure has been extensively studied, less attention has been given to the relationship between ABA, \([\text{Ca}^{2+}]_\text{c}\), and stomatal opening processes.

_**Materials and methods**_

*Plant material and protoplast isolation.* Fava bean (*Vicia faba* L. cv. Long Pod) plants were grown as described by Miedema and Assmann (1996) except that temperatures were 23 °C and 25 °C in the dark and light respectively. Guard-cell protoplasts were isolated as described (Miedema and Assmann 1996).

**Electrophysiology.** The pipette solution contained (mM): 80 K- Glutamate, 20 KCl, 2 MgCl\(_2\), 2 ATP, 10 Hepes (pH 7.8 with 1 N KOH) and sorbitol to a final osmolarity of 500 mosmol kg\(^{-1}\). A stock of 0.5 M Mg-ATP (Sigma, St. Louis, Mo., USA) was prepared daily in 1 M Tris. For ratiometric imaging, pipette solutions contained 60 \(\mu\text{M}\) Indo-1 pentapotassium salt (Molecular Probes, Eugene, Ore., USA). Since in the whole-cell configuration of patch clamping there is diffusional equilibration between the pipette solution, and the cell cytoplasm, the concentration of indicator in the cell will approximate that provided in the pipette solution. Unless otherwise noted, the bath solution contained (Lmmol): 100 KCl, 5 Hepes, 5 MgCl\(_2\), 5 CaCl\(_2\), 5 Ca-ATP, 0.3 M sorbitol (Sigma), and 0.3 M to a final osmolarity of 460 mosmol kg\(^{-1}\). Abscisic acid (+/−cis/trans; Sigma) was added to the bath solution at a final concentration of 15 \(\mu\text{M}\) from a stock in 30 mM TAPS.

Patch pipettes resistances (Kimax 51; VWR Scientific, Boston, Mass., USA) were 6−12 MΩ in the above solutions. Recordings were obtained and analyzed using Axon Instruments hardware and software (Axon Instruments, Foster City, Calif., USA). Data acquisition began 8−10 min after establishment of the whole-cell configuration. Whole-cell data were sampled at 1 kHz and low-pass-filtered (−3 dB) at 0.5 kHz. If a change in series resistance of greater than 10 MΩ was observed during an experiment, the cell was discarded from further study. Membrane potentials were corrected for liquid junction potentials (Ward and Schroeder 1994).

Because current inhibition by ABA was rapid, it was not possible in timecourse experiments to obtain data from a complete current/voltage family and still preserve adequate temporal resolution. Instead, whole-cell currents were elicited by single 1.8-s pulses to −167 mV at 10- or 15-s intervals. Where temporal resolution was not an issue, we observed that ABA effects on \(\text{I}_{\text{Kin}}\) occurred in parallel at all relevant voltages, as previously reported (Lemtiri-Chilieh and MacRobbie 1994). Time-activated whole-cell currents (\(\text{I}_{\text{Kin}}\)) were corrected for a time-independent ("leak") conductance by subtracting the instantaneous current (defined as the current 8 ms into the voltage pulse), from the average steady-state current prevailing between 1,500 and 1,700 ms of the voltage pulse. Whole-cell currents were normalized for membrane surface area as indicated by the whole-cell capacitance and accordingly expressed in pA/pF. Initial K\(^+\) currents across all treatments were variable, ranging from −22 pA/pF to −266 pA/pF at −167 mV, and from 17 pA/pF to 142 pA/pF at +73 mV. The magnitudes of initial \([\text{Ca}^{2+}]_\text{c}\) (0.2−1 \(\mu\text{M}\), see below) and initial \(\text{I}_{\text{Kin}}\) were not correlated (\(r^2 = 0.0637, n = 10\); not shown).

**Confocal ratiometric Ca\(^{2+}\) imaging.** To perform simultaneous patch-clamp measurements and confocal Ca\(^{2+}\) imaging, we attached the patch-clamp setup to a LSM410 laser scanning confocal microscope (Axiovert 100 inverted microscope; Zeiss, Thornwood, N.Y., USA). Images were obtained using a Zeiss 40×, 0.75 NA dry objective. Indo-1 was excited with a scan (1 s unless otherwise noted) of a 364-nm UV laser (Enterprise; Coherent, Auburn, Calif., USA) at 5-s intervals as described in Gilroy (1996). The 400- to 435- and 450- to 505-nm bandpass filters used for Indo-1 emission excluded chlorophyll fluorescence from the measurements. Under these conditions, autofluorescence and dark current represented less than 5% of the Indo-1 fluorescence signal at each detector.

Cells subjected to simultaneous patch-clamp and Ca\(^{2+}\)-imaging protocols showed qualitatively and quantitatively similar K\(^+\) currents as cells subjected to patch-clamp protocols alone (not shown). Visual inspection of protoplasts under study gave no indication of Indo-1 cytotoxicity. Comparison of brightfield and fluorescence images of patch-clamped protoplasts demonstrated that Indo-1 was restricted to the cytoplasm, consistent with microinjection experiments (Gilroy et al. 1991; McAinsh et al. 1992). In addition, when cells were loaded with Indo-1 dye conjugated to a 70,000-M\(_r\) dextran, the dextran-conjugated dye