Ca$^{2+}$-dependent and -independent ABA activation of plasma membrane anion channels in guard cells of *Nicotiana tabacum*.

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Abstract
Drought induces stomatal closure, a response that is associated with the activation of plasma membrane anion channels in guard cells, by the phytohormone ABA. In several species, this response is associated with changes in the cytoplasmic free Ca\(^{2+}\) concentration. In *Vicia faba*, however, guard cell anion channels activate in a Ca\(^{2+}\)-independent manner (Levchenko et al., 2005, PNAS 102; 4203-4208). Because of potential differences between species, *Nicotiana tabacum* guard cells were studied in intact plants, with simultaneous recordings of the plasma membrane conductance and the cytoplasmic free Ca\(^{2+}\) concentration. ABA triggered transient rises in cytoplasmic Ca\(^{2+}\) in the majority of the guard cells (14 out of 19). In 7 out of 14 guard cells the change in cytoplasmic free Ca\(^{2+}\) closely matched the activation of anion channels, while the Ca\(^{2+}\) rise was delayed in 7 other cells. In the remaining 5 cells, ABA stimulated anion channels without a change in the cytoplasmic Ca\(^{2+}\) level. Even though ABA could activate anion channels in *N. tabacum* guard cells independent of a rise in the cytoplasmic Ca\(^{2+}\) concentration, patch clamp experiments showed that anion channels in these cells are stimulated by elevated Ca\(^{2+}\), in an ATP-dependent manner. Guard cells thus seem to have evolved both Ca\(^{2+}\)-independent and -dependent ABA signalling pathways. Guard cells of *N. tabacum* apparently utilize both pathways, while ABA signalling in *V. faba* seems to be restricted to the Ca\(^{2+}\)-independent pathway.
Introduction

The aperture of stomata is determined by two guard cells that surround the stomatal pore. Guard cells are devoid of plasmodesmata (Wille and Lucas 1984) and thus function virtually autonomously. During drought, stomata close to reduce the loss of water via transpiration, a response that depends on the phytohormone ABA (Assmann and Shimazaki 1999; Roelfsema and Hedrich 2005; Schroeder et al. 2001). ABA triggers the efflux of K⁺ salts from the guard cell, thereby reducing the osmotic potential of the cell and causing stomatal closure. The efflux of K⁺ salts is driven through depolarization of the plasma membrane, which allows K⁺ extrusion through outward rectifying K⁺ channels. Two mechanisms could mediate the ABA-induced depolarization of guard cells, activation of anion channels and/or inhibition of H⁺-ATPases in the plasma membrane.

In guard cells of *Vicia faba* studied in intact plants, ABA triggers a transient activation of R- and S-type anion channels (Roelfsema et al. 2001). A peak in anion channel activity is found after ~5 min., during prolonged stimulation the activity of anion channels levels off, but a pre-stimulus value is only observed after removal of ABA. This led to the suggestion that the first phase of this ABA-response induces fast stomatal closure, while the second phase inhibits reopening of the stomata (Roelfsema and Hedrich 2005). The extend to which ABA may inhibit plasma membrane H⁺-ATPases is not known in detail, but ABA was shown to inhibit blue light induced activation of H⁺-ATPases in guard cells (Zhang et al. 2004).

Various lines of evidence predicted an important role of the cytoplasmic Ca²⁺ concentration in ABA-activation of anion channels in guard cells (Hetherington and Brownlee 2004). Both R- and S-type anion channels in *V. faba* guard cells are stimulated by cytoplasmic Ca²⁺ (Hedrich et al. 1990; Schroeder and Hagiwara 1989) and ABA can induce cytoplasmic Ca²⁺ transients in guard cells in isolated epidermal strips or epidermal fragments of *Commelina communis* (Gilroy et al. 1991; McAinsh et al. 1990) as well as in *Arabidopsis thaliana* (Allen et al. 2001; Allen et al. 1999). In contrast to these data, Levchenko et al. (2005) found that ABA activates anion channels without provoking cytoplasmic Ca²⁺ changes, in guard cells of *V. faba*. The differences in result may be due different experimental conditions, since Levchenko et al. (2005) measured guard cells in intact plants, while previous reports were mainly conducted with isolated epidermal peels. Alternatively, the differences may relate to differences between plant species. In some species, ABA-induced stomatal closure could involve alterations in the cytoplasmic Ca²⁺ concentration of guard cells, whereas in others, stomata close in a Ca²⁺-independent fashion.
In search for species specific variation in the anion channel response to ABA, guard cell responses of *Nicotiana tabacum* were compared with those previously obtained for *V. faba* (Levchenko et al. 2005). In contrast to *V. faba*, this plant species can be transformed easily and thus has the advantage that it can be used for molecular biological approaches. The size of *N. tabacum* stomata is similar to that of *V. faba* and *C. communis*, which is a prerequisite for long term intracellular recordings. For these reasons, *N. tabacum* and its close relative *N. benthamiana* already were the species of choice in several studies (Armstrong et al. 1995; Hunt et al. 2003; Leyman et al. 1999; von Caemmerer et al. 2004). We found that ABA activates anion channels in guard cells of intact plants, in a similar manner as previously observed for *V. faba*. However, in contrast to previous results with *V. faba*, the majority of *N. tabacum* guard cells showed an ABA induced rise in the cytoplasmic Ca\(^{2+}\) concentration. Patch clamp experiments with *N. tabacum* guard cell protoplasts, revealed that cytoplasmic Ca\(^{2+}\) as well as ABA enhance anion channel activity. These data suggest species specific differences concerning the role of cytoplasmic Ca\(^{2+}\) changes in guard cell responses to ABA.

**Results**

**Guard cell recordings in intact *N. tabacum* plants**

Guard cells of *N. tabacum* were studied in intact plants using double- and triple barrelled micro electrodes. The adaxial side of a mature leaf was attached to a plexiglass holder, enabling impalement of guard cells at the abaxial epidermis (Fig. 1A and B). Two barrels of the micro electrodes were used to register and manipulate the membrane potential, while the Ca\(^{2+}\) reporter dye FURA2 could be injected with triple barrelled electrodes. After impalement, most electrodes were positioned in the cytoplasm, as judged from the distribution of FURA2 throughout the guard cell (Fig. 1C). In 7 out of 49 cells, however, the dye did not spread trough the cell, but remained in a confined compartment (Fig. 1D). These electrodes were probably placed in the vacuole based on the size and localization of the fluorescent compartment. In these cells, the voltage dependence of K\(^{+}\)-channels was less steep compared to cells in which the electrode was located in the cytoplasm (data not shown). Most likely, the vacuolar membrane acts as a resistor connected in series with that of the plasma membrane. In some cells, the FURA2 initially loaded into the cytoplasm slowly appeared in the vacuole, indicating that *N. tabacum* guard cells are able to transport FURA2 from the cytoplasm to the vacuole (Fig. 1E and F). The FURA2-ratio measurements therefore were only carried out from cytoplasm rich areas.
In the absence of ABA, the conductance of the guard cell plasma membrane in \textit{V. faba} and \textit{A. thaliana} is dominated by two types of K$^+$-selective channels, which were also recognized in guard cells of \textit{N. benthamiana} (Armstrong et al. 1995). In \textit{N. tabacum} guard cells in intact plants, outward rectifying channels activated at potentials positive of -60 mV, while inward channels were observed at potentials negative of -120 mV (Fig. 2B). At a holding potential of -100 mV, both time dependent channels are not active and therefore this potential was chosen to resolve ABA activation of anion channels (Roelfsema et al. 2004). ABA application triggered an inward current, after a lag time of 90 s (SE=12, n=27), that peaked after 268 s (SE=26, n=27) and partially deactivated again during prolonged stimulation with ABA. Before and during the application of ABA, the cells were clamped stepwise to potentials ranging from -180 mV to 0 mV with 20 mV increments (Fig. 2B). ABA stimulated the instantaneous conductance, but had little effect on outward rectifying channels. The effect of ABA on inward rectifying channels was masked by the large activity of instantaneous currents (Fig. 2C and D). The reversal potential of the instantaneous current in the presence of ABA shifted from values negative of -100 mV to -20 mV (Fig. 2D). The current voltage relation was linear, indicating that ABA activated S-type anion channels in these cells. The activation of R-type anion channels by ABA was not observed, even though this channel type was found in \textit{N. tabacum} guard cells with patch clamp (data not shown). These data reveal that ABA stimulates S-type anion channels in guard cells of \textit{N. tabacum} in a very similar manner as previously observed for \textit{V. faba} (Levchenko et al. 2005; Roelfsema et al. 2004).

**ABA-induced changes in cytoplasmic Ca$^{2+}$**

\textit{N. tabacum} guard cells, loaded with the Ca$^{2+}$ reporter dye FURA2, were exposed to 50 µM ABA applied at the cuticle as described above. Guard cells were clamped to -100 mV throughout the experiment, to prevent voltage dependent changes in the cytoplasmic Ca$^{2+}$ concentration (Grabov and Blatt 1998; Levchenko et al. 2005). Under these conditions, ABA induced a transient activation of anion channels just as in \textit{V. faba}. Note, that in contrast to the latter species, this response was accompanied by an increase in the cytoplasmic Ca$^{2+}$ concentration in the majority of cells (14 out of 19). In half of the cells displaying a rise in cytoplasmic Ca$^{2+}$, the increase of the Ca$^{2+}$ concentration mirrored changes in the plasma membrane current (Fig. 3A). False colour images of the 345/390 nm ratio of FURA2 fluorescence, revealed that the free Ca$^{2+}$ concentration rises throughout the cytoplasm, with the most obvious change in the proximity of the nucleus.
Despite the close correspondence between the cytoplasmic Ca\(^{2+}\) concentration changes and changes in current observed for 7 out of 19 cells, this correlation was absent in all other guard cells. In 7 guard cells of \textit{N. tabacum}, the ABA induced rise in the cytoplasmic free Ca\(^{2+}\) concentration was delayed and did not match the activation state of inward current (Fig. 4A). In another subset of 5 cells, ABA did not trigger a change in the cytoplasmic Ca\(^{2+}\), even though ABA stimulated anion channels (Fig. 4B). The absence of an ABA-induced rise in the cytoplasmic Ca\(^{2+}\) concentration could not be linked to differences in Ca\(^{2+}\)-buffering by FURA2. A comparison of the absolute fluorescence of FURA2, revealed a ratio of 0.99 for cells with or without Ca\(^{2+}\) changes, respectively. The magnitude of the current changes in cells that displaying a rise in cytoplasmic Ca\(^{2+}\) was not significantly different (t-test, P>0.05) from those that did not show changes in cytoplasmic Ca\(^{2+}\).

Guard cells of \textit{V. faba} and \textit{A. thaliana} operate Ca\(^{2+}\) permeable plasma membrane channels that activate upon hyperpolarization (Grabov and Blatt 1998; Pei et al. 2000). ABA may activate these channels through protein phosphorylation (Köhler and Blatt 2002) or through an ABA induced rise in reactive oxygen species (Pei et al. 2000). Based on the latter results, the ABA induced rise in cytoplasmic Ca\(^{2+}\) should be voltage dependent and thus more pronounced at negative voltages. Hyperpolarization of the plasma membrane of \textit{N. tabacum} to -250 mV induced a large rise in the cytoplasmic Ca\(^{2+}\) concentration (data not show) indicating the guard cells of this species are equipped with the same Ca\(^{2+}\) uptake machinery as \textit{V. faba} and \textit{A. thaliana}. Guard cells that were left at the free running membrane potential (average E\(_m\)= -55 mV, SE=5, n=6) still displayed ABA induced changes in the cytoplasmic Ca\(^{2+}\) concentration (Fig. 5A). In line with the properties of hyperpolarization activated calcium uptake, the ABA-induced rise in cytoplasmic Ca\(^{2+}\) was smaller at the free running potential, compared to -100 mV (Fig. 5B).

**Patch clamp experiments**

Anion channels in guard cells were ABA-activated through a Ca\(^{2+}\)-independent pathway (Fig. 4), even though these channels were previously found to be stimulated by Ca\(^{2+}\) in \textit{V. faba} (Hedrich et al. 1990; Schroeder and Hagiwara 1989) and \textit{A. thaliana} (Allen et al. 1999). We therefore tested if anion channel activity in \textit{N. tabacum} guard cells is also affected by cytoplasmic Ca\(^{2+}\). These studies were conducted with the patch clamp technique, which allows precise control of the cytoplasmic composition.
Protoplasts of *N. tabacum* guard cells were patched at conditions that block K⁺ channels and allow recordings of anion channels. The nature of the channels recorded under these conditions was tested with voltage clamp protocols from a holding potential of -158 mV to a preconditioning voltage of +62 mV and test potentials ranging from +82 to -218 mV. Lowering the extracellular Cl⁻ concentration from 80 to 8 mM shifted the reversal potential of the plasma membrane (Fig: 6A) from 7.5 mV (SE=0.2, n=4) to 41.3 mV (SE=3.5, n=4). These data show that the channels recorded in these protoplasts have a high permeability for Cl⁻ and a virtually linear current voltage relation, properties that are in line with those of S-type anion channels in other species (Linder and Raschke 1992; Schroeder and Keller 1992).

The effect of cytoplasmic Ca²⁺ on S-type channels was tested in experiments with patch pipettes containing 10 mM ATP and either 0, 110 or 380 nM free Ca²⁺. With pipette concentrations of 0 or 110 nM the activity of anion channels remained virtually constant (Fig. 6B). An increase of the Ca²⁺ concentration to 380 nM activated anion channels during the first 30 s, but during prolonged recordings the activity levelled off to the same value as with 110 or 0 nM Ca²⁺ (Fig. 6B). Apparently, Ca²⁺ stimulates anion channel activity during the first 30 s after establishing the whole cell configuration, but this effect is lost during prolonged recordings. This suggests, that Ca²⁺ diffusing from the patch pipette into the cell initially stimulates anion channels. However, during prolonged recordings cytoplasmic components provoking this response may diffuse from the cytoplasm into the pipette, causing the effect of Ca²⁺ to diminish in time.

Just as with R- and S-type anion channels in *V. faba* and *A. thaliana* (Allen et al. 1999; Hedrich et al. 1990; Schwarz and Schroeder 1998) the activity of anion channels in *N. tabacum* was modulated by nucleotides. At a reduced ATP concentration of 1 mM, anion channels were activated with 0 nM Ca²⁺ in the pipette, during the first 30 s, but their activity decayed thereafter (Fig. 6C). Increasing the cytoplasmic Ca²⁺ concentration to 110 nM inhibited the decay of anion current in time. A further rise to 380 nM enhanced the peak activity of S-type anion channels after 30 s, but afterwards the channels reached the same steady state as with 110 nM Ca²⁺. These data show that depending on the ATP concentration and the free Ca²⁺ level, Ca²⁺ ions activate anion channels either through stimulation of the peak activity (t ~ 30 s) or through inhibition of slow inactivation (half time ~ 6 min.).

ABA probably activates anion channels through an intracellular receptor, since injection of ABA in *V. faba* guard cells of intact plants, activates anion channels right away while external application delayed this response by 1-2 min (Levchenko et al. 2005). In line with these results,
guard cell anion channels in *V. faba*, were activated by ABA applied through the patch clamp pipette (Levchenko et al. 2005). In guard cell protoplasts of *N. tabacum*, ABA did not enhance the peak anion channel activity, but delayed the inactivation of anion channels in time (Fig. 7A). The delayed inactivation of anion channels was not accompanied with changes in voltage dependent gating (Fig. 7B) nor did ABA alter the reversal potential of whole cell current voltage relation (Fig. 7C). The effect of ABA in patch clamp experiments thus was limited to inhibition of S-type anion channel inactivation in *N. tabacum*, while it also enhanced the peak activity these channels in *V. faba*. These data and the ABA induced changes in the cytoplasmic free Ca$^{2+}$ concentration point to species specific differences in ABA signalling of guard cells.

**Discussion**

The role of Ca$^{2+}$ in ABA signalling of guard cells has been discussed, ever since DeSilva et al. (1985) showed synergism between ABA and extracellular applied Ca$^{2+}$. This theory gained support by a number of publications, showing that ABA can trigger a single rises or sustained oscillations in the cytoplasmic free Ca$^{2+}$ concentration of guard cell in several species (Allen et al. 1999; Gilroy et al. 1991; McAinsh et al. 1990; Webb et al. 2001). However, ABA did not trigger a rise of the Ca$^{2+}$ concentration in every guard cell recorded (Allen et al. 1999; Gilroy et al. 1991; McAinsh et al. 1990). Guard cells that did not display a rise in Ca$^{2+}$ still lost turgor (Gilroy et al. 1991) and thus apparently were able to activate anion channels in a Ca$^{2+}$-independent manner. Such a Ca$^{2+}$-independent ABA signalling pathway is supported by recent studies, showing that ABA inhibits inward rectifying K$^{+}$ channels (Romano et al. 2000) and stimulates anion channels (Levchenko et al. 2005), independent of changes in the cytoplasmic Ca$^{2+}$ concentration.

**Ca$^{2+}$-independent ABA-signalling**

Anion channels in the plasma membrane of *N. tabacum* guard cells can be activated by ABA through a pathway that does not involve a rise in the cytoplasmic Ca$^{2+}$-concentration. This pathway is evident from cells that do not display an ABA-dependent increase in cytoplasmic Ca$^{2+}$, but still activate anion channels (Fig. 4B). ABA did induce a rise in the cytoplasmic Ca$^{2+}$ concentration of other cells (Fig. 3), but the change of Ca$^{2+}$ level did not necessarily match that of anion channel activation (Fig. 4A). Even though these data suggest that ABA activation of anion channels occurs through a Ca$^{2+}$-independent mechanism, it could be inhibited in *V. faba* by high
concentrations of BAPTA (Levchenko et al. 2005). Apparently, ABA-activation of anion channels in *V. faba* required a basic level of cytoplasmic Ca\(^{2+}\). ABA may thus stimulate anion channel activity by enhancing the affinity for Ca\(^{2+}\), of components in the pathway that lead to anion channel activation (Levchenko et al. 2005).

OST1 of *A. thaliana* (Mustilli et al. 2002) and its *V. faba* homolog AAPK (Li et al. 2000) resemble Ca\(^{2+}\)-independent protein kinases. A close homolog of OST1, SRK2C, was transiently stimulated by drought stress with a very similar time course as the stimulation of guard cell anion channels by ABA (Yoshida et al. 2002). The OST1 kinase interacts with the ABI1 protein kinase, which is also not Ca\(^{2+}\) sensitive and acts as a negative regulator of ABA-signalling (Bertauche et al. 1996; Merlot et al. 2001). These proteins may thus provide ABA-sensitive, but Ca\(^{2+}\)-independent, key elements for activation of anion channels in guard cells.

**Ca\(^{2+}\)**-dependent ABA-signalling

Even though the ABA-induced activation of plasma membrane anion channels occurs through a Ca\(^{2+}\)-independent pathway, the anion channels activity in *N. tabacum* guard cell protoplasts was stimulated by Ca\(^{2+}\) (Fig. 6B and C). This is in line with early reports, showing that the activity of R- and S-type anion channels in guard cell protoplasts depends on Ca\(^{2+}\) and nucleotides (Hedrich et al. 1990; Schmidt et al. 1995; Schroeder and Hagiwara 1989). Both channel types, however, seem to be stimulated by nucleotides through different mechanisms. Whereas non-hydrolysable ATP analogs stimulate R-type channels in *V. faba* guard cells (Hedrich et al. 1990) as well as in *A. thaliana* hypocotyl cells (Thomine et al. 1997), the activity of S-type channels strictly depends on hydrolysable ATP (Schmidt et al. 1995; Allen et al. 1999). This suggests that S-type channels are stimulated through phosphorylation, which is supported by a reduced activation in presence of protein kinase inhibitors (Allen et al. 1999) and a delayed down regulation by the protein phosphatase inhibitor okadaic acid (Schmidt et al. 1995).

Protein phosphorylation may also stimulate S-type anion channels in *N. tabacum* guard cell protoplasts, since Ca\(^{2+}\) activates these channels in a nucleotide sensitive mechanism. S-type anion channels were stimulated by an elevated Ca\(^{2+}\) concentration of 380 nM in the patch pipette during the first 30s. During prolonged measurement, the effect of Ca\(^{2+}\) was lost, which hints to the diffusion of cytoplasmic proteins that mediate this response. The highest degree of activation was found with 1 mM ATP in the pipette solution, while 10 mM ATP leads to a smaller activation of
S-type anion channels. This indicates that apart of a Ca\(^{2+}\)-dependent activation mechanism, anion channels are also subject to down regulation by nucleotides at high concentrations.

It is likely that Ca\(^{2+}\)-dependent signalling proteins, such as protein kinases, associated with the plasma membrane forward the Ca\(^{2+}\) signal to anion channels. Calcium dependent protein kinases (CDPK) (Hrabak et al. 2003) may represent such regulators, since they seem to be required for the activation of an anion channel in the vacuolar membrane, of A. thaliana guard cells (Pei et al. 1996). Furthermore, these protein kinases are also involved in ABA- and Ca\(^{2+}\)-dependent regulation of S-type anion channels and hyperpolarization activated Ca\(^{2+}\) channels (Mori et al. 2006). Previously, a CDPK has been identified in V. faba guard cells, which was able to phosphorylate the KAT1 channel of Arabidopsis (Li et al. 1998).

Alternatively to CDPK kinases, Calcineurin B-like proteins (CBL) may be involved in Ca\(^{2+}\)-dependent regulation of ion channels (Batistic and Kudla 2004; Hedrich and Kudla 2006). These Ca\(^{2+}\) sensors bind to CBL interacting protein kinases (CIPK), which were found to stimulate the AKT1 channel in root cells (Xu et al. 2006). In guard cells of V. faba, similar proteins may inhibit the inward K\(^{+}\) channels, since this response was repressed by inhibitors of calcineurin (Luan et al. 1993). However, an interaction between CIPK proteins and plasma membrane anion channels in guard cells has not yet been shown.

**Species-specific ABA signalling pathways**

During evolution two signalling pathways seem to have developed that link perception of ABA to guard cell responses. We found that guard cells of V. faba exclusively utilize the Ca\(^{2+}\)-independent pathway (Levchenko et al. 2005), while both Ca\(^{2+}\)-dependent and -independent responses were found in N. tabacum. Based on experiments with the Ca\(^{2+}\) reporters FURA2 (Allen et al. 1999, Hetherington and Brownlee 2004) and cameleon (Allen et al. 2000), ABA induces also Ca\(^{2+}\) signals in guard cells of A. thaliana and Commelina communis, suggesting that Ca\(^{2+}\)-dependent processes also play a role in these species.

Apart from the activation of anion channels, ABA also inhibits blue light-induced activation of H\(^{+}\)-ATPases in guard cells (Goh et al. 1996). This ABA-response depends on the protein phosphatases ABI1 and 2 (Roelfsema et al. 1998) and may be mediated through H\(_2\)O\(_2\) (Zhang et al. 2004). So far, it is not known weather this ABA response, or ABA responses in other cell types, utilize Ca\(^{2+}\)-dependent or independent signalling pathways. Additional information obtained with other species or different cell types may reveal the evolutionary origin of both
pathways and hint to ecological advantages for plants to use one or the other signalling mechanism.

Materials and Methods

Plant material. Nicotiana tabacum (L.) cv. SR1 were grown in a green house under HQL-pressure lamps (Philips, Powerstar HQI-E, 400W) with a day/night cycle of 12/12 hours. For patch clamp experiments, leaves of 3-4-week-old plants were used, while the second or third pair of leaves was used for impalements studies.

Protoplast isolation and patch-clamp experiments. Guard cell protoplasts were isolated from according to (Raschke and Hedrich 1989). Anion currents were studied in the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981), using patch pipettes prepared from Kimax-51 glass (Kimble Products, Vineland, NY, USA) and coated with silicone (Sylgard 184 silicone elastomer kit, Dow Corning, USA). Currents were recorded with an EPC-7 patch-clamp amplifier (HEKA, Lambrecht, Germany), and low-pass-filtered with an eight-pole Bessel filter at a cut-off frequency of 2 kHz and sampled at 2.5-fold filter frequency. Data were digitized (ITC-16; Instrutech Corp. Elmont, N.Y., USA), stored on hard disk and analysed with HEKA and Wavemetrics software (HEKA Elektronik, Lambrecht/Pfalz, Germany ; Wavemetrics Inc., Lake Oswego, OR, USA). The pipette solution contained 150 mM TEACl, 2 mM MgCl2, 10 mM EGTA, 1 mM MgATP, 10 mM Hepes-Tris pH 7.2. The bath solution was composed of 40 mM CaCl2, 10 mM Mes-Tris pH 5.6. Protoplasts were characterized by a mean membrane capacitance of 4.4 ± 0.7 pF (n=140). For ABA experiments, (±)-cis, trans abscisic acid (Lancaster, Newgate, UK) was used from methanol stocks.

Recordings on Guard Cells in Intact Leaves. Guard Cells in intact plants were impaled and recorded with double and triple barrelled electrodes as described (Levchenko et. al 2005, Roelfsema et. al 2001). Two barrels were filled with 300 mM KCl for membrane potential measurements and voltage clamp, the third barrel was filled with 2 mM FURA2 (Fluka, Seelze, Germany) for current injection, and with an additional 50 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate (BAPTA) for calibration of FURA2 in the intact plant (Levchenko et al.
The three barrels of the intracellular electrode were connected to microelectrode amplifiers (VF-102, BioLogic, Claix, France) and the membrane potential was clamped by using a differential amplifier (CA-100, BioLogic). Data were filtered at 250 Hz and sampled at 1 kHz during short pulses, or filtered at 10 Hz and sampled at 33 Hz for long term registration. The solution on the leaf surface contained 5 mM KCl, 5 mM K Citrate (pH 6), 0.1 mM CaCl₂ and 1 mM MgCl₂.

**Ratiometric Fluorescence Spectroscopy**. The dual-excitation wavelength of the Ca²⁺-dependent fluorescent dye FURA2 was used to monitor the cytoplasmic free Ca²⁺ concentration. FURA2 was loaded into the guard cell cytoplasm or vacuole by iontophoretic microinjection from the third microelectrode barrel. A loading current up to -350 pA was applied to inject the dye into the cell, during which the holding potential was kept at -100 mV. The injection current therefore was automatically compensated by a current in the opposite direction through the current injection barrel. Ratiometric fluorescence spectroscopy measurements were carried out by using Metafluor software (Universal Imaging, Downington, PA, USA). FURA2 was excited with 200 ms flashes of UV light at 345 and 390 nm with a time interval of 1s (VisiChrome High speed Polychromator System, Visitron). The emission signal was filtered with a 510 nm bandpass filter (D510/40 M, AF Analysentechnik, Tübingen, Germany) and captured with a cooled charge-coupled device camera (CoolSNAP HQ, Roper Scientific, Tuscon AZ, USA). Background fluorescence levels of both wavelengths were taken from a reference region placed within a part of the unloaded neighbouring guard cell or neighbouring epidermal cells. The intracellular free Ca²⁺ concentration was calculated according to Grynkiewicz et. al (1985) by using the equation:

$$[\text{Ca}^{2+}]_{\text{free}} = K_d \frac{(R - R_{\text{min}})F_{\text{min}}}{(R_{\text{max}} - R)F_{\text{max}}}$$

(Eq. 1)

where $K_d$ represents the binding constant of FURA2 for Ca²⁺, $R$ represents the 345/390 nm excitation ratio, and $R_{\text{min}}$ and $R_{\text{max}}$ correspond to Ca²⁺-free and Ca²⁺-saturated FURA2, respectively. $F_{\text{min}}$ and $F_{\text{max}}$ give the fluorescence intensity measured at 390 nm with Ca²⁺-free and Ca²⁺-saturated FURA2, respectively. We adapted a $K_d$ of 270 nM, determined *in vitro* by Levchenko et. al (2005). $R_{\text{min}}$ and $F_{\text{min}}$ were defined as the values obtained after simultaneously injecting FURA2 and BAPTA at 0 mV into guard cells of intact plants. The values for $R_{\text{max}}$ and
\( F_{\text{max}} \) were obtained by clamping the plasma membrane from 0 mV to -250 mV, inducing a saturating influx of Ca\(^{2+} \) through hyperpolarization activated Ca\(^{2+} \)-channels.

**Legends**

Figure 1. Guard cells in intact *Nicotiana tabacum* plants impaled with triple barreled electrodes. A, Close up of an *N. tabacum* leaf impaled with triple barreled electrode (left), guard cells in the abaxial epidermis were visualized with a water emersion objective (middle). Experimental solutions were perfused through a hypodermic needle (left back) over the cuticle to a suction pipette (right). B, Overview of the experimental setup with the upright microscope (middle), microelectrode amplifiers (left) and the *N. tabacum* plant (right). C, *N. tabacum* guard cell impaled with a triple barreled electrode, through which FURA2 was injected, located with the tip in the cytoplasm. D, Guard cell injected with FURA2 through a triple barreled electrode with the tip in the vacuole. E, Guard cell injected with FURA2 in the cytoplasm, but in which the FURA slowly appeared in the vacuole F, Same guard cell as in E but 15 min. later.

Figure 2. ABA induced changes in the plasma membrane conductance of an *N. tabacum* guard cell. A, Current trace of a guard cell clamped to a holding potential of -100 mV and exposed to 50 \( \mu \)M ABA (as indicated by the bar below the graph). Symbols indicate the time points at which voltage clamp step protocols were applied. B, Current traces from test pulses of the same cell as in A, symbols correlate. Arrows indicate the 0 nA level. Cells were clamped from a holding potential of -100 mV to potentials ranging from -180 to 0 mV with 20 mV increments. Note, that the introduction of ABA (●) caused a dramatic increase of currents measured directly after the capacity compensation peak, while ABA had little effect on time dependent outward currents. The currents virtually recovered to pre-stimulus values after a washout of ABA (□). C, Steady state currents, sampled at the end of the 2 s test pulses, plotted against the clamp voltage (symbols correspond to A). Note, that ABA increased inward current ranging from -40 to -140 mV and caused a large shift of the zero current potential to more positive values. D, Instantaneous currents, sampled directly after termination of the capacity compensation peak, plotted against the clamp voltage (symbols correspond to A). Note, that ABA activated a channel with a linear instantaneous current-voltage relation and a reversal potential at -17 mV.
Figure 3. *N. tabacum* guard cell in intact plant, with an ABA induced rise of the cytoplasmic free Ca\(^{2+}\) concentration, matching an increase of anion currents. A, ABA-responses of a guard cell clamped to -100 mV, loaded with FURA2 and exposed to 50 μM ABA as indicated by the bar below the graphs. *Upper trace*, cytoplasmic free Ca\(^{2+}\) concentration as calculated from the FURA2 F\(_{345}/F_{390}\) fluorescent ratio. *Note*, that ABA induces a transient rise in the free Ca\(^{2+}\) concentration. *Lower trace*, Current trace at -100 mV. *Note*, that ABA induces an inward current that matches the rise in the cytoplasmic free Ca\(^{2+}\) concentration. B, False color cytoplasmic free Ca\(^{2+}\) images of the same guard cell as in A, before during and after the response to ABA (symbols correspond to A). Color codes are linked to free Ca\(^{2+}\) concentration in the bar on the left. *Note*, that ABA triggers a rise in the cytoplasmic free Ca\(^{2+}\) concentration throughout the cell, which is most obvious in the area surrounding the nucleus.

Figure 4. *N. tabacum* guard cells in intact plants, in which ABA induced an increase in anion channel activity, accompanied by a delayed rise in the cytoplasmic free Ca\(^{2+}\) concentration (A) or without a change of the Ca\(^{2+}\) concentration (B). A and B, ABA-responses of guard cells clamped to -100 mV, loaded with FURA2 and exposed to 50 μM ABA as indicated by the bar below the graphs. *Upper traces*, cytoplasmic free Ca\(^{2+}\) concentration as calculated from the FURA2 F\(_{345}/F_{390}\) fluorescent ratio. *Note*, that ABA induces a transient rise in the free Ca\(^{2+}\) concentration that is delayed compared to the increase in current in (A), but no change in the Ca\(^{2+}\) concentration in (B). *Lower traces*, Current trace at -100 mV. *Note*, that ABA induces transient increases in inward current in both cells.

Figure 5. ABA-induced change in free-running membrane potential and cytoplasmic free Ca\(^{2+}\) concentration of a *N. tabacum* guard cell in an intact plant. A, ABA-response of a guard cell at its free running membrane potential, loaded with FURA2 and exposed to 50 μM ABA as indicated by the bar below the graphs. *Upper traces*, cytoplasmic free Ca\(^{2+}\) concentration as calculated from the FURA2 F\(_{345}/F_{390}\) fluorescent ratio. *Note*, that ABA induces a transient rise in the free Ca\(^{2+}\) concentration that matches the change in free running membrane potential. *Lower traces*, Free-running membrane potential of the same cell as in the upper trace. B, Average free Ca\(^{2+}\)-concentration of *N. tabacum* guard cells as calculated from the FURA2 F\(_{345}/F_{390}\) fluorescent ratio before (gray bar) or at the peak of the ABA-response (black bar), cell not showing an ABA-induced change in cytoplasmic free Ca\(^{2+}\) were not included. Guard cells were clamped at a
holding potential of -100 mV (left bars) or at their free-running membrane potential (average $E_m$=-55 mV, SE=5, n=6, right bars). Error bars represent SE, n is indicated below the bars. Note, that the average free Ca$^{2+}$ concentration is lower in cell at their free-running membrane potential and is raised by ABA irrespective of current- or voltage-clamp conditions.

**Figure 6.** Ca$^{2+}$-dependent activation of plasma membrane anion channels in guard cell protoplasts of *N. tabacum*. A, Cl$^-$ induced shift in reversal potential of anion channels. The current-voltage relation was obtained with voltage clamp step protocols from a holding potential of -158 mV to a preconditioning voltage of +62 mV and test potentials ranging from +82 to -218 mV. Measurements were carried out with a pipette solution containing 10 mM ATP, 110 nM free Ca$^{2+}$ and 150 mM Cl$^-$. A change of the extracellular Cl$^-$ concentration from 80 to 8 mM shifted the reversal potential from 7.5 mV (SE=0.2, n=4) to 41.3 mV (SE=3.5, n=4). B and C, Kinetics of activation of anion channels in guard cell protoplasts after establishing the whole cell configuration (t=0) with 10 mM ATP (B) or 1 mM ATP (C) in the pipette solution. The plasma membrane was clamped to -158 mV and Ca$^{2+}$ was given via the patch pipette either at a free Ca$^{2+}$ concentration of 0 (○), 110 (□) or 380 (△) nM. Error bars represent SE, n=6-12. Note, that at 10 mM ATP, 380 nM Ca$^{2+}$ activates anion channels during the first 30 s but the current decays to same value as with 0 or 110 nM during prolonged measurements. At 1 mM ATP, 110 and 380 nM Ca$^{2+}$ activate anion channels during the first 30 s and inhibit slow inactivation.

**Figure 7.** Kinetics of ABA-induced activation of anion channels in guard cell protoplasts from *N. tabacum*. A, Average plasma membrane currents at -158 mV of *N. tabacum* guard cell protoplasts, patched with pipettes containing 1 mM ATP and 0 mM free Ca$^{2+}$. ABA was omitted (○) or applied through the patch pipette at a concentration of 10 µM (●). Note, that ABA inhibits the time-dependent deactivation of anion channels. B, Current traces obtained with voltage clamp step protocols from a holding potential of -158 mV, to a preconditioning voltage of +60 mV and test potentials ranging from +82 to -218 mV. For clarity only current traces of every second voltage step ($\Delta V$=-40 mV) are shown. C, Current-voltage relation of representative protoplasts recorded 4.5 min. after establishing the whole cell configurations in the presence or absence of 10 µM ABA in the pipette. The protoplasts were challenged with fast voltage clamp ramps from a holding potential of -158 mV to +82 mV in 1500 ms.
References


Marten et al., figure 2
Marten et al., figure 3
Marten et al., figure 4
Marten et al., figure 5
Marten et al., figure 6
Marten et al., figure 7