Abscisic acid and 14-3-3 proteins control K⁺ channel activity in barley embryonic root

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Summary

Germination of seeds proceeds in general in two phases, an initial imbibition phase and a subsequent growth phase. In grasses like barley, the latter phase is evident as the emergence of the embryonic root (radicle). The hormone abscisic acid (ABA) inhibits germination because it prevents the embryo from entering and completing the growth phase. Genetic and physiological studies have identified many steps in the ABA signal transduction cascade, but how it prevents radicle elongation is still not clear. For elongation growth to proceed, uptake of osmotically active substances (mainly K⁺) is essential. Therefore, we have addressed the question of how the activity of K⁺ permeable ion channels in the plasma membrane of radicle cells is regulated under conditions of slow (+ABA) and rapid germination (+fusicoccin). We found that ABA arrests radicle growth, inhibits net K⁺ uptake and reduces the activity of K⁺ in channels as measured with the patch-clamp technique. In contrast, fusicoccin (FC), a well-known stimulator of germination, stimulates radicle growth, net K⁺ uptake and reduces the activity of K⁺ out channels. Both types of channels are under the control of 14-3-3 proteins, known as integral components of signal transduction pathways and instrumental in FC action. Intriguingly, 14-3-3 affected both channels in an opposite fashion: whereas K⁺ in channel activity was fully dependent upon 14-3-3 proteins, K⁺ out channel activity was reduced by 14-3-3 proteins by 60%. Together with previous data showing that 14-3-3 proteins control the activity of the plasma membrane H⁺-ATPase, this makes 14-3-3 a prime candidate for molecular master regulator of the cellular osmo-pump. Regulation of the osmo-pump activity by ABA and FC is an important mechanism in controlling the growth of the embryonic root during seed germination.

Keywords: 14-3-3 proteins, abscisic acid, fusicoccin, patch-clamp, radicle, seed germination.

Introduction

Seed germination is characterized by three phases of water uptake and each of these phases represents different processes: imbibition, initiation of metabolic activity, and initiation of growth of the embryonic axis (Bewley, 1997). Imbibition is the uptake of water driven by the very low matric potential of the dry seed and is not subject to physiological control because it is a purely physical process. The second phase, resumption of metabolic activity, starts minutes after imbibition and is evident from the increase in respiratory activity. The third phase completes the germination process when the radicle penetrates the tissues surrounding the embryo. Subsequent events, e.g. mobilization of storage reserves are already associated with growth of the seedling. Dormant seeds, seeds that fail to complete germination although conditions are favorable, progress through the first two phases, but the radicle cells fail to elongate (Bewley, 1997; Carles et al., 2002). Although the phytohormone abscisic acid (ABA) is a key factor controlling
ABA performs important roles not only in germination, but also in seed development. Late in seed maturation, a rise in ABA concentration brings the developing embryo to a dormant state, which is characterized by a cessation of cell division, activation of genes encoding storage proteins and proteins that have a function in desiccation tolerance, and prevention of precocious germination (Bewley, 1997; Finkelstein et al., 2002; Koornneef et al., 2002). Several genes and second messengers have been identified that take part in the ABA signal transduction in developing seeds. Dominant ABA-insensitive mutations abi-1 and abi-2 in Arabidopsis result in ABA insensitivity during both seed germination and vegetative growth (Leung and Giraudat, 1998; Merlot et al., 2001). ABI1 and ABI2 encode type 2C-protein phosphatases. Specific for ABA signaling during seed germination are ABI3, ABI4, and ABI5, genes that encode transcription factors linking ABA signaling with gene expression (Finkelstein and Lynch, 2000; Finkelstein et al., 1998; Giraudat et al., 1992). Mutations in these genes confer ABA insensitivity during seed germination. Analysis of ABA-hypersensitive mutants has identified genes encoding a farnesyl transferase (ERA1) (Cutler et al., 1996) and RNA-binding proteins (HYL1 and ABH1) (Hugouvieux et al., 2001; Lu and Fedoroff, 2000).

Besides the role in seed development, ABA has a wider function in plant adaptation to abiotic stress. It translates the status of water availability of cells and tissues into metabolic and developmental adaptations of plants (Finkelstein et al., 2002; Leung and Giraudat, 1998; Schroeder et al., 2001a) and serves as endogenous signal in the regulation of ion loading of the xylem vessels (Gaymard et al., 1998; Roberts, 1998) and the stomatal aperture (Li et al., 2000; Schroeder et al., 2001a). Many of the above-mentioned mutant plants show defects in the regulation of ion channel activity in stomatal guard cells. Thus, the dominant negative abi1 mutant results in a phosphorylation-dependent loss of sensitivity to ABA of guard cell $k_{\text{in}}$ and $k_{\text{out}}$ activity (Armstrong et al., 1995; Bertauche et al., 1996) and activation of anion channels (Pei et al., 1997). The era1 mutant results in ABA hypersensitivity of anion channel activation and the abh1 mutant shows enhanced slow anion channel and reduced inward potassium channel currents (Hugouvieux et al., 2001). The defects in these mutant plants are in line with the known ABA effects on guard cell ion channels: increase in anion channel activity, enhanced $K^+_{\text{out}}$ and reduced $K^-_{\text{in}}$ currents, and reduced activity of the plasma membrane H$^+$-ATPase (Blatt, 2000; Schroeder et al., 2001b). Ca$^{2+}$ oscillations, changes in cytosolic and apoplastic pH and protein (de)phosphorylation are instrumental in ABA-induced effects.

As mentioned above, stomatal regulation and seed germination are both affected by the mutations that result in ABA insensitivity or hypersensitivity. However, in contrast to our knowledge on the molecular mechanism of ABA-induced stomatal closure, the mechanism of ABA-controlled cell elongation in the emerging embryonic root (radicle) is much more limited. In view of the above-mentioned role of ion channels in guard cell ABA action, we investigated whether in radicle cells ABA targets the plasma membrane K$^+$-transport machinery, notably K$^+$ channel activity, thus providing a mechanism for growth control. We compared the mechanism of ABA action with that of the phytotoxin fusicoccin (FC), a metabolite of the fungus Fusicoccum amygdali (L), well known for its potency to stimulate cell volume increase and as a dormancy breaking agent (Ballio et al., 1981). The comparison between ABA and FC is relevant for cell volume regulation in general as ABA and FC act antagonistically in both reversible (guard cells, pulvini) as well as irreversible (elongation growth) cell volume changes. Although gibberellic acid also stimulates seed germination, we did not include it in this study as it may stimulate radicle growth because it induces a decrease in endogenous ABA levels (Wang et al., 1998).

Our electrophysiological study yields information about the activity and regulation of K$^+$ channels in radicle cells isolated from the germinating seed. Both ABA and FC regulate the activity of inward and outward K$^+$ channels of the radicle cells, what is likely to affect K$^+$ homeostasis and growth of the radicle. In radicle cells, channel activity is also controlled through the action of 14-3-3 proteins and the question whether these proteins are intermediates in the ABA and FC signaling pathway will be discussed.

Results

Growth of isolated radicles, apoplast pH, and K$^+$-uptake are regulated by ABA and FC

The molecular mechanisms that control radicle growth in intact seeds are difficult to study due to the presence of the seed coat and the influence from the attached seed. We therefore chose to study under controlled conditions radicles that were isolated from barley seeds, 22 h after imbibition, just before they protruded through the coleorhiza (Figure 1). To demonstrate that these radicles are still in the germination stage we tested the ABA responsiveness, because the ABA response is highly specific for the germination stage (Schopfer et al., 1979). Indeed, ABA at a concentration typically used in germination experiments (10 $\mu$M) (Wang et al., 1998), inhibited radicle growth (Figure 2a). FC, known for its positive effect on germination, is a very potent molecule in breaking the dormancy of barley seeds as shown in Figure 2(b). As germination is scored as emergence of the radicle, it can be concluded that radicle growth in intact seeds imbibed in FC is much stronger than in seeds imbibed in water alone. As shown in Figure 2(a), isolated radicles retain their responsiveness to FC as well:

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Isolation of radicles from germinating barley seeds 22 h after imbibition. (a) Dry seed. (b) Twenty-two-hour imbibed seed with coleorhiza protruding through the seed coat. (c) Exposure of the radicle flanked by two seminal roots after removal of seed coat and coleorhiza. (d) Isolated radicle (bar = 1 mm). c, coleorhiza; r, radicle; s, seminal root.

Radicles acidify the medium after addition of FC (Figure 2c). In contrast, ABA causes an alkalization of the medium with a lag time of more than 10 min. Besides cell wall relaxation, maintenance of turgor pressure is required for sustained growth, as growth will cease if $P$ drops below $Y$. A constantly growing cell prevents a fall in turgor pressure through a co-ordinated uptake of osmolytes like $K^+$ followed by water (Findlay, 2001). Indeed, the FC-induced growth stimulation is accompanied by a net increase in $K^+$-uptake whereas uptake in the presence of ABA is suppressed, as is growth (Figure 2d; cf. Cocucci and Cocucci, 1977).

**Radicle protoplasts**

Based on these results we concluded that isolated radicles are a good model system to study the molecular mechanisms that control growth of this organ, so vital for seedling establishment. As cell wall loosening is necessary, but not sufficient, to sustain prolonged cell and radicle elongation (Schopfer and Plachy, 1985), we addressed the question whether ABA and FC have a direct effect on transporters that mediate $K^+$ exchange between the cells and medium, like the plasma membrane $K^+$ channels, thus controlling turgor and growth. Using the patch-clamp technique in the whole-cell configuration, these channels were studied in radicle protoplasts. The preparation of protoplasts from barley radicles proved to be rather difficult. A conventional enzyme mixture containing cellulase and pectolyase, successfully used to release protoplasts from the stele of barley nodal roots (Wegner and Raschke, 1994), did not yield any protoplasts when applied to radicles (data not shown). Even cortex cells from primary and nodal roots proved to be resilient to these enzymes, as noticed by (Amtmann et al., 1999), even when supplemented with macerase (data not shown). Only when Driselase, a crude mixture of fungal enzymes, was included, nice protoplasts were released from the radicles. The during the 7 h growth period fresh weight increase in control and FC-treated radicles was 4.4 and 7.0% h$^{-1}$, respectively. Note that the FC effect is especially strong during the first hour of treatment; whereas control and ABA-treated radicles did not grow during the first hour, FC treatment resulted in almost 7% growth. Radicle growth is mainly the result of cell elongation (Antipova et al., 2003). The relative rate of cell elongation growth ($v$) depends on three parameters: wall extensibility ($\phi$), the minimum turgor for cell expansion or yield threshold ($Y$) and turgor pressure ($P$) according to (Ray et al., 1972):

$$v = \phi(P - Y) \text{ for } P > Y.$$  

Because both $\phi$ and $Y$ are pH-sensitive, wall relaxation is a pH-dependent process activated by pH $\leq$ 5. Therefore, elongation growth requires acidification of the apoplast. FC fulfils this prerequisite very well in view of the induction of a rapid (lag time <3 min) and strong acidification (pH < 4) of the radicle medium (Figure 2c). Consequently, ABA causes an alkalization of the medium with a lag time of more than 10 min. Besides cell wall relaxation, maintenance of turgor pressure is required for sustained growth, as growth will cease if $P$ drops below $Y$. A constantly growing cell prevents a fall in turgor pressure through a co-ordinated uptake of osmolytes like $K^+$ followed by water (Findlay, 2001). Indeed, the FC-induced growth stimulation is accompanied by a net increase in $K^+$-uptake whereas uptake in the presence of ABA is suppressed, as is growth (Figure 2d; cf. Cocucci and Cocucci, 1977).
diameter of the protoplasts was typically between 20 and 40 μm (average capacitance 31.2 ± 9.3 (n = 40; ±SE)). Protoplasts used for the experiments contained two to three small vacuoles.

**Plasma membrane inward and outward rectifying K+ channels in radicle cells**

Whole-cell patch-clamp experiments revealed the presence of both inward and outward rectifying currents (Figures 3a and 4b).

**Inward current** Hyperpolarization of the membrane potential from a holding potential of −60 to −180 mV in 10 mV steps resulted in fast activation of time-dependent inward currents, $I_{K,\text{in}}$ (Figure 3a). $I_{K,\text{in}}$ activated with an exponential time course and the majority of the curves could be best fitted with the sum of two exponentials (Figure 3d):

$$I = I_{\text{leak}} + I_{\text{act1}} \exp\left(-\frac{t}{\tau_{\text{act1}}}\right) + I_{\text{act2}} \exp\left(-\frac{t}{\tau_{\text{act2}}}\right),$$

where $I_{\text{leak}}$ is the leak current and $I_{\text{act1}}$ and $I_{\text{act2}}$ are the steady-state currents of the two components after activation. The mean time constants of activation $\tau_{\text{act1}}$ and $\tau_{\text{act2}}$ were in the range of 35 (±3.8) and 325 (±116.5) msec, respectively. Activation of the inward current showed no signs of inactivation. As shown in Figure 3(a), deactivation kinetics was very fast, precluding fitting. Figure 3(c) shows the current/voltage relationship of the steady-state current and it can be seen that the current activates from $V_m$ more negative than −90 mV. Tail current analysis of the inward rectifying current showed a close agreement between $E_{\text{rev}}$ and $E_K$ ($E_{\text{rev}} = +50$ mV and $E_K = +51$ mV), indicating that the inward current is carried mainly by K+ ions. A reduction in extracellular K+ resulted in a decrease in inward current amplitude, leaving the voltage dependence of the current unaffected (data not shown), as has been commonly described for plant inward rectifying K+ channels (Roberts and Tester, 1995; Wegner and Raschke, 1994). These channels are likely candidates to mediate the K+ uptake observed in elongating radicles (Figure 2d).

**Outward current** Depolarizing voltage pulses, starting from a holding potential of −40 mV, caused the activation of a large voltage dependent outward rectifying current ($I_{K,\text{out}}$) (Figure 4a). The activation kinetics of $I_{K,\text{out}}$ were very slow.

![Figure 3. Abscisic acid reduces the inward rectifying K+ current in the plasma membrane of barley radicle protoplasts.](image-url)

(a) Time-dependent whole-cell recording of inward K+ current in the plasma membrane of a radicle protoplast. Inward current was elicited by stepping from a holding potential of −60 to −180 mV in 10 mV decrements. Note the fast (de)activation of the current.

(b) Activation of the inward current in the same cell 20 min after addition of ABA (10 μM) to the bath medium. Voltage protocol as for (a).

(c) Steady-state current/voltage relations of the time-dependent current in the absence (○) and presence (●) of 10 μM ABA, respectively (n = 4; ±SE). The current–voltage relationship in the presence of ABA was determined 20 min after ABA addition. Data were normalized with respect to the current at −180 mV in the control situation.

(d) Kinetics of activation of the inward currents during a voltage pulse from −60 mV (holding potential) to −180 mV. Current traces (dots; data reduction applied) were fitted (continuous line) by the sum of two exponential components (function as given in Eqn 2). Average time constants of activation in the control situation were 35 (±3.8) and 324.5 (±116.5) for $\tau_{\text{act1}}$ and $\tau_{\text{act2}}$, respectively (n = 4). Curves after ABA treatment were sometimes best fitted with a single exponential.
and pulse durations of more than 6 sec were required to reach steady-state levels. Many outward rectifiers from other plant cells and tissues show a sigmoidal time course of activation (Miedema et al., 2000) and the activating outward current ($I_{ac}$) as function of time ($t$) is described adequately with a power function of the order $p$:

$$I_{ac} = I_{leak} + I_{max} \left( 1 - \exp \left( -\frac{t}{\tau_{ac}} \right) \right)^p,$$  \(\text{(3)}\)

where $I_{leak}$ is a leak component, $I_{max}$ and $\tau_{ac}$ represent the maximal steady-state current level at the end of the voltage pulse and the time constant of activation, respectively. The value of the exponent $p$ can be interpreted as the number of independent gating particles which control the opening of the $I_{k,out}$ channels (Hodgkin and Huxley, 1952). Usually, $p$ varies between 2 and 4 (Miedema et al., 2000), but best fits of the activation kinetics of this channel are found with $p = 1$, that is with a single exponential function (Figure 4d). After $I_{k,out}$ activation, stepping back to the holding potential resulted in deactivation of $I_{k,out}$. The majority of the deactivating currents ($I_{de}$) could be described by a single exponential. Current inactivation was not observed, even at prolonged polarization. In Figure 4(c) the time-dependent current at $t = 6$ sec is plotted against the membrane potential and it can be seen that with 100 mM KCl at either side of the membrane the current activates at potentials more positive than +10 mV. Tail current protocols were used to determine the major ion responsible for the outward current. A change in extracellular K$^+$ from 100 to 10 mM, shifted the zero-current voltage (reversal potential, $E_{rev}$) of the current/voltage curve in the direction of the equilibrium potential for K$^+$ ($E_K$), but it remained 30 mV more positive than $E_K$ ($E_K = -51$ mV and $E_{rev} = -21$ mV). This indicates that some other ion with a more positive equilibrium potential does contribute to the current. Moreover, the activation potential of the current does not follow $E_{rev}$, this unlike other outward rectifying K$^+$ currents like SKOR (Gaymard et al., 1998; Roberts and Tester, 1995; Wegner and De Boer, 1997a) or GORK (Ache et al., 2000).

**ABA and FC regulate the activity of radicle $K_{in}^+$ and $K_{out}^+$ channels, respectively.**

As the ABA-induced reduction in net K$^+$ uptake (Figure 2d) is likely to involve channel-mediated K$^+$ transport, we tested whether ABA addition to the bath affected the activity or the properties of the above characterized channels in whole-cell patch-clamp experiments. ABA was added to the bath to...
yield a final concentration of 10 μm. Just like in guard cells (Jacob et al., 1999; Schwartz et al., 1994) the presence of ABA in the bath reduced the $K_{\text{out}}^+$ current in radicle protoplasts up to 50% (Figure 3c). ABA did not affect the voltage dependence of channel gating and activation kinetics was not significantly affected by ABA (Figure 3d). ABA under the conditions used here had no effect on $K_{\text{out}}^+$ channel activity.

In a similar fashion we tested whether the FC induced stimulation of net $K^+$-uptake was mediated by an effect of FC on channel activity. Figure 4(b) shows how addition of FC to the bath medium resulted in a reduction of the outward current, reaching an average reduction of around 60% (Figure 4c). FC did not have a significant effect on the kinetics of activation (Figure 4d) nor on the voltage dependence of channel gating (data not shown). Furthermore, a small (25%) but significant increase in the inward $K^+$ current at hyperpolarized potentials was observed upon FC addition (data not shown).

$K_{\text{out}}^+$ current activity is reduced by 14-3-3 proteins

Because all evidence to date indicates that 14-3-3 proteins are an obligate partner for FC action (De Boer, 1997; Wurtele et al., 2003) and in view of the FC induced reduction in outward current (Figure 4), we investigated whether radicle $K^+$ channels are controlled by 14-3-3 proteins. Thus far, three 14-3-3 genes have been identified and sequenced from barley (De Boer, 2002; Van den Wijngaard et al., 2001). Recombinant 14-3-3B protein (100 nm) was introduced into the cytoplasm through the patch-pipette in the whole-cell patch-clamp configuration. As shown in Figure 6(a), 14-3-3 protein reduces the magnitude of the outward current to the same extent as does FC (60%; cf. Figure 4). Again there was no effect on channel (de)activation kinetics (data not shown).

Because it is known from the Drosophila Slowpoke $K^+$ channel (Zhou et al., 1999) that 14-3-3 affects the gating properties of the channel, we analyzed the steady-state activation of $I_{K_{\text{out}}}$ as a function of the membrane potential, $V_m$. First the chord conductances were calculated from the steady-state $I/V$ relationship according to:

$$G_{ch} = \frac{I_{\text{max}}}{V_m - E_{\text{rev}}}$$

where $I_{\text{max}}$ represents the maximal steady-state current at the end of the voltage pulse and $E_{\text{rev}}$ is the reversal potential (0 mV in this case). Values of $G_{ch}$, as a function of $V_m$, were fitted to a Boltzmann-like expression, which in turn rendered the maximal value of $G_{ch} = G_{\text{max}}$. In Figure 6(c) we plotted $G_{ch}/G_{\text{max}}$ in relation to $V_m$. A Boltzmann distribution was fitted to the mean values according to:

$$G_{ch} = \frac{1}{1 + \exp\left((V_{0.5} - V_m)/S\right)}$$

where $V_{0.5}$ represents the potential at which 50% of the channels are activated and $S$ is a slope factor equivalent to $RT/\delta F$, where $\delta$ is the minimal gating charge and $R$, $T$, and $F$ have their usual meaning. Best fits resulted in $V_{0.5}$ of 82.7 ± 2.6 and 83.8 ± 3.3 mV for the control and 14-3-3 infused protoplasts, respectively. So, 14-3-3 did not affect the half activation potential of $I_{K_{\text{out}}}$. The 14-3-3 infused protoplasts with reduced outward current still respond to FC addition with a current reduction (Figure 6b), but the reduction is much than in cells without extra cytosolic 14-3-3.

$K_{\text{in}}^+$ currents depend on 14-3-3 protein for activation

In contrast to the outward current, the inward $K^+$ current is strongly enhanced (80%) by addition of 14-3-3 protein in the pipette (Figure 7a). In fact, the current seems to be completely dependent upon the availability of 14-3-3 protein. This conclusion is based on experiments with a phosphopeptide, derived from the canonical 14-3-3 interaction motif from barley nitrate reductase (RSTSP TP), which binds to the 14-3-3 protein with high affinity (data not shown) and blocks interaction with its targets (Booij et al., 1999; Bunney et al., 2001). As shown in Figure 7(b), the inward current
Figure 6. Barley 14-3-3B protein reduces the activity of the outward rectifying K+ current in the plasma membrane of radicle protoplasts.
(a) Current/voltage relationship of the time-dependent current at $t = 6$ sec in the absence ($\bullet; n = 14$) and presence ($\Delta; n = 5$) of 100 nM 14-3-3B in the pipette solution. Cytoplasmic introduction of 14-3-3 protein causes a large reduction (60–80%) in outward channel current amplitude.
(b) Current–voltage relationship of the time-dependent current at $t = 6$ sec in the presence of 100 nM 14-3-3B in the pipette solution before ($\bullet$) and after ($\Delta$) the addition of 10 $\mu$M FC ($n = 3$).
(c) The steady-state activation of $I_{\text{K,out}}$, expressed as $G_{\text{ch}}/G_{\text{max}}$, as a function of the membrane potential. Data were fitted to the Boltzmann function given in Eqn (5), resulting in potentials of half activation ($V_{0.5}$) of 82.7 ± 1.2 and 83.8 ± 3.3 mV for the control ($\bullet; n = 9$) and 14-3-3 infused protoplasts ($\Delta; n = 4$), respectively. Data are presented as mean ± SE.

Figure 7. Barley 14-3-3B protein increases the activity of the inward rectifying K+ current in the plasma membrane of radicle protoplasts.
(a) Steady-state current–voltage relationship of the inward K+ current in absence ($\bullet; n = 6$) and presence ($\Delta; n = 6$) of 100 nM 14-3-3B in the pipette solution. Addition of 14-3-3 protein causes an increase (90–130%) in the inward rectifying K+ current.
(b) Current–voltage relationship of the inward current in the presence of 14-3-3 binding peptide (1 $\mu$M) in the pipette solution. $I/V$ curves were generated from measurements at 1.5 ($\bullet$), 2.5 ($\square$) and 5.5 min ($\triangle$) after the establishment of the whole-cell configuration. Cytoplasmic introduction of the 14-3-3 binding peptide (1 $\mu$M) leads to near-complete inactivation of the current, what is ascribed to the binding to and blocking of endogenous 14-3-3 proteins.
(c) The steady-state activation of $I_{\text{K,in}}$, expressed as $G_{\text{ch}}/G_{\text{max}}$, as a function of the membrane potential. Data were fitted to the Boltzmann function given in Eqn (5), resulting in potentials of half activation ($V_{0.5}$) of −142.8 ± 1.6 and −134.6 ± 0.2 mV for the control ($\bullet; n = 3$) and 14-3-3 infused protoplasts ($\Delta; n = 8$), respectively. Data in (a) and (c) are presented as mean ± SE.
disappears within minutes after the pipette solution has gained access to the cytoplasm. Such channel rundown was never observed in control experiments without 14-3-3 binding peptide. These results can be explained by binding of the phospho-peptide to endogenous 14-3-3, thereby preventing the interaction between 14-3-3 and other proteins. As a control, we mixed the peptide with recombinant 14-3-3 protein before introducing it into the pipette and this also blocked the effect of recombinant 14-3-3 protein. The outward current slightly increased with peptide in the pipette when compared with the control situation (data not shown). These results lead to the conclusion that also in vivo 14-3-3 proteins regulate the activity of the inward and outward K⁺ channels of radicle cells. In order to test whether the presence of 14-3-3 affected the gating properties of k⁺, we calculated the steady-state activation curve from the chord conductance in a similar fashion as shown above for k⁺ (Figure 7c). Best fits resulted in V½ of −142.8 ± 1.6 and −134.6 ± 0.2 mV for the control and 14-3-3 infused protoplasts, respectively. From this analysis it can be concluded that activation of k⁺ by 14-3-3 proteins results in a small positive shift in the potential of half-activation. Activation kinetics of k⁺ were not significantly affected by 14-3-3 (data not shown).

Discussion

Elongating embryonic roots from germinating seeds have not been used thus far to study the activity and regulation of ion transporters at the molecular level. Therefore, no direct comparison is possible with other studies. Our study describes a number of new findings that will be discussed below: (i) radicles as a new model system to study molecular processes during seed germination, (ii) some of the radicle K⁺ channel characteristics differ from other monocot K⁺ channels, (iii) radicle channel activity is affected ABA and FC, and (iv) 14-3-3 proteins are channel regulators in radicle cells.

Isolated radicles as model system

Detachment of the radicle from the seed has the advantage that the radicles can be supplied with nutrients and growth regulating compounds of choice, without the (unknown) influence of the rest of the seed. The isolated radicles continued to grow and retained their ABA responsiveness (Figure 2), comparable to the response of whole seeds to ABA (Schofer et al., 1979; Wang et al., 1998). Although standard enzyme mixtures failed to produce protoplasts from the radicles, stable protoplasts amenable for patch-clamp studies could be produced using Driselase, a crude enzyme mixture containing laminaranase, xylanase, and cellulase. We conclude that the radicles and the protoplasts used in the electrophysiological experiments can provide valuable information about the processes that determine the success of germination.

Plasma membrane ion channels

Typical features of the outward rectifying channel are slow activation with single exponential kinetics, poor selectivity for K⁺-ions, and an activation potential that does not shift with Eᵦ. The well-characterized outward rectifying K⁺ channel in barley root xylem parenchyma cells activates much faster, exhibits sigmoidal activation kinetics, is more selective for K⁺-ions than the radicle channel (Erev closely follows Eᵦ) and the activation potential shifts with changes in Eᵦ (Wegner and De Boer, 1997b, 1999). A channel with similar characteristics was described for maize xylem parenchyma cells (Roberts and Tester, 1995) and these barley and maize channels are likely to be encoded by genes homologous to the Arabidopsis gene SKOR (Gaymard et al., 1998). A second outward rectifying channel in barley root xylem parenchyma cells, called NORC, does not resemble this radicle channel. In a number of studies on barley cells no outward currents were reported or reported to be very small; for example aleurone cells (Bush et al., 1988; Van Duijn et al., 1996) and suspension culture cells (Amtmann et al., 1999). Besides SKOR, a second gene called GORK encodes a K⁺-selective delayed outward rectifier (Ache et al., 2000), but the properties of this channel do not resemble those of the radicle outward rectifier. The radicle channel does have a number of properties in common with the outward current of Zea mays guard cells (Fairley-Grenot and Assmann, 1992): this current also exhibits very slow activation kinetics and the difference between the observed Erev and Eᵦ was around 30 mV as well. Characteristics of the hyperpolarization-activated inward rectifier, like the time-dependent activation with single exponential time course, fast activation and de-activation kinetics and high K⁺-selectivity, are characteristic of other inward rectifying K⁺ channels characterized in monocots: barley aleurone cells (Bush et al., 1988; Van Duijn et al., 1996), barley suspension culture cells (Amtmann et al., 1999), and maize guard cells and root cortex cells (Fairley-Grenot and Assmann, 1992; Roberts and Tester, 1995). The kinetics of activation of the K⁺ current in barley and maize xylem parenchyma cells are notably slower than those of the radicle channel (Roberts and Tester, 1995; Wegner and De Boer, 1997a; Wegner and Raschke, 1994). In cDNA isolated from barley radicles, a gene (KHV1) was identified and sequenced (P.W.J. van den Wijngaard, M.P. Sinnige and A.H. De Boer, unpublished data) showing the highest homology to the Z. mays inward rectifier KZM1 (Philippar et al., 2003) and to the Arabidopsis KAT7 gene. The KHV1 gene was expressed in leaves and radicles, but not in roots. Thus far the KZM1 transcripts were identified in guard cells, subsidiary cells and vascular/
ATPase. Inhibition of H+-ATPase is likely to cause depolarization (Figure 2c) after ABA addition has been reported for a variety of tissues and may be the result of inhibition of the H+-ATPase. Bunney et al. (2003) showed that FC has an effect on animal cells and that just like in plant cells 14-3-3 proteins take part in the formation of an FC-receptor complex. However, the databases do not show an equivalent of the plant H+-ATPase in animal cells with a motif at the C-terminal end (-YTp V, whereby Tp denotes a phosphorylated threonine residue) that is essential for the formation of a functional FC receptor site (Wurtele et al., 2003). Secondly, FC stimulates leakage of cytochrome c from mitochondria resulting in programmed cell death with apoptotic features, independent of the activation of H+-ATPase. Inhibition of H+-ATPase is likely to cause depolarization of the membrane, as was observed in cells in the embryonic axis of radish (Ballarin-Denti and Cocucci, 1979). The reduced driving force for K+ uptake in combination with a reduction in K+ in channel activity may explain the observed growth arrest and reduced radicle K+ uptake (Figure 2a,d). A twofold reduction in K+ current by ABA might be the result of a reduction in mean open time of the channels. However, this would have resulted in a change in the macroscopic current kinetics, and this was not observed. As ABA did not affect the voltage dependence of gating either, we conclude that ABA inactivates K+ channels thus reducing the pool of functional and voltage-activated channels. Blatt (1990) reached a similar conclusion when he studied the mechanism of ABA-induced closure of stomata in intact guard cells. Detailed analysis of single channel characteristics will be necessary to understand the mode of ABA action.

Reduction of inward current by ABA

For roots, only one study describes an effect of ABA on K+ channels when added during a patch-clamp experiment (Roberts, 1998). ABA reduced the magnitude of outward K+ currents in protoplasts isolated from the root stele, but had no effect on inward K+ channel activity (Roberts, 1998). In contrast, radicle protoplasts respond to ABA with a reduction in inward current (Figure 3c), whereas the outward current was not affected. Such a ‘direct’ inhibitory effect of ABA on K+ currents is also found in guard cells, but in these cells activation of K+ channel activity was observed at the same time (Armstrong et al., 1995). The reduction in net H+ extrusion (Figure 2c) after ABA addition has been reported for a variety of tissues and may be the result of inhibition of the H+-ATPase. Inhibition of H+-ATPase is likely to cause depolarization of the membrane, as was observed in cells in the embryonic axis of radish (Ballarin-Denti and Cocucci, 1979). The reduced driving force for K+ uptake in combination with a reduction in K+ channel activity may explain the observed growth arrest and reduced radicle K+ uptake (Figure 2a,d). A twofold reduction in K+ current by ABA might be the result of a reduction in mean open time of the channels. However, this would have resulted in a change in the macroscopic current kinetics, and this was not observed. As ABA did not affect the voltage dependence of gating either, we conclude that ABA inactivates K+ channels thus reducing the pool of functional and voltage-activated channels. Blatt (1990) reached a similar conclusion when he studied the mechanism of ABA-induced closure of stomata in intact guard cells. Detailed analysis of single channel characteristics will be necessary to understand the mode of ABA action.

Reduction of outward current by FC

Since the discovery of FC, attention has focused overwhelmingly on its ability to activate the plasma membrane H+-ATPase, the more so after the finding that 14-3-3 proteins were involved in the activation (Baunsgaard et al., 1998; Korthout and De Boer, 1994). As a consequence, dogma is that the H+-ATPase is the only direct target for FC action. Although it is evident that FC can stimulate K+ uptake indirectly through hyperpolarization activated inward K+ channels (because of hyperpolarization of the membrane potential following activation of the H+-pump), there is the intriguing possibility that FC increases net K+ uptake also because it inactivates outward K+ channels (Blatt and Clint, 1989). Indeed FC is able to inactivate the outward K+ current in the radicle protoplasts (Figure 4). It is deemed unlikely that, in the patch-clamp configuration used here, the FC-induced outward K+ channel inactivation is the consequence of H+-ATPase activation, because membrane potential changes are clamped by the amplifier and intra- and extra-cellular pH are clamped by the buffers. This corroborates the results of Vicia faba guard cell impalement studies wherein the outward rectifying K+ channels were identified as one site of action for FC (Blatt and Clint, 1989). Recently, two other reports were published that provide evidence that FC targets proteins other than the plant plasma membrane H+-ATPase. Bunney et al. (2003) showed that FC has an effect on animal cells and that just like in plant cells 14-3-3 proteins take part in the formation of an FC-receptor complex. However, the databases do not show an equivalent of the plant H+-ATPase in animal cells with a motif at the C-terminal end (-YTp V, whereby Tp denotes a phosphorylated threonine residue) that is essential for the formation of a functional FC receptor site (Wurtele et al., 2003). Secondly, FC stimulates leakage of cytochrome c from mitochondria resulting in programmed cell death with apoptotic features, independent of the activation of H+-ATPase (Malerba et al., 2003, 2004). All these reports warrant a renewed search for new FC target proteins. The conventional view is that FC stimulates net K+ uptake (cf. Figure 2d) due to the combined effect of increased activity of hyperpolarization-activated K+ channels and larger electrical driving force after H+-ATPase activation. In light of this, the physiological relevance of the FC-induced reduction of the outward current in the radicle cells is not directly obvious because the membrane potential will be far negative of the activation potential of the outward current (Figure 4). However, tracer flux analysis with 86Rb showed that FC addition to guard cells not only resulted in an increase in influx, but also in a large reduction in net 86Rb efflux (Clint and Blatt, 1989). Voltage clamp experiments of the guard cells revealed that FC reduced the activity of outward rectifying K+ channels independent of H+ pump activation (Blatt and Clint, 1989).

14-3-3 effects on plasma membrane currents

Thus far, two papers report a stimulatory effect of 14-3-3 proteins on plasma membrane outward rectifying K+ currents (Booij et al., 1999; Saalbach et al., 1997), contrary to the effect on the outward current in radicle protoplasts (Figure 6). A comparison of the outward currents in tomato suspension cells with those reported here shows that we are probably dealing with different channels; the tomato channels activate faster with a sigmoidal instead of single exponential time course and are more selective for K+ (Booij, 2000; Booij et al., 1999). Detailed information on the tobacco currents is lacking. Booij et al. (1999) concluded that the increase in current was due to the recruitment of channels to the pool of activatable channels, rather than to an increase in single channel conductance, open probability, or a shift in voltage dependence of gating. In animal cells a number of

outward-rectifying K⁺ channels are modulated by 14-3-3 interaction. The voltage sensitivity of the Drosophila Slow-poke K⁺ channel (dSlo) is drastically modulated by 14-3-3 via phosphorylation-dependent interaction with the channel-interacting protein SloB (Zhou et al., 1999). The shift in voltage required for half maximal activation elicited by 14-3-3 was +61 mV. A different mode of action has been suggested for the modulation of the two-pore-domain potassium channels TASK1 by 14-3-3 (Rajan et al., 2002). The C-terminal pentapeptide of TASK1 (RRSSV) interacts with 14-3-3 and co-injection of 14-3-3 cRNA with TASK cRNA in Xenopus oocytes increased the TASK1 current by about 70%. The authors conclude that the biophysical characteristics of the channel are unaltered, but that the increase in current was related to an increase in the number of functional channels. The suggestion that 14-3-3 proteins control ion channel assembly and release from the endoplasmic reticulum was confirmed for the K⁺ channel complex consisting of an α (Kir6.2) and β (SUR1) subunit (Yuan et al., 2003). This function of 14-3-3 in the quality control system of the cell should result in more outward current and this is contrary to what we see.

Activation of the inward rectifying K⁺ current by 14-3-3 and complete loss of activity with introduction of the P-peptide (Figure 6) is consistent with the hypothesis that channel activity fully depends on 14-3-3 interaction. The interaction may be direct, as inward rectifying channels like KAT1 are phosphorylated (Li et al., 1998), or indirect through interaction with channel subunits or channel-interacting kinases or type 2C phosphatases (Cherel et al., 2002). The observed decrease in current amplitude after ABA addition may be explained by ABA activation of type 2C protein serine/threonine phosphatases, ABI1 and 2, resulting in channel dephosphorylation, dissociation of 14-3-3 protein, and loss of activity (Armstrong et al., 1995). Our current model to explain the mechanism of FC action on the outward current is analogous to how FC modulates H⁺-ATPase activity: FC increases the affinity of 14-3-3 for the channel or its subunits that results in more interaction and reduction of the current. Increasing the cytosolic 14-3-3 concentration has the same effect (cf. Figures 4c and 5b). Effects of the peptide or 14-3-3 protein in the patch pipette were already evident within minutes after establishing the whole-cell configuration (e.g. Figure 7b). These observations are in line with the theoretical considerations of Goh et al. (2002). These authors determined a time constant for solute exchange between patch pipette and cytosol of guard cells. Using the formula given (τ = 8.8 ⋅ M⁰.31, where τ is the time constant in seconds and M the molecular mass in g mol⁻¹) time constants for the peptide and 14-3-3 protein exchange are in the range of 1.5 and 5 min, respectively, in line with our measurements.

We have reported here on the characterization of ion channels in protoplasts derived from radicle cells. Studies like this are very difficult to perform on the model plant Arabidopsis, often the plant of choice because of the wide range of molecular tools available, due to the small seed size. Although at first sight a comparison between radicle cells and guard does not speak for itself, it should be noted that some of the radicle plasma membrane current characteristics resemble those of guard cell currents. Moreover, ABA plays an important role in the physiology of both cell types as a hormone controlling ion and water uptake. Both cell types may use the same ion transport machinery and the regulatory systems to achieve tight control over ion and water uptake. It is therefore of interest that we find expression of KHV1, a homolog of guard cell-expressed KZM1 and KAT1 genes, in the radicles. The presence of a canonical mode-2 14-3-3 target sequence (Yaffe et al., 1997) in the C-terminus of KHV1 makes KHV1 a good candidate for regulation by 14-3-3 proteins, and this is currently under study. Based on the rapid growth of the radicles we expected that plasma membrane aquaporins might take part in the osmotic pump. However, the water permeability of the plasma membrane of radicle protoplasts is in the range of the permeability of the lipid bilayer only, also after treatment with FC (R. Morillon, University of California, CA, USA, unpublished data). As a new paradigm for 14-3-3 action we postulate that these proteins, together with cytoplasmic Ca²⁺ and pH, control the running of the osmotic pump and co-ordinate the respective components like the plasma membrane H⁺-ATPase and K⁺ channels and tonoplast V-type ATPase (A.H. De Boer, unpublished data) and K⁺ channels (De Boer, 2002). Co-ordination can be achieved through activation/inhibition of one single phosphatase (or kinase) and the phosphorylation-dependent 14-3-3 interaction with its targets. Our model predicts that one essential factor that holds seeds in a dormant state is that ABA keeps the osmotic pump ‘switched off.’ Lack of radicle growth in dormant seeds precludes a direct test, but consistent with our model the inward K⁺ conductance in aleurone protoplasts from isogenic dormant and non-dormant barley was strongly reduced (more than 65%) in the dormant protoplasts (Van Duijn et al., 1996). The molecular identification of genes encoding the proteins making up the osmotic pump in the radicle will open new ways to unravel the complexity of cell volume regulation and germination.

**Experimental procedures**

**Germination and isolation of barley radicles**

Radicles were isolated from barley (*Hordeum distichum* L. cv. Alexis) seeds 22 h after imbibition. Seeds were imbibed as a single layer between filter paper wetted with water. Seeds with a visible coleorhiza were selected for radicle isolation. The coleorhiza layer was removed and the radicle was mechanically isolated from the seed. The radicles were then agitated in a petri dish on a rotary shaker in a buffer containing 10 mM KCl, 20 mM sucrose, Gamborg's B5 Vitamins and Micronutrients, and 5 mM Mes/Ca(OH)₂.
Radicle elongation, $K^+$ uptake and medium pH

After the recovery period, radicles were transferred to fresh buffer of the same composition and vigorously agitated for maximal aer-ation. At different time points the fresh weight of the radicles was determined. The $K^+$ concentration of the bathing buffer was deter-

mined by Atomic Absorption Spectroscopy. To determine the effect

of FC and ABA on medium acidification, radicles (30) were added to

1 ml buffer (1 mM KCi, 1 mM CaCl$_2$ and 0.2 mM Mes/Tris pH 6.5) in a

cell cuvet and aerated with oxygen. The pH of the buffer was

recorded with a miniature pH electrode.

Radicle protoplast preparation

The preparation of protoplasts from barley radicles proved to be

very difficult. A conventional enzyme mixture containing cellu-

lase and pectolyase, successfully used to release protoplasts from the

stele of barley nodal roots (Wegner and Raschke, 1994), did not give

any protoplasts when applied to radicles (data not shown). Even
cortex cells from primary and nodal roots proved to be resilient to
these enzymes (Amtmann et al., 1999), even when supplemented
with macerase (data not shown). Only when Driselase, a mixture of
fungus enzymes, was included nice protoplasts were released from
the radicles. The following procedure was used for radicle proto-

plast preparation: radicles, after the recovery period, were cut lon-
gitudinally with a sharp razor blade and incubated in an enzyme
solution with the following composition: 10 mM Mes/Tris pH 5.5,
1 mM CaCl$_2$, 400 mM sorbitol, 0.5% (w/v) polyvinylpyrrolidone, 1%
(w/v) BSA, 0.8% (w/v) Cellulase (Onozuka Yakult Honsha Co., Tokyo,
Japan), 0.1% (w/v) Pectolyase (Sigma, St. Louis, MO, USA) and 1%
(w/v) Driselase (Sigma). Cut radicles were incubated for 2.5 h at
28°C in the dark and vigorously agitated. After 2.5 h radicles were
put on ice and left in the enzyme solution. When required, radicles
were taken from the enzyme solution and protoplasts were isolated from
the radicles mechanically by gently squeezing the tissue on a
fine mesh in buffer containing 10 mM Mes/KOH pH 5.5, 100 mM KCl,
2 mM MgCl$_2$, 1 mM CaCl$_2$ and sorbitol to a final osmolarity of
500 mosm kg$^{-1}$. The protoplast suspension was transferred directly to
a 200-μl recording chamber for patch-clamp measurements.

Electrophysiological experiments

Channel currents of the radicle protoplasts were measured by using
the whole-cell patch-clamp technique. Currents were measured with
an Axopatch 200 patch-clamp amplifier (Axon Instruments,
Union City, CA, USA) and low-pass filtered using a 4-pole Bessel
filter (internal filter of the Axopatch). The data were digitized using
a Digidata 1320A (Axon Instruments) and analyzed with pClamp 8.0
software (Axon Instruments). Pipette pullers were pulled from
Kimax-51 glass (Kimble Products, Vineland, NJ, USA) and briefly
fire-polished. Pipettes were filled with buffer containing 10 mM
Hepes/KOH pH 7.2, 100 mM KCi, 2.0 mM MgCl$_2$, 0.55 mM CaCl$_2$, 2.0 mM EGTA (free Ca$^{2+}$ concentration 150 mM), 5 mM MgATP and sorbitol to a final osmolarity of 500 mosm kg$^{-1}$. Recombinant 14-3-3 protein and 14-3-3 binding peptide were added to the pipette solution to a final concentration of 100 nM and 1 μM, respectively. The bath was flushed continuously (0.2 ml min$^{-1}$) with buffer containing 10 mM Mes/KOH (pH 5.5), 100 mM KCi, 2 mM MgCl$_2$, 1 mM CaCl$_2$, adjusted with sorbitol to 500 mosm kg$^{-1}$. Fusicoccin and ABA were added from methanol stock (10$^{-2}$ M) and in control experiments methanol alone was added. Before addition of FC and ABA bath perfusion was stopped and a number of recordings were made to test the stability of the currents. Variations in current levels are given as standard errors of the mean (SE).

Recombinant 14-3-3B

An infrane construct of barley 14-3-3B isoform in the pRSET vector was
constructed and the orientation and reading frame were checked by sequencing. BL21(DE3) pLysS cells were grown at 37°C in 2xYT supplemented with 50 μg ml$^{-1}$ ampicillin and 1% glucose until the OD$_{600}$ had reached 0.8. Expression was induced by adding isopropyl-$β$-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The cells were grown for 4 h at 37°C in the presence of IPTG without glucose. Subsequently the cells were pelleted at 5000 $g$ for 15 min and the manufacturer’s instructions were followed for cell lysis and recombinant protein purification. Briefly, the His-tag-
gg recombinant 14-3-3B was purified using a Hitrap chelating Ni$^{2+}$ column, desalted and concentrated yielding a stock solution of
7.5 μM 14-3-3 in 1 mM Hepes, pH 7.5. Purified protein was run on SDS-PAGE gel to control purity by means of Coomassie staining. The sequence of the phosphorylated peptide derived from barley nitrate reductase is C-P-G-L-K-R-S-T-pS-T-P-F-M-N, where pS is the phosphorylated serine and the binding motif is underlined.

Determination of 14-3-3 proteins levels

Recovered radicles (20) were frozen in liquid $N_2$ and ground with mortar and pestle in 100 μl 20 mM Hepes pH 7.5 with Complete protease inhibitors (Roche, Mannheim, Germany). Debris was pel-
leted by centrifugation for 1 min at 13 400 × $g$. Total protein (15 μg) was separated on 12% SDS-PAGE. The separated proteins and control recombinant 14-3-3 proteins were transferred to Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with isoform-specific Anti-14-3-3 antibodies (Testerink et al., 1999) and 14-3-3 isoforms were quantified using the recombinant 14-
3-3 standards and Multi-Analist (Bio-Rad).

Quantitative real-time RT-PCR

Total RNA was isolated from 10 radicles using TRIzol Reagent
(Invitrogen, Carlsbad, CA, USA). RNA was treated with DNase and first-strand cDNA was produced using SuperScript reverse tran-
scriptase (Invitrogen) and oligo(dT) primers. Quantitative real-time
RT-PCR (DNA Engine Opticon; MJ Research, Waltham, MA, USA) was carried out with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) using primers: 14-3-3A fw (5′-gtagtgatgctgtcagcaagac-3′), rev (5′-actaagaagccagatgtcaca-
a-3′), 14-3-3B fw (5′-ggaagctcctaatggtgtgta-3′), rev (5′-acagagaaggtcacaagagc-3′), 14-3-3C fw (5′-gtgattcatggtagctgtcagca-3′), rev (5′-aacctcggcagcaca-3′) and GAPDH fw (5′-ctcctcagctcctactactg-3′), rev (5′-ggttccttactcctactactg-3′). Genbank accession numbers are as follows: 14-3-3A (X62388), 14-3-3B (X93170), 14-3-3C (Y14200) and GAPDH (M36650). All kits were used according to the manufacturer’s protocol.

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References


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