Abscisic acid maintains S-type anion channel activity in ATP-depleted *Vicia faba* guard cells

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Abstract The plant hormone abscisic acid (ABA) regulates important developmental and stress responses. Recent data show that ABA activates phosphorylation events, but whether dephosphorylation events are post-translationally regulated by ABA or whether these are constitutive remains unknown. Slow anion channels in the plasma membrane of guard cells have been proposed to play an important role during ABA-induced stomatal closing. Anion channels are deactivated by removal of cytosolic ATP. However, when guard cells were treated with ABA and depleted of ATP, anion currents remained active. Subsequent removal of extracellular ABA caused deactivation of currents. Deactivation of currents was reversed by reintroduction of cytosolic MgATP. These data show that anion channels are regulated by ABA even in the absence of cytosolic ATP required for kinase-induced phosphorylation events and that anion channel activity is maintained by ABA under conditions that favor dephosphorylation-induced deactivation. Furthermore, channel activation proceeded at high ATP concentrations with nanomolar cytosolic Ca^{2+} showing a Ca^{2+}-independent final step in anion channel activation.

Key words: Abscisic acid; Cl^- channel; Stomate; Protein kinase; Protein phosphatase; Abscisic acid-insensitive

1. Introduction

The plant hormone abscisic acid (ABA) plays important roles in the regulation of diverse plant growth and developmental responses [1,2]. ABA is synthesized during drought stress [3] and induces closing of stomatal pores in leaves [4]. Stomatal closing reduces transpirational water loss in plants. ABA-induced stomatal closing is mediated by efflux of K^+ and anions across the plasma membrane of guard cells and by organic osmolyte metabolism. Anion channels have been proposed to provide an important control mechanism for the regulation of ion efflux from guard cells during stomatal closing [5–8]. Biophysical studies have shown that the slow and sustained ('S-type') anion channels in guard cells can produce sustained anion efflux, which would result in a long-lasting depolarization of the plasma membrane [5,7,8]. Depolarizations, can activate outward-rectifying K^+ channels proposed to mediate K^+ release during stomatal closing [9,10]. Recent studies in *Arabidopsis* and tobacco guard cells have shown that ABA regulates S-type anion channels [11,12], while revealing new regulation mechanisms in *Arabidopsis*, which act in addition to those studied here (see Section 4). However, no direct evidence for ABA regulation of S-type anion channels has been shown in *Vicia faba*, despite many correlative studies in this species [5,7,8,13–15].

Anion channel regulation has been proposed to play important roles in physiological processes of other plant cells, including hypocotyl growth, root transport, and blue light and red light signaling [16–19]. In *Vicia faba* guard cells, S-type anion channels were shown to be activated by phosphorylation events: depletion of the intracellular ATP pool leads to a strong down-regulation of S-type anion currents, which cannot be restored by even 4 mM of non-hydrolyzable ATP analogues or GTP analogues [13]. The protein kinase inhibitors K252a and H7 abolished slow anion channel activity in the presence of excess ATP and also abolished ABA-induced stomatal closing, supporting central roles of phosphorylation events for ABA signaling [13,15]. Biochemical studies have recently directly shown a rapid enhancement of a 48 kDa protein kinase activity by ABA in *Vicia faba* guard cell extracts [20,21] and ABA activation of additional types of protein kinases has been found in other tissues [22,23].

The protein phosphatase inhibitor okadaic acid (OA) inhibits the activity of inward-rectifying K^+ channels in *Vicia faba* guard cells, providing evidence for modulation by OA-sensitive protein phosphatases in guard cells [24,25]. Furthermore, OA maintained slow anion current activity even in the absence of intracellular ATP [13]. In correlation to this finding, ABA-induced stomatal closing is enhanced by OA in *Vicia faba* and *Commelina communis* [13,15]. ABA-regulated S-type anion channels in tobacco guard cells show stimulation by OA [12]. Together, these data suggest that ABA signaling and S-type anion channel regulation in *Vicia faba* are mediated by protein kinase activation and that OA-sensitive dephosphorylation events are negative regulators of this pathway [13].

The recent identifications of the *Arabidopsis ABI1* and *ABI2* (ABA-insensitive) genes as protein phosphatases type 2C (PP2C) show that at least two additional OA-insensitive protein phosphatases affect ABA signaling [26–29]. PP2Cs are insensitive to OA, as was demonstrated in vitro for the ABI1 and ABI2 proteins [28,30]. Although several distinct protein phosphatase activities appear to affect ABA signaling (*abi1*, *abi2* and two opposing OA-sensitive phosphatases, see Section 4), no information exists whether ABA post-translationally up- or down-regulates any of these phosphatases or whether their constitutive activity affects the ABA signaling pathway, as is proposed for yeast kinase signaling cascades [31]. For example, because both stomatal closing and activation of slow anion channels in the presence of ABA were further stimulated by simultaneous addition of OA in *Vicia faba*, *Commelina communis* and tobacco guard cells [12,13,15],

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the question whether ABA down-regulates OA-sensitive phosphatases, in addition to kinase activation, remains unknown.

The findings of ABA signaling and anion channel regulation in guard cells without resolving changes in cytosolic Ca^{2+}, has led to models that include important Ca^{2+}-independent steps and pathways in stomatal closing [12,32–34]. Elevation in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) can cause enhancement of guard cell slow and rapid anion currents [5,6]. This Ca^{2+} effect on S-type channels was hypothesized to be indirect, based on 'wash-out' [5]. The strong activation of S-type anion channels by phosphorylation events at resting $[Ca^{2+}]_{cyt}$, led to suggestions that important steps in this regulation may be Ca^{2+}-independent [11–13]. However, the question whether directly buffering of $[Ca^{2+}]_{cyt}$ to nanomolar levels, under strongly phosphorylating conditions, can activate S-type anion channels remains unknown and was analyzed here.

The present study has specifically focused on the following pertinent questions relating to S-type anion channel regulation and ABA signaling:

1. Does ABA regulate S-type anion channels in *Vicia faba* guard cells?
2. Is the strong deactivation of S-type anion channels by depletion of cytosolic ATP reversible?
3. Can ABA maintain anion channel activity under ATP-free conditions in *Vicia faba*, which abolish effects of ABA-activated kinases [20–23]? ABA regulation under ATP-free conditions would allow initial cell biological insight into the question whether ABA post-translationally regulates dephosphorylation events or whether these activities are constitutive.
4. Can activation of anion channels under strongly phosphorylating conditions occur with cytosolic Ca^{2+} buffered to nanomolar levels?

The present study demonstrates that ABA maintains S-type anion channel activation in *Vicia faba* guard cells under ATP-free dephosphorylating conditions, that this regulation is reversible and that a Ca^{2+}-independent final step exists in anion channel activation.

2. Materials and methods

Guard cell protoplasts were isolated from leaves of *Vicia faba* by enzymatic digestion as described previously [7]. Pipette solutions contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA-$(Tris)_2$, 3.35 mM $CaCl_2$, 10 mM HEPES adjusted to pH 7.1 with Tris and 5 mM MgATP unless otherwise indicated. Chloride activities were 114 mM after correction for ionic activities, which lies in the upper physiological range measured in guard cells [35,36]. The osmolality was adjusted to 510 mosmol/kg by adding D-mannitol. MgATP was omitted from the pipette solution, when indicated in the text. Guard cells were bathed either in a 'Ca^{2+} bath' medium of 40 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM MES-Tris, pH 5.6 or in a 'Cs^+ bath' of 30 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM MES-Tris, pH 5.6, adjusted to 490 mosmol/kg using D-mannitol. ABA ([±]*cis-trans* isomer, Sigma) was prepared as a 50 mM stock solution in 100% ethanol and added at the indicated concentrations to the bath and/or pipette solution. The final concentration of ethanol in all experiments did not exceed 0.2% (v/v) and ethanol control experiments did not show effects.

Whole-cell recordings were performed, with an Axopatch-1D amplifier as previously described [7]. For time course analyses, 8-s voltage pulses were applied over a period of 30 min. For current-voltage analyses a voltage protocol as in the top inset of Fig. 1A was used. Peak currents at −85 mV of each individual guard cell at the indicated times were normalized relative to the maximum current amplitude measured during the whole-cell recording and expressed in percent of maximum current. Peak currents were measured 100 ms after the onset of each individual voltage pulse.

3. Results

Effects of ABA on S-type anion currents were analyzed under cytosolic ATP-free conditions. To quantitatively determine the time course of anion channel deactivation upon removal of cytosolic ATP, slow anion channel currents were recorded immediately after gaining access to the cytosol of guard cells with pipette solutions containing zero ATP. At time zero, ATP was not yet removed from the cytosol and

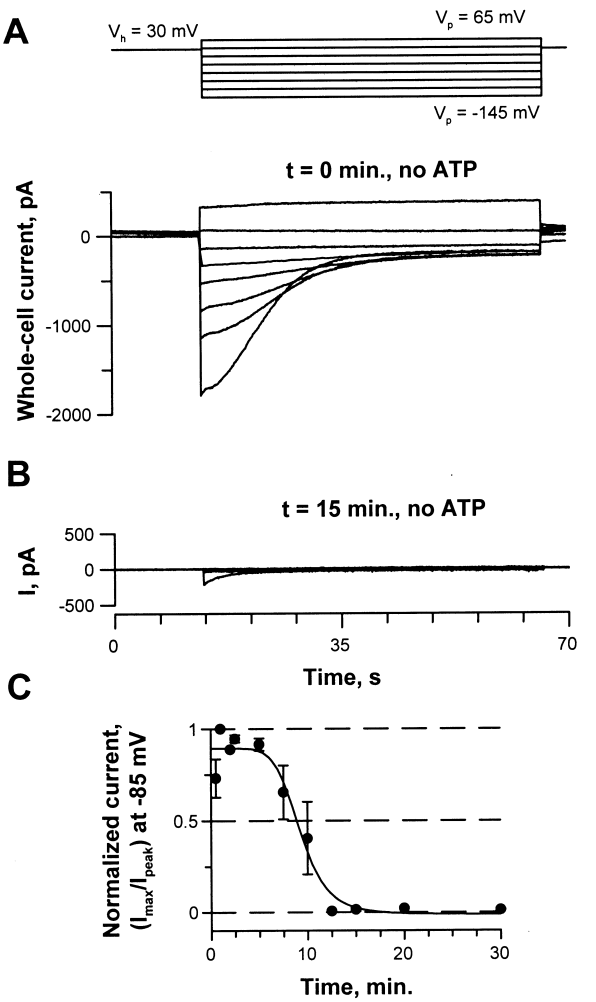

Fig. 1. Deactivation time course of slow anion currents in guard cells during depletion of cytosolic MgATP. A: Typical slow anion channel currents were observed when recordings were performed immediately after gaining access to whole cells. The membrane potential was clamped from a holding potential of $V_h = +30$ mV to potentials starting at $V_p = -145$ to +65 mV in +30 mV increments. Negative currents correspond to anion efflux in all figures. B: After 15 min of perfusing the same guard cell with MgATP-free pipette solution slow anion channel currents were deactivated. C: Time course of channel deactivation upon ATP removal ($n = 7$ guard cells averaged). Experiments were performed in the Ca bath solution (see Section 2).

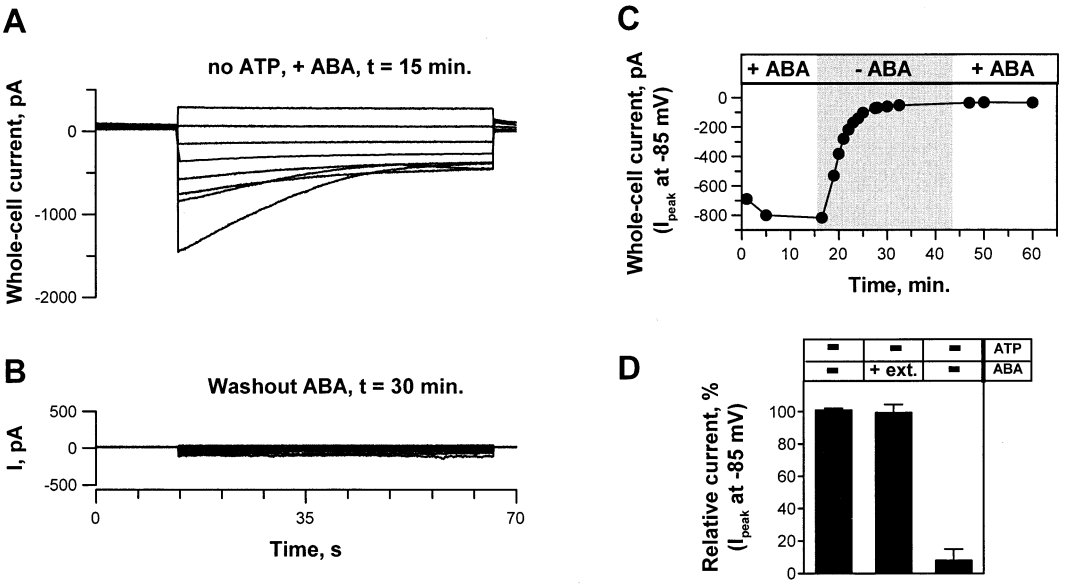

Fig. 2. ABA maintains slow anion current activity in ATP-depleted guard cells. A: Typical slow anion channel currents remain active after 15 min of gaining whole-cell access with MgATP-free solutions when guard cells were incubated in 100 μM extracellular ABA. B: Removal of extracellular ABA deactivates anion currents in the same guard cell within 15 min (30 min total recording time). C: Time course of slow anion current deactivation upon wash-out of extracellular ABA. Anion current activity was not recovered by reapplication of ABA to ATP-depleted guard cells. D: Average effects of external ABA on slow anion currents in ATP-depleted guard cells. Left column: 2 min after gaining whole-cell access with zero ATP in the pipette, anion currents were not yet deactivated. Middle column: Cells incubated in ABA and 0 ATP still show 99.3 ± 5.2% of the initial current after 15 min. Right column: Guard cells with 0 ATP which were not incubated in ABA showed strong deactivation of anion currents after 20 min of gaining whole-cell access. Average data from 23 guard cells are shown. A Cs-containing bath solution was used (see Section 2).

large S-type anion currents were fully activated by depolarization of guard cells followed by hyperpolarizing voltage pulses (Fig. 1A) as reported previously [5]. However, as the cytosol of guard cells equilibrated with the ATP-free pipette solution, anion channel current activities declined over time until >98% of the current disappeared (Fig. 1B). This strong deactivation had an average half-maximal deactivation time of 9.4 min (Fig. 1C). Controls in the presence of ATP showed reduction of anion currents by only 19 ± 8% after 15 min in this set of experiments.

Subsequent experiments were pursued to determine whether ABA can maintain S-type currents in the absence of cytosolic ATP. In the absence of cytosolic ATP, ABA-induced kinase activation [20–22] is abolished, and therefore ABA effects on dephosphorylation events can be separately analyzed. In the absence of cytosolic ATP, but in the presence of ABA, large S-type anion currents were recorded even 15 min after gaining access to the cytosol (Fig. 2A). These data suggest that anion channel deactivation events that occur under ATP-free cytosolic conditions [13] may be down-regulated by ABA. If ABA indeed down-regulates these deactivation events, subsequent removal of extracellular ABA should result in S-type channel deactivation. Removal of extracellular ABA after 15 min of sustained activity resulted in a complete decline of anion currents after a further 15 min (Fig. 2B,C). Re-application of

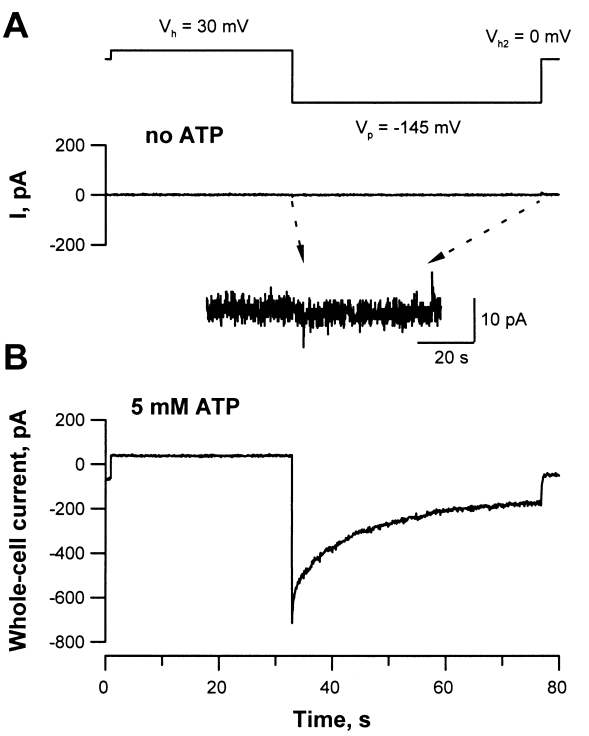

Fig. 3. Reactivation of slow anion currents in ATP-depleted guard cells by reintroducing MgATP into the cytosol. A: The whole-cell current in an ATP-depleted guard cell is shown 15 min after gaining access to the cytosol. The lower inset in A shows the same current at a higher current magnification. The arrows and spikes in the current trace indicate the on/off-set of the applied voltage pulse from +30 mV to −145 mV. B: 'Repatching' of the same cell with a 5 mM ATP-containing pipette solution shows a recovery after 8 min of gaining whole-cell access. Guard cells were bathed in 40 mM Ca solution and the same voltage pulse as in A was applied (top inset). The three guard cells tested showed reversibility of ATP depletion effects.

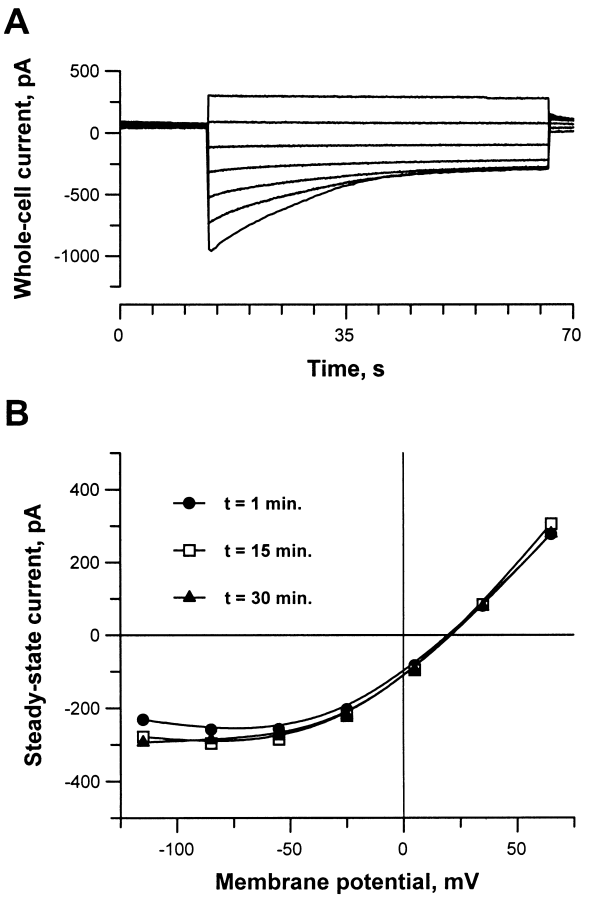

Fig. 4. A: Dialysis of guard cells with 10 mM ATP allows anion channel activation 30 min after whole-cell access, even in the presence of 10 mM BAPTA. B: Current-voltage curves showing anion current activities over time during cytosolic dialysis with ATP and BAPTA. Voltage pulses were as in Fig. 1. Solutions were as in Section 2 with 0 $CaCl_2$, 10 mM BAPTA and 10 mM ATP in the pipette solution and the Ca^{2+} bath solution was used. Seven of nine cells showed similar responses (see text).

extracellular ABA to the bath solution did not lead to a renewed activation of slow anion currents (Fig. 2C), as can be expected under cytosolic ATP-free conditions, because ATP acts as the substrate for phosphorylation [13]. On average, ATP-free cells, exposed to extracellular ABA in the Cs^+ bath solution (see Section 2) showed 99.3 ± 5.2% of the initial current after 15 min of whole-cell recording (Fig. 2D, middle column). However, cells not exposed to ABA showed a strong deactivation of slow anion currents (Fig. 2D, right column). After only 2 min of whole-cell recording deactivation had not yet occurred (Fig. 2D, left column; Fig. 1C).

The reversibility of the ATP-free-induced deactivation of S-type currents has not yet been analyzed. In order to establish whether anion channel deactivation can be reversed, the same guard cells that were depleted of ATP were patch-clamped again with new patch pipettes containing 5 mM ATP. Fig. 3A displays the lack of anion currents in guard cells which had been depleted of cytosolic ATP. The continuous access to whole guard cells was confirmed by capacitance measurements before and after all voltage pulses. After reintroduction of ATP into the cytosol of the same guard cells, large slow anion channel currents slowly appeared within 8 min, showing reversibility (Fig. 3B). Interestingly, immediately after gaining access to guard cells previously depleted of ATP, slow anion channel currents were negligible and anion currents recovered over time, which would be consistent with ATP-dependent phosphorylation events [13,20,21] governing the observed reactivation of S-type currents (Fig. 3B).

The question whether high ATP conditions can activate S-type anion currents even when $[Ca^{2+}]_{cyt}$ is buffered to nanomolar levels was analyzed. When the cytosol of guard cells was dialyzed with solutions containing 10 mM MgATP and the strong Ca^{2+} chelator BAPTA, S-type anion currents remained active over periods of 30 min in guard cells (Fig. 4A,B; $n = 7$ of 9 cells). In some cells, anion currents were reduced in the presence of high ATP and BAPTA ($n = 2$ cells, data not shown).

We then conducted a series of experiments to test whether intracellularly supplied ABA can maintain anion currents (Fig. 5). In the absence of any added ABA, the large average activity of S-type anion currents shortly after gaining access to whole cells with zero ATP in the pipette is shown in Fig. 5, column 1 for this series of experiments using the Ca^{2+} bath solution. Control cells with 5 mM MgATP added to the cytosol showed large anion current activity after 15 min (column 2). Removal of cytosolic ATP from guard cells resulted in strong deactivation of S-type anion channel currents (Fig. 5, column 3). When guard cells were exposed to extracellular ABA and cytosolic ATP was removed, 53.3 ± 22% of the initial current remained activated after 15 min in the high Ca^{2+} bath solution (Fig. 5, column 4). Note that a slow run-down of S-type anion currents was consistently observed in these experiments with high (40 mM) Ca^{2+} [6] in the bathing solution. When Cs^+ was the major cation in the bath solution, less run-down was observed with ATP in the pipette solution (e.g. compare Fig. 2D, column 2 and Fig. 5, column 4; see Section 2 for bath solutions). In spite of this Ca^{2+}-bath-medi-

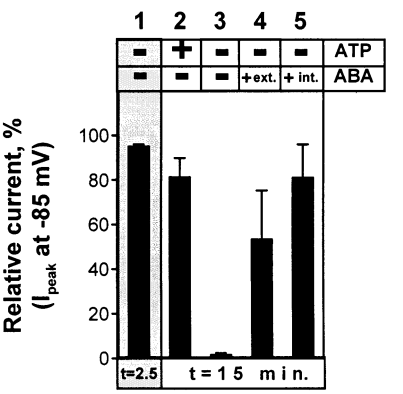

Fig. 5. Average effects of internal and external ABA on slow anion currents in ATP-depleted guard cells in the Ca^{2+} bath solution. Zero ATP control (column 1) shows the average anion current activity 2.5 min after the start of whole-cell recordings in the absence of MgATP in the pipette solution. Control guard cells supplied with 5 mM MgATP to the cytosol (ATP-control) showed after 15 min of whole-cell access an average of 81 ± 8.5% anion current activity (column 2) of the maximal current. In contrast, guard cells depleted of cytosolic ATP showed negligible currents after 15 min (column 3). When cells were incubated in a 100 μM ABA-containing bath solution and ATP was depleted from the cytosol, S-type anion currents averaged 53.3 ± 22.6% of the initial current when recordings were performed in the high Ca^{2+} bath solution (see text) (column 4), whereas guard cells in which 50 μM ABA was introduced into the cytosol via the pipette maintained 81 ± 14.9% of the initial current (column 5). Averaged data from 21 cells are illustrated.

ated run-down, when ABA was applied only to the cytosol of guard cells in the absence of ATP, 81 ± 14.9% of the initial current was maintained after 15 min (column 5). These data show that intracellularly supplied ABA could counteract S-type anion channel deactivation upon ATP depletion.

4. Discussion

Biophysical, second messenger regulation, genetic, pharmacological and stomatal movement studies have indicated an important role for anion channels during ABA signaling (see Section 1). Furthermore, recent studies have shown effects of several distinct protein phosphatases during ABA signaling in guard cells (*abi1*, *abi2*, two distinct OA-sensitive phosphatases – see below) [11–13,24–29]. However, the question has not yet been addressed as to whether ABA post-translationally regulates these phosphatase activities or whether they represent constitutive background activities impinging upon the ABA signaling pathway as proposed for yeast and mammalian kinase cascades (e.g. [31]). The fact that hydrolyzable ATP activates S-type anion channels, and that ATP-free conditions strongly deactivate these ion channels in *Vicia faba* guard cells [13] could be exploited here to analyze ABA signaling in the absence of ATP and kinase regulation.

4.1. ATP-independent ABA regulation of anion channels

Vicia faba guard cells provide an opportune system to characterize negatively regulating dephosphorylation events, because these become rate-limiting under ATP-free conditions [13]. The findings that OA enhances both ABA-induced stomatal closing and ABA activation of S-type currents [12,13,15], even after addition of saturating ABA concentrations, leaves open the question whether ABA down-regulates dephosphorylation events (see Section 1). The results obtained here show that, in ATP-depleted *Vicia faba* guard cells, ABA treatment was able to maintain S-type anion currents after 15 min of whole-cell recording at levels comparable to control cells supplied with ATP (Figs. 2 and 4). The finding that ABA mimics the previously reported OA-induced maintenance of S-type currents with zero ATP in the cytosol (Fig. 2) supports a hypothesis in which ABA down-regulates protein dephosphorylation events during stomatal closing in *Vicia faba* guard cells. This hypothesis is further supported by the fact that ABA activation of protein kinase activities in *Vicia faba* guard cells [20,21] was abolished in the present study by removal of cytosolic ATP. Thus the presented data provide cell biological evidence that ABA can post-translationally inactivate protein dephosphorylation events, while at the same time ABA activates protein kinases [20–23] during signaling.

Biochemical studies with the ABI1 and ABI2 PP2C proteins demonstrate that ABI1 and ABI2 are not sensitive to OA [28,30]. Note that because no inhibitors or null mutants for these PP2Cs are available, the question whether ABA post-translationally activates, down-regulates or does not affect the wild-type ABI1 and ABI2 phosphatase activities remains to be determined.

4.2. Evidence for counter-acting phosphatases in ABA signaling

Recent findings have shown that OA can either inhibit or enhance ABA responses in *Pisum* leaf guard cells, depending on the experiment [37]. In *Pisum*, ABA-induced stomatal closing was enhanced by OA, as previously shown in *Vicia* and *Commelina*. But in the same study, OA inhibited ABA effects on stomatal opening and also inhibited ABA induction of dehydrin mRNA [37]. OA further inhibits ABA induction of the *PHAV1* gene in barley aleurone [38]. These data suggest regulation by two distinguishable OA-sensitive phosphatase activities during ABA signaling (positively and negatively regulating OA-sensitive dephosphorylation events). In correlation with these findings, in *Arabidopsis* guard cells OA partially inhibited ABA responses [11], confirming the presence of additional OA sensitive phosphatases in the guard cell signaling cascade with opposite roles to those studied here. These recent studies on ABA signaling implicate both positively and negatively regulating protein phosphatases activities, in the early ABA transduction cascade [11,13,15,37–39]. Also, while OA inhibits inward-rectifying K^+ channels in guard cells [24,25], specific inhibitors of calcineurin phosphatases ('PP2Bs') stimulate the same K^+ channels [40]. These K^+ channel studies likewise support models of counteracting phosphatases in guard cell signaling [24,25,40]. Outward-rectifying K^+ channels have also been shown to be both activated or inhibited by phosphorylation events, depending on conditions [41].

Interestingly, in the *abi1* mutant background, the activity of negatively regulating protein kinases becomes evident [11,39], in addition to the kinase activities that positively transduce ABA signals [13,22,23]. These novel negatively regulating kinase activities seem to become clearly apparent in the *abi1* mutant background [11,39], further suggesting that they act at distinct locations from the positively regulating kinase activities [13,22,23] (for discussion see [11]). Further research will be required to determine the relative locations of these elements in the ABA signaling network, and whether ABA post-translationally modulates their activities.

A recent study showing that Ca^{2+} activation of ABA-induced genes is inhibited by the kinase inhibitor k252a has led to the suggestion that positively regulating kinase activities are either Ca^{2+}-dependent or lie further downstream as Ca^{2+}-independent steps [42]. The finding that S-type anion channel activation by large cytosolic ATP concentrations proceeds, even when cytosolic Ca^{2+} is buffered to nanomolar levels by 10 mM BAPTA provides direct evidence that final channel activation steps are Ca^{2+}-independent (Fig. 4). This result provides direct support for the hypothesis that Ca^{2+} regulation of S-type anion channels is indirect [5] and may play a role in findings showing that ABA-induced guard cell signaling can proceed without resolution of increases in $[Ca^{2+}]_{cyt}$ [12,32,33]. Thus Ca^{2+}-dependent regulation of S-type channels occurs either further upstream or in a parallel pathway (for review see [34]).

4.3. ABA application

Experiments using immunohistochemical, fluorescence staining, and photo-affinity labeling have suggested that ABA may bind to a membrane bound receptor protein [43]. Studies have provided evidence for both extracellular and intracellular sites of ABA action, with some studies not ruling out both extracellular and intracellular sites of action in guard cells [33,44–48]. ABA is taken up and accumulated by an inward directed proton gradient across the plasma membranes of guard cells [49]. We found extracellular ABA regulation of S-type anion currents at an extracellular pH of 8.0 ($n = 4$ of 8 cells), which would limit ABA uptake to a final cytosolic concentration of $\sim$60 nM [49]. The ABA sensitivity of *Vicia*

guard cells is very weak, such that 50 μM ABA, even at pH 5.5, is needed to see a partial stomatal closing response (e.g. [14,50]). Removal of ABA from the bath solution led to deactivation of S-type channels (Fig. 2B,C). On the other hand, intracellular ABA was effective at maintaining S-type anion currents in the absence of cytosolic ATP (Fig. 5). The present study shows S-type anion channel stimulation by intracellular ABA and by externally applied ABA.

4.4. Physiological regulation

Both in the present study and in recent studies of *Arabidopsis* and tobacco guard cells large dynamic ranges in ABA regulation of S-type anion channels have been demonstrated (Figs. 2 and 4) [11,12]. Stomatal opening and closing assays have suggested that the actual degree of anion channel activity in vivo in guard cells depends on the physiological state of the stomatal apertures. For example several lines of evidence suggest that complete down-regulation of guard cell anion channels in vivo may occur mainly when stomatal apertures are opened very wide [13–15,50]. Furthermore, stomata open wider when anion channels are blocked or deactivated [11,13–15,50]. These stomatal movement studies suggest that when stomata are opened to intermediate apertures by light, significant residual anion channel activities may exist, which act as negative regulators of stomatal opening (see [13–15,50]). The fine-tuning of anion channel activity depending on kinase and phosphatase activities, that can both be regulated by ABA, may be a factor contributing to stomatal apertures under different physiological conditions in vivo.

In conclusion, the present study provides direct evidence for ABA regulation of S-type anion channels in *Vicia faba* guard cells in the absence of cytosolic ATP and implicates ABA down-regulation of protein dephosphorylation events during ABA signaling, in addition to previously reported ABA activation of protein phosphorylation events [13,20–23]. Furthermore, the strong down-regulation of S-type anion channels by ATP removal is shown here to be reversed by reintroduction of ATP and final steps in ATP-dependent anion channel activation are shown to be Ca^{2+} independent suggesting that Ca^{2+} acts further upstream or in a parallel pathway.

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References

[1] Zeevaart, J.A.D. and Creelman, R.A. (1988) Annu. Rev. Plant Physiol. Mol. Biol. 39, 439–474.
[2] Giraudat, J. (1995) Curr. Opin. Cell Biol. 7, 232–238.
[3] Harris, M., Outlaw, W., Martens, R. and Weiler, E. (1988) Proc. Natl. Acad. Sci. USA 85, 2584–2588.
[4] Mittelheuser, C.J. and Steveninck, R.F.M.v. (1969) Nature 221, 281–282.
[5] Schroeder, J.I. and Hagiwara, S. (1989) Nature 338, 427–430.
[6] Hedrich, R., Busch, H. and Raschke, K. (1990) EMBO J. 9, 3889–3892.
[7] Schroeder, J.I. and Keller, B.U. (1992) Proc. Natl. Acad. Sci. USA 89, 5025–5029.
[8] Linder, B. and Raschke, K. (1992) FEBS Lett. 131, 27–30.
[9] Schroeder, J.I., Raschke, K. and Neher, E. (1987) Proc. Natl. Acad. Sci. USA 84, 4108–4112.
[10] Blatt, M.R. and Armstrong, F. (1993) Planta 191, 330–341.
[11] Pei, Z.-M., Kuchitsu, K., Ward, J.M., Schwarz, M. and Schroeder, J.I. (1997) Plant Cell 9, 409–423.
[12] Grabov, A., Leung, J., Giraudat, J. and Blatt, M.R. (1997) Plant J. 12, 203–213.
[13] Schmidt, C., Schelle, I., Liao, Y.J. and Schroeder, J.I. (1995) Proc. Natl. Acad. Sci. USA 92, 9535–9539.
[14] Schwartz, A., Ilan, N., Schwarz, N., Scheaffer, J., Assmann, S.M. and Schroeder, J.I. (1995) Plant Physiol. 109, 651–658.
[15] Esser, J.E., Liao, Y.-J. and Schroeder, J.I. (1997) J. Exp. Bot. 48, 539–550.
[16] Skerett, M. and Tyermann, S.D. (1994) Planta 192, 295–305.
[17] Thomine, S., Zimmermann, S., Guern, J. and Barbier-Brygoo, H. (1995) Plant Cell 7, 2091–2100.
[18] Cho, M. and Spalding, E. (1996) Proc. Natl. Acad. Sci. USA 93, 8134–8138.
[19] Elzenga, J.T.M. and Volkenburgh, E.V. (1997) Plant Physiol. 113, 1419–1426.
[20] Li, J. and Assmann, S. (1996) Plant Cell 8, 2359–2368.
[21] Mori, I. and Muto, S. (1997) Plant Physiol. 113, 833–840.
[22] Knetsch, M.L.W., Wang, M., Snaar-Jagalska, B.E. and Heimovaara-Dijkstra, S. (1996) Plant Cell 8, 1061–1067.
[23] Sheen, J. (1996) Science 274, 1900–1902.
[24] Li, W., Luan, S., Schreiber, S. and Assmann, S. (1994) Plant Physiol. 106, 963–970.
[25] Thiel, G. and Blatt, M.R. (1994) Plant J. 5, 727–733.
[26] Meyer, K., Leube, M.P. and Grill, E. (1994) Science 264, 1452–1455.
[27] Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F. and Giraudat, J. (1994) Science 264, 1448–1452.
[28] Leung, J., Merlot, S. and Giraudat, J. (1997) Plant Cell 9, 759–771.
[29] Rodriguez, P.L., Benning, G. and Grill, E. (1998) FEBS Lett. 421, 185–190.
[30] Bertauche, N., Leung, J. and Giraudat, J. (1996) Eur. J. Biochem. 241, 193–200.
[31] Shiozaki, K. and Russell, P. (1995) EMBO J. 14, 492–502.
[32] Gilroy, S., Fricker, M.D., Read, N.D. and Trewavas, A.J. (1991) Plant Cell 3, 333–344.
[33] Allan, A.C., Fricker, M.D., Ward, J.L., Beale, M.H. and Trewavas, A.J. (1994) Plant Cell 6, 1319–1328.
[34] Trewavas, A.J. and Malho, R. (1997) Plant Cell 9, 1181–1195.
[35] MacRobbie, E.A.C. and Lettau, B. (1980) J. Memb. Biol. 53, 199–207.
[36] Raschke, K. and Schnabl, H. (1978) Plant Physiol. 62, 84–87.
[37] Hey, S.J., Bacon, A., Burnett, E. and Neill, S.J. (1997) Planta 202, 85–92.
[38] Kuo, A., Cappelluti, S., Cervantes-Vervantes, M., Rodriguez, M. and Bush, D.S. (1996) Plant Cell 8, 259–269.
[39] Armstrong, F., Leung, J., Grabov, A., Brearley, J., Giraudat, J. and Blatt, M.R. (1995) Proc. Natl. Acad. Sci. USA 92, 9520–9524.
[40] Luan, S., Li, W., Rusnak, F., Assmann, S.M. and Schreiber, S.L. (1993) Proc. Natl. Acad. Sci. USA 90, 2202–2206.
[41] Moran, N. (1994) Plant Physiol. 105, 101.
[42] Wu, Y., Kuzma, J., Marechal, E., Graeff, R., Lee, H.C., Foster, R. and Chua, N.-H. (1997) Science 278, 2126–2130.
[43] Hornberg, C. and Weiler, E.W. (1984) Nature 310, 321–324.
[44] Gilroy, S. and Jones, R.L. (1994) Plant Physiol. 104, 1185–1192.
[45] Anderson, B.E., Ward, J.M. and Schroeder, J.I. (1994) Plant Physiol. 104, 1177–1183.
[46] Schwartz, A., Wu, W.-H., Tucker, E.B. and Assmann, S.M. (1994) Proc. Natl. Acad. Sci. USA 91, 4019–4023.
[47] MacRobbie, E.A.C. (1995) Plant J. 7, 565–576.
[48] Schultz, T.F. and Quatrano, R.S. (1997) Plant Sci. 130, 63–71.
[49] Kaiser, W.M. and Hartung, W. (1981) Plant Physiol. 68, 202–206.
[50] Schroeder, J.I., Schmidt, R.C. and Sheaffer, J. (1993) Plant Cell 5, 1831–1841.