
Plant 14-3-3 proteins assist ion channels and pumps

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Abstract

Turgor pressure is a cellular parameter, important for a range of physiological processes in plants, like cell elongation, gas exchange and gravitropic/phototropic bending. Regulation of turgor pressure involves ion and water transport at the expense of metabolic energy (ATP). The primary pump in the plasma membrane (the H⁺-ATPase) is a key player in turgor regulation since it provides the driving force for ion uptake, followed by water influx through osmosis. Using the phytotoxin fusicoccin (a well-known activator of the ATPase) as a tool, 14-3-3 proteins were identified as regulators of the H⁺-ATPase. Since fusicoccin has a dramatic effect on K⁺ accumulation and cellular respiration as well, we studied whether 14-3-3 proteins play a role in the regulation of the mitochondrial F₀F₁-ATP synthase and ion channels in the vacuolar and plasma membranes. Besides the plasma membrane H⁺-ATPase, we have identified thus far at least four other transport proteins that are regulated by 14-3-3 proteins. The mechanism of regulation will be described and the possibility that 14-3-3 proteins act as coordinators of ion transporters with varied but interdependent functions will be discussed.

Introduction

Regulation of cell volume is an essential element of everyday plant life. Cell volume changes can be reversible as well as irreversible. In stomata, the volume of the guard cell pair is controlled in a reversible manner. In the light the volume rapidly increases and the specialized structure of the cell walls creates an opening between the cells, thus facilitating the exchange of CO₂ and water [1,2]. Examples of other processes driven by reversible cell volume changes are leaf movements in response to darkness (nyctinasty, e.g. in *Samanea saman*) or touch (thigmonasty, e.g. in *Mimosa pudica*). In these movements so-called motor organs or pulvini positioned at the base of a leaf

play a crucial role [3]. Differential volume changes of cells in the upper (flexor) and lower (extensor) half of a pulvinus change the curvature of the pulvinus and thus the position of the leaf. Also, the very rapid closure of leaves of the carnivorous venus flytrap (*Dionaea muscipula*) is due to a sudden change in cell volume.

On the other hand, normal plant growth is accomplished by an irreversible increase in cell size in combination with cell division. Cell division does not by itself constitute growth, it merely increases the potential for growth by increasing the number of cells that can grow. Differential, but irreversible, volume changes also underly the growth response of a plant to environmental cues. Thus roots and shoots respond to gravity (gravitropism), shoots grow towards the light (phototropism) and tendrils of cucumber twist around an object with which they come in contact (thigmotropism).

Turgor pressure, i.e. the hydrostatic pressure inside a plant cell, which is balanced by the wall pressure, is an essential element in all of the above-mentioned cell volume changes. In stomata or pulvini, changes in turgor pressure cause relatively rapid cell and organ movement. During growth, individual cell expansion is determined by the amount of turgor pressure and the extensibility of the cell walls. Turgor pressure in a plant cell develops as a result of osmosis: water molecules diffuse across the membrane down a water potential gradient. Cells can affect the water potential by means of selective uptake/release of osmotically active substances like ions, sugars, organic acids etc. Membrane-localized transport proteins (pumps, channels and carriers) are key components of this 'osmotic motor' and in view of the importance of cell volume control for the overall growth and development of a plant it is not surprising that these transporters are subject to sophisticated regulation mechanisms. Paradigm for this is the complex regulation mechanism of guard cell transporters, which respond to almost all known plant hormones, and sense light of different wavelengths and the availability of CO₂ [2,4]. In summary, when the osmotic motor is geared towards an increase in turgor pressure,

Key words: K⁺ channel, osmotic motor, patch-clamp, turgor.
Abbreviations used: SV, slow vacuolar; FV, fast vacuolar.
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primary pumps in the plasma membrane and tonoplast are activated to provide the driving force for ion uptake through ion channels or carriers and water influx follows through osmosis facilitated by the activity of aquaporins. This increase in transport must be fuelled by ATP, so an increase in oxidative phosphorylation (in the mitochondria) or photophosphorylation (chloroplasts) is required.

Fusicoccin, blue light and osmotic stress stimulate the osmotic motor

Fusicoccin, a diterpene glucoside produced by a fungus, stimulates the osmotic motor through activation of the primary pump in the plasma membrane, the H⁺-ATPase [5–8]. In 1994 it was discovered that fusicoccin binds to 14-3-3 proteins, thereby increasing the affinity of the 14-3-3 proteins for the autoinhibitory C-terminal end of the plasma membrane H⁺-ATPase [9–11]. 14-3-3 proteins are acidic proteins with a molecular mass of around 30 kDa, forming homo- and possibly hetero-dimers. After their initial discovery in 1967 a function was assigned to them in the late 1980s as regulators of tyrosine and tryptophan hydroxylases (for a review see [12]). Since then a near exponential increase in publications has shown 14-3-3 proteins to be involved in a wide range of cellular functions, thereby acting as activators/repressors, adapters and chaperones [13,14]. Phosphorylation of a serine or threonine residue within a conserved motif in the target protein ensures the physical association with a 14-3-3 dimer. This also holds for the H⁺-ATPase–14-3-3 interaction [15,16], except that the 14-3-3-binding motif in the H⁺-ATPase (QQHYpTV; where pT is phosphothreonine) is very different from the canonical motifs, RSXpSXP (where pS is phosphoserine) and RXY/FXpSXP, found in most 14-3-3 target proteins [17].

It is not only fusicoccin that affects the pump, but also environmental factors like light and osmotic stress. Kinoshita and Shimazaki [18,19] showed that during blue-light-stimulated opening of stomata in *Vicia faba* leaves, the increase in pump activity correlated well with an increase in the amount of 14-3-3 protein bound to the H⁺-ATPase. Osmotic stress, resulting in a drop in turgor, rapidly activated the pump activity (not the hydrolytic activity) of the plasma membrane H⁺-ATPase in suspension-cultured cells of sugar beet (*Beta vulgaris* L.) [20]. The 14-3-3 content of the plasma membrane strongly increased (2–3-

fold) during this treatment and it was concluded that regulation of H⁺-ATPase in the plant plasma membrane by osmotic stress is achieved via modulation of the coupling between H⁺ transport and ATP hydrolysis, and that such regulation involves 14-3-3 proteins [20].

Fusicoccin not only affects reversible volume changes of guard cells, but irreversible changes in volume, i.e. growth, as well. Examples of this are cell elongation in coleoptiles [21] and stimulation of seed germination through a promoting effect on radicle growth [22]. Besides the above-mentioned stimulatory effect on the H⁺ pump, the influence of fusicoccin on other components of the osmotic motor are well described: the net uptake of K⁺, the influx of water and increase in respiration [23]. We therefore addressed the question whether 14-3-3 proteins (alone or in combination with fusicoccin) play a role in the regulation of these components.

Mitochondrial and chloroplast ATP synthases

Although the mitochondrial and chloroplast H⁺-ATPases are evolutionarily unrelated to the plasma membrane P-type H⁺-ATPase, they do interact with 14-3-3 proteins, as was shown in barley [24]. The amount of 14-3-3 co-purifying with the synthases increased in the presence of phosphatase inhibitors, which suggests a phosphorylation-dependent interaction. Using tagged recombinant 14-3-3 protein in an overlay assay, we demonstrated that the 14-3-3 proteins interact with the catalytic β -subunit present in the extrinsic F₁ complex. Functionally, the binding of 14-3-3 seems to reduce the production or hydrolysis (the pumps are reversible) of ATP. The role of this 14-3-3 mechanism in fusicoccin-induced increase in respiration warrants further study.

Plasma membrane K⁺ channels

The first indication that 14-3-3 proteins play a role in the regulation of plasma membrane K⁺ channels came from a study wherein a 14-3-3 gene was overexpressed in tobacco [25]. The overexpressing plants showed an increase in outward K⁺ currents. Booi et al. [26] provided further evidence for such a mechanism by adding recombinant 14-3-3 protein to the cytosolic side of tomato suspension cells, which resulted in an exactly 2-fold increase in outward K⁺-currents. Since neither the voltage sensitivity nor the activation kinetics of the channel were affected, it was concluded that 14-3-3 increases the current by recruiting a population of

'sleepy' channels. Outward K^+ channels play a role in the release of K^+ ions (resulting in loss of turgor) and Blatt and Clint [27] concluded that the physiological effect of fusicoccin was partly due to the inactivation of K_{out}^+ channels. Currently we are trying to make a link between the observations

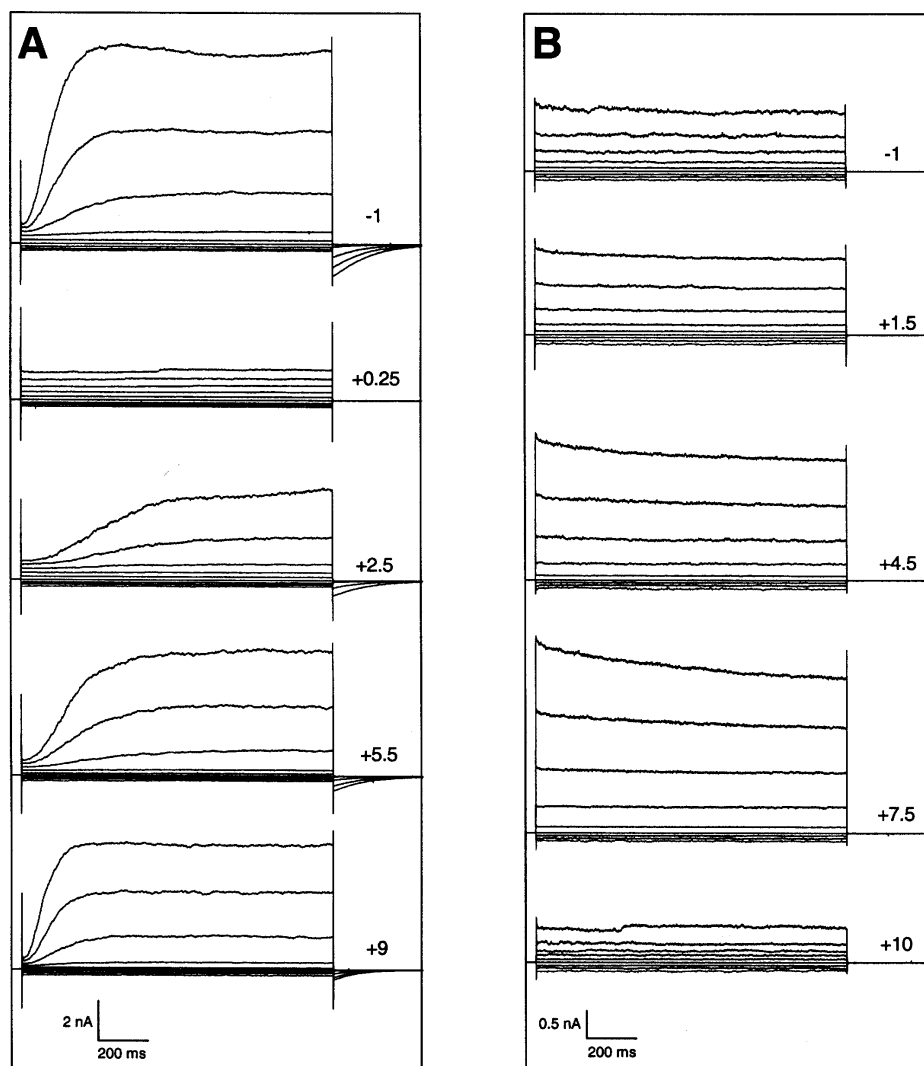
made by Blatt and Clint and the role of 14-3-3 in K_{out}^+ regulation.

Also in animal cells evidence has been provided that 14-3-3 proteins regulate the activity of ion channels. For example, the activity of the slowpoke K^+ channel (dSlo), present in *Drosophila*

Figure 1

14-3-3B protein inhibits the SV channel and activates the FV channel

(A) Modulation of SV current by 14-3-3. Protoplasts were prepared from barley mesophyll cells as described [33] and a small aliquot was released in the measuring chamber filled with SV bath solution (in mM): 95 KCl, 10 Hepes/KOH (pH 7.5), 2 $CaCl_2$ (total $[K^+]$, 101 mM). The pipette solution contained (in mM): 95 KCl, 5 EGTA, 10 Hepes/KOH (pH 7.5) (total $[K^+]$, 113 mM). The latter solution was identical to the FV bath solution. Osmolarities of all solutions were adjusted to 525 mOsm with sorbitol. Test voltages ranged from +100 to -60 mV in steps of 20 mV. Before and after test steps vacuoles were held at 0 mV. Recording and analysis conditions were essentially as described in [33]. When a stable recording was obtained, bath perfusion (0.25 ml/min) was stopped, several recordings were made to test whether this did not affect the SV or FV current and then at $t = 0$ recombinant 14-3-3B protein was added (final concentration, 100 nM). As observed before [33] the inhibitory effect of 14-3-3 was very rapid, but a partial recovery of the current was observed within 5 min. At $t = 6.5$ the bath was washed again with SV solution and the recording at 9 min shows that the magnitude of the current did not increase but the kinetics of channel activation were restored (see the -1 and +9 min recordings). (B) Modulation of the FV current by 14-3-3. Recording of the whole-vacuole FV current. After the addition of 100 nM 14-3-3 to the bath solution ($t = 0$) a steady increase in current density was observed. The 14-3-3 effect was reversible after washing (started at $t = 9.5$ min) the bath with 14-3-3 free FV solution. The numbers refer to the time (in min) before and after the moment of 14-3-3B addition ($t = 0$ min) to the bath.



presynaptic nerve terminals, is regulated by 14-3-3 proteins [28], just like Ca^{2+} -activated Cl^- channels of *Xenopus* oocytes [29].

Vacuolar channels

In most plant cells, the vacuole makes up a large fraction of the cell volume. Selective accumulation of ions or sugars across the vacuolar membrane (the tonoplast) increases the osmotic pull of water into the cell, raising the turgor pressure. So, the vacuole is an essential component of the osmotic motor. Three K^+ -conducting ion channels have been characterized in the tonoplast: VK (vacuolar K^+ selective; thus far only measured in vacuoles from guard cells), SV (slow vacuolar) and FV (fast vacuolar) channels [30].

14-3-3 down-regulates the SV channel

Regulation of the SV channel, the opening of which is time- and voltage-dependent, is highly complex. Not only is it Ca^{2+} -dependent and activated by reducing conditions [31], it is also phosphorylation-dependent [32]. The latter was for us the reason to investigate a role for 14-3-3 in SV regulation by means of the patch-clamp technique. Vacuoles were isolated from barley mesophyll cells and SV currents were recorded before and after the addition of recombinant barley 14-3-3 B protein (Figure 1A). As shown before [33], the SV current is rapidly inhibited by 14-3-3. However, after almost complete inhibition the current spontaneously recovered within minutes,

Figure 2

Time-course of SV and FV current (in)activation upon addition of 14-3-3B protein

The steady-state currents at +100 mV are shown before and after the addition of 14-3-3. 14-3-3 protein was removed by washing the bath with SV (start at $t = 6.5$) or FV (start at $t = 9.5$) solution.

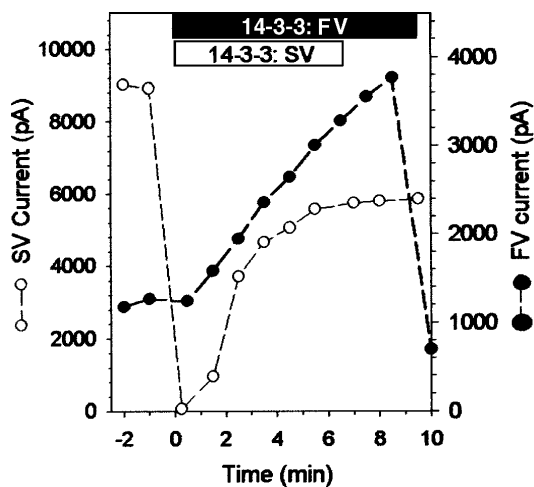
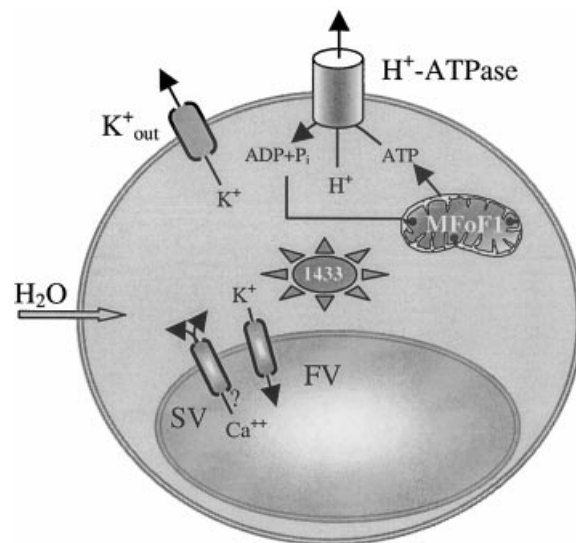


Figure 3

Model showing a number of components essential for the osmotic motor that are regulated by 14-3-3 proteins

(i) The plasma membrane H^+ -ATPase, providing the driving force for secondary transport systems, (ii) the mitochondrial or chloroplast F_0F_1 -ATP synthase (MF_0F_1) providing the fuel for the H^+ -ATPase, (iii) plasma membrane outward K^+ channels, important for the shut down of the motor, (iv) the vacuolar SV channel that may affect the activity of the FV channel through Ca^{2+} release into the cytoplasm (although this is still a matter of debate) and (v) the FV channel that may play a role in vacuolar K^+ accumulation.



although only partially. This recovery was not seen in our earlier study, which may be due to a difference in the Ca^{2+} and Mg^{2+} concentrations in the bath solution: 2 and 0 mM here, respectively, versus 0.1 and 10 mM in the study by Van den Wijngaard et al. [33]. However, even though the current recovered partially, its properties were not the same as before 14-3-3 addition. The activation kinetics of the current were markedly slower and only after washing the bath did kinetic parameters return to their initial values. Interestingly, this difference in activation kinetics resembles the 'fast' and 'slow' mode of the SV channel described by Gambale et al. [34]. The SV channel is virtually impermeable to anions [35,36], but conducts K^+ as well as Ca^{2+} . Whether it has a function in Ca^{2+} signalling or K^+ -uptake is still a matter of debate [37,38].

14-3-3 activates the FV channel

The activity of the FV channel becomes manifest at physiological Ca^{2+} concentrations, i.e. concentrations < 100 nM [30,39]. So, after recording the SV current, the FV current was activated in the

same vacuole by switching to a bath solution containing 5 mM EGTA (Figure 1B). At the same time, the SV current disappeared because it was fully Ca^{2+} -dependent. The FV channel conducts monovalent cations [40]. The current was outwardly rectifying under the conditions used (Figure 1B), which allows uptake of monovalent cations from the cytosol into the vacuole.

After stabilization of the FV current, 14-3-3B protein (100 nM) was added to the bath. In contrast to the inhibitory effect on the SV current, 14-3-3 activated the FV current (4-fold after 7.5 min) in a time-dependent fashion. Washing the bath showed that this effect was reversible. Figure 2 shows the time lag of the effect of 14-3-3 on the SV and FV currents. Whereas the inhibitory effect on the SV current was almost instantaneous (in one recording full inhibition occurred between two consecutive sweeps; < 3 s), the activation of the FV current was relatively slow.

Summary

In animal cells 14-3-3 proteins attract intense attention because of their role in numerous signalling pathways, apoptosis, gene expression, embryo development etc. In plants they have a major function in the regulation of carbon and nitrogen metabolism [41]. From our recent studies a new function for plant 14-3-3 proteins is emerging in the regulation of key ion transporters: the plasma membrane H^+ -ATPase and outward rectifying K^+ channel, the ATP synthases and the ubiquitous SV and FV channels in the vacuolar membrane. All these transporters participate in one way or another in the functioning of the osmotic motor in plants [42]. Figure 3 gives an overview of those components of the osmotic motor that thus far have been shown to be regulated by 14-3-3 proteins. Currently, we are studying the regulation of inward rectifying K^+ channels and aquaporins by 14-3-3 to complete this picture. So far, we can only speculate whether the up- or down-regulation of the osmotic motor is achieved through the concerted control of 14-3-3 over the different components. This and the question of 14-3-3 isoform specificity is the challenge of the near future.

This work was supported by grant 00-1021 from INTAS in the framework of the Aral Sea Basin call and STW/ALW grant, nos. 790.43.850/805.22.765. Rainer Hedrich and Gerald Schönknecht are gratefully acknowledged for providing the facilities and support to initiate the study of 14-3-3 regulation of vacuolar ion channels.

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Received 14 March 2002