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Slow vacuolar channels from barley mesophyll cells are regulated by 14-3-3 proteins

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Abstract **The conductance of the vacuolar membrane at elevated cytosolic Ca^{2+} levels is dominated by the slow activating cation selective (SV) channel. At physiological, submicromolar Ca^{2+} concentrations the SV currents are very small. Only recently has the role of 14-3-3 proteins in the regulation of voltage-gated and Ca^{2+}-activated plasma membrane ion channels been investigated in *Drosophila*, *Xenopus* and plants. Here we report the first evidence that plant 14-3-3 proteins are involved in the down-regulation of ion channels in the vacuolar membrane as well. Using the patch-clamp technique we have demonstrated that 14-3-3 protein drastically reduces the current carried by SV channels. The current decline amounted to 80% and half-maximal reduction was reached within 5 s after 14-3-3-addition to the bath. The voltage sensitivity of the channel was not affected by 14-3-3. A coordinating role for 14-3-3 proteins in the regulation of plasma membrane and tonoplast ion transporters is discussed.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: 14-3-3 protein; Barley mesophyll; Patch-clamp; Plant vacuole; Slow activating vacuolar channel

1. Introduction

Plant vacuoles are multipurpose compartments with a wide range of functions, which are dictated by the respective cell-type, organ or developmental stage. These functions range from the sequestration of (toxic) ions/metabolites, the generation of turgor pressure, pH homeostasis, protein degradation or the formation of flower color. Membrane transport processes are instrumental in all of these functions and not surprisingly vacuolar membranes contain a large number of transporters [1,2]. The slow activating vacuolar (SV) channel was the first tonoplast channel to be characterized [3]. The channel is ubiquitous in most higher plants and in view of the multitude of factors that regulate this channel, it is likely an essential element in most cells. Despite extensive research over the past years the physiological function of the SV channels is not clear yet. The channel is permeable for both K^+ and Ca^{2+} ions, although the selectivity may vary with cell-type, developmental stage or experimental conditions [4–6]. Ward and Schroeder [6] proposed a role for the SV channel in Ca^{2+}-induced Ca^{2+} release, but Pottosin et al. [7] concluded that the SV channel is not suited for this. One problem in assigning the SV channel a physiological role is the voltage range over which the SV channel activates. The SV channel is voltage-gated and in whole-vacuole patch-clamp experiments it activates at (cytosol) positive voltages [4,6–10]. However, in most plant cells ATP- and PPi-dependent H^+-pumps generate a small cytosol-negative electrical potential across the vacuolar membrane [11].

A second factor that influences the SV channel is Ca^{2+}. Cytosolic Ca^{2+} strongly stimulates channel activity [4,6,7, 10,12], whereas vacuolar Ca^{2+} decreases single channel current in barley mesophyll vacuoles [7]. In *Fava* bean guard cell vacuoles Mg^{2+} in the cytosol sensitized the SV channels to more physiological Ca^{2+} concentrations and shifted the half-activation potential to more negative (physiological) potentials [12]. Other factors known to affect the activity of the SV channel are calmodulin, protein phosphatases and kinases, and the redox state of the channel [5,10,13–16].

14-3-3 proteins have been shown to be involved in the regulation of K^+ and Cl^- channels in plant, *Drosophila* and *Xenopus* cells [17–20]. Plant 14-3-3 proteins are produced by a family of highly conserved genes of around 10 members [21,22]. The recurrent theme in the mechanism of 14-3-3 action is that 14-3-3s physically interact with target proteins specifically phosphorylated on a conserved serine or threonine residue [23,24]. Here we report that the SV channel of barley mesophyll cells is affected by the barley 14-3-3B protein, resulting in an 80% reduction of current.

2. Materials and methods

2.1. Expression and purification of 14-3-3B

A *Bam*H1 fragment of the barley 14-3-3B isoform was cloned into the pPinpoint vector (Promega, USA) and the orientation and reading frame checked by sequencing. JM109 cells were grown overnight at 37°C in 20 ml LB containing 50 μg/ml ampicillin. 10 ml was used to inoculate 500 ml LB containing 50 μg/ml ampicillin and the cells were grown at 37°C for 8–10 h until the OD_{600} was between 0.6 and 0.8. Expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM. Cells were grown for 4 h at 28°C in the presence of IPTG. Cells were pelleted at $5000\times g$ for 15 min and subsequently the manufacturer's instructions were followed for the production of cell lysate and purification of the recombinant protein. Briefly, the biotin-labeled recombinant 14-3-3B was purified using monomeric avidin and the biotinylated peptide of

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13 kDa was removed by overnight incubation with protease Factor Xa. The 14-3-3B was purified from the biotinylated peptide and protease by anion exchange chromatography. Fig. 1 shows a Coomassie stained SDS–PAGE gel with the purified 14-3-3B–biotin and 14-3-3B after Factor Xa cleavage.

2.2. Isolation of barley mesophyll vacuoles

Barley (*Hordeum vulgare* cv. Alexis) plants were grown in a controlled environment growth chamber (Weis Technik, Germany), with a 14 h light/10 h dark cycle and a day/night temperature of 20 and 18°C, respectively. Protoplasts were isolated from the primary leaf of 7–13-day-old plants. First the epidermal layer was stripped and then the leaves were floated with the exposed mesophyll cell layer on an enzyme solution containing 1 mM $CaCl_2$, 500 mM sorbitol, 0.05% (w/v) polyvinylpyrrolidone, 15 mM MES/Tris pH 5.5, 0.2% (w/v) bovine serum albumin, 1% (w/v) cellulase, 0.5% (w/v) Macerozym, 0.01% (w/v) pectolyase, and agitated for 30 min. To release protoplasts the leaves were gently pressed on a mesh and protoplasts were suspended in 100 mM KCl, 10 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, sorbitol to 525 mOsm/kg. For the patch-clamp experiments vacuoles from spontaneously bursting mesophyll protoplasts were used.

2.3. Electrophysiological experiments

Channel currents were studied using, in analogy to the whole-cell configuration, the 'whole-vacuole' configuration [25]. Currents were measured with an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA) and low-pass filtered using a four pole Bessel filter (internal filter of the Axopatch). The data were digitized using a Digidata 1320A (Axon Instruments, USA) or CED 1401 (Cambridge Electronic Design, UK) and analyzed with pClamp 8.0 (Axon Instruments, USA) or Patch and Voltage Clamp (Cambridge Electronic Design, UK) software. Patch pipets were pulled from Kimax-51 glass (Kimble Products, USA) and briefly fire-polished. Pipettes were filled with 100 mM KCl, 10 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, sorbitol to 525 mOsm/kg. The bath was flushed with the same solution supplemented with 100 μM $CaCl_2$ using a peristaltic pump at 0.2 ml/min. For the Ca^{2+} experiments the bath contained different concentrations of $CaCl_2$ as indicated in the text. Before 14-3-3 protein was added to the bath, the perfusion was stopped and a number of pulse protocols were run to test whether the SV current remained stable. Then, 5 μl 14-3-3 was added to the bath (volume 0.2 ml) from a stock solution (0.25 mg/ml in 10 mM HEPES/KOH, pH 7.5) giving a final concentration of 100 nM 14-3-3B protein. As control, the same volume of buffer was added and this had no effect on SV characteristics. The voltage convention described for plant endomembranes was used [26]. Data were fitted using SigmaPlot 5.0 (SPSS, USA). Data are given as mean ± S.D.

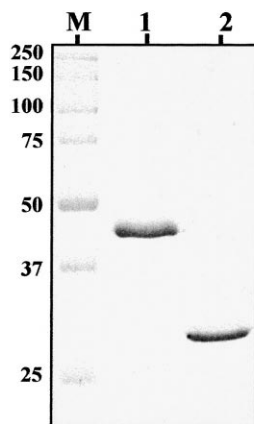

Fig. 1. Purification of biotin-tagged and tag-free 14-3-3B protein from barley. Coomassie-stained SDS–PAGE gel showing biotin-labeled 14-3-3B eluted from monomeric avidin and 14-3-3B after cleavage of the biotin label by Factor Xa. Lane M, molecular weight markers; lane 1, 14-3-3B-biotin; lane 2, cleaved 14-3-3B.

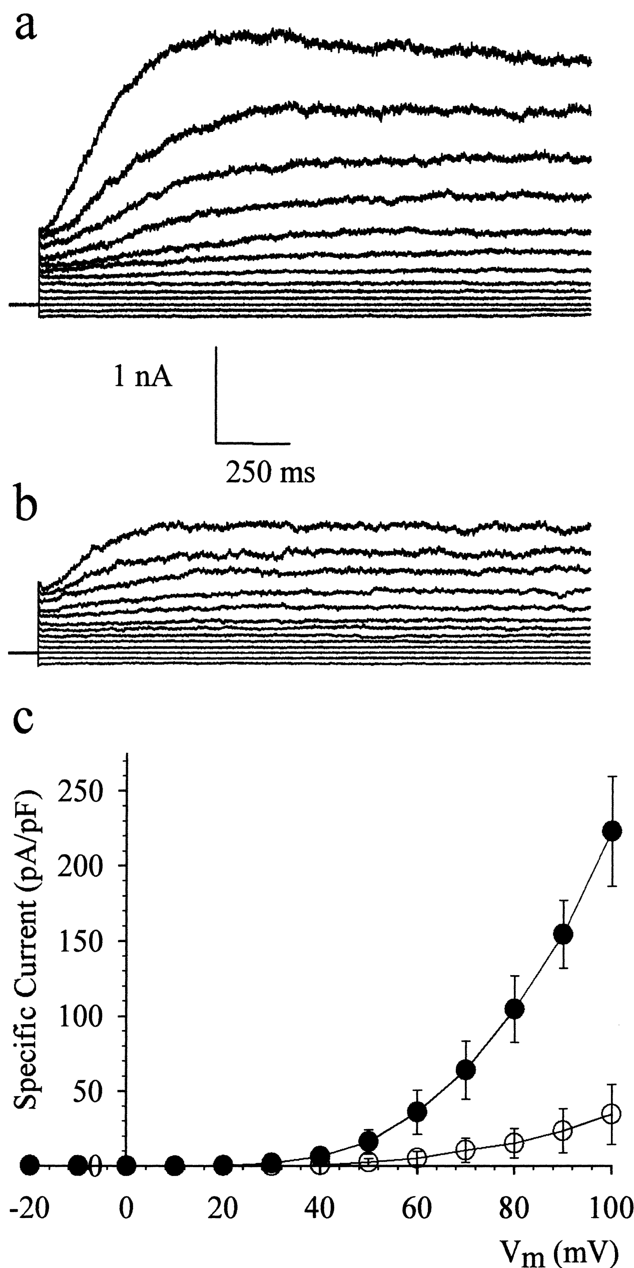

Fig. 2. Whole-vacuole SV channel current is drastically reduced by 14-3-3B. Whole-vacuole current recordings in the absence (a) and presence (b) of 100 nM 14-3-3B. The potential was held at 0 mV and subsequently stepped from +100 to −20 mV in 10 mV increments. (c) Current–voltage relationship of the time-dependent SV current in the absence (●) and presence (○) of 14-3-3B ($n = 5$).

3. Results

3.1. 14-3-3 reduces the SV current

To determine the effect of 14-3-3 proteins on the activity of the SV channel, the recombinant barley 14-3-3B was added to the bath solution, i.e. the cytosolic side of the vacuole, to a final concentration of 100 nM. The addition of 14-3-3B to the bath resulted in a dramatic decrease in SV channel current to 0.20 ± 0.14 of the current in the control situation (Fig. 2). Fig. 2a,b show typical 'whole-vacuole' current recordings of a barley mesophyll vacuole in the absence and presence of 14-3-3B, respectively. The current–voltage relationship of the SV chan-

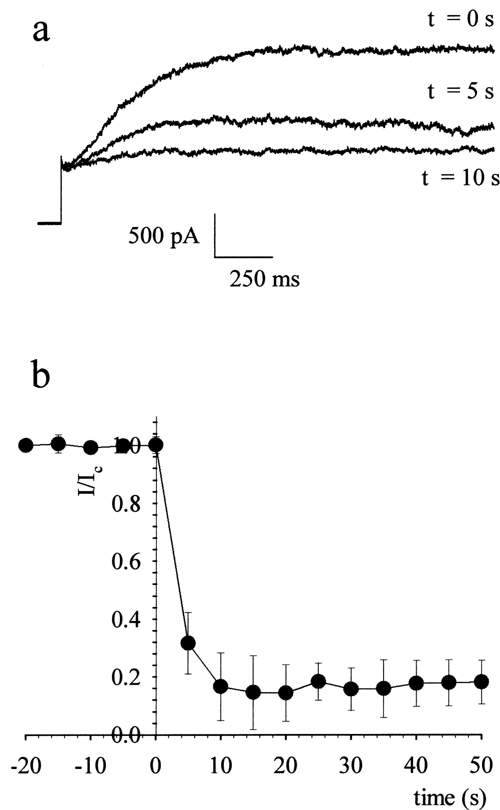

Fig. 3. Time course of 14-3-3 action. (a) Whole-vacuole current recordings at $t = 0$, 5 and 10 s after the addition of 14-3-3B to the cytosolic side of the vacuole. The vacuole was held at 0 mV and repetitively stepped to +100 mV. (b) Relative SV current before and after the addition of 14-3-3B to the cytosolic side of the tonoplast. 14-3-3B was added at $t = 0$ s and currents are expressed relative to the current at $t = 0$ s ($n = 5$).

nel in the absence and presence of 14-3-3B is shown in Fig. 2c. The block of the SV channel caused by 14-3-3B is voltage independent (Fig. 2). The kinetics of activation and deactivation were also not affected by the addition of 14-3-3B to the cytosolic side of the vacuole (Fig. 2). The decrease in SV channel current caused by 14-3-3B reached its maximum within 10 s after the addition of 14-3-3B (Fig. 3). The biotinylated recombinant 14-3-3B caused a similar decrease in SV channel current (result not shown).

3.2. *The voltage sensitivity of the SV channel is not affected by 14-3-3B*

The addition of 14-3-3B to the cytosolic side of the vacuole does not result in a change in the voltage sensitivity of the SV channel (Fig. 4). The relative degree of activation was determined from tail currents at −40 mV after activation pulses from +100 to −20 mV (Fig. 4a,b). Fig. 4c shows the voltage sensitivity of the SV channel in the absence and the presence of 14-3-3B. The $V_{1/2}$ of the SV channel was determined by fitting a Boltzmann equation to the data of Fig. 4c. This fit shows that 14-3-3B does not cause a shift in the $V_{1/2}$ of the SV channel. The fitted values for $V_{1/2}$ were 74 and 71 mV in the absence and presence of 14-3-3B, respectively. For both fits the slope factor is 16 mV.

3.3. *[Ca^{2+}]-dependence of SV activity*

Since plant 14-3-3 proteins are able to bind Ca^{2+} [27,28], we studied whether a reduction in cytosolic Ca^{2+} affected SV channel properties in a similar fashion as 14-3-3B. The Ca^{2+}-dependence of barley mesophyll SV channel has been described [7]. However, in the experiments described here a high concentration of Mg^{2+} was present, therefore the Ca^{2+}-dependence of the SV channel under these conditions was determined. Fig. 5a shows the strong reduction in SV current when the Ca^{2+} concentration is reduced from 500 to 10 μM. In contrast to the 14-3-3-induced current reduction, this Ca^{2+}-dependent reduction in current is accompanied by a change in voltage sensitivity of the channels as shown in Fig. 5b. A decrease in cytosolic Ca^{2+} was accompanied by an increase in τ_{act} 119, 170 and 234 ms at 500, 100 and 10 μM Ca^{2+}, respectively, at an activation potential of +100 mV (results

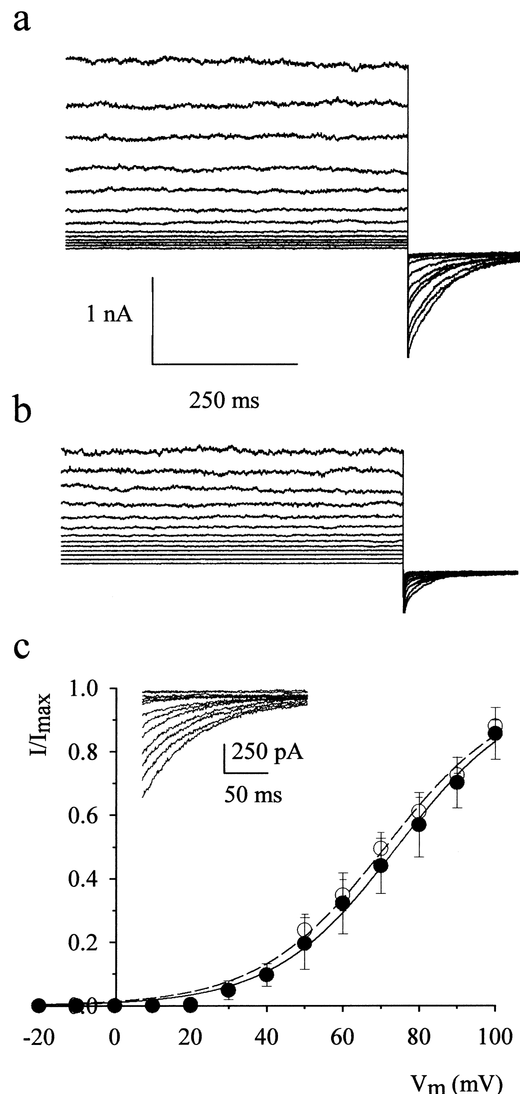

Fig. 4. Tail current analysis of the voltage sensitivity of the SV channel. Whole-vacuole current recordings in the absence (a) and presence of 14-3-3B (b), used for tail current analyses. The potential was held at 0 mV and stepped to activating potentials ranging from +100 to −20 mV in 10 mV increments. Tail currents were determined directly after stepping the voltage from the activating potential to −40 mV. (c) Voltage sensitivity of the SV channel in the absence (●) and presence (○) of 14-3-3B. The $I_{tail}/I_{tail,max}$ was determined from tail currents as shown in the inset. Lines represent the fits of the Boltzmann equation to the data ($n = 5$).

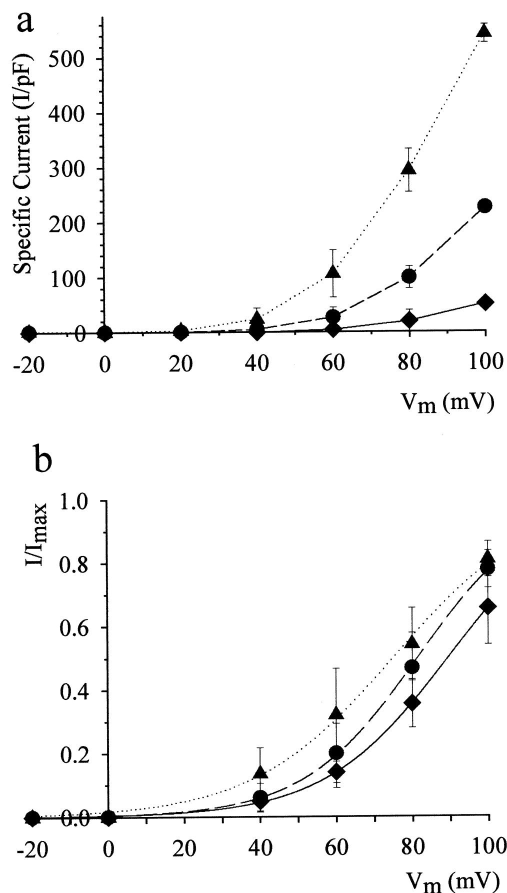

Fig. 5. A reduction of SV current at low Ca^{2+} concentrations is accompanied by a shift in activation potential. (a) Current–voltage relation of the SV channel at three different Ca^{2+} concentrations of the bath solution: 10 μM (◆), 100 μM (●) and 500 μM (▲) Ca^{2+}. (b) Voltage sensitivity of the SV channel at the three different Ca^{2+} concentrations of the bath solution. $I_{tail}/I_{tail,max}$ was determined as in Fig. 4c. Lines represent the fits of the Boltzmann equation to the data ($n = 5$).

not shown). The Ca^{2+} sensitivity of the barley mesophyll SV channel in high Mg^{2+} concentration is similar to the Ca^{2+}-dependence described [7].

4. Discussion

4.1. 14-3-3 rapidly reduces SV currents

Thus far, in plant cells only outward rectifying K^+ channels in the plasma membrane were shown to be regulated by 14-3-3 proteins [17,19]. The data presented here strongly suggest a novel role for 14-3-3 proteins in the regulation of SV channel of barley mesophyll cells. However, the effects on both channel types are very different. Whereas, the K^+_{out} current *increased* two-fold when 14-3-3 was brought into the cytosol of the cell, the addition of 14-3-3 to the cytosolic side of the tonoplast resulted in a five-fold *decrease* in SV channel current (Fig. 2). Besides this drastic reduction, the rate of the 14-3-3 effect was noteworthy: within 10 s after the addition of 14-3-3B a new steady-state was reached (Fig. 3). As a control just buffer was added to the bath and in no case was an effect observed on SV characteristics. The reduction in SV channel current caused by 14-3-3B could reflect a decrease in channel open probability and or a decrease in the single channel conductance. The experiments described here do not provide conclusive evidence for either one of these possibilities. Noteworthy is that the tagged 14-3-3 protein has the same effects as the 14-3-3 protein alone. The tag consists of a 13 kDa biotinylated peptide attached to the N-terminal end of the 14-3-3 protein, which forms the dimerization domain. In view of the size of this tag the attachment of larger constructs like green fluorescent protein to the N-terminus (e.g. for fluorescence resonance energy transfer) might be possible without compromising the 14-3-3 function.

4.2. 14-3-3 does not affect the voltage sensitivity of SV channel gating

In tomato suspension cells, 14-3-3 had no effect on the voltage sensitivity of the plasma membrane K^+_{out} channel [17]. In contrast, in *Drosophila* tsA201 cells 14-3-3ζ strongly affected the voltage sensitivity of the calcium-dependent potassium Slowpoke channel, thereby shifting the voltage at which half of the channels are activated ($V_{1/2}$) to more positive values [20]. As shown in Fig. 4, 14-3-3B does not affect the voltage sensitivity of the SV channel. The SV and Slowpoke channel share the strong reduction in current in the presence of 14-3-3: the Slowpoke current evoked by depolarization to +30 mV was inhibited by about 65% by 14-3-3ζ [20].

4.3. Mechanism of 14-3-3 action

The regulation of the *Drosophila* Slowpoke K^+ channel by 14-3-3ζ has been shown to require a third protein, the so-called Slowpoke binding protein or Slob [20]. Slob binds to the carboxy-terminus of Slowpoke and 14-3-3ζ interacts with Slob, where serine54 (RSNS^{54}AI) and serine79 (RSAS^{79}SE) were shown to be essential for binding to 14-3-3. Both serines are canonical consensus sites for phosphorylation by calcium/calmodulin-dependent kinase II. The regulation of the SV channel activity by 14-3-3 proteins can be the result of a direct interaction between the 14-3-3 protein and the channel or maybe an intermediate protein is involved as is the case with Slowpoke. On the basis of the experiments described above it is not possible to distinguish between these two models of 14-3-3 action.

4.4. Is Ca^{2+} involved in the 14-3-3 effect?

It has been shown that barley and *Arabidopsis* 14-3-3 proteins can bind Ca^{2+} ions [27,28]. If the effect of 14-3-3 is due to a local decrease in Ca^{2+} concentration in the proximity of SV channels, then it is to be expected that all parameters change in the same way. The current reduction brought about by 14-3-3 and a [Ca^{2+}] change from 100 to 10 μM (cf. Figs. 2 and 5 is about the same, but the change in voltage sensitivity is not (cf. Figs. 4 and 5). The addition of 14-3-3B did not cause a shift in $V_{1/2}$ of the SV channel. A decrease in cytosolic Ca^{2+} concentration did cause a shift in $V_{1/2}$ towards more positive potentials, similar to what has been described in the absence of Mg^{2+} [7]. Moreover, whereas 14-3-3B did not significantly affect the activation time constant (τ_{act}), a decrease in cytosolic Ca^{2+} was accompanied by an increase in τ_{act}. However, the most convincing argument that the regulation of SV by 14-3-3 and cytosolic Ca^{2+} is mediated by different mechanisms is the observation that the 14-3-3-induced effects were also observed with 2 mM Ca^{2+} in the bath (data not shown). From this we conclude that the regulation of the SV

channel by 14-3-3B cannot be explained by a (local) lowering of the Ca^{2+} concentration.

The SV channel is regulated by many cytosolic factors including the aforementioned Ca^{2+} concentration and phosphorylation state [4–7,10,12–15,29]. The high density of the SV channel in the tonoplast together with the high conductance of this channel, make a proper regulation of channel activity very important. All the factors that influence SV channel activity must be therefore tightly regulated to ensure proper functioning of the channel. Possibly, 14-3-3 proteins have a role in the interplay between the different factors regulating the SV channel activity. The regulation by 14-3-3 proteins could also provide a mechanism of linking the activity of plasma membrane K^+ channels [17,19], H^+-ATPase [30,31] and the tonoplast channels. Recently it has been shown that binding of 14-3-3 to vimentin in human cells can lead to complete sequestering of all available 14-3-3 [32]. A similar mechanism in plant cells could function to coordinate the tight regulation of different transporters by 14-3-3. The possibility that the regulation of both plasma membrane and tonoplast transporters by 14-3-3 proteins function as a way to couple the activity of these transporters is an attractive hypothesis for future investigation.

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