



14-3-3 protein regulation of proton pumps and ion channels

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Abstract

In addition to their regulation of cytoplasmic enzymes, the 14-3-3 proteins are important regulators of membrane localised proteins. In particular, many of the cells' ion pumps and channels are either directly or indirectly modulated by 14-3-3 proteins. Binding of 14-3-3 can lead to the activation of pump activity as in the case of the plasma membrane H⁺-ATPase or inhibition as in the case of the F-type ATP synthase complexes. 14-3-3 binding can also lead to surprising results such as the recruitment of 'sleepy' outward rectifying K⁺ channels in tomato cells. Our present knowledge extends to an initial understanding of isoform-specific binding of 14-3-3 to certain membrane proteins and a perception of the protein kinases and phosphatases that maintain the regulatory process in a state of flux.

Introduction

The role of phosphorylation in the regulation of enzyme activity and protein function is an area of great interest in biochemistry. Theoretically, the phosphorylation of a protein could have a direct effect on its activity but in practice phosphorylation of one protein can create a binding site for another. In recent years the 14-3-3 family of proteins have been the subject of intense study due to their high affinity for the motifs, R(S/Ar)XpSXP and RX(Ar/S)XpSXP in which pS denotes pSer/pThr and Ar denotes aromatic residues (Yaffe and Elia, 2001). The list of putative 14-3-3 binding targets increases by the week, and binding targets have been identified that do not require phosphorylation for binding (Fu *et al.*, 2000; Zhai *et al.*, 2001). In view of these findings it is important to retain a broad perspective on prospective binding partners and an understanding of the affinity of 14-3-3 protein interaction for their targets. Investigations into the number of genes coding for 14-3-3 proteins in plants has recently culminated in an article using data mining to show that 15 genes are present

in *Arabidopsis* and at least 12 expressed (Rosenquist *et al.*, 2001). Data from our own laboratory suggest that monocot plants also contain more isoforms (at least five) than at present have been documented (Sinige *et al.*, unpublished data). In plants many of these binding proteins are involved in metabolic functions such as nitrate reductase (NR) (Moorhead *et al.*, 1996) and sucrose phosphate synthase (SPS) (Toroser *et al.*, 1998; (see also Huber *et al.*, this issue). However, as we will show, a large number of membrane proteins are also regulated through reversible serine/threonine phosphorylation and subsequent 14-3-3 binding. The challenge of future years will be to show how 14-3-3 protein binding to targets is coordinated with the relevant serine/threonine kinase activation to regulate the diverse processes at the membrane and in the cytoplasm. The purpose of this review will be to analyse our present knowledge of these protein pumps and ion channels and to discuss what they tell us of the extensive roles of 14-3-3 proteins in plant cells in particular and eukaryotic cells in general.

H⁺-ATPases

The plasma membrane H⁺-ATPase

The first membrane localised protein shown to be 14-3-3-regulated is the P-type H⁺-ATPase (Korthout and de Boer, 1994; Marra *et al.*, 1994; Oecking *et al.*, 1994). This protein is an electrogenic pump that couples ATP hydrolysis to proton transport out of the cell. The resulting proton gradient provides the driving force for uptake of various nutrients such as potassium, nitrate, sulphate, sucrose and amino acids, while also playing a role in maintaining cytoplasmic pH (Sze *et al.*, 1999). In line with its physiological importance, the pump is regulated by a range of endogenous and environmental factors including hormones, cytosolic pH, light, gravity, osmotic stress and pathogens (Lohse and Hedrich, 1992; Xing *et al.*, 1996; Kinoshita and Shimazaki, 1999; Babakov *et al.*, 2000). There has been enormous attention in recent years in the elucidation of structure and function of the pump. Extensive information relating to these topics can be found in several of the following reviews (Palmgren, 1998, 2001; Morsomme and Boutry, 2000). Here, we will concentrate on the role of 14-3-3 proteins in the regulation of the pump activity and the physiological factors that modulate the 14-3-3/H⁺-ATPase interaction.

The C-terminal tail is the regulatory domain of H⁺-ATPase

The C-terminal region of the plant H⁺-ATPase is longer than its yeast counterpart and has little structural similarity (Portillo, 2000). Trypsin treatment of plant plasma membranes results in the cleavage of this C-terminal domain and the concomitant near irreversible activation of the pump (Palmgren *et al.*, 1990, 1991). These data were later supported by genetic experiments where the *Arabidopsis* H⁺-ATPase (AHA2) was expressed in yeast with and without the C-terminal 92 amino acid residues. The mutant protein supported yeast growth while the wild-type did not, which suggested the pump possesses an autoinhibitory C-terminal domain (Palmgren and Christensen, 1993). It had earlier been shown that the phytotoxin fusicoccin (FC) cause uncontrollable stomatal opening due to H⁺ extrusion followed by solute and water uptake (Aducci *et al.*, 1990). Various methods were used to isolate the FC receptor and it was shown to consist of 14-3-3 protein (Korthout and de Boer, 1994; Marra *et al.*, 1994; Oecking *et al.*, 1994). Interestingly, there

was a remarkable similarity between the physiological effect of FC and the functioning of the mutant C-terminally deleted H⁺-ATPase (Lanfermeyer and Prins, 1994). This led to a study of the role of 14-3-3 protein in the regulation of the pump, where FC was used as a tool to manipulate the interaction between the proteins and illustrate binding *in vitro*.

14-3-3 binding to the H⁺-ATPase is stimulated by FC

There is a correlation between the amount of FC applied to plants and the stimulation of the H⁺-ATPase (DeMichelis *et al.*, 1996). FC application also leads to an increase in membrane bound 14-3-3 (Korthout and de Boer, 1994; Marra *et al.*, 1996). A direct interaction was then shown to exist between the C-terminal tail of the pump and 14-3-3 protein, where fusicoccin stabilises the interaction (Fullone *et al.*, 1998; Jahn *et al.*, 1997; Oecking *et al.*, 1997; Fullone *et al.*, 1998). Thus FC was responsible for creating an activated state of the pump through an interaction with 14-3-3, which has a very high affinity for FC (Olivari *et al.*, 1998). Furthermore, co-expression in a heterologous system of a plasma membrane H⁺-ATPase and 14-3-3 protein was shown to be necessary and sufficient for the generation of a fusicoccin receptor and fusicoccin-stimulated proton pumping (Baunsgaard *et al.*, 1998; Piotrowski *et al.*, 1998). The question remained, however, of whether the phytotoxin was necessary for the interaction between the two proteins or whether 14-3-3 and the C-terminal domain formed a complex in a physiological situation that is fusicoccin-independent.

14-3-3 protein is a physiologically relevant effector of H⁺-ATPase activity

Further research localised the 14-3-3 binding site to the extreme C-terminus of the pump. It was shown that phosphorylation of the penultimate residue, threonine, was necessary for 14-3-3 binding (Fuglsang *et al.*, 1999; Svennelid *et al.*, 1999) and that addition of FC created a binding site that was virtually irreversible. Furthermore, it was shown that purified recombinant H⁺-ATPase (possessing a 6-His-tag) expressed in yeast co-purified with 14-3-3 (Maudoux *et al.*, 2000). A hypothesis was proposed where FC could somehow substitute for this threonine phosphorylation in creating a 14-3-3 binding site (Camoni *et al.*, 2000), whereas phosphothreonine was essential for FC-independent 14-3-3 binding. A recent article provides evidence to suggest that phosphorylation of the C-terminal threonine is essential for FC-independent

and -dependent 14-3-3 binding in guard cells (Kinoshita and Shimazaki, 2001). The implication is that FC stabilises the H⁺-ATPase/14-3-3 interaction in such a way that phosphatases can no longer remove the phosphate from the threonine residue. The result is that more H⁺-ATPase becomes phosphorylated which attracts more 14-3-3 to bind. This leads ultimately to irreversible activation of all the proton pumps in the cell. In a sense FC perturbs the delicate protein kinase/phosphatase balance in the cell that ensures fine-tuning of the regulation of the H⁺-ATPase.

The C-terminal 14-3-3-binding site, YTpV, is atypical as when compared to all other known 14-3-3-binding domains, RSXpSXP and RXY/FXpSXP (Yaffe *et al.*, 1997). It is argued that the proline normally associated with the binding site is not necessary in this case, as the 14-3-3 protein interacts with the terminus of the protein. Normally the α -helix of the binding protein requires the proline to exit from the 14-3-3-binding groove (Yaffe and Elia, 2001).

The presence of a second 14-3-3 binding site in H⁺-ATPase between transmembrane domains 8 and 9 has been suggested (Marra *et al.*, 2000). Whether this domain co-operates with the C-terminus in binding 14-3-3 or competes is not known. Neither is it clear whether the same or different isoforms of 14-3-3 bind to these distinct sites.

14-3-3 isoform specificity in regulation of H⁺-ATPase

Initial data suggested that any 14-3-3 protein was capable of binding to the pump in a fusicoccin-dependent manner (Baunsgaard *et al.*, 1998). However, more recently a number of techniques have been applied to look at the question of isoform-specific binding *in vitro* and *in vivo* (see also Sehnke *et al.*, this issue). Rosenquist *et al.* (2000) generated a phosphopeptide corresponding to the last 15 amino acids of the C-terminal domain of AHA2. They quantified the binding affinity of the various recombinant *Arabidopsis* 14-3-3 isoforms to the peptide by means of surface plasmon resonance analysis (Biacore). The difference in binding affinity to the peptide was used as an estimate of a difference in function between the various isoforms. The differences were striking, and in the light of previous attempts at investigating isoform specificity, rather unexpected. 14-3-3 φ gave the highest binding affinity while 14-3-3 λ did not bind at all. Rosenquist *et al.* related these differences in affinity to differences in the primary amino acid sequence of the isoforms. This conclusion was based on the good

correlation, which was obtained when the homology of seven isoforms (compared to 14-3-3 φ) was plotted against the affinity of these isoforms for the peptide (compared to 14-3-3 φ); the only exceptions being 14-3-3 ϵ and 14-3-3 ω . Considering the high homology between 14-3-3 isoforms, these results suggest that single amino acid residue changes can have large effects on function. It should be noted that in studies where the affinity of different recombinant proteins is determined by means of surface plasmon resonance analysis, mass spectrometry data and silver-stained gels should be included to be sure that the proteins have been synthesized correctly and are intact. Otherwise, observed differences in affinity may be due to the presence of inactive or degraded protein.

In *Vicia faba* it has been shown that H⁺-ATPase (VHA1) immunoprecipitates from fusicoccin-stimulated mesophyll protoplasts contain just one 14-3-3 isoform (Emi *et al.*, 2001). This is despite the fact that mesophyll protoplasts contain at least two other 14-3-3 isoforms. These data strengthen the argument that the large number of 14-3-3 isoforms in plants has a functional significance.

14-3-3 protein-mediated blue light regulation of the H⁺-ATPase

The elucidation that 14-3-3 protein is a regulator of the H⁺-ATPase in the absence of fusicoccin (Carnoni *et al.*, 2000) leads to the question of what the physiological trigger(s) is(are) for 14-3-3 binding and activation of the H⁺-ATPase.

Stomatal opening is stimulated by blue light and mediated by an accumulation of K⁺ salts in guard cells. K⁺ enters the cells through a voltage-gated K⁺ channel driven by an inside negative electrical potential created by the H⁺-ATPase. A number of elements of the signal transduction pathway leading from blue light to proton pumping have been identified (Zeiger, 2000). Blue light photons are absorbed by the carotenoid zeaxanthin, found in the antenna bed of the guard cell chloroplasts, that initiates the sensory transduction cascade (Frechilla *et al.*, 1999). Zeaxanthin undergoes an isomerization that is the first step in the signal transduction cascade. Precisely how this signal exits the chloroplast and affects the H⁺-ATPase in the plasma membrane is not known. There must however be at least two protein kinases involved in the cascade since inhibitors to different families of kinases all have their effect on blocking the cascade. The fact that more than one kinase is involved with the regula-

tion of the pump with respect to defence responses has been known for some time (Xing *et al.*, 1996). The final step is the phosphorylation of the C-terminus of the pump and subsequent binding of 14-3-3 protein (a specific isoform as mentioned above) to this region. The subsequent activation of the pump and uptake of K^+ leads to the opening of the stomata. A correlation between the amount of blue light and the amount of 14-3-3 protein bound to the pump has been shown (Kinoshita and Shimazaki, 1999). Furthermore, there was a good correlation between the amount of 14-3-3 and the activity of the H^+ -ATPase. This is compelling evidence that the 14-3-3/ H^+ -ATPase interaction plays a physiological role in the blue-light response and not just in the FC-initiated activation of the pump. This blue-light-stimulated activation of the H^+ -ATPase is not limited to guard cells, since hypocotyls of *Arabidopsis* are sensitive to blue light as well and bend in response to unidirectional blue light (Jin *et al.*, 2001). Chloroplasts have been shown to be important for this response but the role of 14-3-3 protein and phosphorylation of the H^+ -ATPase has yet to be elucidated.

Regulation of H^+ -ATPase through protein kinases and phosphatases: a role for 14-3-3 proteins?

14-3-3 protein modulates the activity of the H^+ -ATPase only in response to phosphorylation by a protein kinase. There have been numerous studies of the protein kinases that phosphorylate the pump but there is as yet no evidence of individual kinases phosphorylating the pump in response to specific signals in the cell (Figure 1). One candidate for a kinase that is involved with the 14-3-3 protein regulation of the pump is the lysophospholipid activated kinase (Scherer, 1994). The production of lysophospholipids by a phospholipase A_2 activates a membrane bound kinase that in turn activates the H^+ -ATPase. The plant growth substance auxin is the activator for the phospholipase A_2 , which is reasonable evidence for a signal transduction chain leading from auxin to activation of the proton pump. Secondly, there is the blue-light-activated serine/threonine kinase mentioned above (Kinoshita and Shimazaki, 1999). Whether this is one and the same protein kinase is unknown as this kinase has not been further characterised. Another protein kinase has been shown to be associated with the purified FC receptor fraction (van der Hoeven *et al.*, 1996). This kinase is activated by Ca^{2+} and *cis*-unsaturated fatty acids and inhibitors of the kinase (calphostin C

and chelerythrin) reduced the H^+ extrusion induced by FC treatment of plant material. This protein kinase is most likely related to the Ca^{2+} -dependent protein kinase (CDPK) family of enzymes. To complicate the picture even more another CDPKCa has been identified whose working inhibits pump activity (Lino *et al.*, 1998). This latter protein kinase most likely phosphorylates the pump at another region which leads to deactivation of pump activity. Whether 14-3-3 protein is involved in this deactivation is not known. There is also evidence in tomato of a protein kinase cascade regulating pump activity, wherein the role of 14-3-3 proteins is not known (Xing *et al.*, 1996). Tomato cells treated with race-specific fungal elicitors showed marked dephosphorylation of H^+ -ATPase and concomitant pump activation. Thus, this combination suggests that in this case 14-3-3 protein interaction with the C-terminus is not relevant. The first stages of rephosphorylation of the H^+ -ATPase were enhanced by protein kinase C (PKC) activators and prevented by PKC inhibitors (bisindolylmaleimide, calphostin C, chelerythrin and *N*-(2-guanidinoethyl)-5-isoquinolinesulfphonamide). Appearance of a second kinase was detected after 1 h and was responsible for continued phosphorylation of the proton pump. This second kinase was a CDPK and inhibited by calmodulin (CaM) antagonists (W-7) and inhibitors of Ca^{2+} /CaM-dependent protein kinase II (KN-62 and EGTA). Interestingly, the activity of the CDPK depended on the prior activation of the PKC-like kinase.

Our knowledge of phosphatases controlling the pump activity is not as extensive as for the kinases. At present a membrane-associated phosphatase has been implicated in the activation of the H^+ -ATPase (Desbrosses *et al.*, 1998). In this study the activity of partially purified H^+ -ATPase was found to be 40% by alkaline phosphatase treatment, which reduced the phosphothreonine content by 92%. The phosphoserine content was unaffected. Since lysophosphatidylcholine and trypsin treatment had no effect on the activation of the purified enzyme, it was concluded that the alkaline phosphatase treatment regulates the enzyme at a site outside the C-terminal domain. This again suggests another point of regulation besides the 14-3-3 site. There is evidence that pump dephosphorylation is necessary for pump activation in response to fungal elicitors (Vera-Estrella *et al.*, 1994). In this study fungal elicitors led to a 4-fold increase in ATPase activity of the plasma membrane and this increase could be prevented by addition of the phosphatase inhibitor okadaic acid, but not by the kinase inhibitor

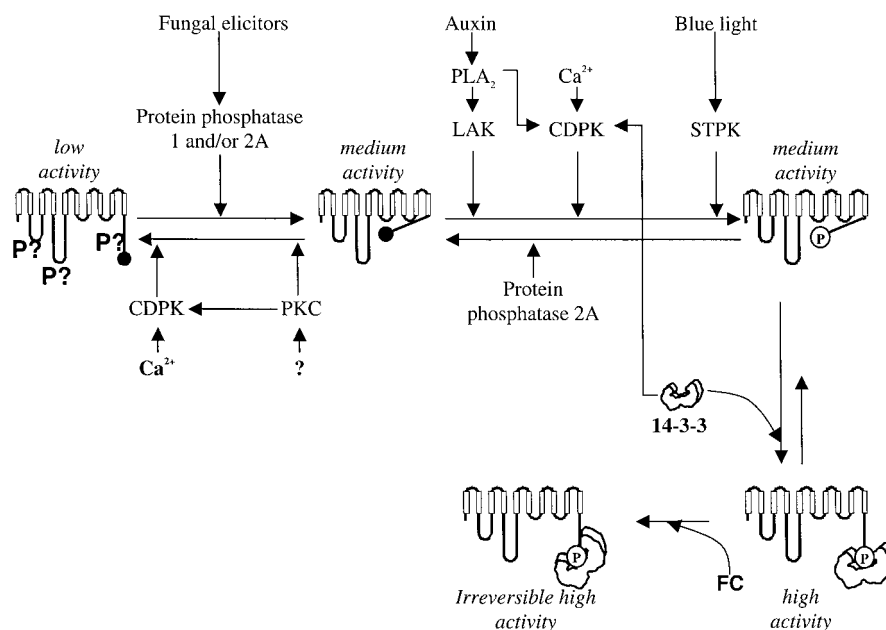


Figure 1. Schematic representation of our present knowledge of the regulation of the H^+ -ATPase. The diagram illustrates the complexity of the regulation of the proton pump and the reversibility of most of the steps involved in its regulation. The role of fusicoccin is to irreversibly activate the proton pump due to the high-affinity 14-3-3/ H^+ -ATPase interaction that is generated. LAK, lysophospholipid-activated kinase; CDPK, Ca^{2+} -dependent protein kinase; STPK, serine/threonine protein kinase; PKC, protein kinase C; P, phosphorylated serine or threonine residue; FC, fusicoccin.

staurosporine. It is clear that regulation of the H^+ -ATPase by 14-3-3 proteins and their associated kinases and phosphatases is only a fraction of the regulatory features possessed by the pump. A protein phosphatase 2A was identified in maize roots whose activity impairs the interaction between the H^+ -ATPase and 14-3-3 proteins (Camoni *et al.*, 2000). This phosphatase is likely to be a regulator of the interaction between these two proteins.

Regulation of H^+ -ATPase by auxin

The physiological relevance of the 14-3-3 protein interaction with the H^+ -ATPase has been recently shown to be linked to the blue light stimulation of the pump in guard cells and hypocotyls. There may be many more stimuli that lead to phosphorylation of the C-terminus of the H^+ -ATPase and binding of 14-3-3 protein. However, 14-3-3 proteins may not be the only regulatory factor that can bind to this C-terminal region. Recent data suggests that an auxin-binding protein (ABP₅₇) can directly regulate the H^+ -ATPase by binding to the protein (Kim *et al.*, 2001). This protein is a 57 kDa monomer (Kim *et al.*, 1998) that exists in two isoforms in the plant. The root and the shoot isoform have different auxin-binding affinities

that mirror the auxin-induced effects on tissue elongation in both the stem and the root. Cross-linking studies showed that the ABP₅₇ binds directly to the H^+ -ATPase. The ABP₅₇ exists in three conformations. In state I no IAA is bound, in state II one molecule of IAA is bound and in state III two molecules of IAA. States I and III have low affinity for the H^+ -ATPase while state II has a higher affinity. The binding characteristics of the protein are thought to be responsible for the bell-shaped auxin response curve. It still remains to be shown whether the ABP₅₇ binds at the same location as 14-3-3 and thus in essence competes for binding at this site. It is possible that the ABP₅₇ binds at a second regulatory site in the protein and its modulation of the H^+ -ATPase is unrelated to the 14-3-3 regulation.

The F-type H^+ -ATPases

It is not only the ATP consuming pumps in plant cells that are regulated by 14-3-3 proteins but also the pumps that produce the vast majority of cellular ATP. Mitochondrial and chloroplast H^+ -ATPase are evolutionarily unrelated to the plasma membrane P-type H^+ -ATPase. They are composed of an extrinsic catalytic F₁ complex, containing 5 different

subunits ($\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$), and a proton pathway, F_o , which is formed from a variable number of subunits, the stoichiometry of which is still under investigation (Schemidt *et al.* 1998; Groth and Strotmann, 1999). The use of 14-3-3 protein coated affinity columns identified the β -subunit of chloroplast ATP synthase as one of many 14-3-3-binding proteins in plant extracts (Moorhead *et al.*, 1999). It has recently been shown that 14-3-3 protein inhibits the activity of the ATP synthase in isolated mitoplasts and broken chloroplasts isolated from barley (Bunney *et al.*, 2001). Furthermore, 14-3-3 protein is associated in a phosphorylation-dependent manner with isolated ATP synthase complexes from both organelles. Far-western overlay assays identified the interacting subunit to be the β -subunit, which has recently been shown to be phosphorylated *in vivo* by a casein kinase II enzyme (Kanekatsu *et al.*, 1998). In the case of chloroplasts, it seems clear that the 14-3-3-modulated down-regulation of the ATP synthase is involved in light-dark regulation of the enzyme. This ensures that ATP is not hydrolysed in the dark when the H^+ gradient across the chloroplast membrane is not maintained by the photosynthetic H^+ pumping complexes. The implication of 14-3-3 protein within the matrix of the mitochondria (Bunney *et al.*, 2001) and the stroma of the chloroplasts (Sehnke *et al.*, 2000; Riedel *et al.*, 2001) also has implications for cross-talk between these compartments and the cytoplasm. Time will tell how many other membrane pumps are regulated by 14-3-3 and more importantly how this regulation is inter-related.

Regulation of ion channel activity by 14-3-3 proteins

Animal ion channels

Recently 14-3-3 proteins have been shown to regulate the activity of several ion channels in animal cells. In *Drosophila* presynaptic nerve terminals 14-3-3 protein regulates the activity of the slowpoke K^+ channel (dSlo) (Zhou *et al.*, 1999). This channel is an outwardly rectifying Ca^{2+} -dependent K^+ channel. A yeast two-hybrid screen identified a protein that binds to the carboxy-terminal tail of dSlo (Schopperle *et al.*, 1998). This protein was termed Slowpoke-binding protein (Slob). *In vivo* interaction between dSlo and Slob was shown by co-immunoprecipitation. Binding of Slob to dSlo increased the open probability of the

channel and this increase was the result of a shift in voltage dependence of dSlo on Slob binding (Zhou *et al.*, 1999). The channel could be activated by less depolarised voltages in the presence of Slob.

In a yeast two-hybrid screen with Slob as bait a 14-3-3 protein was identified as a Slob-binding protein (Zhou *et al.*, 1999). Co-immunoprecipitation confirmed an interaction between Slob and 14-3-3 protein. These experiments furthermore showed that 14-3-3 protein can interact with dSlo, but only in the presence of Slob. dSlo, Slob and 14-3-3 were found to be co-localised in the *Drosophila* neuromuscular junction. Heterologous expression of dSlo and 14-3-3 protein together did not result in altered activity of the ion channel. When, however, dSlo, 14-3-3 protein and Slob were expressed, the current evoked by a depolarizing step to +30 mV was inhibited by 65% as compared to the current in cells only expressing dSlo or dSlo and 14-3-3 protein. Expression of a mutant Slob, that can bind dSlo, but not 14-3-3 protein, results in currents that are identical to those in cells expressing only dSlo and Slob. Based on these results it was concluded that the effect of 14-3-3 protein on dSlo currents is due to 14-3-3 protein binding to Slob. The reduction in dSlo current at +30 mV by 14-3-3 protein was shown to be the result of a shift in voltage dependence of the channel. 14-3-3 protein binding to Slob shifts the activation of dSlo to more depolarised potentials, whereas in the absence of 14-3-3 protein Slob shifts the activation of the channel in the other direction, to less depolarised potentials.

The interaction between Slob and 14-3-3 protein was shown to be dynamically regulated *in vivo* by the Ca^{2+} /calmodulin-dependent kinase II (CaMKII) (Zhou *et al.*, 1999). In transgenic *Drosophila* constitutively expressing rat CaMKII, increased binding between Slob and 14-3-3 protein was observed. The inhibition of endogenous CaMKII by expression of a peptide inhibitor of the protein kinase resulted in a decreased binding between 14-3-3 protein and Slob. The modulation of dSlo by 14-3-3 protein can in this way be dynamically regulated by CaMKII phosphorylation of Slob and subsequent 14-3-3 protein binding.

In contrast to the direct binding of 14-3-3 protein to the dSlo-Slob complex, the Ca^{2+} -activated Cl^- channels (CaCC) of *Xenopus* oocytes are regulated by 14-3-3 protein indirectly (Chan *et al.*, 2000). Injection of 14-3-3 antisense oligonucleotides resulted in a significant increase in ionomycin-induced CaCC currents. The enhanced CaCC in the antisense injected oocytes could be inhibited by injection of 14-3-3 pro-

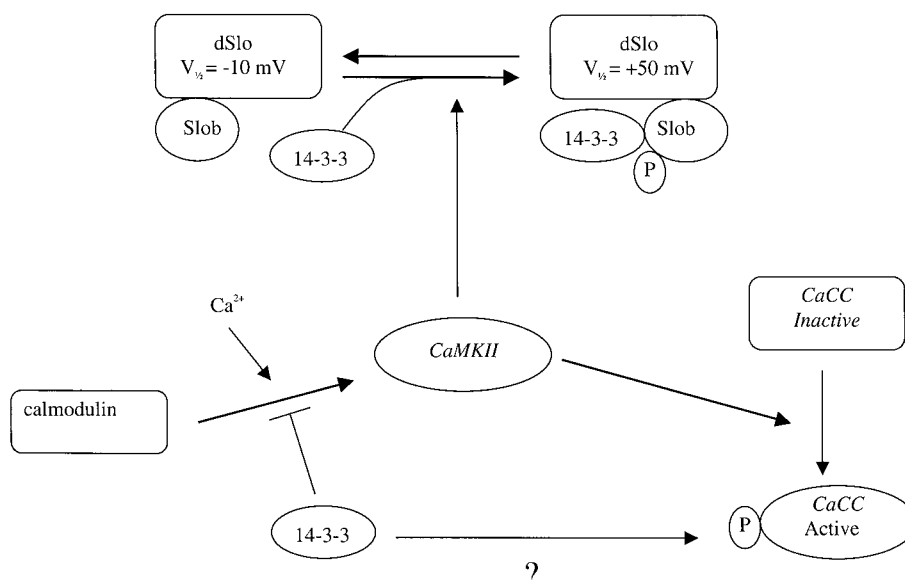


Figure 2. Schematic representation of the mechanisms of the regulation of dSlo and CaCC activity by 14-3-3 proteins. CaMKII is indicated as having a central role in the possible cross-talk between these regulation pathways. The regulator of CaMKII by 14-3-3 also provides a possible feedback loop for the regulation of dSlo activity by 14-3-3 proteins.

tein in these oocytes. This led to the conclusion that CaCC currents are inhibited by 14-3-3 protein. It was shown that the enhanced currents, in the antisense injected cells, could be reduced again by the addition of the CaMKII inhibitor W13. One explanation is that the increase in CaCC current by 14-3-3 antisense oligonucleotides is caused by an increase in CaMKII activity, because of the absence of 14-3-3 protein. It was shown that 14-3-3 proteins *in vitro* interact with calmodulin in a Ca^{2+} -dependent manner (Luk *et al.*, 1999). The regulation of CaMKII activity is thought to be the result of the interaction between 14-3-3 protein and calmodulin. Lowering the amount of 14-3-3 protein in the oocyte, by injection of antisense oligonucleotide, would release the inhibiting effect of 14-3-3 protein on the calmodulin cascade of CaMKII activation, ultimately leading to a higher activity of CaMKII and CaCC.

The interaction of 14-3-3 protein with calmodulin and thereby the regulation of the activity of CaMKII by 14-3-3 protein suggests an interesting regulation mechanism for 14-3-3 protein binding and regulation of ion channel activity. As is mentioned above, the interaction of 14-3-3 protein with dSlo-Slob and thereby the regulation of the *Drosophila* slowpoke channel is regulated by CaMKII (Zhou *et al.*, 1999). The regulation of 14-3-3 protein binding to the G-protein regulator phosducin was also shown to be regulated

by CaMKII (Savage *et al.*, 2001). However, the regulation of CaCC currents by 14-3-3 demonstrates that the activity of the kinase CaMKII itself can also be regulated by the interaction between calmodulin and 14-3-3 protein (Chan *et al.*, 2000). These findings lead to the hypothesis that the interaction between 14-3-3 and their binding proteins is modulated upstream in the signal transduction cascade by other 14-3-3 proteins interacting with kinase regulating proteins. The activity of the kinase CaMKII is regulated by 14-3-3 protein via calmodulin and this in turn regulates the phosphorylation of 14-3-3-binding partners, leading to the regulation of 14-3-3 protein binding. A schematic representation of this mechanism is shown in Figure 2, integrating the mechanisms of regulation of dSlo and CaCC by 14-3-3 proteins. The regulation of the CaMKII, the protein kinase that controls the binding of 14-3-3 protein to the dSlo/Slob complex, by 14-3-3 proteins has not been ruled out (Zhou *et al.*, 1999) nor has a direct interaction between 14-3-3 protein and CaCC (Chan *et al.*, 2000). This means that the mechanism shown in Figure 2 could function in the regulation of both dSlo and CaCC.

Plant ion channels

In plant cells 14-3-3 proteins also regulate the activity of ion channels, although the mechanisms of regulation are less well understood. Over-expression

of 14-3-3 in tobacco leads to an increase of outward K^+ currents (Saalbach *et al.*, 1997). The outward K^+ current in tomato suspension cells is also enhanced by 14-3-3 proteins (Booij *et al.*, 1999). Addition of 14-3-3 protein to the cytosolic side of tomato suspension cells led to a two-fold increase in $I_{K^+,out}$. The addition of 14-3-3 did not affect the voltage sensitivity or the activation kinetics of the channel. This led to the conclusion that 14-3-3 increases the current by recruiting a population of 'sleepy' channels, that can only be activated in the presence of 14-3-3 protein. For interaction of proteins with 14-3-3 protein, phosphorylation of the 14-3-3-binding protein is shown to be of vital importance. Phosphorylation of outward K^+ channels does influence the activity of the channels. In *Vicia faba* mesophyll and *Samanea saman* motor cells K^+ currents were shown to be enhanced by phosphorylation (Li *et al.*, 1994; Moran, 1996). There are indications that 14-3-3 protein binding protects transport proteins from dephosphorylation and thereby stabilises the phosphorylation status of the protein (Lehoux *et al.*, 2001).

Regulation of the tonoplast slow vacuolar (SV) channel by 14-3-3 protein has also been described (van den Wijngaard *et al.*, 2001). It was found that addition of 14-3-3 protein to the cytosolic side of the vacuolar membrane led to the reduction in SV channel currents. 14-3-3 protein induces an 80% reduction of this current. The voltage dependence of the SV channel was not affected by 14-3-3 proteins. The molecular mechanism of SV channel regulation by 14-3-3 is still unknown.

The molecular basis for regulation of plant ion channels by 14-3-3 proteins is less well understood than the regulation of animal ion channels. Mechanisms similar to the ones observed in animals are possibly involved. It was shown that 14-3-3 protein can regulate the activity of the plant specific calcium-dependent protein kinase (CDPK) (Camoni *et al.*, 1998). This kinase possesses a C-terminal calmodulin-like regulatory domain. It is thought that CDPKs have functions in plant cells that are comparable to the Ca^{2+} -calmodulin-dependent kinases in animal cells. This finding raises the possibility that a mechanism similar to 14-3-3 regulation of CaCC could function in plant cells. The SV channel of barley aleurone cells was shown to be regulated by CDPK (Bethke and Jones, 1997).

The regulation of the $I_{K^+,out}$ in tomato suspension cells by 14-3-3 protein does not appear to be the result of the activation of a kinase or phosphatase by 14-3-

3 protein leading to an altered phosphorylation state of the K^+ channel (Booij *et al.*, 1999). This regulation is therefore more likely to be the result of the direct interaction between the channel protein and 14-3-3 protein, either with or without the involvement of a third channel-binding protein as is the case for the regulation of dSlo (Zhou *et al.*, 1999).

Conclusions

Our present knowledge of 14-3-3-regulated membrane proteins is concentrated on the H^+ -ATPase and a number of membrane ion channels. We now have a good understanding of the role of fusicoccin in the activation of the H^+ -ATPase and know that 14-3-3 proteins are also physiologically relevant regulators at least in the context of guard cells and blue-light responses. In the context of our understanding of 14-3-3 regulation of the pump and the importance of the phosphothreonine it is important to re-evaluate and further study the importance of other serine/threonine phosphorylation of the protein. Undoubtedly, research during the next few years will uncover a wider range of membrane proteins such as transporters, antiporters as well as pumps and channels that undergo phosphorylation and 14-3-3 binding. The challenge will be to understand the protein kinases and phosphatases that are the real regulators of the pump/channel activity and to link different stimuli to cell signal transduction cascades.

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