CMLS, Cell. Mol. Life Sci. 55 (1999) 183–203
1420-682X/99/020183-21 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Ion channels in plant signaling

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Abstract. Plant ion channel activities are rapidly modulated in response to several environmental and endogenous stimuli such as light, pathogen attack and phytohormones. Electrophysiological as well as pharmacological studies provide strong evidence that ion channels are essential for the induction of specific cellular responses, implicating their tight linkage to signal transduction cascades. Ion channels propagate signals by modulating the membrane potential or by directly affecting cellular ion composition. In addition, they may also be effectors at the end of signaling cascades, as examplified by ion channels which determine the solute content of stomatal guard cells. Plant channels are themselves subject to regulation by a variety of cellular factors, including calcium, pH and cyclic nucleotides. In addition, they appear to be regulated by (de)-phosphorylation events as well as by direct interactions with cytoskeletal and other cellular proteins. This review summarizes current knowledge on the role of ion channels in plant signaling.

Key words. Abscisic acid (ABA); ion channel; membrane transport; patch-clamp; second messenger.

Introduction

Electrogenic transport processes through membranes play important roles in cells of all living organisms, from microorganisms to animals and plants. Integral membrane proteins facilitate and regulate such transport processes, thus providing a system for fine-tuning of cellular salt and metabolite concentrations. Among the different transport proteins, ion channels constitute a very large and divergent class, divided into various (sub)classes due to the transported ions or mechanisms of their regulation. For example, according to stimuli leading to activation (or deactivation), voltage-dependent, ligand-dependent and mechanosensitive ion channels can be distinguished. The functions of such channel proteins diverge, as do their regulatory mechanisms, thus providing every cell type with a specific and adapted set of transport units. Today, ion channels are thought to be involved in a number of cellular processes such as membrane potential maintenance, volume regulation and signal transduction. In animals, the propagation of excitation in nerves has been explained by a coordinated pattern of ion conductances (Na^+, K^+) leading to action potentials (for review see [1]). Since that time knowledge about ion channels and their function has been rapidly growing and recently increased with the determination of the first three-dimensional structure of a K^+ channel protein [2]. Electrophysiological methods like the patch-clamp technique [3] allow study of the electrical currents resulting from the activity of single ion channels or ensembles of channels under controlled conditions.

Compared with the impressive progress in animal cell research, knowledge concerning ion channels of plants, their structure-function relationships and their physiological role has only begun to emerge in the last few years. At present, the most extensively studied system in higher plants is the stomatal guard cell, where a set of ion channels, in association with pumps, has been shown to participate in cell volume regulation underlying the opening or closing movement of guard cells [4, 5]. In addition to this motor function in cell movement, plant ion channels are probably involved (besides pumps and carriers) in signal transduction chains responsible for the reception and transmission of stimuli essential for plant development and arising from the environment [6, 7]. As a matter of fact, signaling processes caused, for example, by plant hormones or by

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elicitors of defense responses often include early changes in membrane potential and modulation of ion fluxes [8–11]. In most cases, the transport proteins involved still have to be identified on the molecular level. By now, characterization of plant ion channels and investigation of their regulatory mechanisms constitute essential steps towards understanding their physiological roles in plant cells and their positions in signaling networks.

In this review we give an overview of the role of plant ion channels in signaling processes as well as of the regulatory mechanisms controlling their function. We summarize results concerning internal and external signals such as hormones, elicitors, light and mechanical stress which are received by plants and which are at least in part mediated by ion channel activities. In the second part, ion channel regulation by Ca^{2+}, 1,4,5-inositol trisphophate, phosphorylation and dephosphorylation, nucleotides, guanosine-5′-triphosphate (GTP)-binding proteins, pH and interactions with cytoskeletal and other proteins is discussed. Finally, what we know about ion channel action will be integrated into a picture of stomatal guard cell physiology. Besides this prominent model, of course, other experimental systems are employed to unravel the role of ion channels in plant signaling, such as *Arabidopsis* hypocotyl cells (stem elongation), *Samanea saman* pulvinar cells (leaf movements), xylem parenchyma cells (long-distance transport) and suspension culture cells (plant-pathogen interactions).

Transduction of external and internal signals mediated by ion channel activity

A series of experimental results has demonstrated the involvement of membrane potential changes and/or ion flux modulations in early responses triggered by hormones [7, 8], elicitors [10, 12], wounding [13, 14] and light [15, 16]. In terms of an involvement of ion channels in plant cell signaling, the most classic and intensively evoked idea concerns the role of calcium channels in generating membrane depolarization and intracellular calcium signals [17]. However, several examples show that the situation is more complex, suggesting also an important role of, for example, anion channels in the transduction of signals in plant cells (for review see [18]). One objective has been to gain insight into the elementary components of such signal transduction chains, namely the ion transport units involved. Given the increasing number of plant ion channel genes, questions arise concerning on the one hand electrophysiological characteristics of these channels and the regulation of their activities, and on the other, their physiological role within plant cells (fig. 1). In relation to the second point, it will be particularly interesting to understand the function of ion channels within signaling cascades. The modulation of plant ion channel activities by specific signals (elicitors, hormones etc.) may occur via direct regulation by ligand binding to the channel protein or by an indirect mechanism involving a separate receptor. In either case, direct interactions between channels and regulatory proteins can be anticipated. Such interactions may be detected by biochemi-

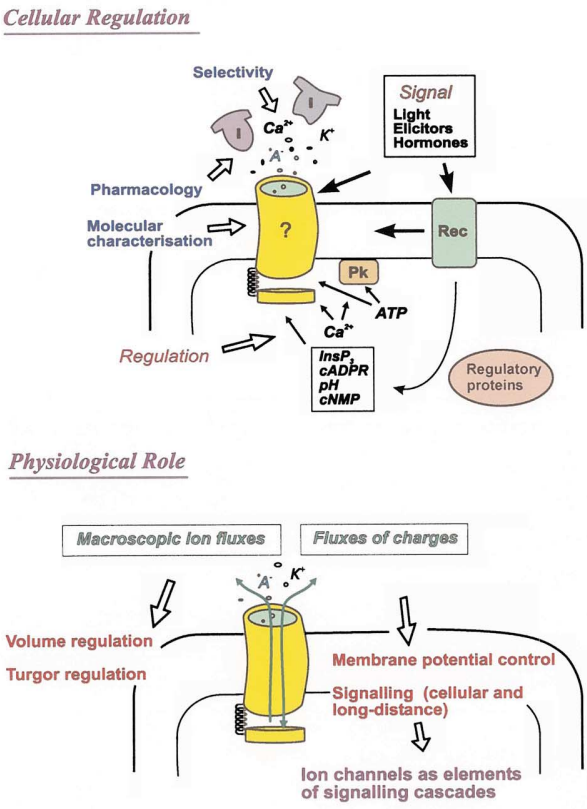

Figure 1. Cartoon depicting some of the cellular factors regulating ion channels in plants (top) and the physiological roles they have (bottom). In many cases the molecular structures of the ion channels are not yet known. Based on electrophysiological and pharmacological studies, however, a considerable number of different channel proteins exist in plant cells. Ions passing these channels are often Ca^{2+} ions, K^+ ions and anions (A^-). Regulatory proteins may include protein kinases and phosphatases, G proteins, cytoskeletal components and additional, less well characterized cellular proteins. Note that receptors (Rec) may not only be located at the plasma membrane, as shown here, but may also be present within the cellular compartment, which is, for example, the case for light-recepting molecules. Large ion fluxes are involved in the regulation of cell volume and turgor. Changes in charge distribution place ion channels at key positions within signaling cascades. $InsP_3$, inositol 1,4,5-trisphosphate; cADRP, cyclic adenosine 5′-diphosphoribose; cNMP, cyclic nucleotides; I, ion channel inhibitor; Pk, protein kinase; ATP, adenosine-5′-triphosphate.

cal techniques or by newly developed molecular–biological methods, including, for example, the yeast-based two-hybrid system [19].

Hormones

Plant hormones control plant growth, differentiation and development both synergistically and antagonistically (reviewed in [20]). Phytohormones activate cellular signaling chains after binding to specific receptors, but the precise molecular mechanisms underlying reception and signal transduction are largely unknown [21]. Despite this, electrophysiological measurements have revealed a number of effects of plant hormones on membrane potential, cellular pH, cytosolic Ca^{2+} concentrations and ion channel activities [7].

Abscisic acid (ABA), which is a phytohormone involved in plant adaptation to environmental stresses such as water deficiency, triggers signal transduction cascades leading, for instance, to reduction of stomatal aperture, thereby lessening transpirational water loss [22]. In guard cells, the activity of three major classes of ion channels (inwardly rectifying potassium [K^+_{in}], outwardly rectifying potassium [K^+_{out}] and anion channels, respectively) is modulated by ABA treatment to achieve a net ion efflux and finally stomatal closure [5]. These changes have been shown by tracer flux measurements, electrophysiological experiments and intracellular Ca^{2+} monitoring using Ca^{2+}-sensitive fluorescent dyes. ABA was demonstrated to activate nonselective Ca^{2+}-permeable plasma membrane ion channels [23]. ABA-induced elevation in cytoplasmic Ca^{2+} [24] leads to inhibition of plasma membrane K^+_{in} channels [25, 26] and to activation of tonoplast K^+ channels involved in ABA-induced K^+ release from the vacuole [27]. Additionally, Goh et al. [28] demonstrated that ABA inhibits plasma membrane H^+-ATPase, thus reducing the driving force for K^+ uptake. In patch-clamp experiments, application of ABA to the cytosol of *Vicia faba* L. guard cell protoplasts inhibits K^+_{in} currents, demonstrating an intracellular locus of phytohormone reception [29]. The activation of the plasma membrane K^+_{out} channel is mediated by an ABA-induced cytosolic alkalinization [26, 30–33]. Therefore, net loss of K^+ seems to be achieved by ABA acting at multiple sites outside and inside the cell [34]. The K^+ fluxes are tightly linked to the activation of voltage-dependent anion channels contributing to membrane depolarization and stomatal closure [35]. Activity of S-type anion channels in *Arabidopsis* guard cells was found to be upregulated by application of ABA at 1–50 μM [36]. Furthermore, pharmacological tests revealed that anion channel blockers inhibiting the activity of these slow anion channels in *V. faba* guard cells were also able to block stomatal closing in response to ABA, strongly suggesting the involvement of the *S*-type anion channel in ABA signaling [37].

New hints for transduction of the ABA signal to the channel proteins arose from the analysis of *Arabidopsis abi1-1* and *abi2-1* mutants, which were genetically identified during a screen for insensitivity to ABA. The affected genes in these two mutants encode type 2C serine/threonine phosphatases [38–40]. Both proteins play an important role in the (direct or indirect) regulation of ion channels, since activation of the *S*-type anion channel by ABA was strongly impaired in these mutants [36]. To study the influence of these phosphatases on ion channels, K^+_{in}, K^+_{out} and anion currents were monitored in guard cells of *Nicotiana benthamiana* stably transformed with the *abi1-1* dominant mutant allele. The activity of K^+_{out} channels as well as the sensitivity of both K^+_{in} and K^+_{out} channels to ABA was reduced in these transgenic plants [41]. Broad-range protein kinase antagonists restored sensitivity to ABA, implying that the ABI1 protein is part of a phosphatase/kinase pathway that modulates the sensitivity of guard cell K^+ channels to ABA-evoked signaling. In addition to these phosphatases, Li and Assmann biochemically identified a Ca^{2+}-independent serine/threonine kinase, designated AAPK, which becomes autophosphorylated and activated in dependence on ABA [42]. Thus, AAPK might play a role in the ABA signaling pathway and contribute to the phosphorylation of channel protein(s). ABA signals are transduced through both a Ca^{2+}-dependent and -independent pathway [43], and AAPK would participate in Ca^{2+}-independent signaling. A protein kinase activated by ABA in *V. faba* guard cells was also identified by Mori and Muto [44]. Very recently Cousson and Vavasseur suggested at least two possible Ca^{2+}-dependent ABA signal transduction pathways, depending on the extracellularly applied ABA concentration [45]. Both pathways require protein phosphorylation and different kinetics in the increase of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), and are thought to be initiated by distinct receptors.

The plant hormone *auxin* is involved in a number of physiological processes such as elongation growth, control of cell division and vascular tissue differentiation (reviewed in [20]). Auxin modulates $[Ca^{2+}]_{cyt}$ and cellular H^+ concentrations [46]. Electrical membrane responses to auxins preceding growth induction in various plant tissues are characterized by a transient depolarization followed by a sustained hyperpolarization [47–49], suggesting the involvement of different ion transporters. Auxin-dependent stimulation of the H^+-ATPase may result in the observed sustained hyperpolarization [50], whereas auxin-induced depolarization might be mediated by the activation of anion channels, leading to an

anion efflux. Recently, Thomine et al. [51] demonstrated that anion channel inhibitors interfere with the auxin-dependent hypocotyl elongation of *Arabidopsis* seedlings.

Auxin was shown to regulate the activity of voltage-dependent anion channels in *V. faba* guard cells [52] and tobacco suspension cells [53] indicating their involvement in auxin signaling pathways. Activation potentials were shifted in such a way that the anion channels could be active at more hyperpolarized voltages near the cellular resting potential (usually around -130 to -180 mV). The tobacco suspension cell anion channel (TSAC [53]) and the fast anion channel from stomatal guard cells (GCAC1 [54]) share certain similarities concerning voltage dependence, kinetics and modulation by auxin. Taken together, their strong voltage dependence and modulation by the hormone auxin may suggest that TSAC and GCAC1 play a role in the fine regulation of the membrane potential or in signaling cascades connected with depolarization of the cellular resting potential.

Studies on guard cells have shown that external auxin modulated the voltage gating of the fast anion channel (GCAC1) even in excised membrane patches, implying a membrane-delimited regulation of the channel [52]. Interestingly, the extracellularly applied antibody D16 raised against a synthetic peptide mimicking the putative auxin-binding site of the most abundant auxin binding protein from maize [55, 56] shifted the activation potential of TSAC like the auxin naphthalene acetic acid (1-NAA) [53]. This agonist activity of D16 provides evidence for the recognition of the auxin signal at the outer face of the plasma membrane.

In addition to the auxin-dependent regulation of GCAC1 and plasma membrane, H^+-ATPase, the activity of K^+_{in} and K^+_{out} channels has been shown to be dependent on auxin via the activation of an auxin-binding protein and the induction of cytosolic pH changes [57–59]. An auxin-induced acidification of the cytosol was found to be due to H^+ release from the vacuolar compartement in *Vicia* mesophyll and guard cells [60].

Direct evidence for the involvement of ion channels in signaling of other plant hormones is still missing, but can be anticipated in at least some cases. *Cytokinins* may be involved in stomatal movement in *V. faba* [61]. Cytokinin-induced bud formation in moss (*Physcomitrella patens*) protoplasts was explained by Ca^{2+} influx through plasma membrane Ca^{2+} channels [62, 63]. A role of Ca^{2+} entry stimulated by cytokinin was also shown for the budding process in another moss, that is *Funaria* [64]. *Brassinosteroids* were demonstrated to induce membrane hyperpolarization in the leaves of *Egeria densa* [65] and to inhibit stomatal opening in *Commelina communis* [66]. *Jasmonic acid*, described to be involved in stress and defense responses [67], was shown to inhibit stomatal opening in rice seedlings [68] as well as to induce stomatal closure in tomato [14], equally suggesting a modulation of ion channel activities.

Plant-microbe interactions

Plants must react to a wide range of biotic stimuli originating from symbiotic and pathogenic microorganisms. However, the molecular bases of the recognition events at the plant cell surface and of signaling cascades finally leading to successful interactions in the case of symbiosis or to defense responses against pathogens are largely unknown.

Plant defense reactions triggered by pathogen-derived signals, referred to as elicitors, are organized in a pattern of responses including the infected cell, the surrounding cells and a so-called systemic response. Intracellular signal transduction involves changes in the ion permeability of the plasma membrane, a generation of reactive oxygen species ('oxidative burst') and alterations in the phosphorylation status of proteins, giving rise to gene activation that leads to the synthesis of pathogenesis-related (PR) proteins. In addition, long-distance signals are produced, triggering enhanced resistance in unchallenged parts of the plants [69–71]. Active elicitor molecules have now been identified as oligosaccharides, oligopeptides, glycoproteins or lipids. Both their specific recognition and the triggering of signal-specific defense reactions point to the existence of highly specialized receptors and signal transduction chains [11, 72–74]. Although some of the early events in the signal cascades such as membrane potential changes and a variety of ion fluxes have been described, their exact role within the network of defense responses remains to be elucidated.

The nonhost resistance response of cultured parsley cells (*Petroselinum crispum*) to *Phytophthora sojae*-derived elicitor preparations (Pmg) constitutes a model system for studying such processes [75], mimicking the infection of intact leaves with zoospores. Enzymatically isolated protoplasts from parsley suspension cell cultures retain their sensitivity to the Pmg elicitor [76]. Recognition of the elicitor by its receptor, a 91-kDa plasma membrane protein, rapidly stimulated large, transient influxes of Ca^{2+} and H^+, and effluxes of K^+ and Cl^- [12, 77]. Pharmacological studies revealed that this pattern of ion fluxes is necessary for oxidative burst, gene activation and phytoalexin production [78]. Furthermore, it was recently demonstrated that the activation of a specific kinase, related to animal MAP (mitogen-activated protein) kinases, by the same recognition event also relies on these early ion fluxes [79]. Among a wide range of tested effectors anion channel

inhibitors were able to simultaneously block all investigated elicitor responses, that is Cl^-, K^+, Ca^{2+} and H^+ fluxes, as well as phytoalexin accumulation, oxidative burst, MAP kinase activation and expression of defense-related genes [77, 79]. Similar effects have also been observed in the defense reaction of soybean to the *Phytophthora* hepta-β-glucoside elicitor [69]. These results suggest a dominating role of anion channels in a network which links the different ion currents and controls the specific terminal defense reaction.

Part of the signaling chain leading to pathogen resistance is a transient influx of Ca^{2+} into the cytosol. Both omission of Ca^{2+} from the extracellular medium and application of inhibitors of Ca^{2+} channels abolished the plant defense response [11]. Patch-clamp analysis of parsley protoplasts identified a novel Ca^{2+}-permeable, La^{3+}-sensitive plasma membrane cation channel of large conductance (80 pS), even at a low extracellular Ca^{2+} concentration of 1 mM [80], which is typical for the plant cell apoplast. This channel, called LEAC (for large conductance elicitor-activated ion channel), has long open times of up to several seconds. LEAC is an excellent candidate for a transport protein mediating effective and rapid ion concentration/potential changes in signaling processes. Most important, this ion channel was reversibly activated upon treatment of parsley protoplasts with an oligopeptide elicitor (Pep-13) derived from a cell wall glycoprotein of *P. sojae*. Structural features of the peptide elicitor found to be essential for activation of LEAC were identical to those required for receptor binding, transcriptional activation of defense-related genes, and phytoalexin production in parsley cells and protoplasts [12, 81]. Thus, receptor-mediated stimulation of LEAC appears to be causally involved in the signaling cascade, triggering pathogen defense in parsley. This hypothesis is further substantiated by the fact that Ca^{2+} channel inhibitors, which efficiently inhibit LEAC, block elicitor-induced oxidative burst and phytoalexin production as well. Long open times of LEAC, its cation permeability, a large unitary Ca^{2+} conductance at physiological extracellular Ca^{2+} concentrations and the elicitor-dependent activation may account for the increase in cytoplasmic Ca^{2+} concentration and the significant macroscopic ion fluxes in elicitor-treated parsley cells. Alternatively, membrane depolarization by Ca^{2+} influx through LEAC could activate Ca^{2+} and/or voltage-dependent anion channels as well as K^+_{out} channels. The important role of Ca^{2+} permeable ion channels for signal transduction during pathogen recognition was also demonstrated for the race-specific interaction between tomato and the fungus *Cladosporium fulvum* [82]. In the reported case, only elicitor preparations from an avirulent fungal strain elicited a plasma membrane Ca^{2+}-permeable ion channel, whereas a preparation from a virulent strain did not. Since comparable macroscopic ion fluxes have also been detected in several other plants upon elicitor treatment [10, 83–87], the existence of similar Ca^{2+}-permeable ion channels can be anticipated in these species.

Using tobacco cell suspensions Mathieu et al. [10] have shown that among other early responses oligogalacturonides elicited a K^+ as well as an anion efflux. An outwardly rectifying K^+ channel in tobacco protoplasts was found to mediate the observed cellular K^+ efflux induced by the elicitor [88]. However, a direct influence of oligogalacturonides on ion channel activity could not be demonstrated in patch-clamp experiments (S. Thomine et al. unpublished results). This may reflect either an indirect regulation of the channel by the elicitor or a loss of responsiveness of the enzymatically isolated protoplasts. The tightly connected anion efflux may be mediated by the voltage-dependent anion channel TSAC [53].

Besides pathogenic interactions ion channels also seem to play a role during nodulation, through which a symbiosis between nitrogen-fixing rhizobia and legumes is established. Early recognition events include the excretion of lipochitooligosaccharides (*Nod factors*) by the bacteria, and in response to that the induction of specific legume genes involved in nodule development on the side of the plant [89, 90]. Besides that, very little is known about Nod signal perception and transduction, but it has been suggested that changes in plasma membrane potential and ion fluxes are of importance.

In this context Ehrhardt et al. [91] described the depolarization of the plasma membrane in alfalfa root hairs as one rapid response to the Nod factors of *Rhizobium meliloti*. These findings were extended by experiments performed by Felle et al. [92] and Kurkdjian [93], who could demonstrate that the rapid plasma membrane depolarization in alfalfa root hairs displays a high degree of specificity for the cognate Nod factors from *R. meliloti*. Such specificity argues for an important role of membrane potential changes in early steps of nodulation signaling.

Another hint about the role of ion channels in the establishment of symbiotic interactions came from the analysis of the nodulation signaling protein NodO from *Rhizobium leguminosarum* biovar *viciae*. It was shown that this protein, when added to planar lipid bilayers, forms cation-selective channels that allowed the movement of monovalent cations (K^+ and Na^+) across the membrane [94]. Such fluxes might participate and/or amplify the response induced by the lipooligosaccharide nodulation factor.

Although signaling processes in symbiotic interactions are less well analyzed compared with interactions between plants and pathogens, the available data point to a similar important role for ion channels during the establishment of symbiosis.

Light

Light, relevant to numerous physiological and developmental processes, rapidly modulates electrogenic transport systems in plant cells, resulting in large changes in membrane potential [15, 16, 95, 96]. These changes are mediated by activation or deactivation, respectively, of plasma membrane H^+-ATPase as well as of voltage-dependent ion channels [97, 98]. Light of different spectral qualities is recepted by different cellular molecules. Absorption of blue light may occur via cryptochrome [99, 100] or may alternatively involve zeaxanthin, as outlined by Zeiger et al. [101–103]. Red light and far-red light are absorbed by phytochromes, which have been extensively investigated at the biochemical and genetic level [104, 105].

Blue light and membrane hyperpolarization

Blue light regulates many aspects of plant development, and in several cases affects gene expression [106] and rapidly modulates plasma membrane electrical potential in various cellular systems. In *V. faba* guard cell protoplasts irradiated continuously with photosynthetically saturating red light, blue light induced the extrusion of H^+ ions [107]. A short blue light pulse of 30 s was sufficient to induce a long-lasting (10 min) H^+ extrusion, which indicated that a continuous input of blue light energy was not needed for a sustained response. The kinetics of the blue light-dependent acidification of the external medium closely matched that observed for stomatal opening in intact leaves. It was postulated that blue light activates the plasma membrane H^+-ATPase via Ca^{2+}-dependent pathways, probably also including protein phosphorylation [108–110]. The H^+ extrusion observed by Shimazaki et al. [107] was in accordance with electrophysiological studies demonstrating membrane hyperpolarization in response to blue light [97]. Acidification of the apoplastic pH in response to light was observed in other experiments as well [111, 112], although some reports also demonstrate a biphasic response to light, where medium acidification is preceded by an alkalinization, most likely involving photosynthetic processes [113, 114].

Blue light and hypocotyl elongation

In another physiological process blue light rapidly inhibits stem elongation in seedlings of many dicotyledonous species e.g. [115, 116]. In cucumber and *Arabidopsis* hypocotyls, a large and transient depolarization of the plasma membrane was evident before growth suppression [16, 117]. Blue light-induced membrane depolarization involves inhibition of plasma membrane H^+-ATPase [118] and stimulation of a plasma membrane anion channel [117]. In *Arabidopsis* the anion channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) reversibly inhibited blue light-induced channel activation and membrane depolarization and also decreased the inhibitory effect of blue light on hypocotyl elongation. The strong inhibitory effect NPPB has on blue light-activated anion channels contradicts the previous conclusion that the proton pump significantly contributes to membrane depolarization [16]. Therefore, as pointed out by Cho and Spalding [117] more direct evidence of the contribution of H^+-ATPase is needed before its contribution to the blue light-induced transient membrane depolarization can be fully assessed [117]. NPPB also strongly inhibited the blue light-induced accumulation of anthocyanin pigments in *Arabidopsis* seedlings, although expression levels of genes and proteins participating in anthocyanin synthesis were not affected by the presence of NPPB [119]. These data led to the model that blue light-induced anthocyanin accumulation involves previously undiscovered posttranslational modifications that activate the biosynthetic enzymes. Alternatively, changes in the cytosolic milieu resulting from anion channel activation, such as a change in pH, may be involved [119].
Recently it was demonstrated that the *Arabidopsis* hypocotyl anion channel depends on $[Ca^{2+}]_{cyt}$ within a concentration range of 1 to 10 μM [120], raising the possibility that blue light stimulates this channel by an elevation of $[Ca^{2+}]_{cyt}$. However, irradiation with blue light did not affect $[Ca^{2+}]_{cyt}$ in hypocotyl cells as demonstrated by luminescence measurements on transgenic *Arabidopsis* plants cytosolically expressing the Ca^{2+}-indicator protein aequorin. These experiments indicated that Ca^{2+} does not serve as a second messenger in blue light-induced anion channel activation [120]. In contrast, treatment with cold triggered an increase in $[Ca^{2+}]_{cyt}$ and transient plasma membrane depolarization. It was therefore concluded that the hypocotyl anion channel can be activated by both a Ca^{2+}-independent (blue light) and a Ca^{2+}-dependent (cold) pathway. However, it cannot be excluded that subtle changes not detectable by the aequorin system may still play a role in the blue light signal transduction pathway leading to activation of the hypocotyl anion channel.

Red light-induced membrane depolarization mediated by phytochrome

Processes regulated by the red-light receptor phytochrome include seed germination, deetiolation and photoperiodic aspects of flowering [104], as well as induction of side branch initials in caulonemal filaments of the moss *Physcomitrella patens* [121]. Several reports indicate that phytochrome exerts some of its effects via an elevation of $[Ca^{2+}]_{cyt}$ [122–124], though cyclic

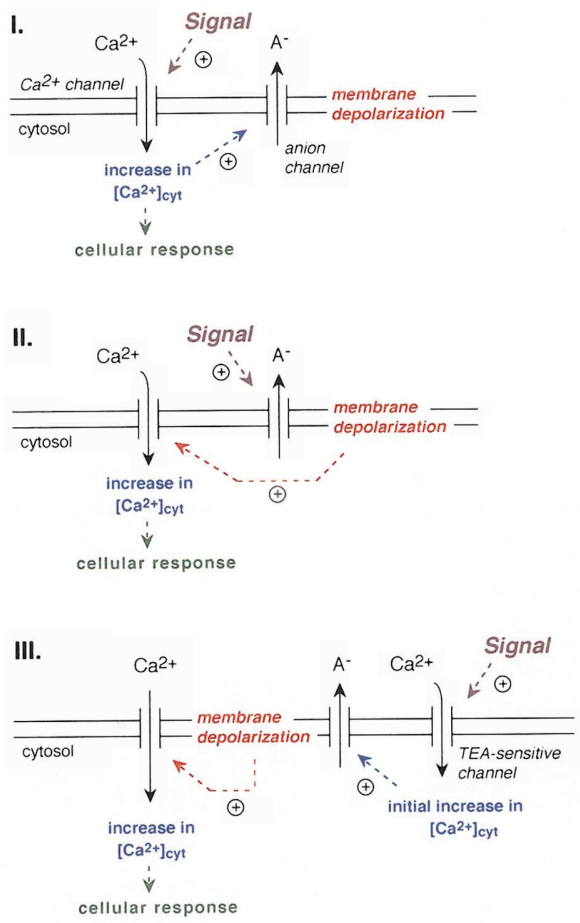

Figure 2. Models for the interactions of ion channels at the plasma membrane upon signal perception. In the first model, activation of anion (A^-) channels and thereby membrane depolarization results from an elevation of $[Ca^{2+}]_{cyt}$, which is caused by signal-induced opening of Ca^{2+} channels. In the second model, the signal stimulates anion channels, leading to membrane depolarization, which in turn triggers Ca^{2+} channel opening, allowing influx of Ca^{2+} from the extracellular medium. The third model suggests signal-dependent stimulation of a TEA-sensitive ion channel, through which an initial influx of external Ca^{2+} may occur. The resulting (small) increase in $[Ca^{2+}]_{cyt}$ subsequently activates the anion channels which, through membrane depolarization, open voltage-gated Ca^{2+} channels. The resulting further elevation in $[Ca^{2+}]_{cyt}$ induces downstream cellular events. This figure was modified from an original presentation in Ermolayeva et al. [128].

guanosine-5′-monophosphate (cGMP) has been shown to be involved in others [125]. An important point is that early events in phytochrome-mediated signaling often involve a transient depolarization of the plasma membrane e.g. [96, 126]. Models have been developed to explain the precise relationship (i.e. temporal order) between membrane depolarization and elevation in $[Ca^{2+}]_{cyt}$ (fig. 2). In one scheme entry of Ca^{2+} occurs first, followed by the activation of Ca^{2+}-sensitive anion channels [127]. In an alternative figure presented by Ward et al. [18], anion channels activate in response to a stimulus (such as red light), and thereby depolarize the plasma membrane potential, which in turn activates voltage-gated Ca^{2+} channels. Based on a detailed investigation of phytochrome-mediated membrane depolarization during *Physcomitrella* caulonemal side branch initial formation, Ermolayeva et al. [128] evolved a more complex scheme, adding a nonselective, TEA-sensitive cation channel (TEA = tetraethylammonium) as a further component of the reaction chain [128]. In this model, red light activates the nonselective cation channel, allowing an initial (small) influx of Ca^{2+} from the external medium. A minimal rise in $[Ca^{2+}]_{cyt}$ activates the anion channel, which in turn leads to membrane depolarization and thereby triggers the opening of voltage-gated Ca^{2+} channels, resulting in a further elevation of $[Ca^{2+}]_{cyt}$. This model therefore places the anion channel at a central position within a signal transduction chain that employs Ca^{2+}-induced Ca^{2+} influx for initiating the phytochrome response. As pointed out by the authors [128], such a model could form the basis for responses to stimuli other than red light, such as ABA, elicitors and blue light.

In another experimental system, that is the alga *Mougeotia*, red light regulates the positioning of the single large chloroplasts present in these cells via a phytochrome-dependent pathway that most likely involves a modulation of $[Ca^{2+}]_{cyt}$ [129, 130]. Although an activation of ion channels has been demonstrated to occur in response to red light irradiation [131, 132], the precise relationship between perception of red light, channel activation and chloroplast movement has so far not been elaborated in great detail.

White light and photosynthesis

Chlorophyll serves as the predominant light-absorbing pigment in photosynthetic energy production. In *Arabidopsis thaliana* leaf mesophyll cells white light caused a transient plasma membrane depolarization [133]. The electrical response to light was abolished when photosynthesis was inhibited by chemical treatment or in the albino mutant *alb-1*, which is unable to perform photosynthesis [134]. Three different types of K^+-transporting ion channels can be distinguished in *Arabidopsis* mesophyll protoplasts on the basis of their different current-voltage relations in single-channel recordings. PKC1 and PKC2 were selective for K^+, whereas PCC1 was found to be a nonselective cation channel. At least one of these channels (i.e. PKC1) was activated by white light, suggesting that channel activation may be subject to control by metabolic intermediates [133, 134]. In accordance with this assumption, all three *Arabidop-*

sis channels, but not a stretch-activated channel, were activated by cytoplasmic ATP applied at physiologically relevant concentrations [134]. Is is presently unknown whether ATP exerts its effect by simply binding to the channel proteins without being hydrolyzed or whether protein kinases and phosphatases associated with the channels are involved. Other factors like photosynthetically generated changes in the cytosolic redox potential may also be of importance, as artificial reducing agents were found to activate the *Arabidopsis* channels [133]. A contribution of chloroplasts to regulation of plasma membrane ion transport has been reported by other groups as well, although the nature of the regulatory factor was not investigated [98, 135]. In pea (*Pisum sativum*) mesophyll cells a Ca^{2+}-stimulated and light-controlled anion channel was detected [136]. Although a change in the cytosolic free Ca^{2+} concentration may mediate the activation by light, other light-dependent processes, for example photosynthetic ATP production, may be equally important.

Photosynthetically active light (red light) was also found to stimulate an electrogenic proton pump at the plasma membrane of *V. faba* guard cell protoplasts [137]. This pump appeared to be modulated by the guard cell chloroplasts because inhibition of photosynthetic electron transport by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibited red light-stimulated pump current in a dose-dependent manner. The role of chloroplasts in this reaction is probably to supply ATP to fuel the H^+-ATPase, although application of excess ATP to the cytosol in the presence of DCMU did not fully restore H^+ extrusion, indicating that additional metabolites delivered from the chloroplast may be required as well [137].

Mechanical stimuli

Physical forces are transduced to changes in plasma membrane ion fluxes leading to changes in cell turgor or electrical impulses (reviewed in [138, 139]). Stretch-activated (SA) ion channels have been described in animal cells, where they might serve as mechanoreceptors and baroreceptors [140–143] or osmoreceptors [144], as well as in yeast [146] and bacteria [147], where osmoregulatory functions have been suggested. More recently, mechanosensitive ion channels have also been characterized in plant cells where they could sense gravity, friction, internal tissue stress or changes in the osmotic pressure of the environment. In contrast to SA channels in animals, which are rather fine-regulated with respect to selectivity, conductance and open times, SA channels in walled cells generally seem to be poorly selective, with high conductances and long open times. In tobacco suspension cells a large-conductance, SA anion channel was discovered and thought to be involved in osmoregulation [145]. Stretch-operated cation channels have been identified in the plasma membrane of several plants and found to be weakly selective for Ca^{2+}, also allowing K^+ permeation [148]. Complex behaviours of adaptation and interrelation have been ascribed to these channels, which suggest multiple pathways of regulation [149]. A possible example of an osmoreceptor for plant cells is represented by the hydrostatic- and osmotic pressure (HOP)-activated channel in the membrane of red beet vacuoles [150]. This channel, permeable for K^+ and Cl^-, was very sensitive to an osmotic gradient as the only driving force when applied to the vacuolar membrane.

Using the touch-sensitive tendrils of *Bryonia dioica* as a system to study the transduction of mechanical stimuli, Klüsener et al. [151] identified a calcium-selective, strongly rectifying channel, designated BBC1, in the endoplasmatic reticulum of this plant. This channel as well as the response of the tendrils to touch are blocked by Gd^{3+}, suggesting that BBC1 represents a key element of the underlying signal transduction chain. Although Gd^{3+} is known to block SA channels [152], it is not clear if BBC1 is directly responsive to the mechanical force since studies revealed that Gd^{3+} cannot be regarded as selective for SA channels [153].

A mechanosensitive ion channel activity in the plasma membrane of parsley suspension cells most likely identical to the elicitor-activated cation channel LEAC (see before) was evoked upon membrane suction [53], arguing for possible cross-points of different signal cascades. *V. faba* guard cell protoplasts (see below) possess several SA channels with distinct selectivities for anions, cations and Ca^{2+} [154, 155], in this case resembling SA channels found in animal cells. The guard cell SA channels were distinguishable from the other voltage-dependent ion channels with respect to conductance, selectivity, voltage dependence, kinetics and sensitivity to membrane tension. They are proposed to participate in volume and turgor regulation, thereby controlling leaf gas exchange.

Beside these data, electrical events similar to action potentials from animal cells have been described to play a role in the systemic propagation of wounding signals in tomato plants, leading to the expression of proteinase inhibitor genes in unchallenged parts of the plant [13].

Modulators of ion channel activities

As mentioned before, ion channels react to a number of internal and external stimuli, thereby controlling cellular functions. In turn, they are themselves subject to regulation by various effectors, thus allowing the cell to use the ion channel activities as fine-tuners of mem-

brane potentials and ionic concentrations and as coupling elements in signaling cascades. Up to now there is certainty about the involvement of ion channels in signal-transducing pathways, but their position and exact functions remain largely speculative. Unraveling the regulatory mechanisms will allow ion channels to be defined as elements in the interacting network of signal transduction and as putative targets of modulators issued from other channels and pumps.

Calcium remains one of the most important signals within the cell, implicated in a series of responses. The release of Ca^{2+} from internal stores (in plants, mainly the vacuoles) or its entry through the plasma membrane is mediated by strictly controlled transporters which themselves can be modulated by $[Ca^{2+}]_{cyt}$ in a feedforward or feedback manner.

The slow vacuolar (SV) calcium-sensitive channel ubiquitously found in the tonoplast of guard cells shows dominant K^+ permeability but also a considerable conductance for Ca^{2+}. The SV channel is obviously gated by a calcium gradient across the tonoplast [156] rather than by elevated $[Ca^{2+}]_{cyt}$, as previously suggested [157–159]. This channel type may contribute to calcium-induced calcium release [27]. Inhibitors of the calcium-binding protein calmodulin (CaM), a ubiquitous regulator of several enzymes and ion channels, decrease SV channel activity in storage protein vacuoles of *Chenopodium rubrum* suspension cells and barley aleurone cells [160, 161]. In the latter case exogenously applied CaM sensitized the channels for Ca^{2+}. Both cytosolic Ca^{2+} and CaM concentrations rise in these cells after treatment with the phytohormone gibberellic acid, indicating a role for CaM as a modulator of SV channels in hormonal signal transduction [161]. In *V. faba* guard cell tonoplasts, SV channels are also modulated by CaM inhibitors [159], and their activity was decreased by low concentrations of Ca^{2+}/CaM-activated protein phosphatase 2B (calcineurin), which may establish a feedback regulation of SV channel activity [162]. However, within a physiological range of cytosolic Ca^{2+} (100 to 1000 nM), Pottosin et al. [156] reported that calmodulin had no effect on SV channel activity.

Furthermore, voltage independent, Ca^{2+}-gated K^+ channels at the tonoplast of fava bean guard cells, so-called VK channels, have been implicated in Ca^{2+}-dependent potassium release during stomatal closure [27, 163]. Additionally, other ion channels in the tonoplast and plasma membranes are modulated by $[Ca^{2+}]_{cyt}$, even though they are not permeable for Ca^{2+}. Calcium ions could act at the channel proteins themselves, for example by binding to specific domains of the protein, or through more indirect mechanisms.

Regarding the plasma membrane, regulation by calcium has been reported for some outwardly rectifying K^+ channels. In *Zea mays* protoplasts activation of such currents was observed after elevating $[Ca^{2+}]_{cyt}$ to 400 nM [164], which is in the physiological range of calcium signals. As has been suggested, such channels may participate in the repolarization of the membrane after Ca^{2+}-induced depolarization signals. Another report presented activation of nonselective cation channels in *Haemanthus* and *Clivia* endosperm protoplasts by much higher calcium concentrations (200 μM) [165]. Further, a Ca^{2+}-dependent deactivation of K^+ channels has been described in *V. faba* mesophyll cells, guard cells of *Zea mays* and the pulvini motor cells of *Mimosa pudica*; however, all measurements were executed at Ca^{2+} concentrations exceeding physiological significance [166–168]. Moran et al. [169] reported inhibition of the outward-rectifying potassium current in *Samanea saman* motor cell protoplasts by the Ca^{2+} channel blocking ions Cd^{2+} and Gd^{3+}. These blockers were presumed to act either at the K^+ channels themselves or to block Ca^{2+} channels which elevate $[Ca^{2+}]_{cyt}$ on which the K^+ channels might depend. However, since the specificity of blocking ions on plant channels is largely unknown, the interpretation of these data remains difficult. Furthermore, the way in which Ca^{2+} acts, mainly regarding the question of whether or not it directly affects the described channels, is still unanswered. Initial structural evidence for direct regulation of plant ion channels by Ca^{2+} was given by the finding that the recently cloned 'two-pore' channel KCO1 from *Arabidopsis* possesses two C-terminal Ca^{2+}-binding motifs (EF hands) which display calcium-binding properties in vitro (K. Czempinski and B. Müller-Röber, unpublished results). Indeed, KCO1 elicited outwardly directed potassium currents which were dependent on physiologically relevant, elevated $[Ca^{2+}]_{cyt}$ [170]. These results, initially obtained in baculovirus-infected insect cells, await confirmation in independent expression systems, and furthermore, determination of the target membrane of KCO1 is necessary to reveal its position in signal transduction events and its physiological role.

Another interesting effect of cytosolic Ca^{2+} on ion channels has been described for xylem parenchyma cells of barley seedlings. In these cells the ratio of the activity of two distinct outwardly rectifying cation channels, namely KORC and NORC, depended on the intracellular Ca^{2+} concentration [171]. The effect of Ca^{2+} was only found in the whole-cell patch-clamp mode but not in inside-out patches, suggesting the participation of cytosolic components in these processes.

Activation of the anion conductance in guard cells (GCAC1) is thought to be due to an increase of $[Ca^{2+}]_{cyt}$ resulting from an influx of external calcium [54]. Because anion channels can contribute substantially to plasma membrane depolarization in order to activate K^+_{out} channels, the activation of anion channels by calcium may be an important early signal in stomatal

closure [18]. Furthermore, as a prerequisite to stomatal closure, elevation of free calcium in the cytoplasm to concentrations of ≥ 400 nM has been shown to inhibit inwardly rectifying K^+ channels in *V. faba* guard cell plasma membranes by shifting their activation potential to more negative voltages [172, 173]. In the case of ABA-induced stomatal closure Ca^{2+} appears to originate from internal stores (mainly vacuoles) [26].

Release of calcium from the plant vacuoles can be triggered by second messengers known from animal systems. Calcium channels controlled by voltage, inositol 1,4,5-trisphosphate (*$InsP_3$*) and cyclic adenosine 5′-diphosphoribose (*cADPR*) have been found to coexist in the vacuolar tonoplast [174, 175]. $InsP_3$, involved in Ca^{2+}-based signal transduction pathways [176], opens a Ca^{2+}-selective channel residing in the tonoplast of isolated red beet vacuoles [177]. Stimulation of plant cells by the growth hormone auxin [178] or by light [179] is followed by an increase of $InsP_3$ via the pathway of phosphatidylinositol lipid metabolism. This elevation in $InsP_3$ concentration in turn leads to an increase in $[Ca^{2+}]_{cyt}$ by induction of Ca^{2+} release from the endoplasmic reticulum and from the vacuole [180]. Microinjection of caged $InsP_3$ into guard cells followed by flash photolysis triggered elevation in $[Ca^{2+}]_{cyt}$ as well as stomatal closing [181] and inhibited inwardly rectifying K^+ channels [182].

A cADPR-activated calcium channel, coexisting with the $InsP_3$-sensitive channel, has been reported to reside in the vacuolar membrane [175]. In animal cells cADPR targets the ryanodine receptor involved in calcium release. In plants cADPR appears to mediate an elevation in $[Ca^{2+}]_{cyt}$, for example required for ABA-induced changes in gene expression [183].

Changes in the *pH* value of living cells are part of many physiological processes, for example in early responses to elicitors [10] or auxins [184]. Some examples for the regulation of ion channel activities by pH are known from animal [185] and plant cells (reviewed in [27, 31, 186]). Because the cytosolic pH is strictly controlled, it can serve as a second messenger. The vacuole is mainly implicated in short-term homeostasis of the cytosolic pH and in hormonally evoked H^+ signaling [60]. Cytosolic alkalinization along with an increase of $[Ca^{2+}]_{cyt}$ displays an intermediate role in ABA-mediated closure of stomata and directly or indirectly modulates K^+ channel activities. The K^+_{out} channel of guard cell protoplasts was shown to be activated after experimental alkalinization of the cytosol in the whole-cell patch-clamp mode, whereas the K^+_{in} channel was inhibited, implying reciprocal regulation of these channels [33, 173]. The effect on K^+_{out} survived patch excision [187], indicating either pH sensitivity of the channels themselves or a membrane-delimited signal transduction pathway in pH sensing (for review see also [188]).

Modulation of the K^+_{in} channel by intracellular pH fits nicely with the pH sensitivity of the cloned K^+_{in} channel KAT1 from *Arabidopsis*, which is predominantly expressed in guard cells. After expression in *Xenopus* oocytes Hoshi found a shift of the voltage dependence of KAT1 by ca. 50 mV to more positive voltages by reducing the cytosolic pH from 7.0 to 6.2, which would render the channel more active at low pH [189]. The anion channel GCAC1 in the guard cell plasma membrane also exhibits a pH dependence, titrable with a pK value of about 6.9, thus enabling it to be regulated by physiological significant pH changes [190]. pH sensitivity is not restricted to ion channels of the plasma membrane, since also the tonoplast SV channel was shown to be inhibited by a cytoplasmic pH shift from 7.3 to 6.3 by changing its voltage dependence [159].

Furthermore, the extracellular pH has an influence on K^+_{in} but not on K^+_{out} channels [31]. Extracellular acidification activates the cloned inwardly rectifying K^+ channels KST1 and SKT1 from potato [191, 192] and KAT1 from *Arabidopsi*s [193], suggesting that this pH dependence is an intrinsic property of plant K^+_{in} channels. Sequence analysis and site-directed mutagenesis led to the identification of two histidine residues residing in the external loops of KST1 which are involved in sensing extracellular pH[194]. The structural basis for the effect of regulation by cytosolic pH has not yet been investigated.

In many cases signal transduction events include the posttranslational modification of regulatory proteins by phosphorylation. Evidence is accumulating that protein *phosphorylation* and *dephosphorylation* as in animal cells (reviewed in [195]) are of major importance for the regulation of ion channel activity in plant cells, too. In this respect the anion channel TSAC provides an original example of a phosphorylation-controlled conversion between different gating states. Using tobacco protoplasts in the whole-cell configuration, it was shown that the phosphorylation state, which can be modulated by ATP and inhibitors of phosphorylation or dephosphorylation, determines the deactivation kinetics and thus the voltage dependence of TSAC [53]. In this case identification of the regulating kinases and phosphatases as well as of internal factors modulating these enzymes (Ca^{2+}, CaM, cyclic nucleotides) may help to determine in which signaling cascades these anion channels are involved. More detailed data, however, are available for protein phosphorylation and dephosphorylation events modulating ion channel activities during ABA-mediated stomatal closure, which in *A. thaliana* employs the two type-PP2C protein phosphatases that are encoded by the *abi1* and *abi2* genes (see above, 'Hormones').

Phosphatase inhibitors have also been used to demonstrate the modulation of current amplitudes of K^+_{in} and

K^+_{out} channels. Cyclosporin A und FK-506, which in animals specifically inhibit calcium-dependent protein phosphatases of the calcineurin type (PP2B), prevent the inhibition of K^+_{in} channels by calcium in *V. faba* guard cell protoplasts, indicating participation of a PP2B in downregulation of these channels by the calcium signal [196]. Inhibitors for protein phosphatases of the PP1A and PP2A classes modulated K^+_{in} and K^+_{out} channels in guard cells and mesophyll cells of *V. faba* [197, 198]. Yet it remains unclear how these dephosphorylation-mediated regulation events are connected to signal transduction chains.

Biochemical studies also led to the identification of several ABA- and light-activated protein kinase activities with calcium-dependent as well as -independent properties [42, 44, 199]. Molecular cloning revealed the presence of several serine/threonine protein kinases as well as calcium-dependent/calmodulin-independent protein kinases (CDPKs) in different plant species. To evaluate their relation to plant ion channels, treatment of protoplasts and vacuoles with recombinant or purified CDPKs and subsequent electrophysiological analysis has been performed. By this approach Pei et al. [200] identified a new vacuolar chloride and malate conductance (VCL) activated only in the presence of ATP, calcium and a recombinant CDPK from *Arabidopsis*. These results suggested a CDPK-dependent anion conductance, leaving open, however, the question whether the channel itself is subject to phosphorylation. Using a similar approach the guard cell K^+_{in} channel KAT1 from *Arabidopsis* has been shown to be phosphorylated in vitro by a CDPK, isolated from *V. faba* guard cell protoplasts, in a calcium-dependent manner [201]. A role of CDPK-mediated KAT1 phosphorylation in calcium-mediated K^+_{in} channel inhibition during stomatal closing is supported by a report of Kamasani et al. [202]: coexpression of KAT1 and a CDPK from soybean in *Xenopus* oocytes led to a 10-fold reduction of KAT1-elicited currents. The demonstration of such a direct link between K^+ channel phosphorylation and regulation would be a major step forward towards establishing a signal transduction chain in guard cells.

In recent years GTP-binding proteins (*G proteins*) turned out to participate in the regulation of K^+ channels in various plant cells. Our present knowledge is mainly based on pharmacological attempts at studying the effect of guanosin nucleotide analogues and bacterial toxins on ion channel activities in *V. faba* guard cells. Treatment of guard cell protoplasts with guanosine 5′-[γ-thio]trisphosphate (GTPγS), which leads to sustained G protein activation in animals, resulted in reduced K^+_{in} currents in the presence of cytosolic Ca^{2+} [203, 204]. These data imply a calcium-dependent G protein regulation pathway tuning K^+_{in} channel activity in addition to the inhibition of K^+_{in} channel activity by elevated $[Ca^{2+}]_{cyt}$, as mentioned above. These results were confirmed in experiments with excised membrane patches arguing for a membrane-associated regulatory mechanism [205]). Studies utilizing guanosine 5′-[β-thio]bisphosphate (GDPβS), which locks G proteins in their inactivated state, gave conflicting results, in one case leading to activation [203], in another case to inactivation [204], of K^+_{in} currents at low $[Ca^{2+}]_{cyt}$. Consequently, G protein regulation of guard cell K^+_{in} channels appears to be rather complex, possibly involving several signaling pathways. This interpretation is strengthened by the presence of a further calcium-independent, G protein-controlled K^+_{in} channel inhibition pathway that may work via seven transmembrane-spanning (7TMS) receptors, which in animal systems play a prominent role in signal transduction. A synthetic compound (mas7), thought to stimulate G proteins coupled to 7TMS receptors in animals, inhibited K^+_{in} currents in *Vicia* guard cells [206]. However, the in vivo signals as well as the hypothesized receptors remain to be identified. Further questions concerning the role of G proteins in stomatal movements remain open. Although K^+_{in} channel inhibition in *V. faba* guard cells should reduce stomatal opening, treatment of epidermal peels from *Commelina communis* with GTPγS promoted opening of stomata [207]. More recently, two K^+_{in} conductances from barley xylem parenchyma cells, designated KIRC1 and KIRC2, have been described to be regulated by G proteins. Contrary to the situation in guard cells, these channels were activated by the nonhydrolyzable GTP analogue guanosine 5′-[β,γ-imido]triphosphate [Gpp(NH)p], whereas the K^+_{in} channel KIRC3 was insensitive to Gpp(NH)p [208]. To date the only example of a K^+_{out} channel modulated by G proteins was found in *V. faba* mesophyll protoplasts, where GTPγS inhibited K^+_{out} currents [167].

Although the present data clearly argue for G proteins participating in plant K^+ channel regulation, the number of G proteins and their mode of action in plants remain unclear. Biochemical and molecular approaches have identified several small and some heterotrimeric G proteins and their respective genes, but no connection to physiological processes involving ion channels has been elucidated yet (see [209] for review). Thus the integration of the pharmacological results on ion channel regulation into their physiological context appears to be a major goal for the near future. Possibly transgenic approaches may help to address this important question.

Besides phosphorylation-dependent voltage regulation shown, for example, for TSAC, the dependence of ion channel activity on *nucleotides* may provide a sensor reflecting the metabolic state of a cell, for example the balance between ATP and ADP/AMP (adenosine-5′-diphosphate/adenosine-5′-monophosphate) [210]. A

prominent example for a bimodal control is given by the chloride channel connected with cystic fibrosis (CFTR) in mammals, possessing both protein kinase A phosphorylation and nucleotide binding sites [211]. For the voltage-dependent guard cell anion channel GCAC1, nucleotides have been shown to activate the channel [54]. From dose–response measurements a model was proposed with four nucleotide binding sites regulating ion channel activity [190]. Additionally, an anion channel from *Arabidopsis* hypocotyl cells has been shown to be regulated by ATP. Thomine et al. [212] reported that channel inhibition by a shift in voltage dependence was mediated by ATP binding rather than channel phosphorylation because nonhydrolyzable ATP analogues were also effective. The cloned K_{in}^+ channels from different plant species possess a putative nucleotide binding site e.g. [191, 213], the function of which still has to be proven. Their activity in heterologous expression systems as well as the K_{in}^+ currents measured in guard cell protoplasts require hydrolyzable ATP to remain in the activated state [189, 191, 192, 214]; however, the question is open whether this nucleotide binds directly to the channel or whether its presence is a prerequisite for channel phosphorylation by protein kinases (for discussion see [205]). Other examples of ATP-activated channels comprise the cation channels PKC1, PKC2 and PCC1 from *Arabidopsis* mesophyll cells (see above).

In mammalian heart and other tissues ATP-inhibited inwardly rectifying potassium (K-ATP) channels link the cellular metabolic charge to electrical signaling. Up to now the only example of an ATP- (and AMP-) inhibited cation channel in plants has been reported from single-channel recordings on protoplasts isolated from a characeaen alga [215]. The mammalian K-ATP channels are composed of the channel protein itself bound to the so-called sulfonylurea receptor, which comprises the target for a class of sulfonamides. Very recently, pharmacological studies performed in plants revealed a target for *sulfonylureas* in guard cells and its connection to stomatal movements. Leonhardt et al. [216] found that sulfonylureas inhibit K_{out}^+ activity in guard cell protoplasts and induce stomatal opening in epidermal strips of *V. faba*. The authors also detected transcripts of a sulfonylurea receptor homologue in guard cell preparations leading them to suggest the presence of homologous proteins in guard cells as well as their participation in K_{out}^+ channel regulation.

Furthermore, *cyclic nucleotides* may act as second messengers on plant ion channels [217]. In *V. faba* mesophyll cell protoplasts K_{out}^+ currents were specifically activated by cAMP in a dose-dependent fashion, most likely involving cAMP-dependent protein kinases [218]. The cloned plant K_{in}^+ channels possess a domain with homology to binding sites for cyclic nucleotides. Studies on the channels KAT1 and AKT1 after expression in *Xenopus* oocytes and insect cells, respectively, indicate a cGMP-dependent shift of their activation potentials towards more negative voltages [189, 214]. Although suggestive, direct binding of cyclic nucleotides to the channel proteins still has to be proven. Recently, a calmodulin-binding protein with high similarity to K^+ and animal olfactory channels has been cloned from barley aleurone cells. This membrane protein contained a putative cyclic nucleotide binding domain at its C terminus [219]. Unfortunately, functional activity of this novel protein has not yet been unequivocally demonstrated, leaving open the question whether it is indeed regulated by cyclic nucleotides. However, the molecular cloning of an adenylate cyclase gene from tobacco [220] as well as experiments on phytochrome- and gibberellic acid-regulated gene expression [125, 221, 222] provide convincing evidence that cyclic nucleotides, including cAMP and cGMP, indeed act as second messengers in higher plant cells.

The cytoskeleton and other cellular proteins regulating ion channels

The cytoskeleton represents a complex and highly dynamic network of microtubules, actin filaments, and intermediate filaments which are decorated with accessary and regulatory proteins, including signaling enzymes [223, 224]. The plant cytoskeleton has been shown to reorganize in response to various biotic and abiotic factors, including hormones, pathogens and light [225–227], and has been suggested to serve as a sensing element in response to mechanical stress [228]. In animal cells, strong evidence indicates that components of the cytoskeleton bind directly to membrane transport proteins in the plasma membrane, such as Na^+ channels, anion exchanger, Na^+, K^+-ATPase, and H^+-ATPase. Stimulus-induced alterations in the organization of the cytoskeleton may not only change the cellular distribution of transport proteins along the cellular membrane but may also modify transport activities [229]. Recent experiments indicate that membrane transport may also be regulated by the cytoskeleton in higher plants. Thion et al. [230] demonstrated that perturbation of microtubules in carrot cells by two different agents, colchicine and oryzalin, enhanced plasma membrane voltage-dependent Ca^{2+} currents and reduced the current rundown typically observed [230]. By contrast, compounds that interfere with the polymerization status of actin filaments did not have any effect on intensity and stability of Ca^{2+} currents in carrot cell protoplasts. It was therefore concluded that cytoskeletal regulation of Ca^{2+} channels is mainly due to microtubule organization and not to microfilaments.

Actin filaments have been demonstrated to be involved in the regulation of another class of plant ion channels, namely the K_{in}^+ channels in guard cells of *V. faba*. The pharmacological agent cytochalasin D, which depolymerizes filamentous actin, stimulated K_{in}^+ currents [231]. In addition it promoted light-induced stomatal opening, consistent with the important role of K_{in}^+ channels in stomatal movements. Phalloidin, a stabilizer of actin filaments, inhibited K_{in}^+ currents as well as stomatal opening in response to light [231]. Interestingly, a subclass of plant K_{in}^+ channel proteins contains so-called ankyrin repeats within their C-terminal halves [192, 213, 232–234]. These repeats represent potential domains for interactions with the cytoskeleton, as has been shown in animal cells [229]. However, none of the plant K_{in}^+ channels harboring ankyrin repeats is known to be expressed in guard cells. The guard cell K_{in}^+ channels KAT1 (*Arabidopsis*) and KST1 (potato) lack such ankyrin sequences, suggesting that other channel domains participate in regulation by cytoskeletal proteins. It should be noted that a highly conserved protein domain was detected at the extreme C terminus of all plant K_{in}^+ channel proteins identified in higher plants. The role of this domain for channel activity within the context of a plant cell is currently unknown. It could, however, be demonstrated that the conserved C termini mediate direct interactions between individual channel polypeptides. Heterologous expression of the plant channels in insect cells revealed channel clustering mediated by the conserved homology domain, leaving open the possibility that this domain may participate in the regulation of channel distribution [234].

Recently, proteins with significant homology to β-subunits of animal K_{in}^+ channels [235] have been identified in *Arabidopsis* [236] and potato (our group, unpublished). Although the plant β-subunit protein in vitro associates with KAT1 protein [237], the physiological role of this interaction remains unknown. Besides such β-subunits, so-called 14-3-3 proteins have been identified as candidates for regulators of plant ion channels [238]. In animal cells, 14-3-3 proteins bind to various signaling proteins, modifying their activities [239]. In plants, these proteins constitute the fusicoccin receptor [240, 241], which mediates the stimulatory effect of the fungal toxin fusicoccin towards the plasma membrane H^+-ATPase. Overexpression of two different *V. faba* 14-3-3 proteins in transgenic tobacco led to an enhancement of K^+ conductance in mesophyll cell protoplasts. The precise mechanism underlying this enhancement was, unfortunately, not investigated [238]. Clearly, further experiments are required to clarify whether 14-3-3 proteins regulate K^+ channel activities by direct protein-protein interactions or, alternatively, via intermediary steps involving, for example, protein kinases.

In conclusion, there is considerable evidence that plant ion channels directly interact with a variety of cellular proteins. Some of these proteins may have structural functions, including cytoskeletal components, allowing a defined distribution of channels along the plasma membrane and thereby permitting localized electrical responses likely to be involved in numerous physiological and developmental processes. It is probably fair to assume that additional as yet unknown or poorly defined proteins interacting with plant ion channels will be discovered in the near future.

Stomatal guard cells

Guard cells regulating gas exchange of higher plants are intensively studied because of their physiologically important stomatal movements [242, 243]. Stomata are composed of two symmetrical guard cells which form a diffusive pore through the epidermal cell layer in leaves and other green tissues. Regulation of stomatal aperture, that is pore size, is needed to balance CO_2 uptake with water release. Changes in cell volume are brought about by osmotic processes which are controlled in large part by release and uptake of ions through finely tuned ion channel and pump activities at the plasma and vacuolar membranes [4, 18, 242, 244–246].

To fulfil their physiological function, guard cells have to integrate a large number of external and internal signals [247, 248]. Light quality and quantity [249, 250], CO_2 concentration [251], temperature [252], and water status [253, 254] are such external parameters acting in tight interaction. The reception of environmental signals is only beginning to be understood. Zeaxanthin, for example, is thought to act as a blue light receptor [102].

Hormones, which can be regarded as internal factors, are produced at elevated levels as a response to external parameters or as part of developmental programs. The best-studied example in relation to stomatal movements is ABA [255, 256] reviewed in [257]. ABA, the concentration of which increases in leaves under conditions of water shortage, itself induces stomatal closure. It may therefore participate in signaling processes mediating drought stress which is often originally sensed by the roots. It is at present unclear whether ABA acting in vivo on guard cells is produced by the guard cells themselves or whether it originates from other parts of the plant. There is evidence that ABA can be received at the plasma membrane as well as within the guard cells [25, 34]. The signaling pathways are less clear for the other plant hormones. Auxin, for instance, modulates stomatal behavior with a bimodal concentration dependence. At low concentrations (below 10 μM) auxin leads to an increase in stomatal aperture, whereas at higher concentrations it is less effective or even inhibits

stomatal opening [50]. In addition to hormones, metabolites of primary carbon metabolism, namely extracellular malate, have been implicated to modulate stomatal behavior in response to environmental stimuli (i.e. elevated CO_2 concentration; [258]), although a role of malate in guard cell CO_2 sensing was questioned by others [259]. Several of the ion channels mentioned throughout this review appear to contribute to stomatal closure in response to ABA treatment, via a coordinated activation and inhibition of individual channel types. Voltage-dependent Ca^{2+} channels, Ca^{2+}-dependent anion channels as well as K^+_{out} channels located at the guard cell plasma membrane are all activated by the closing signal, whereas K^+_{in} channels are inhibited [18, 259, 260]. Additional channels residing in the vacuolar membrane, including the SV and VK channels, possibly need to be modulated in their activity to allow efficient ion efflux, although their exact role is still a matter of debate.

Elevation of the cytosolic Ca^{2+} concentration appears to involve release of Ca^{2+} ions from internal stores (the vacuole), although initial (and most likely localized) changes in Ca^{2+} concentrations may occur at the plasma membrane upon activation of the voltage-dependent Ca^{2+} channels [31, 32] reviewed in [257]. $InsP_3$, but possibly also cADPR, seems to act as an intracellular Ca^{2+}-releasing compound acting on ion channels coexisting at the tonoplast membrane. One of the many questions that still needs to be addressed in relation to stomatal functioning is how the specificity of the Ca^{2+}-signaling system is encoded, because not only ABA but also auxin at stomata-opening concentrations employ Ca^{2+} as an intracellular second messenger. In many cases spikes and oscillations in cytosolic Ca^{2+} concentrations have been observed in guard cells (as well as in other systems). Importantly, the spatial and temporal patterns of changes in $[Ca^{2+}]_{cyt}$ appear to be stimulus-dependent, thereby allowing introduction of specificity into the cellular response [261, 262].

Besides Ca^{2+} many other typical elements of signal transduction cascades including $InsP_3$, diacylglycerol, cytosolic pH changes, phosphatases and kinases, G proteins as well as cyclic nucleotides have been implicated in stomatal responses to environmental and hormonal stimuli e.g. [35, 263] reviewed in [242, 264]. Moreover, a decrease in the extracellular pH, which is achieved through proton efflux mediated by the plasma membrane H^+-ATPase upon opening signals, activates the guard cell K^+_{in} channels KST1 and KAT1 [191, 193], as already mentioned above, introducing a further mechanism of channel regulation in guard cells.

Several mechanosensitive anion and cation channels [155] may contribute to multiple, highly specialized feedback mechanisms for sensing and controlling cell volume and turgor [265]. Certainly, the activation of SA channels in response to cellular turgor has to be tightly linked to the regulation of the other guard cell non-SA channels to allow precise control of stomatal aperture.

The transport processes involved in stomatal movement have mainly been investigated in *V. faba* guard cell protoplasts but recently also in *Arabidopsis* guard cells [36, 266, 267]. Additional progress in stomatal functioning has been obtained by using molecular–genetic techniques (reviewed in [268]).

Conclusions

In this review we summarized current knowledge on the role of ion channels in plant signal transduction. The application of electrophysiological techniques in combination with pharmacological knowledge, derived from studies on signaling events in animal cells, has proven to be very powerful in ion channel analysis, although in many cases their exact positions in signaling pathways remain uncertain. In order to address this important issue, the molecular identification of channel proteins involved in signaling cascades is pivotal. A considerable number of ion channel genes, most notably those from K^+ channels, have been identified from higher plants in recent years, and it is almost certain that numerous additional channels will soon be uncovered on the molecular level. Novel channels need to be carefully characterized with respect to their electrophysiological and biochemical properties through expression in heterologous systems, such as *Xenopus* oocytes and insect cells, or plant tissues as recently examplified by Bei and Luan [269]. New clues about the composition of signaling cascades can be expected from the application of modern techniques developed to identify and characterize protein-protein interactions. In addition, the determination of gene expression patterns might help to assign cell type-specific functions to plant ion channels. Furthermore, individual cell types may be marked by the expression of fluorescent molecules, such as the green fluorescent protein, driven by cell type-specific promoters in genetically modified plants. Such an approach allows selection of specific cell types otherwise not easily identified and analysis of their particular ion channel activities. Finally, 'reverse genetics' can be used to produce mutant plants deficient for individual channels or regulatory proteins, helping to elucidate the specific roles these channels play in defined physiological processes.

Acknowledgments. We are grateful to Steffi Hartje for helpful advice and discussions while preparing this review. We also thank Ulrich Schneider for critically reading the manuscript.

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