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Review

Calcium channels in higher plants

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Abstract

Calcium channels are involved principally in signal transduction. Their opening results in increased cytoplasmic Ca^{2+} concentration, and the spatial and temporal variations in this are thought to elicit specific physiological responses to diverse biotic and abiotic stimuli. Calcium-permeable channels have been recorded in the plasma membrane, tonoplast, endoplasmic reticulum, chloroplast and nuclear membranes of plant cells. This article reviews their electrophysiological properties and discusses their physiological roles. Emphasis is placed on the voltage-dependent and elicitor-activated Ca^{2+} channels of the plasma membrane and the depolarisation-activated (SV), hyperpolarisation-activated, IP_3- and cADPR-dependent Ca^{2+} channels of the tonoplast. The closing of stomatal guard cells in the presence of abscisic acid (ABA) is used to illustrate the co-ordination of Ca^{2+} channel activities during a physiological response.

Keywords: Calcium; Channel; Endoplasmic reticulum; Permeation; Plasma membrane; Vacuole

1. Introduction

A Ca^{2+} channel can be defined as a channel permeable to Ca^{2+} whose physiological role is to mediate rapid Ca^{2+} transport across cellular membranes [1]. Although a role in catalysing nutritional fluxes can be envisaged [1,2], these channels are predominantly involved in cell signalling. The physiological poise of a plant cell is determined, in part, by its cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). In a resting cell, $[Ca^{2+}]_{cyt}$ is maintained at about 100 nM through the activity of Ca^{2+}-ATPases and Ca^{2+}/H^+ antiporters in cell membranes (reviewed in [3,4]). However, many stimuli elevate $[Ca^{2+}]_{cyt}$ and the interplay between Ca^{2+} channels and Ca^{2+} efflux mechanisms on a variety of cell membranes, together with cytoplasmic Ca^{2+} buffering, determines the spatial and temporal changes in $[Ca^{2+}]_{cyt}$ [3–6]. The specificity of the $[Ca^{2+}]_{cyt}$ signal in initiating an appropriate physiological response is thought to be encoded by different amplitude, temporal or spatial changes in $[Ca^{2+}]_{cyt}$ [4,6–8].

Calcium channels have been characterised in the plasma membrane, endoplasmic reticulum (ER), tonoplast, nuclear and plastid membranes of plant cells. They can be classified according to their voltage

Abbreviations: ABA, abscisic acid; cADPR, cyclic ADP-ribose; $[Ca^{2+}]_{cyt}$, cytoplasmic Ca^{2+} concentration; CaM, calmodulin; CDPK, calmodulin-like domain protein kinase; CICR, Ca^{2+}-induced Ca^{2+} release; DACC, depolarisation-activated Ca^{2+}-permeable channel; E_{Ca}, Nernst (equilibrium) potential for Ca^{2+}; E_K, Nernst (equilibrium) potential for K^+; ER, endoplasmic reticulum; E_{rev}, zero-current (reversal) potential; IP_3, inositol triphosphate; IP_6, phytic acid; NAADP, nicotinic acid adenine dinucleotide phosphate; PK, protein kinase; P_o, probability of an active channel being open; PP2B, protein phosphatase type 2B; PP2C, protein phosphatase type 2C; SV, slow vacuolar

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Table 1
Calcium-permeable channels in the plasma membrane of plant cells

Name	Tissue	Conductance	Permeability	Inhibitors of Ca^{2+} flux	Ref.
Depolarisation-activated					
DACC	Carrot cell suspension	13 pS (40 mM Ca)	Ca, Ba, Sr, Mg		[12,13]
DACC	*Arabidopsis* root		Ca	Mibefradil	[2]
	Vicia faba guard cell	10 pS (30 mM Ca)	Ca, K		[37]
rca	Wheat root	29 pS (40 mM Ca)	Na, Cs, K, Li, Rb	Ruthenium red, verapamil, diltiazem, Ni^{2+}, Al^{3+}, La^{3+}, Gd^{3+}, TEA^{+}	[1,20]
			Ba, Sr, Ca, Co, Mg, Zn, Mn, Ni, Cd, Cu		
VDCC2	Rye root	40 pS (100 mM Ca)	Cs, K, Rb, Na Ba, Ca	Verapamil, La^{3+}, TEA^{+}	[1]
Maxi-cation	Rye root	135 pS (100 mM Ca)	K, Rb, Cs, Na, Li Ba, Sr, Ca, Mg, Co, Mn	Ruthenium red	[1]
Hyperpolarisation-activated					
Mechano-sensitive	*Vicia faba* guard cell	3 pS (30 mM Ca)	Ca		[37]
Mechano-sensitive	Onion bulb epidermis	6 pS (200 mM Ca)	Ca, K	Al, Gd^{3+}, La^{3+}	[31]
	Vicia faba guard cell		Ca	Gd^{3+}, calcicluidine	[39,40]
	Arabidopsis root		Ca	Al^{3+}	[2]
	Arabidopsis leaf mesophyl	23 pS (50 mM Ba)	Ba, Ca	$[Ca^{2+}]_{cyt}$	[69]
Elicitor-activated	Tomato cell suspension	4 pS (20 mM Ca)	Ba, Ca	La^{3+}, nifedipine	[42]
Voltage-independent					
LEAC	Parsley cell suspension	245 pS (50 mM Ca)	Ca, K	La^{3+}, Gd^{3+}	[44]

dependence and may be regulated by additional factors such as membrane stretching, interactions with the cytoskeleton, the binding of ligands or covalent modification. Different stimuli appear to utilise different pools of calcium to effect characteristic changes in $[Ca^{2+}]_{cyt}$ and signals may be transduced through various Ca^{2+} channels on different cell membranes. This article reviews the electrical properties of Ca^{2+} channels in plant cell membranes and speculates on their physiological roles. The voltages cited follow the physiological convention and refer to the potential on the cytoplasmic side of the membrane relative to the non-cytoplasmic side [9].

2. Calcium channels in the plasma membrane

Calcium channels in the plasma membrane have been characterised electrically by patch-clamping plant-cell protoplasts and following the incorporation of plasma-membrane vesicles into planar lipid bilayers (PLB). Several Ca^{2+} channel activities have been observed. These can be divided into depolarisation-activated, hyperpolarisation-activated and voltage-insensitive channels (Table 1 and [10]). Thereafter, they may be subdivided on the basis of their modulation by a variety of secondary effectors.

2.1. Depolarisation-activated Ca^{2+} channels

Depolarisation-activated Ca^{2+}-permeable channels (DACC) appear to be present in the plasma membrane of all plant root cells [1,2,11], leaf mesophyll cells [11] and suspension-cultured cells [12–14]. At least three distinct classes can be defined on the basis of their differential sensitivities to La^{3+}, Gd^{3+} and verapamil [1]. It is noteworthy that several can be inhibited by ruthenium red (Table 1), rendering this

pharmaceutical useless as a diagnostic of Ca^{2+} mobilisation from intracellular stores.

A characteristic depolarisation-activated Ca^{2+} current was identified first in protoplasts from suspension-cultured cells from carrot [12–14] and later in protoplasts from leaf mesophyll and root cells of *Arabidopsis* [2,11]. In protoplasts from *Arabidopsis* root cells this current was inhibited by mibefradil (E. Kiegle, Cambridge, UK, personal communication). The channel mediating this current is permeable to divalent cations, including Ca^{2+}, Ba^{2+}, Sr^{2+} and Mg^{2+} [12,13], and has a unitary conductance of 13 pS with 40 mM calcium in the external solution [13]. The Ca^{2+} current is activated by membrane depolarisation to voltages more positive than about −140 mV, and exhibits slow and reversible inactivation at extreme negative voltages [12,13]. It shows 'rundown', Ca^{2+} currents decreasing with time after establishment of the whole-cell configuration [12–14] and a phenomenon termed 'recruitment' [12], which has been attributed to the activation of quiescent channels upon extreme depolarisation to positive voltages. Rundown probably results from channels entering the quiescent state since currents can be repetitively and fully recovered by recruitment [12]. Thus, both are likely to be a consequence of voltage-dependent conformational changes in protein structure, rather than manifestations of protein phosphorylation and dephosphorylation events. The recruitment of Ca^{2+} channels may serve to modulate the Ca^{2+} signal in response to the magnitude and repetitiveness of plasma-membrane depolarisation.

The depolarisation-activated Ca^{2+}-current in protoplasts is stabilised by pharmaceuticals, such as colchicine and oryzalin, which disorganise microtubules [11,14]. Furthermore, these Ca^{2+} channels appear to be constitutively recruited in protoplasts from the *Arabidopsis ton2* mutant, in which the anchoring proteins that bind microtubules to the plasma membrane appear to be defective [11]. It has long been known that the cytoskeleton can regulate the activities and/or the distribution of membrane proteins. Cortical microtubules have been implicated in osmosensing, mechanical sensing, morphogenesis and cell division, and their rearrangement is an early response to biotic and abiotic stimuli (for reviews see [15,16]). Recently, the microtubule cytoskeleton has been associated with regulating the direction of root hair growth [17]. Depolarisation-activated Ca^{2+} channels have been implicated in the acclimation of chilling-resistant plants to growth at low temperatures [1]. This is thought to be initiated by Ca^{2+} influx across the plasma membrane and mediated by the consequent increase in $[Ca^{2+}]_{cyt}$. Disruption of cortical microtubules results in a significant increase in the $[Ca^{2+}]_{cyt}$ elicited by chilling and the response is suppressed when 10 mM La^{3+}, but not 10 mM Gd^{3+}, is present in the extracellular medium [18].

Two depolarisation-activated Ca^{2+}-permeable channels have been recorded when plasma-membrane vesicles from cereal roots were incorporated into PLB. These are termed the *rca* [19,20] or VDCC2 [21,22] channel and the maxi cation channel [22,23]. For PLB studies a high-purity plasma-membrane fraction was obtained by aqueous-polymer two-phase partitioning [19,24] and the plasma-membrane origin of the *rca*/VDCC2 and maxi cation channels was confirmed by their colocalisation with a plasma-membrane marker-enzyme (vanadate-sensitive ATPase) when microsomal membranes were separated on a linear sucrose gradient [25].

In the absence of Ca^{2+}, the *rca* channel is permeable to both monovalent and divalent cations (Table 1). These include cations that are relatively impermeant (Mg^{2+}, Mn^{2+}) or block (Cd^{2+}, Co^{2+}, Ni^{2+}) the L-type Ca^{2+} channels in animal cell membranes [19,20,26]. The *rca* channel is inhibited by a range of pharmaceuticals including micromolar concentrations of Al^{3+}, La^{3+}, Gd^{3+}, verapamil, diltiazem and ruthenium red, but it is insensitive to 1,4-dihydropyridines and bepredil [1,20]. A model for cation permeation through the pore of the *rca* channel has been developed [26] and is presented in Section 2.2. The unitary conductance of the *rca* channel was 27 pS in symmetrical 1 mM $CaCl_2$ [19] and the predicted unitary conductance is 34 pS under the ionic conditions employed in patch-clamp experiments with 30 to 40 mM extracellular calcium [26]. The kinetics of the *rca*/VDCC2 channel are dominated by voltage-dependent inactivation [20,21]. Inactivation is more rapid at voltages further displaced from the reversal potential of the current through the channel (E_{rev}) and apparently modulated in parallel with changes in E_{rev} [21]. Under physiological ionic conditions the majority of the inward current through the *rca* channel will be carried by Ca^{2+}, which justifies the

classification of the *rca* channel as a Ca^{2+} channel [26]. The *rca* channel would be closed at root-cell resting potential, but would open upon plasma-membrane depolarisation and catalyse Ca^{2+} influx.

The maxi-cation channel in the plasma membrane of rye roots is permeable to a wide variety of monovalent and divalent cations [22,23] and a model for the permeation of K^+, Na^+, Ca^{2+} and Ba^{2+} through the pore of this channel has been developed [27]. Under physiological ionic conditions, Ca^{2+} influx is likely to dominate the ionic fluxes through the channel. The channel exhibits a complex pharmacology [1]. Cation influx through the channel is inhibited by micromolar concentrations of ruthenium red, whereas cation efflux is inhibited by ruthenium red, diltiazem, verapamil and quinine at micromolar concentrations and TEA^+ at millimolar concentrations.

The kinetics of the maxi-cation channel are dominated by its increasingly rapid inactivation as membrane potential is displaced from E_{rev} [23]. In the resting cell the maxi-cation channel will be closed, but it will open upon plasma-membrane depolarisation to facilitate Ca^{2+} influx sufficient for cell signalling [27]. White and Ridout [22] have enumerated the gating kinetics of the maxi-cation channel by assuming that the voltage dependence of both activation and inactivation vary in parallel with changes in E_{rev} and that the probability of an active channel being open (P_o) approaches unity. Although these assumptions are not precisely correct, they have allowed qualitative temporal estimates of the probability of finding an activated channel and, by coupling to an appropriate permeation model (reviewed in Section 2.2), have enabled the currents and ionic fluxes through the maxi-cation channel to be determined as the membrane potential fluctuates. Combining kinetic and permeation models for the maxi-cation channel has suggested that depolarisation-activated Ca^{2+} channels may be responsible for the transient increase in $[Ca^{2+}]_{cyt}$ elicited by cooling [27], which probably initiates acclimation to low-temperatures.

As a class, depolarisation-activated Ca^{2+}-channels are thought to transduce general stress-related signals. Plasma-membrane depolarisation is elicited by many environmental, developmental and pathological stimuli, it may occur by one of many diverse mechanisms, and it is likely to generate a global signal. Unless depolarisation-activated Ca^{2+} channels are clustered or regulated by additional factors, $[Ca^{2+}]_{cyt}$ will be increased throughout the periphery of the cell [1]. The subsequent pattern of $[Ca^{2+}]_{cyt}$ changes will then depend upon local dampening or regeneration of the Ca^{2+} signal through the influence of intracellular organelles and/or cytoplasmic constituents.

2.2. Permeation models for depolarisation-activated Ca^{2+} channels

Cation permeation through both the maxi-cation [27] and *rca* [26] channels has been described using models based on the concept of single-file permeation through 'multi-ion' pores [28,29]. These models suggest that cations traverse a corrugated free-energy profile as they pass through the pore (Fig. 1). The energy maxima represent energy-requiring processes, such as the transition from free diffusion in the external solutions to single-file movement within the pore itself, and energy minima (or energy 'wells') represent cation binding sites. These are placed at discrete positions within the electric field across the pore. The rates at which cations cross an energy barrier (defined as the difference between an energy minimum and an energy maximum) are calculated according to traditional Eyring rate theory [28,29]. They are related to the magnitude of the energy barrier, the electrical driving force and, when cations are entering the pore, their concentration in the external solution. The pores of both the maxi-cation and *rca* channels have been modelled as having two high-affinity binding sites for cations separated from each other, and from the external solution, by energy barriers (Fig. 1A,B). Both binding sites can be occupied simultaneously by cations, but cations within the pore will repel each other. Similar models have been used to describe cation permeation through L-type Ca^{2+} channels in animal cell membranes (Fig. 1C). From these permeation models several inferences have been made regarding pore structure.

Both the maxi-cation and the *rca* channels are predicted to have considerable negative surface charge in the vestibules to their pores [26,27]. This appears to be a general characteristic of cation channels. It serves to concentrate cations in the vestibules and increase cation fluxes at low concentrations in the

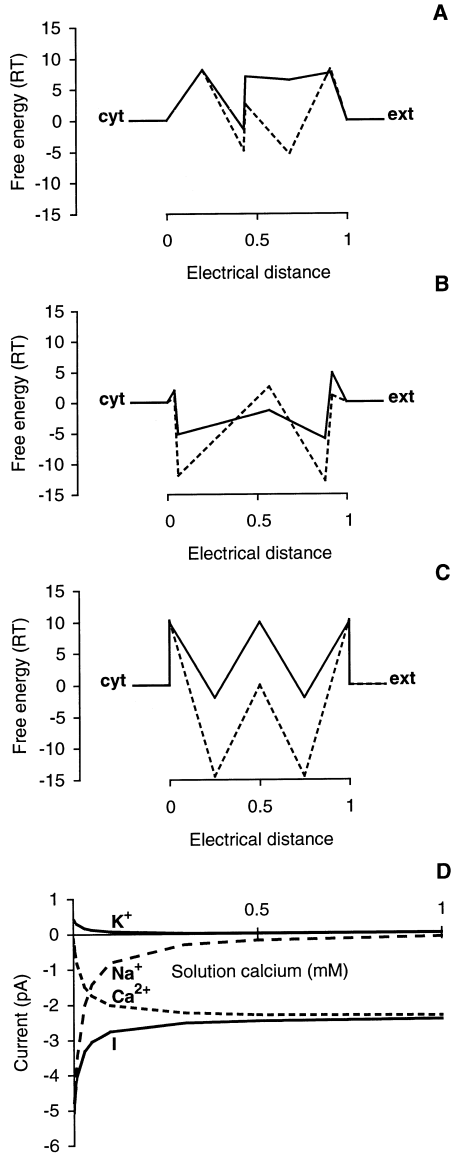

Fig. 1. Free energy profiles for the permeation of K^+ (——) and Ca^{2+} (- - -) through the pores of (A) the maxi-cation channel in the plasma membrane of rye (*Secale cereale* L.) roots (data from [27]) and (B) the *rca* channel in the plasma membrane of wheat (*Triticum aestivum* L.) roots (data from [26]). (C) Free energy profiles for the permeation of Na^+ (——) and Ca^{2+} (- - -) through the pore of a typical plasma-membrane L-type Ca^{2+} channel from animal cells [29]. The solution reference state for all free energy profiles is 1 M. (D) The predicted dependence of total (I), K^+, Na^+ and Ca^{2+} currents at −100 mV through the *rca* channel in the plasma membrane of wheat roots on extracellular Ca^{2+} activity. The extracellular K^+ and Na^+ activities were 0.90 and 0.93 mM, and the cytoplasmic K^+, Na^+ and Ca^{2+} activities were 71.5 mM, 3.5 mM and 100 nM, respectively.

bulk solution [28–30]. In the absence of Ca^{2+}, the flux of monovalent cations is rapid [19,21,23]. For example, a large Na^+ influx is predicted to occur through the *rca* channel under physiological conditions in the absence of extracellular Ca^{2+} (Fig. 1D). However, since the cation binding sites have a higher affinity for Ca^{2+} than for monovalent cations, when Ca^{2+} is present it preferentially occupies these sites and restricts the flux of monovalent cations (e.g., Fig. 1D). Thus, the selectivity of these channels is created by their affinity for Ca^{2+}. Under physiological ionic conditions, with 1 mM Ca^{2+} activity in the extracellular solution, Ca^{2+} influx dominates the ionic currents through these channels (Fig. 1D; see also [26,27]), justifying their classification as Ca^{2+} channels. Considerable electrostatic repulsion is predicted to occur between cations simultaneously within the pore of these channels, which will accelerate cation conduction at high cation concentrations.

The cation binding-sites in both the maxi-cation and the *rca* channels have a lower affinity for divalent cations, and a higher affinity for monovalent cations, than L-type calcium channels (Fig. 1). This suggests that the pore structure of the plant plasma-membrane Ca^{2+} channels and the L-type channels of animal cell membranes are different. The plant Ca^{2+} channels are less selective for Ca^{2+} than the L-type Ca^{2+} channels of animal cell membranes. However, this difference in selectivity may reflect the contrasting ionic conditions and voltages under which plant and animal channels operate. The pore structure of the plant Ca^{2+} channels is suited to catalysing Ca^{2+} influx whilst maintaining the extremely negative membrane potentials typical of plant cells.

2.3. Hyperpolarisation-activated Ca^{2+} channels

Hyperpolarisation-activated Ca^{2+} channels have been reported in protoplasts from various cell types, where they are proposed to fulfil various physiological functions. The hyperpolarisation-activated Ca^{2+} channels in the plasma membrane of onion epidermal cells are stretch-activated and proposed to act as mechanoreceptors. In guard cells hyperpolarisation activated Ca^{2+} channels appear to trigger plasma-membrane depolarisation to coordinate the loss of solutes and initiate stomatal closure. In root endo-

dermal cells they may serve a role in mineral nutrition and in suspension-cultured tomato cells they are implicated in elicitor signalling pathways. These channels will be considered sequentially in the following paragraphs.

Mechanosensitive ion channels are likely to be involved in the regulation of turgor and mechanosensitive Ca^{2+} channels may transduce the mechanical stresses induced by gravity, touch or flexure [31]. In association with the reorientation of cortical microtubules, it is possible for mechanosensitive Ca^{2+} channels to determine the allometry of cell expansion and morphogenesis by coordinating the expansion of neighbouring cells. By analogy with tip-growth in algal rhizoids and fungal hyphae, specific roles for mechanosensitive Ca^{2+} channels in the elongation of root hairs [1] and pollen tubes [32] have been proposed. The suggested sequence of events for polarised cell growth is: cell wall yielding, local turgor-driven evagination and stretching of the plasma membrane, local activation of stretch-activated Ca^{2+} channels, Ca^{2+} influx and generation of a tip-high $[Ca^{2+}]_{cyt}$ gradient, secretory-vesicle movement to and exocytosis in the region of elevated $[Ca^{2+}]_{cyt}$. However, little is known about the Ca^{2+} channels involved in these processes. Although their pharmacology has apparently been inferred from the effects of pharmaceuticals on internal $[Ca^{2+}]_{cyt}$ gradients, Mn^{2+} influx or cell elongation, these might not reflect direct interactions with the Ca^{2+} channel itself. In root hairs the Ca^{2+} current, internal $[Ca^{2+}]_{cyt}$ gradient and elongation are all eliminated in the presence of external La^{3+} [33] or verapamil [34]. In pollen tubes, the influx of Mn^{2+} (used as a tracer for Ca^{2+}) and elongation are inhibited by Gd^{3+} and La^{3+}, but nifedipine and verapamil are relatively ineffective [35,36].

Mechanosensitive Ca^{2+}-permeable channels have been reported in protoplasts from guard cells [37] and onion bulb epidermal cells [31]. However, only the channel from onion bulb epidermal cells has been characterised in any detail. This channel appears to be functionally linked in clusters and exhibits bursting kinetics, which have a complex dependence on both pressure and voltage. Its P_o is increased by increasing pressure and by plasma-membrane hyperpolarisation within the physiological range. Its activity in isolated membrane patches increases as temperature is reduced from 25°C to 6°C, but drops abruptly and irreversibly at 5°C. The channel is permeable to both Ca^{2+} and K^+. The inward unitary conductance was 6 pS with 200 mM Ca^{2+} and 35 pS with 200 mM K^+ as the sole cations in the extracellular solution. Channel activity is decreased by acidifying the extracellular medium from pH 7 to pH 5, and the channel is inhibited by micromolar concentrations of Gd^{3+}, La^{3+} and aluminium. It has been suggested that this channel may act as a sensor integrating electrical, thermal and chemical signals within the framework of transducing mechanical stimuli. However, for a channel to be certified as a physiological mechanotransducer it will be necessary to demonstrate that single channel experiments agree with macroscopic currents and, following Jaffe's criteria [38], that Ca^{2+} influx through these channels is coupled to a cellular and physiological response.

A role in cell signalling has also been proposed for hyperpolarisation-activated Ca^{2+} channels in the plasma membrane of guard cells. The membrane potential of guard cells oscillates between a hyperpolarised state (about −200 mV) and a voltage slightly more positive than E_K, the equilibrium potential for K^+ [39]. In an unchallenged guard cell hyperpolarisation elicits both Ca^{2+} influx, and a subsequent rise in $[Ca^{2+}]_{cyt}$, with a voltage threshold of approximately −120 mV [39,40]. The increase in $[Ca^{2+}]_{cyt}$ is sensitive to $[Ca^{2+}]_{ext}$, to Gd^{3+} and to calcicluidine, an inhibitor of animal L-type Ca^{2+} channels. The application of abscisic acid (ABA) displaces the voltage threshold for the rise in $[Ca^{2+}]_{cyt}$ to more positive voltages, and increases its amplitude and duration. This suggests that hyperpolarisation-activated Ca^{2+} channels are an integral component of the response of the guard cell to ABA. Their regulation by ABA will effectively poise the guard cell for the loss of solutes. The application of ABA will increase the likelihood of plasma-membrane depolarisation to voltages more positive than E_K, not only by Ca^{2+} influx through hyperpolarisation-activated Ca^{2+} channels but also by anion efflux through Ca^{2+}-activated anion channels (see Section 6). This will promote K^+ efflux, solute loss and stomatal closure. If the voltage is already positive to E_K prior to the application of ABA then this depolarisation is superfluous and, as noted by Grabov and Blatt [39], an increase in $[Ca^{2+}]_{cyt}$ may not be required for stomatal

closure. This observation may resolve the longstanding debate of Ca^{2+}-dependent and Ca^{2+}-independent ABA-signalling pathways.

Recently, Kiegle et al. [2], using the molecular-genetic cell-marking approach pioneered by Maathuis et al. [41], compared Ca^{2+} currents across the plasma membrane of various cell types from *Arabidopsis* roots. They observed a depolarisation-activated Ca^{2+}-current in all cell types, and a hyperpolarisation-activated Ca^{2+} current in a subgroup of cells in the elongation zone including endodermal cells. This current was inhibited by 10 μM Al^{3+} whereas the depolarisation-activated Ca^{2+} current was insensitive to this concentration of Al^{3+} (E. Kiegle, personal communication). Based on the tissue location of the voltage-dependent Ca^{2+} currents, it has been suggested [2] that the ubiquitous depolarisation-activated Ca^{2+} channels are likely to be involved in cell signalling, whereas the hyperpolarisation-activated Ca^{2+} channels may have a role in mineral nutrition. Specifically, the hyperpolarisation-activated Ca^{2+} channels could open a symplastic route for Ca^{2+} movement to the xylem by allowing Ca^{2+} to circumvent the Casparian band through the endodermal cell.

2.4. Elicitor-activated Ca^{2+} channels

Two elicitor-activated, plasma-membrane Ca^{2+}-permeable channels have been documented and Ca^{2+} influx through these channels appears to be an early event in the initiation of defence responses to pathogens. The first, a Ca^{2+} channel activated at voltages more negative than −120 mV, was characterised in protoplasts from tomato (*Lycopersicon esculentum* L.) suspension-cultured cells [42,43]. It has inward unitary conductances of 11 pS with 20 mM Ba^{2+} and 4 pS with 20 mM Ca^{2+} in the extracellular medium, and is inhibited by micromolar concentrations of La^{3+} and nifedipine. The second Ca^{2+} channel, termed a large-conductance elicitor-activated channel (LEAC), was characterised in protoplasts from parsley (*Petroselinum crispum*) suspension-cultured cells [44]. The activity of this channel did not appear to depend on the membrane potential (between −30 and −150 mV). The channel was permeable to Ca^{2+} and K^+, and had an inward unitary conductance of 186 pS with 5 mM extracellular $CaCl_2$. The LEAC channel was inhibited by micromolar concentrations of La^{3+} and Gd^{3+}.

The resistance of tomato to the leaf mould pathogen *Cladosporium fulvum* involves the products of plant (*Cf*) genes that respond specifically to the products of single avirulance (*avr*) genes in the pathogen. The induction of defence responses is critically dependent upon G-protein activation, changes in the phosphorylation status of proteins and Ca^{2+} influx (see [45] for a review). Treating suspension-cultured *Cf5* tomato cells with the *afr5* elicitor not only increased the P_o of the hyperpolarisation-activated Ca^{2+} channels through a membrane-delimited, heterotrimeric G-protein-dependent activation of a protein kinase [43,46] but also resulted in membrane hyperpolarisation through the activation of the plasma-membrane H^+-ATPase by heterotrimeric G-protein-dependent activation of a protein phosphatase. These regulatory cascades ensure Ca^{2+} influx in response to an appropriate elicitor and the induction of pathogen defence responses.

The defence responses elicited in parsley suspension cells by an oligopeptide elicitor derived from a cell wall protein of the phytopathogenic fungus *Phytophthora sojae* are also mediated through Ca^{2+} influx [44]. They are not observed in the absence of extracellular Ca^{2+} and are prevented by inhibitors of the LEAC channel. The elicitor activates the LEAC channel in protoplasts, but not in excised membrane patches, and the structural features of the elicitor required for the activation of the LEAC channel are also essential for both receptor binding and induction of defence-related gene expression. Thus, Zimmermann et al. [44] argue that indirect receptor-mediated activation of the LEAC channel is causally related to the induction of defence-responses.

3. Vacuolar calcium channels

The vacuole, which may occupy more than 90% of the volume of a plant cell, is a major source of Ca^{2+}. The Ca^{2+} electrochemical gradient across the tonoplast is large. Both the resting membrane potential (−20 mV to −50 mV) and the difference between $[Ca^{2+}]_{cyt}$ (100 to 500 nM) and $[Ca^{2+}]_{vac}$ (micromolar to millimolar) favour Ca^{2+} entering the cytoplasm. It

Table 2
Calcium-permeable channels in the tonoplast of plant cells

Name	Tissue	Conductance	Permeability	Inhibitors of Ca^{2+} flux	Ref.
SV	Various	50–80 pS (100 mM K^+ 100 μM Ca^{2+})	Ca, Ba, Mg	Amiloride	[48,54,64, 66,67]
			K, Na, Rb, Cs	CTX, 9-AA, quinacrine, quinine TEA^+, Ba^{2+}, $Tris^+$, polyamines Zn^{2+}, DIDS, SITS, EA Pyridoxal phosphate, A9C, C114	
SV	Beet root	40 pS (100 mM $[Ba^{2+}]_{cyt}$)		La^{3+}, verapamil	[52,65]
SV	Guard cell	240–280 pS (100 mM K^+ 100 μM Ca^{2+}) 16 pS (5 mM $[Ca^{2+}]_{cyt}$)			[48,59]
Depac 2	Tobacco suspension	19 pS (50 mM Ca^{2+}) 30 pS (50 mM Ba^{2+})	Ba, Sr, Ca, Mg	Cd^{2+}	[68]
	Beet root	15 pS (50 mM Ba^{2+})	Ba		[68]
	Arabidopsis leaf mesophyll	20 pS (50 mM Ba^{2+})	Ba		[69]
Hypac 1	Beet root	6 pS (10 mM $[Ca^{2+}]_{vac}$)	Ba, Sr, Ca	Nifedipine, verapamil, La^{3+}, $[Ca^{2+}]_{cyt}$	[71]
	Vicia faba guard cell	14 pS (5 mM $[Ca^{2+}]_{vac}$)			[72]
Hypac 2	Beet root	12 pS (5 mM $[Ca^{2+}]_{vac}$)	Ca, Ba, Sr, Mg, Mn, K	Zn^{2+}, Gd^{3+}	[70,73,74]
	Vicia faba guard cell	27 pS (5 mM $[Ca^{2+}]_{vac}$)	Ca, Ba, Sr, Mg, K	Gd^{3+}, nifedipine, DIDS	[72]
	Commelina guard cell	25 pS (5 mM $[Ca^{2+}]_{vac}$)			[73]
IP_3-Dependent	Beet root	30, 50 pS (5 mM $[Ca^{2+}]_{vac}$)	Ca, Ba, Sr	Verapamil, heparin, TMB8	[5]
		11, 51, 182 pS (5 mM $[Ca^{2+}]_{vac}$)			[78]
cADPR-Dependent	Beet root		Ca	Ruthenium red, procaine	[77]
	Vicia faba guard cell		Ca	$[Ca^{2+}]_{cyt}$	[82]

is thought that Ca^{2+} efflux from the vacuole could sustain large and prolonged elevations in $[Ca^{2+}]_{cyt}$ by propagating and amplifying an initial signal. When this initial signal is a rise in $[Ca^{2+}]_{cyt}$ the process has been termed Ca^{2+}-induced Ca^{2+} release (CICR) and the role of various vacuolar Ca^{2+} channels in CICR is greatly debated (see [47] for an overview).

There are several classes of vacuolar Ca^{2+} channels, which co-reside in the tonoplast (Table 2). Depolarisation-activated Ca^{2+} channels, including the slow vacuolar (SV) channels, have a reasonable selectivity for divalent cations but their physiological role is unclear. Hyperpolarisation-activated Ca^{2+} channels also have a reasonable selectivity for divalent cations and catalyse Ca^{2+} influx to the cytoplasm. These channels may have a role in cell signalling. There are also several classes of second messenger-activated Ca^{2+} channels. These include Ca^{2+} channels activated by inositol triphosphate (IP_3) and cyclic ADP ribose (cADPR). These channels are highly selective for divalent cations over monovalent cations.

3.1. Slow vacuolar (SV) channels

At $[Ca^{2+}]_{cyt}$ greater than 300–600 nM the conductance of the vacuole is dominated by currents activating slowly at positive membrane potentials. These are termed slow vacuolar (SV) currents and have been observed in many tissues (see [48–51] for reviews).

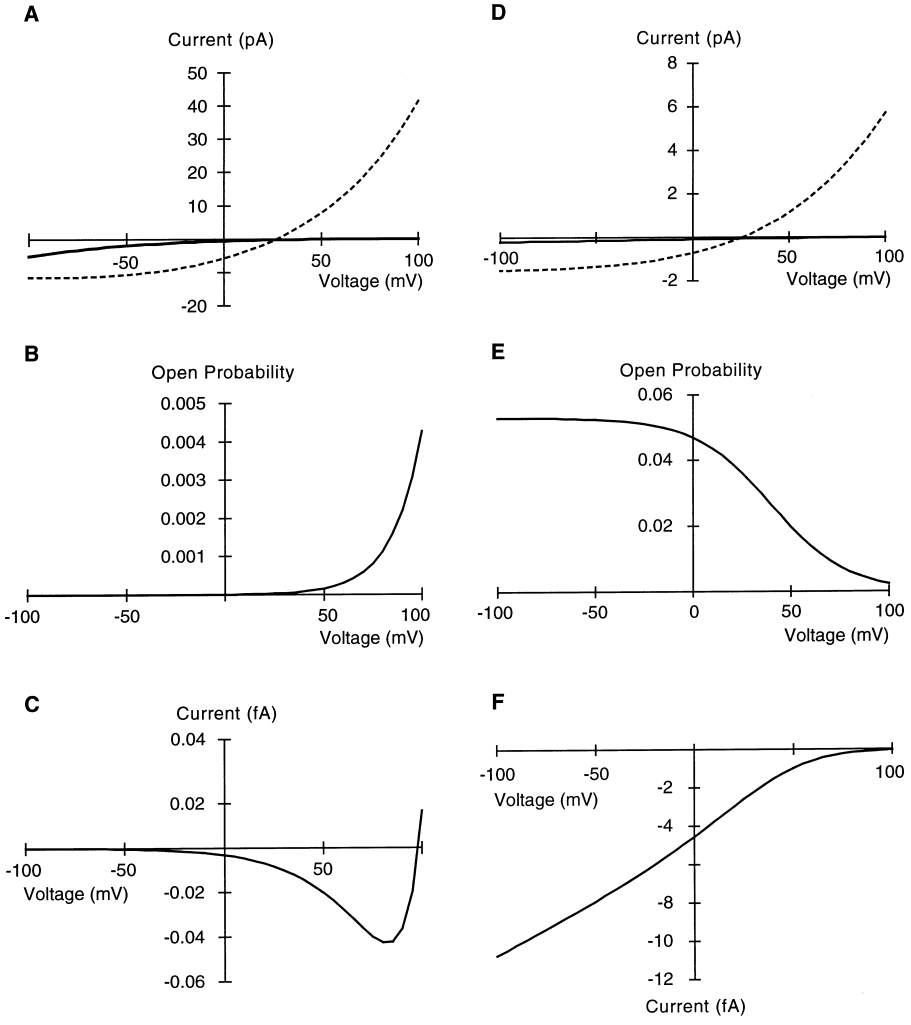

Fig. 2. (A) The Ca^{2+} (———) and K^+ (- - -) currents through a single open SV channel predicted under ionic conditions approximating the guard cell of an open stomate using the reaction-kinetic model of Allen et al. [60]. Cation activities were $[K^+]_{cyt} = 70$ mM, $[K^+]_{vac} = 200$ mM, $[Ca^{2+}]_{cyt} = 0.001$ mM, $[Ca^{2+}]_{vac} = 1$ mM. (B) The relationship between the probability of finding an open SV channel and tonoplast voltage calculated for physiological ionic conditions at 20°C using Equation 1 of Pottosin et al. [57], with $A = 1$, $z = 1.68$ and $V_o = 182$ mV. (C) The voltage dependence of the Ca^{2+} current through an average SV channel under physiological conditions. The data were obtained by multiplying the Ca^{2+} current through an open channel and its open probability. (D) The Ca^{2+} (———) and K^+ (- - -) currents through a single open hyperpolarisation-activated Ca^{2+}-permeable channel in the tonoplast predicted under ionic conditions approximating the guard cell of an open stomate using the reaction-kinetic model of Gradmann et al. [76]. (E) The relationship between the probability of finding an open hyperpolarisation-activated channel and tonoplast voltage calculated for physiological ionic conditions at 20°C using Equation 4 of Johannes and Sanders [74], with $P_o\max = 0.053$, $z = 1.3$ and $V_{0.5} = 40$ mV. (F) The voltage dependence of the Ca^{2+} current through an average vacuolar hyperpolarisation-activated Ca^{2+} channel under physiological conditions. The data were obtained by multiplying the Ca^{2+} current through an open channel and its open probability. This figure is drawn from the unpublished simulations of M.S. Ridout and P.J. White.

The underlying SV channel was initially characterised as being K^+ selective, but with a significant permeability to Cl^- [48]. However, recent studies have demonstrated that the SV channel is permeable to Ca^{2+}, Ba^{2+} and Mg^{2+}, as well as K^+, Na^+, Rb^+ and Cs^+ [49,52–57], but is impermeable to Cl^-. The unitary conductance of the SV channel at positive voltages is frequently estimated as 50 to 100 pS in solutions containing 100 mM KCl and 100 μM $CaCl_2$ (summarised in [49,54]). However, lower unitary conductances have occasionally been reported [57,58] and the SV channel in the tonoplast of guard

cells appears to have a higher unitary conductance [48,54,59].

The conductance properties of the SV channel suggest that cation permeation can be described by a model incorporating interactions within a multi-cation-binding pore [55,56]. The apparent cationic permeability ratios for the SV channel depend on the ionic composition of the solutions, the concentration dependence of unitary conductance displays a maximum, and an anomalous mole fraction effect (a minimum in the relationship between E_{rev} and the ratio of Ca^{2+}/total cations in either the cytoplasmic or vacuolar solutions) has been reported [55,56]. However, a quantitative, multi-site energy-barrier model has not been developed. Instead, Ca^{2+} and K^+ permeation through the SV channel has been described by a reaction-kinetic (dynamic pore) model [60]. This may generate little insight into the structure of the channel pore, but it does allow the prediction of ionic fluxes. Under physiological conditions (Fig. 2A), the SV channel is predicted to facilitate Ca^{2+} and K^+ influx to the cytoplasm. However, the SV channel could facilitate K^+ efflux to the vacuole with appropriate membrane potentials and K^+ gradients between cytoplasm and vacuole.

The physiological cation fluxes through the SV channel will depend not only on the permeation of cations through the pore but also on the gating of the channel. The voltage-dependent gating of the SV channel is modulated by Ca^{2+} and pH. The relationship between P_o and voltage for the SV channel shifts to more negative voltages with increasing $[Ca^{2+}]_{cyt}$ and to more positive voltages with an increase in $[Ca^{2+}]_{vac}$ (see [57] and references therein). Cytoplasmic calmodulin (CaM) sensitises the SV channel to Ca^{2+}, allowing activation at lower $[Ca^{2+}]_{cyt}$ [54,58]. In some studies the SV channel could also be activated by increasing $[Mg^{2+}]_{cyt}$ to millimolar levels [55], but this is not universally observed. Pottosin et al. [57] suggested that this was a consequence of an increase in $[Ca^{2+}]_{cyt}$ following the addition of Mg^{2+} to media with insufficient Ca^{2+} chelation. Cytoplasmic alkalinisation over the physiological range (pH 6 to pH 8) activates the SV channel by shifting the relationship between P_o and voltage to more negative voltages [54]. Vacuolar acidification, in the presence of subphysiological $[Ca^{2+}]_{vac}$, decreases channel activity by shifting the P_o versus voltage relationship to more positive voltages [54,57]. In addition, increasing $[Cl^-]_{cyt}$ specifically increases the P_o of the SV channel in vacuoles from sugar beet [61].

Ward et al. [49] suggested that SV channels might allow CICR. They suggested that a small, initial increase in $[Ca^{2+}]_{cyt}$ through 'trigger' channels, such as ligand- or voltage-gated Ca^{2+} channels at the vacuole, ER or plasma membrane, might activate Ca^{2+}-dependent K^+ channels (VK channels) in the tonoplast. This would depolarise the tonoplast through K^+ efflux and, combined with the initial rise in $[Ca^{2+}]_{cyt}$, this depolarisation could activate SV channels. Activation of SV channels would then result in Ca^{2+} efflux, further depolarisation and the perpetuation of the cycle. However, this sequence of events has been questioned. A quantitative study of the relationship between the P_o of the SV channel and the electrochemical gradient for Ca^{2+} (performed by Pottosin et al. [57] at $[Ca^{2+}]_{cyt}$ of 12.5 μM to 2 mM and $[Ca^{2+}]_{vac}$ of 50 μM to 2 mM) suggests that its P_o may be as low as 0.004 at the Nernst (equilibrium) potential for Ca^{2+} (E_{Ca}; Fig. 2B) and that higher P_o requires a net Ca^{2+} motive force that would drive Ca^{2+} movement into the vacuole. Thus, Pottosin et al. [57] argued that the SV channel was not suited to CICR. However, the SV channel can sustain a small Ca^{2+} influx to the cytoplasm under physiological conditions (Fig. 2C). For example, if there are 560 SV channels per vacuole (vacuolar surface area = 1.4×10^{-9} m^{-2} and channel density = 4×10^{11} m^{-2}) Ca^{2+} influx would approach 2.4×10^{-20} mol s^{-1} at 25 mV (E_K), which corresponds to an increase in $[Ca^{2+}]_{cyt}$ of 5 nM s^{-1} if a cytoplasmic volume of 5×10^{-16} m^{-3} and a buffering capacity of 90% of the Ca^{2+} influx are assumed. This may approach the rates at which $[Ca^{2+}]_{cyt}$ increases in response to many stimuli [3,6].

The SV channel can also be regulated by phosphorylation at two sites, which may be on the channel or on separate regulatory proteins [62,63]. One site is phosphorylated by a protein kinase sensitive to H-7 ([1-(5-isoquinolinesulfonyl)-methylpiperazine]) and dephosphorylated by an endogenous protein phosphatase sensitive to okadaic acid or exogenous calcineurin in the presence of calmodulin. Phosphorylation at this site inhibits channel activity. The second site is possibly phosphorylated by a CaM-like

domain protein kinase (CDPK), and dephosphorylated by a Ca^{2+}-dependent protein phosphatase. Phosphorylation at this site stimulates SV channel activity. It has been proposed that Ca^{2+}-dependent inhibition of the SV channel might prevent an excessive increase in $[Ca^{2+}]_{cyt}$ [62] and/or regulate the frequency of oscillations in $[Ca^{2+}]_{cyt}$ [4,8].

The SV channel has a complex pharmacology. It is inhibited by micromolar verapamil and millimolar La^{3+} [52]. It is inhibited by pharmaceuticals that act also on anion transporters: Zn^{2+}, DIDS, SITS, pyridoxalphosphate, ethacrinic acid (EA), anthracene-9-carboxylic acid (A9C) and C144 [54,64]. It is inhibited by amiloride, an inhibitor of Na^+ transporters [64,65]. It is inhibited by nanomolar charybdotoxin (CTX) in the cytoplasm and by micromolar (+)-turbocurarine, 9-amino-acridine, quinacrine or quinine [66]. In addition, the SV channel is blocked by millimolar concentrations of TEA^+ and Ba^{2+} [64,66], which both permeate the SV channel, as well as Tris and the polyamines spermine, spermidine and putrescine [67].

3.2. Other depolarisation-activated Ca^{2+} channels

A pharmacologically distinct depolarisation-activated Ca^{2+} channel was observed in vacuoles from tobacco suspension cells [68]. This channel was blocked by 100 μM cytoplasmic Cd^{2+} and was insensitive to similar concentrations of Ni^{2+}, La^{3+}, Gd^{3+}, verapamil and nifedipine. It was permeable to Ba^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+}, and had a unitary conductance of 19 pS with 50 mM $[Ca^{2+}]_{cyt}$ and 30 pS with 50 mM $[Ba^{2+}]_{cyt}$ as the charge carrier. Another depolarisation-activated Ba^{2+}-permeable channel was reported in the tonoplast of *Arabidopsis* leaf mesophyll cells, which had a lower unitary conductance of 20 pS with 50 mM $[Ba^{2+}]_{cyt}$ as the charge carrier [69].

3.3. Hyperpolarisation-activated Ca^{2+} channels

Hyperpolarisation-activated Ca^{2+} channels, which open at negative voltages within the physiological range (−20–−70 mV), have been recorded in vacuoles from beet (*Beta vulgaris* L.) taproot [70,71] and guard cells from *Vicia faba* [72] and *Commelina communis* [73]. These channels mediate Ca^{2+} efflux from the vacuole. Two distinct types have been observed. The first type is inhibited by $[Ca^{2+}]_{cyt}$ greater than 1 μM [71]. This may ensure that the activity of the channel is curtailed before $[Ca^{2+}]_{cyt}$ rises excessively. The second type is insensitive to $[Ca^{2+}]_{cyt}$, but increasing $[Ca^{2+}]_{vac}$ increases the P_o of the channel under physiological conditions by shifting the threshold for activation to more positive voltages [70,72,74]. Analysis of its voltage- and $[Ca^{2+}]_{vac}$-dependence suggests that the binding of two Ca^{2+} at a site located 30% of the electrical field across the membrane from the vacuolar side are required for activation of the channel from beet [74]. Vacuolar acidification also decreases the P_o of this type of channel [70,72] and it has been suggested that this might be a feedback mechanism to prevent uncontrolled Ca^{2+} efflux during short-term responses to environmental signals [51].

The first type of hyperpolarisation-activated Ca^{2+} channel had inward unitary conductances of 6 pS in the presence of 100 mM $[K^+]_{cyt}$: 10 mM $[Ca^{2+}]_{vac}$ [71] and 14 pS in the presence of 50 mM $[K^+]_{cyt}$: 5 mM $[Ca^{2+}]_{vac}$ [72]. These channels passed no outward K^+ current. The channel studied in beet was permeable to Ba^{2+}, Sr^{2+} and Ca^{2+}, and was inhibited by micromolar concentrations of nifedipine added to the vacuolar solution and verapamil, La^{3+} or Ca^{2+} added to the cytoplasmic solution. It was insensitive to IP_3.

The second type of hyperpolarisation-activated Ca^{2+} channel had an inward (Ca^{2+}) unitary conductance (measured at nonsaturating voltages between E_{rev} and approximately −40 mV) of 12 pS in beet [70,74–76], 27 pS in *Vicia faba* guard cells [72] and 25 pS in *Commelina communis* guard cells [73] in the presence of 50 mM $[K^+]_{cyt}$: 5 mM $[Ca^{2+}]_{vac}$. Under these ionic conditions these channels had outward (K^+) unitary conductances between 120 and 200 pS. The outward current through the 12 pS channel from red beet was completely blocked by 0.03 mM $[Ca^{2+}]_{cyt}$ [74] and the channel was inhibited by micromolar Zn^{2+} and Gd^{3+}, but was insensitive to IP_3, heparin, TMB8, ruthenium red, ryanodine and La^{3+} [70]. The 27 pS channel from *Vicia faba* guard cells was inhibited by micromolar Gd^{3+}, nifedipine and DIDS [72].

Both the 12 pS channel from beet root and the 27 pS channel from guard cells were permeable to Ca^{2+}, Ba^{2+}, Sr^{2+}, Mg^{2+} and K^+ [72,73]. The channel from beet was also shown to be permeable to Mn^{2+},

but not to Zn^{2+}, Ni^{2+} or Co^{2+} [74]. Their cation selectivity based on permeability ratios differed from that based on conductance ratios [72,74], suggesting that there were interactions between cations within the pore. However, cation permeation through the 12 pS channel was initially interpreted in terms of an energy-barrier (rigid-pore) model with a (single) binding site for Ca^{2+}. The binding site was located 9% through the electrical field within the pore from the vacuolar side and had a K_m of 0.3 mM at 0 mV [73,75]. Subsequently, the permeation of Ca^{2+} and K^+ through the pore was described using a single-binding-site dynamic-pore model [76]. The latter model predicted that, under physiological conditions, Ca^{2+} influx through the channel was about 10% of the K^+ influx at negative voltages (Fig. 2D). It also predicted that Ca^{2+} currents reversed at a voltage more positive than E_{Ca} due to a partial coupling between Ca^{2+} and K^+ fluxes (Fig. 2C and [76]). It should be noted that this phenomenon cannot occur with a rigid-pore model.

In the absence of other regulatory phenomena, these channels would catalyse significant Ca^{2+} influx to the cytoplasm at all physiological membrane potentials (Fig. 2F). However, both the 12 pS channel from beet root and the 27 pS channel from guard cells exhibited spontaneous transitions between states with distinct kinetic behaviour [72,75]. In general, only the states with high P_o were analysed in detail. For the 12 pS channel from beet the transition from the less active state resulted in a threefold increase in unitary conductance (from 4 pS to 12 pS) and a positive displacement of the relationship between P_o and voltage [75]. It is speculated that such regulation may play a role in activation of the channel during signal transduction and it has been suggested that the phenomenon may result from the dissociation of a regulatory ligand.

3.4. IP_3-Dependent channels

The application of IP_3 releases Ca^{2+} from vacuoles and tonoplast vesicles isolated from many plant species and tissues (see [5,50,51,77] for reviews). However, only in vacuoles from beet storage roots have IP_3-dependent Ca^{2+} channels been characterised electrically [5,78]. The competence of vacuoles from this tissue to respond to IP_3 is enhanced by osmotic stress, which may indicate a role in cell water relations and turgor regulation [78].

The IP_3-dependent Ca^{2+} channels are electrically silent in the absence of IP_3 and can be activated half-maximally at IP_3 concentrations as low as 200 nM [5]. Their absolute requirement for IP_3 strongly suggests that these channels are involved in Ca^{2+}-mediated signal transduction. The channels appear highly selective for divalent cations and permeable to Ca^{2+}, Ba^{2+} and Sr^{2+} [5,78]. They can be inhibited by verapamil, TMB8 and heparin, the latter compounds being inhibitors of the IP_3-dependent channels in animal cells, but they are unaffected by ruthenium red and ryanodine [5,77]. Channel gating is insensitive to $[Ca^{2+}]_{cyt}$ between 0.1 μM and 1 mM. The unitary conductances reported for the IP_3-dependent Ca^{2+} channel in isolated membrane patches vary greatly. Alexandre and Lassalles [5] observed substates at 30 and 50 pS and Allen and Sanders [78] observed conductance levels of 11, 51 and 182 pS in media containing 1 mM $[Ca^{2+}]_{cyt}$ and 5 mM $[Ca^{2+}]_{vac}$.

The IP_3-dependent Ca^{2+} channels activate at physiological tonoplast membrane potentials and their P_o is increased by hyperpolarisation [5,78]. They will mediate Ca^{2+} influx to the cytoplasm and excessive cytoplasmic Ca^{2+} loading might be prevented because Ca^{2+} influx will depolarise the tonoplast and shut the channels. The opening of IP_3-dependent Ca^{2+} channels has been implicated in turgor regulation in beet root in response to hyperosmotic stress [78], the closing of stomata in response to ABA (Section 6) and the self-incompatibility and reorientation responses of pollen tubes [79,80].

3.5. IP_6-Dependent channels

The application of physiological concentrations of phytic acid (IP_6) to the cytoplasm of guard cell protoplasts results in electrical events consistent with an increase in $[Ca^{2+}]_{cyt}$ [81], suggesting the presence of IP_6-dependent Ca^{2+}-permeable channels in guard cells. The membrane location(s) of these putative Ca^{2+} channels are unknown.

3.6. cADPR-Dependent channels

Cyclic ADP ribose (cADPR) activates inward rec-

tifying currents in vacuoles from red beet taproots (K_m of 24 nM; see [51,77] for reviews) and guard cells [82]. Rectification is induced by the presence of Ca^{2+} in the vacuole [51]. The channels underlying this current appear to be fairly selective for Ca^{2+} over K^+, and will mediate Ca^{2+} efflux from the vacuole. Their pharmacology is similar to the cADPR-activated, ryanodine-receptor channels in the endomembranes of animal cells. They are activated by ryanodine and caffeine, and blocked by ruthenium red and procaine. The channels in the vacuole of guard cells were inhibited at $[Ca^{2+}]_{cyt}$ greater than 600 nM, suggesting that these channels are unlikely to sustain CICR. Heparin and TMB8, characteristic inhibitors of the IP_3-dependent channel, were without effect on the cADPR-dependent channel.

A role for cADPR-dependent Ca^{2+} channels in two ABA-dependent signalling pathways has been proposed. A link between the induction of *kin2* (cold responsive) and *rd29A* (desiccation responsive) genes by ABA in tomato hypocotyl cells and cADPR-dependent Ca^{2+} release is suggested by the observations: (i) that cADPR is increased in response to ABA, (ii) microinjection of cADPR or Ca^{2+} induced gene expression, and (iii) inhibition of cADPR production and buffering $[Ca^{2+}]_{cyt}$ to low concentrations prevented gene expression [83]. A role of cADPR-dependent channels in ABA-induced stomatal closure will be discussed below (Section 6).

4. Calcium channels in the endoplasmic reticulum

Calcium efflux from the ER has been implicated in cell signalling. However, since such arguments are largely based on the sensitivity of stimulus-induced perturbations in $[Ca^{2+}]_{cyt}$ to pharmaceuticals, they should be regarded with caution. Biochemical studies indicate that Ca^{2+} can be released from cauliflower (*Brassica oleracea* L.) ER vesicles by the application of cADPR, NAADP (nicotinic acid adenine dinucleotide phosphate; L. Navazio, D. Sanders, York, UK, personal communication), a metabolite of NADP that releases Ca^{2+} from endomembrane stores in animal cells [84], and (possibly) IP_3 [85]. This suggests the presence of cADPR-, NAADP- and IP_3-dependent Ca^{2+} channels in the ER. However, no electrophysiological demonstration of these channels has yet been published.

Calcium channels from the ER have been studied electrically following the incorporation of ER vesicles from either the touch-sensitive tendrils of *Bryonia dioica* (BCC1; [86,87]) or the root tips of *Lepidium sativum* (LCC1; [88]) into PLB. Both channels are strongly rectifying and open at more positive voltages. The relationship between P_o and voltage is shifted to more negative voltages when a Ca^{2+} gradient ($[Ca^{2+}]_{ER} > [Ca^{2+}]_{cyt}$) is applied. Both channels show a moderate selectivity for Ca^{2+} over K^+, and are permeable to Ba^{2+}, Sr^{2+} and Mg^{2+}. Their single channel conductances were 29 pS (BCC1) and 24 pS (LCC1) in 50 mM $CaCl_2$. Cytoplasmic acidification increased both the mean conductance and P_o of BCC1 [87]. Both BCC1 and LCC1 were blocked by micromolar concentrations of Gd^{3+}, La^{3+} and Erythrosine B applied on the lumenal side of the channel. Verapamil had no effect on LCC1 but blocked BCC1. In addition, BCC1 was blocked by micromolar concentrations of Cu^{2+}, Cd^{2+} and Zn^{2+}, but 1,4-dihydropyridines (< 70 μM), IP_3 (< 10 μM) and ryanodine (< 30 μM) were ineffective. The opening of BCC1 was inhibited by the presence of H_2O_2 on the cytoplasmic side, which may be due to loss of channel function [87].

It is likely that the physiological activity of BCC1 is governed primarily by the Ca^{2+} activity in the lumen of the ER, although modulation by cytoplasmic pH can also be envisaged [86,87]. At the resting membrane potential of the ER, which is assumed to be close to zero, and with submicromolar lumenal Ca^{2+} activities, BCC1 is likely to be closed. If the lumenal Ca^{2+} activity is raised, perhaps by the activity of an ER calmodulin-dependent Ca^{2+}-ATPase [89], the relationship between P_o and voltage will be shifted to more negative voltages and the channel will open to release Ca^{2+} into the cytoplasm. This will reduce the lumenal Ca^{2+} and the channel recloses. This cycle could generate transient elevations of $[Ca^{2+}]_{cyt}$ and the frequency of such elevations could be determined by modulation of the BCC1 channel, the Ca^{2+}-ATPase or the Ca^{2+} gradient across the ER [86]. Thus, the BCC1 channel might give rise to signal-specific temporal and spatial changes in $[Ca^{2+}]_{cyt}$.

5. Calcium channels in chloroplast and nuclear membranes

A channel permeable to Ca^{2+}, Mg^{2+} and K^+ has been recorded in swollen thylakoids from spinach chloroplasts [90]. This channel was activated at membrane potentials promoting cation movement to the intrathylakoid space. Its unitary conductance at such voltages was 62 pS with 100 mM K^+ and 21 pS with 5 mM Ca^{2+} as the charge carrier. The physiological role of this channel may be to provide compensatory cation movements during light-driven H^+ uptake into thylakoids. Incidentally, Ca^{2+} uptake into intact chloroplasts is stimulated by illumination and is inhibited by ruthenium red [91]. This is thought to stimulate anabolic metabolism.

A channel permeable to K^+, Na^+, Cs^+ and Ca^{2+} has been recorded in the nuclear envelope of nuclei from red beet [92]. The unitary conductance of this channel was 110 pS in symmetrical 150 mM KCl, which was reduced to 15 pS by replacing KCl on one side with 50 mM $CaCl_2$. Most nucleus-attached patches were quiescent, but excision of the patch resulted in an immediate activation of the channel. The channel was activated by membrane hyperpolarisation when the $[Ca^{2+}]$ on the side of the channel originally facing the perinuclear space (the lumen of the nuclear envelope) approached 1 μM. Increasing the perinuclear $[Ca^{2+}]$ shifted the relationship between channel P_o and voltage to more positive voltages, thereby increasing channel P_o at physiological membrane potentials. Channel activity was unaffected by changes in $[Ca^{2+}]_{cyt}$. The channel was blocked by 1 mM Zn^{2+} when applied to the cytoplasmic side of the channel, and by 2 mM amiloride when applied to the side facing the perinuclear space. Based on the sensitivity of this channel to the $[Ca^{2+}]$ of the periplasmic space, a role for this channel in regulating Ca^{2+}-dependent nuclear processes has been proposed [92].

6. The co-ordination of Ca^{2+} channel activities

An illustration of the co-ordination of Ca^{2+} channel activities in effecting a physiological response is provided by the closing of stomata in response to ABA. Stomata are pores in the leaf epidermis through which CO_2 diffuses for photosynthetic carbon fixation and water vapour is lost to the atmosphere. This exchange is regulated by the movements of two guard cells, which surround the stomatal pore. Under drought conditions, the plant growth regulator abscisic acid (ABA) closes stomata by promoting the efflux of K^+ and anions (Cl^- or malate, depending on the plant and the environmental conditions) from the guard cells. The mechanism by which ABA triggers stomatal closure has been reviewed extensively [47,93–96] and only a skeletal scheme is outlined here.

It is proposed that ABA binds to (unidentified) receptor proteins in the plasma membrane and/or intracellular membranes (Fig. 3A) to initiate a sequence of events leading to cytoplasmic alkalinisation (from a resting value of pH 7.3 to between pH 7.5 and 7.7), an increase in the cytoplasmic concentrations of signalling metabolites (cADPR, IP_3, IP_6), an increase in $[Ca^{2+}]_{cyt}$ (to concentrations in excess of 1 μM) and plasma-membrane depolarisation (to values more positive than E_K) of cells in the (hyperpolarised) P-state.

Cells in the P-state are depolarised through the activation of ion channels mediating inwardly directed currents. Central to this response appears to be an hyperpolarisation-activated Ca^{2+} channel (Fig. 3B; see [39]). The application of ABA displaces the voltage threshold for the activation of this channel to more positive voltages, thereby increasing the likelihood of its opening. Plasma-membrane depolarisation will then proceed by Ca^{2+} influx through this channel and by anion efflux through $[Ca^{2+}]_{cyt}$-dependent anion channels. If the guard cell is already in the (depolarised) K-state when challenged by ABA, the opening of the hyperpolarisation-activated Ca^{2+} channel may not be required for stomatal closure.

The $[Ca^{2+}]_{cyt}$ may also rise through Ca^{2+} release from internal stores, such as the ER and vacuole (Fig. 3C). The major Ca^{2+} influx may be through cADPR-activated channels. Microinjection of cADPR increases $[Ca^{2+}]_{cyt}$ in guard cells, and the application of pharmaceuticals that inhibit cADPR synthesis or the opening of cADPR-dependent channels reduce the ABA-induced rise in $[Ca^{2+}]_{cyt}$ [95] and stomatal closure [82]. However, since the cADPR channel is inhibited by $[Ca^{2+}]_{cyt}$ greater than 600 nM it is unlikely to effect the observed

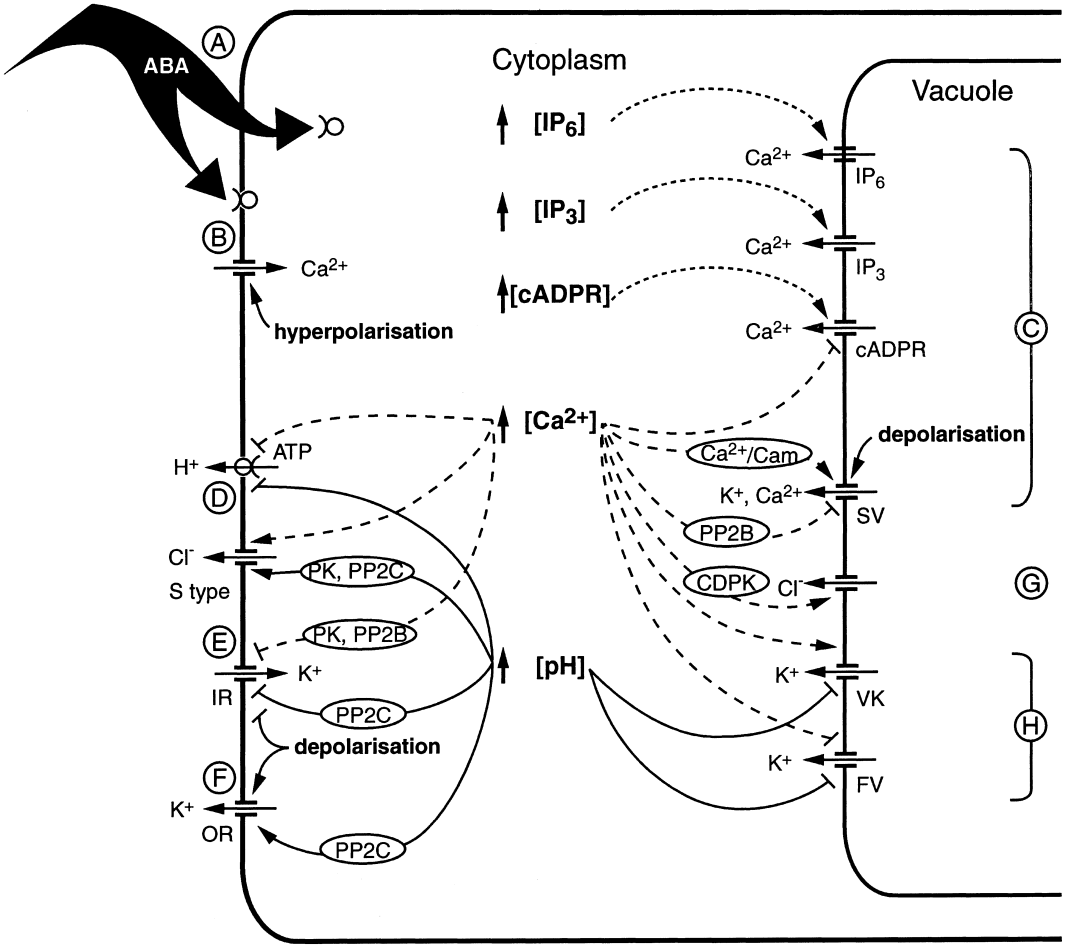

Fig. 3. A hypothetical sequence of events leading to the efflux of K^+ and Cl^- from guard cells in response to abscisic acid (ABA). (A) Binding of ABA to receptor proteins. (B) Opening of a hyperpolarisation-activated plasma-membrane Ca^{2+} channel. (C) Increase in $[Ca^{2+}]_{cyt}$ through the opening of vacuolar Ca^{2+} channels. (D) Opening of Ca^{2+}-dependent Cl^- channels in the plasma membrane and inhibition of the plasma-membrane H^+-ATPase. (E) Inactivation of inward rectifying (IR) K^+ channels in the plasma membrane. (F) Opening of outward rectifying (OR) K^+ channels in the plasma membrane. (G) Opening of vacuolar Cl^- channels. (H) Opening of vacuolar (VK and FV) K^+ channels.

increase in $[Ca^{2+}]_{cyt}$, which may be in excess of 1 μM, by itself. The opening of IP_3-dependent channels has also been implicated in stomatal closure. Upon the application of ABA the turnover of IP_3 is increased in guard cells, the application of exogenous IP_3 increases $[Ca^{2+}]_{cyt}$ and inhibitors of IP_3 metabolism inhibit stomatal closure [97]. In addition, evidence that increasing $[IP_6]_{cyt}$ to levels comparable to those observed when guard cells are challenged by ABA mimics the effects of ABA and acts by increasing $[Ca^{2+}]_{cyt}$, suggests the involvement of IP_6-dependent Ca^{2+} channels [81]. A Ca^{2+} influx through SV channels may also occur (see Section 3.1), but these channels might be subject to feedback inhibition through the activity of a Ca^{2+}-dependent protein phosphatase type 2B (PP2B).

Increasing $[Ca^{2+}]_{cyt}$ activates Ca^{2+}- and depolarisation-activated S-type anion channels in the plasma membrane (Fig. 3D). These channels can also be activated by phosphorylation, but the exact mechanism(s) of their modulation following the application of ABA remains unclear. In *Arabidopsis* guard cells, the anion channels activated by ABA can be activated in the absence of a rise in $[Ca^{2+}]_{cyt}$, suggesting

that anion efflux can be a Ca^{2+}-independent process. This may be effected through the activation of a protein phosphatase type 2C (PP2C) by increasing cytoplasmic pH. The efflux of anions depolarises the plasma membrane to voltages more positive than E_K. Increasing $[Ca^{2+}]_{cyt}$ and cytoplasmic alkalinisation probably also inhibit plasma-membrane H^+-ATPase activity, preventing plasma-membrane repolarisation.

The rise in $[Ca^{2+}]_{cyt}$, increase in cytoplasmic pH and depolarisation of the plasma membrane, all inactivate inward-rectifying (IR) K^+ channels (Fig. 3E). This is not essential for stomatal closure but could accelerate it. The inhibition of IR K^+ channels may proceed through dephosphorylation of either the channel itself or of a regulatory intermediate by pH-modulation of PP2C, Ca^{2+}-dependent activation of PP2B and/or phosphorylation by a protein kinase. Cytoplasmic alkalinisation and plasma-membrane depolarisation also activate a Ca^{2+}-independent outward-rectifying (OR) K^+ channel (Fig. 3F), possibly also through a pH-sensitive PP2C activity. This facilitates K^+ efflux from the cell.

The simultaneous opening of K^+ and anion channels in the plasma membrane will facilitate solute efflux from the guard cell. However, most of the K^+ and anions lost from guard cells must first be transported from the vacuole to the cytoplasm. An anion channel in tonoplast of guard cells (Fig. 3G), activated by a Ca^{2+}-dependent protein kinase, is likely to mediate anion efflux from the vacuole [98]. At least three K^+-permeable channels coexist in the guard-cell tonoplast, namely the VK, SV and FV channels (Fig. 3C,H). The VK channel probably mediates the ABA-induced K^+-efflux from the vacuole. Increasing $[Ca^{2+}]_{cyt}$ above 100 nM activates the VK channel and although the VK channel is downregulated by increasing cytoplasmic pH the effect will be minor [59,93]. Under certain conditions the FV channel might also contribute to ABA-induced K^+ efflux from the vacuole [93]. However, the FV channel is inhibited by raising $[Ca^{2+}]_{cyt}$ to micromolar levels and by increasing pH above 7.3. By these mechanisms both K^+ and anions can be released from the vacuole and effluxed from the guard cell. This results in the reduction of turgor, and the closing of stomata.

7. Future directions

A wide variety of Ca^{2+} channels on all major plant cell membranes have been characterised electrically. For many, a physiological role has been suggested based on their cation permeability, voltage dependence and regulation by cellular effectors. However, the physiological stimuli that activate specific Ca^{2+} channels are unknown. To implicate a specific Ca^{2+} channel in signal transduction it will be necessary to demonstrate at least: (i) that physiological responses are preceded by the opening of the Ca^{2+} channel, (ii) that the specific blockade (or absence) of the Ca^{2+} channel eliminates the physiological responses, and (iii) that changes in $[Ca^{2+}]_{cyt}$ comparable to those generated by the active Ca^{2+} channel initiates a comparable physiological response. Unfortunately, specific inhibitors of particular Ca^{2+} channels are not available at present (see Tables 1 and 2). The pharmaceuticals commonly used to inhibit Ca^{2+} channels, with (perhaps) the exception of heparin, inhibit a wide variety of plant ion channels [1]. Furthermore, many plant Ca^{2+} channels exhibit atypical pharmacologies. For example, consider the diversity of pharmaceuticals acting on the SV channel (Table 2). Thus, plant Ca^{2+} channels cannot be readily classified on the basis of their pharmacology alone. These facts make the task of elucidating the physiological role of a particular Ca^{2+} channel by pharmacological dissection difficult.

To date, no genes for plant Ca^{2+} channels have been identified unequivocally. This remains a priority. Several strategies to identifying Ca^{2+} channel genes have been undertaken. Initially, protein purification and sequencing was considered as a starting point to obtain the genes for the phenylalkylamine-binding protein [99] or the heparin-binding protein [100]. More recently, strategies based on sequence homologies have been pursued. For example, it was known that the selectivity of animal Ca^{2+} channels is generated by a ring of four glutamate residues that coordinate Ca^{2+} [29]. A search of the yeast *Saccharomyces cerevisiae* genome revealed a single gene with significant sequence homology to the $\alpha 1$ subunit of the animal L-type Ca^{2+} channel termed CCH1 [101,102]. Mutants lacking CCH1 were defective in Ca^{2+} uptake, but there has been no electrophysiolog-

ical confirmation that CCH1 is a Ca^{2+} channel. Unfortunately, no comparable sequence has yet been revealed in the plant database. The observation that heterologously expressed transporters allowing Ca^{2+} influx can rescue yeast mutants disrupted in either the CCH1 or MID1 genes, which both encode non-homologous proteins required for Ca^{2+} influx during recovery from the mating response, suggests a useful functional assay to screen for plant Ca^{2+}-transporter genes (cf. [103]). In addition, the approach pioneered by Hartje et al. [104], to assay for Ca^{2+}-channels through the appearance of an endogenous Ca^{2+}-dependent Cl^- current in oocytes following the injection of plant poly(A)$^+$ RNA, may prove fruitful. Finally, screening for mutants in physiological processes thought to involve Ca^{2+} signals could reveal the genes for Ca^{2+} channels or their effectors [1]. Once the genes for plant Ca^{2+} channels are obtained, their physiological role(s) might be ascertained through gene-knockout approaches.

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