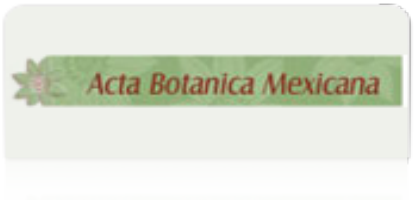

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HIGHER PLANT VACUOLAR IONIC TRANSPORT IN THE CELLULAR CONTEXT

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ABSTRACT

The vacuole, occupying up to 95% of the volume of a mature higher plant cell, serves as a main store of solutes and as the key element of intracellular Ca^{2+}- and pH-stat. The role of the vacuole in the ionic homeostasis of the cell is also evident under K^+-starving conditions and under salt stress. It is involved in the regulation of turgor and cell volume, and in specialized cells, such as stomatal guard cells, a large central vacuole is a principle component of the cell osmotic motor. For more than a decade, the guard cell has gained the status of a higher plant cell model, intensely studied by physiological, cell and molecular biology techniques. Despite tremendous progress in the understanding of signal transduction events taking place at plasma membrane of guard cells, the involvement of vacuolar ion transporters remains a significant challenge. Like in a plasma membrane, the ionic transport across the vacuolar membrane, the tonoplast, is directed and coordinated through a complex set of specific transport proteins, including pumps, transporters, and ionic channels. During recent years, the application of modern electrophysiological techniques (principally, the patch-clamp) has enabled numerous individual ion channels to be functionally characterized, although none of them has been structurally identified. The work of our group is focused on the characterization of so called slow (SV) and fast (FV) vacuolar ion channels, which have a wide, if not ubiquitous, distribution in higher plants, and can be detected in stems, leaves, as well as in root storage tissue. Their ionic selectivity, gating by membrane voltage and by physiologically abundant cations, as well as their possible roles in signal transduction and ionic balance are discussed.

Key words: intracellular calcium, ion channels, ionic transport and homeostasis, patch-clamp, polyamines, signal transduction, vacuole.

RESUMEN

La vacuola ocupa hasta 95% del volumen celular en las plantas superiores, es un reservorio de solutos y un organelo clave en el control de la concentración de Ca^{2+} y del pH intracelulares. El papel de la vacuola en la homeostasis iónica celular es también evidente en condiciones ambientales de falta de potasio y estrés salino. La vacuola central está involucrada en la regulación de la turgencia y el volumen celular en células especializadas como las estomáticas, constituyendo

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el principal componente del motor osmótico celular. Desde hace más de una década, la célula estomática ha ganado un status de modelo celular en plantas superiores, siendo extensamente estudiada mediante técnicas fisiológicas y de biología molecular y celular. A pesar del gran avance en el conocimiento de la transducción de señales en la membrana plasmática, el entendimiento de los mecanismos implicados en el transporte iónico en la membrana vacuolar continúa siendo un gran reto. Al igual que en la membrana plasmática, el transporte iónico en la membrana vacuolar o del tonoplasto, está dirigido y coordinado a través de un complejo sistema integrado por proteínas de transporte que incluye bombas (H^+-ATPasa, pirofosfatasa, bomba de Ca^{2+}), transportadores (antiportadores Na^+/H^+ y Ca^{2+}/H^+) y varios canales iónicos. En años recientes, con la aplicación de las técnicas modernas de electrofisiología (patch clamp), numerosos canales iónicos han sido caracterizados funcionalmente, aunque ninguno de ellos ha sido estructuralmente identificado. El trabajo de nuestro grupo se ha enfocado a la caracterización de los canales iónicos vacuolares lentos (SV) y los canales iónicos vacuolares rápidos (FV); estos canales tienen una amplia, si no es que ubicua, distribución en plantas superiores, se les encuentra en células troncales, en células diferenciadas de las hojas o de los tejidos de almacenamiento de las raíces. En este trabajo se discuten las características funcionales de estos canales en cuanto a selectividad iónica, mecanismos de compuerta dependientes de voltaje e influencia de cationes de importancia fisiológica (calcio, magnesio, protones, poliaminas), así como su posible papel fisiológico. Asimismo, se plantean los principales temas que están por resolverse. Particularmente, se analiza la participación de los canales aniónicos en la función de la vacuola como motor osmótico. Se sabe que el tonoplasto presenta una gran permeabilidad para varios aniones, sin embargo, los canales iónicos involucrados no han sido claramente identificados. El otro problema es la identificación de los canales activados por ligandos intracelulares. A pesar de que las células vegetales poseen receptores intracelulares para inositol 1,4,5 trifosfato (IP_3) y adenosinadifosfatoribosa cíclica (cADPR), su ubicación celular no está definida. Por el momento no son confiables las evidencias que indican la existencia de canales iónicos vacuolares activados por estos factores.

Palabras clave: calcio intracelular, canales iónicos, transporte y homeostasis iónica, patch-clamp, poliaminas, transducción de las señales, vacuola,

VACUOLE, IONIC HOMEOSTASIS AND OSMOTIC MOTORS OF PLANT CELLS

Vacuole and ionic homeostasis. The vacuole controls cell volume and turgor pressure of a plant cell, plays a key role in the regulation of cytosolic Ca^{2+} and pH, serves as a dynamic store of sugars, amino acids and nutrients, and sequesters xenobiotics and toxic ions (e.g., heavy metals). The ion transport ability of the vacuolar membrane, the tonoplast, controls all these functions, either directly, via release or uptake of certain ions, or indirectly, by setting the membrane voltage and H^+ gradient to define the direction of H^+-coupled secondary transport of non-ionic compounds.

The vacuole is the central storage compartment of a higher plant cell, and, as such, is used to deposit nutrient ions, as for instance nitrate (Zhen & Leigh, 1990), inorganic phosphate (Bielski, 1968), and potassium (Walker et al., 1996). In plants with crassulacean acid metabolism (CAM), the only way for the temporal storage of CO_2 is in the form of organic acids (malate) in the vacuole (Cheffings et al., 1997; Raven, 1997).

There is a well-defined example of K^+ homeostasis in a vacuolated plant cell (Leigh & Wyn Jones, 1984; Walker et al., 1996). Vacuolar K^+ is thought to play a sheer osmotic role; cytosolic K^+, besides this, regulates the activity of several enzymes, particularly, of those involved in the protein synthesis (Evans & Sorger, 1966). Therefore, K^+ concentration

in the cytosol needs to be maintained at about the 100 mM level. At K^+ deficiency, K^+ in the cytosol is kept fairly constant at the expense of the vacuolar K^+ until the latter reaches a critical level ~20 mM, and only at this point the decline in cytosolic K^+ is initiated (Walker et al., 1996). Similarly, when the extracellular supply of NO_3^- is limited, the cytosolic NO_3^- pool is refilled by NO_3^- stored in the vacuole (Zhen & Leigh, 1990). In barley roots, cytosolic nitrate is kept around 4 mM, whereas vacuolar NO_3^- shows a large variation due to the accumulation of nitrate or its mobilization at nitrate-limiting conditions (Van der Leij et al., 1998). Phosphate in maize root cells also behaves in a similar fashion: whereas in cytosol its concentration is independent of nutritional status and kept in the range 4-6 mM, in vacuoles of phosphate-replete plants, it is between 4 and 20 mM, and under phosphate starvation it is vanishing low (Lee et al., 1990).

Whilst the aforementioned examples deal with essential nutrients, whose concentration in cytosol needs to be kept at a certain level regardless of the growing conditions, another example of ionic homeostasis, based on the vacuolar transport capacity, is provided by a plant's response to high salinity. Excess Na^+ in the cytosol is toxic, so external Na^+ entering the cytosol through cationic channels needs to be extruded from the cell (Shi et al., 2000) or sequestered into the vacuole. Extreme halophytes, such as the ice plant, *Mesembryanthemum crystallinum*, can accumulate up to 1 M of Na^+ in the vacuole (Adams et al., 1992). This is possible via the function of the Na^+/H^+ antiporter of the vacuolar membrane energized by the H^+ pumping V-ATPase. Both, the Na^+/H^+ antiporter and the H^+ -ATPase are known to be stimulated by salt stress (Barkla et al., 1995; Barkla & Pantoja, 1996; Bethke & Jones, 2000; Ratajczak, 2000).

Finally, the vacuole plays a pivotal role in H^+ and Ca^{2+} homeostasis. Due to its large size, the vacuole serves as a main sink for these ions in the plant cell. Whereas cytosolic pH is normally maintained in the 7-7.5 range, vacuolar pH of 5 to 5.5 is typical, and in acidic fruits and in leaves, accumulating oxalic acid, vacuolar pH as low as 2.7 may be observed (Smith & Raven, 1976). The resting Ca^{2+} activity in the cytosol of most living cells is in the range of 100-300 nM. An increase of free calcium up to 500 nM-1 µM serves as a trigger, firing a cascade of signal transduction events. The vacuolar free Ca^{2+}, as reported by ion-selective microelectrodes, is in the range of 0.2-2 mM (Bethmann et al., 1995; Allen & Sanders, 1997). As the transtonoplast electrical potential is close to zero (+5- -20 mV), this implies that huge electrochemical gradients, 100-fold for H^+ and 10^3 -10^4 fold for Ca^{2+}, are therefore actively generated. K^+ seems to be distributed almost equally between the cytosol and the vacuole (Bethmann et al., 1995). Thus, it can be driven passively into and out of the vacuole, depending on the nutrient supply for this ion. For inorganic anions (Cl^- and NO_3^-), a moderate 3-10 fold cytosol-directed concentration gradient was detected. Phosphate is distributed evenly, and malate may be concentrated in the vacuole up to the 100 mM level, as compared to a few millimolar in the cytosol (Bethmann et al., 1995; Allen & Sanders, 1997; Barbier-Brygoo et al., 2000). A summary of transtonoplast electrochemical gradients for main inorganic ions is presented in Fig. 1. In conclusion, it should be noted that despite existing transtonoplast ionic gradients, the vacuole is at any moment in osmotic equilibrium with the cytosol due to the extremely high water conductivity of the tonoplast (Maurel et al., 1997; Kjellbom et al., 1999).

Generation of turgor pressure. Functional morphology and anatomy, especially in non-woody plants, may not be maintained without generation of turgor pressure in every

plant cell. This forms a basis of the hydrostatic skeleton of the whole plant. The cell becomes turgid due to the accumulation of solutes in its large central vacuole. This creates a driving force for the influx of water, whereas the resulting volume expansion is delimited by the rigidity of the cell wall. On the energetic costs grounds, it is much more efficient to accumulate inorganic ions in the vacuole via H^+-coupled transport than to synthesize organic compounds de novo to be used as a pure osmoticum (Raven, 1997). However, for stomatal guard cells this consideration is valid only under the energy (solar light) deficient conditions that occur in the morning, whereas during high photosynthetic activity in the afternoon, potassium salts in the vacuolar solution are almost completely substituted by sucrose (Amodeo et al., 1996; Talbott & Zeiger, 1996). Another advantage of turgor generation via ion uptake is the possibility to release turgor pressure through high-capacity passive ion transport pathways: ion channels. Indeed, ion channels can rapidly transfer small inorganic ions but generally not organic compounds. The latter are transported instead by means of at least 1,000-fold-slower carriers. Thus, fast (in seconds to minutes) volume changes may occur in specialized cells, such as pulvinar motor cells guiding leaves movements (e.g., *Mimosa pudica*) but also, and this is of general importance for all land plants, in stomatal guard cells.

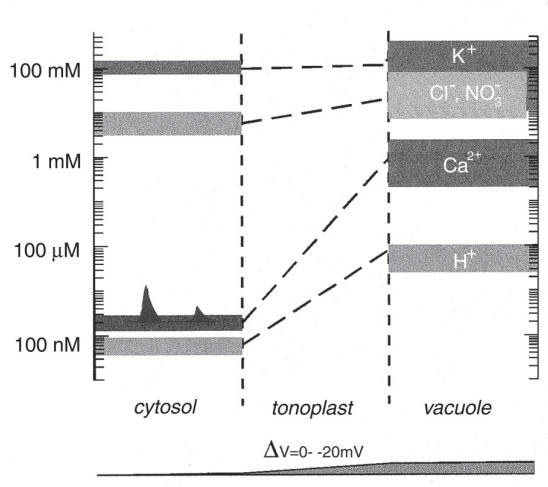

Fig. 1. Electrical and chemical gradients for main inorganic ions across the vacuolar membrane (tonoplast). Values are expressed as activities. Shadowed areas correspond to reported concentration ranges for different species (Allen & Sanders, 1997; Barbier-Brygoo et al., 2000) and dashed lines connect mean values for the cytosol and vacuole, respectively. Cytosolic Ca^{2+} resting range is indicated, whereas Ca^{2+} activity (free concentration) spikes can be observed. Special instances of very acidic pH in vacuoles, e.g., of immature fruits, are not shown. The transtonoplast electrical potential convention is according to Bertl et al. (1992), cytosol minus vacuole ($+V=V_{cyt}-V_{vac}$). Thus, a negative potential favors the release of cations from the vacuole and uptake of anions, respectively.

Function of the stomata. Stomata are pores formed by two bean-shaped cells, located primarily on the lower leaf epidermis. They serve as main gates for water and gas (CO_2) exchange with the environment. Performance of this function is of vital importance for the two main physiological functions of plants, photosynthesis and transpiration. Stomatal complexes can be traced in early land plants down to the Silurian period, ca 400 millions years ago (Edwards et al., 1998). There are no significant differences between the morphology of fossil stomata and those of extant plants, though stomatal density has increased since ancient times due to a decrease in the atmospheric CO_2 concentration (Beerling et al., 1998; Edwards et al., 1998). Due to a unique radial arrangement of actin filaments in the cytoskeleton of the guard cell, turgor pressure changes drive cell volume modifications, which in turn are transformed into changes in size of the stomatal aperture (Kim et al., 1995). Stomatal closure is associated with a decrease of volume, cell shrinkage, and, occasionally, with the fragmentation of a large central vacuole. There are no plasmodesmal contacts between adjacent cells in mature stomatal complexes (Wilmer & Fricker, 1996). Guard cells are quite resistant to acid treatment, so it is possible therefore to analyze stomatal movements *in situ*, in epidermis strips or even in whole leaves, by killing cells other than the guard cells. The conventional voltage-clamp, as well as ion-selective microelectrode techniques, tracer flux measurements, and ratiometric fluorescence measurements, may be then applied to study ionic fluxes and concentration changes. On the other hand, guard cell protoplasts, and further, vacuoles, may be easily isolated and utilized for patch-clamp recordings of the activity of individual ion transport proteins (Raschke & Hedrich, 1989). Therefore, guard cells serve as an excellent experimental model to study the ion basis of signal transduction in plants. Stomatal opening is elicited by blue light, auxin, and some fungal toxins (fusicoccin), whereas stomatal closure is induced by high CO_2, darkness, oxidative stress, abscisic acid (ABA) and water deficiency, among other factors (McAinsh et al., 1997; Assmann & Shimazaki, 1999). The closing and opening of stomata is accompanied by massive changes in ionic contents. Concentration of K^+ and Cl^- in open stomata are ~450 mM and ~120 mM, whereas in closed ones these concentrations are ~100 mM and ~30 mM, respectively (Penny & Bowling, 1974; Penny et al., 1976). Every 1 µm change of stomata aperture diameter is associated with an approximately 30 mM change in K^+ content (Wilmer & Fricker, 1996). The difference in volume between open and closed states of a *Vicia faba* guard cell is 200-300%; the osmotic potential changes by 200-300 mOsm; and ~2 pmole of KCl is released over a period of 30 min (closing) or is taken up in 2-3 h (opening) (Blatt, 2000). Even for the relatively rapid process of stomatal closure, a cell must maintain a K^+ and Cl^- release across vacuolar and plasma membranes of just ~100 pA in each guard cell, which translates to few tens of open K^+ and anion channels. In recent years a number of excellent reviews have appeared devoted to the regulation and co-ordination of the ion transporter function during stomatal response (MacRobbie, 1998; Grabov & Blatt, 1998; Pei et al., 1998; Assmann & Schimazaki, 1999; Blatt, 2000; Schroeder et al., 2001). In this paper we will feature only some mechanisms leading to stomatal closure in response to the application of ABA, the “drought-hormone” (Fig. 2). Ion transport across the plasma membrane of a guard cell is well characterized. It is mediated by a handful of key transporters, including two K^+-selective channels (one mediating K^+ influx (K_{in}) and another K^+ efflux (K_{out})), the slow anion channel, the Ca^{2+}-influx channel, and the vanadate-sensitive H^+ ATPase (Fig. 2). However, our understanding of the function of the tonoplast

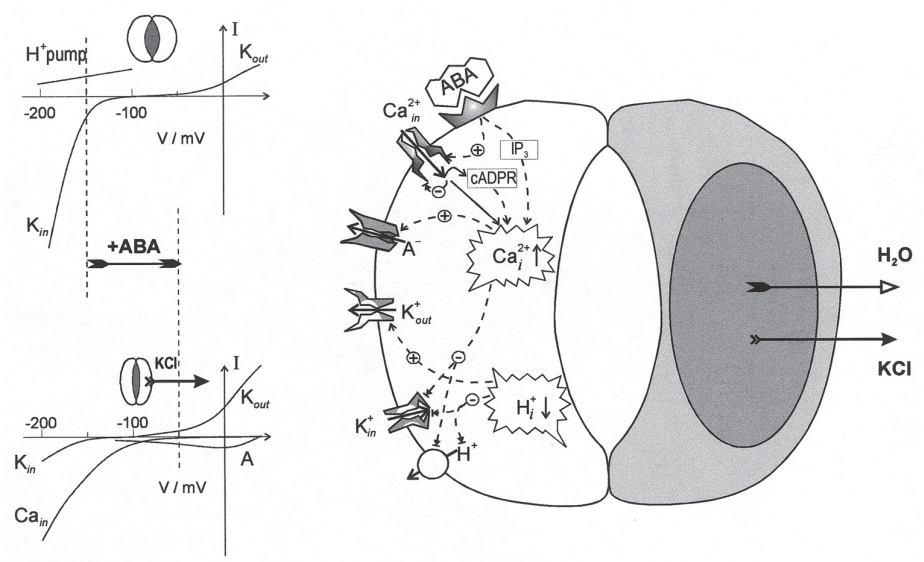

Fig. 2. ABA-induced stomatal closure: electrocoupling of ionic currents and communication via intracellular Ca^{2+} and pH trigger KCl loss across the guard cell membrane. Left: whole cell ionic currents before and after addition of ABA. Switch of membrane potential level from KCl uptake conditions to the conditions of net KCl loss in response to ABA is indicated by an arrow between two dashed lines. Relative amplitudes of ionic currents carried by H^+-pump, inwardly rectifying K^+ channels (K_{in}), outwardly rectifying K^+ channels (K_{out}), slow anion channels (A), and Ca^{2+}-influx channels (Ca_{in}) are collected from the original measurements in *Vicia faba* guard cell membrane by Blatt (1987), Schroeder & Keller (1992), Hamilton et al. (2000), Grabov & Blatt (1997,1999). For simplicity, it is assumed that anion channels are not active, and the H^+ pump is only active at resting $Ca^{2+}{}_i$. Right: ABA binds to an unidentified receptor and activates the Ca^{2+} influx channel and Ca^{2+} release from IP_3-sensitive intracellular stores, possibly via a G-protein-phospholipase C pathway (Blatt, 2000). A rise in intracellular Ca^{2+} is amplified and prolonged by the activation of Ca^{2+} release through cADPR-receptor channels. Independently, ABA causes the alkalization of the cytosol. A decrease in $H^+{}_i$ and an increase in $Ca^{2+}{}_i$ concentration down-regulates inward K^+ channels and H^+-ATPase (Kinoshita et al., 1995; Grabov & Blatt, 1999), whereas outward K^+ channels are stimulated by alkaline pH_i (Grabov & Blatt, 1997). An increase in $Ca^{2+}{}_i$ causes the activation of anion channels, which, together with outward rectifier K^+ channels, causes membrane depolarization, thus a release of KCl from the cell is promoted. The reversibility of this process is secured by inactivation of Ca^{2+} channels due to increase of cytosolic Ca^{2+} and membrane depolarization; ABA may evoke several cycles (oscillations) of Ca^{2+} channels activity (Hamilton et al., 2000). Ion fluxes are shown by solid arrows, modulating by dashed arrows, positive and negative regulation are indicated by (+) and (-), respectively.

ion channels and the means of their regulation is much less satisfactory. Based on tracer flux measurements, up to 90% of K^+ salts lost during stomatal closure come from the vacuole (MacRobbie, 1998). Therefore, some yet non-identified vacuolar K^+- and anion-release channels must operate in conjuction with their plasma membrane counterparts. Meanwhile, some specific hypotheses involving vacuolar K^+ and Ca^{2+}-permeable channels have been generated (e.g., Ward & Schroeder, 1994) and will be further discussed.

ROUTES OF THE IONIC TRANSPORT ACROSS THE TONOPLAST

General features of transporters. Ions can not pass through biological membranes via simple diffusion and always require a specialized transporter. Transporters are classified into three categories: primary pumps, carriers (porters), and channels. The transport of H^+ the vacuolar membrane by primary pumps is coupled to the hydrolysis of high-energy compounds, ATP (by an ATPase) and inorganic pyrophosphate (by a PPase). Carriers can transport ions either passively, down the electrochemical gradient (all uniporters), or utilize the gradient of one ion to transport another one, even against the electrochemical gradient of the latter (co-transporters). Tonoplast carriers, e.g., the Na^+/H^+ antiporter, generally utilize a H^+ gradient created by H^+ pumps[1]. Ion channels, when open, are water-filled pores which transport ions exclusively downhill with respect to their electrochemical gradients. The difference between ion channels and uniporters is their capacity as well as their mechanism of conduction. However, in the absence of a specific substrate, the carrier may operate as a high-conductance ion channel. An example of such a phenomonan reported for plant systems was the conversion of the chloroplast's inner membrane triose phosphate/ phosphate translocator into a high-conductance anion channel upon replacement of natural substrates by Cl^- (Schwarz et al., 1994). In general terms, a passive ion transporter may be represented as an ion channel with a switch inside, which binds one ion at any one moment and translocates it from one side of the membrane to the other. The conformational transition of the switch, if occurring in a "true" ion channel, needs to be not rate-limiting, i.e., faster than or comparable to the diffusion of the ion, although in certain occasions it is supposed to be the case (Hansen et al., 1997; Allen et al., 1998b). Therefore, ionic channels, normally operating a few tens of millivolts away from their equilibrium potential, may generate currents in pA-range, where 1 pA is equivalent to $6x10^6$ ions/s. In comparison, the maximum velocity of 250 Na^+ ions/sec can be estimated for the red beet vacuolar Na^+/H^+ antiporter from the data of Barkla et al. (1990), whereas V-ATPases transport 60-180 H^+/sec assuming a $2H^+$/ATP stoichiometry (Ratajczak, 2000). In general, channels are responsible for a rapid, low-affinity ion transport, whereas carriers and pumps mediate a low-rate, high-affinity ion transport. The latter is particularly important when the substrate, for instance H^+, naturally occurs at low concentrations. Although V-ATPase is highly abundant, reaching 6.5-35% of the total tonoplast protein (Ratajczak, 2000), within physiological range of the transtonoplast

[1] For the sake of completeness, it should be noted that secondary compounds, such as glutathione-conjugates, gluconurides, bilic acids, catabolites of chlorophyll, sulfated or sulfonated compounds, as well as some glycosides, are transported into vacuoles by means of direct energization (ATP-hydrolysis), mediated by so called ABC transporters (Martinoia et al., 2000).

potentials, the whole vacuole H^+-pump current reached only 10-30 pA at optimal conditions (Davies et al., 1994; Gambale et al., 1994). In other words, few open ion channels per vacuole may electrically balance the net H^+ current generated by the whole V-ATPase population. The total number of copies of the dominant ion channels in plant vacuoles ranges from 2,000 to 10,000 (Schulz-Lessdorf & Hedrich, 1995, Tikhonova et al., 1997, Pottosin et al., 1997). These numbers are comparable to the number of Na^+/H^+ antiporter units: 10,000 per vacuole (Barkla et al., 1990). Keeping in mind the huge difference in transport numbers between ion channels and porters, together with the low selectivity of vacuolar cation channels (see below) and the physiological demand to keep low Na^+ (1-10 µM) in the cytosol, the maintenance of about a 10-fold vacuole to cytosol Na^+ concentration gradient (Martinoia et al., 1986; Barkla et al., 1995) requires a tight control of the ion channel function. Permanent opening of a single non-selective cation channel per vacuole could in principle dissipate the Na^+ gradient across the tonoplast.

MEMBRANE ENERGIZATION BY H^+ PUMPS

The function of tonoplast H^+ pumps is to take up protons into the vacuole, thus contributing to the cytosol pH-stat and energizing the vacuolar membrane for secondary transports against electrochemical gradients for corresponding ions. It is not clear why the tonoplast possesses two H^+-translocation pumps: V-ATPase and PPase (inorganic pyrophosphatase). The possession of these two pumps is unique feature of vacuole among eucaryotic organelles (Bethke & Jones, 2000).

V-ATPase. Plant V-ATPase is found mainly in the vacuolar membrane, although in some occasions V-ATPase activity can be detected in endoplasmic reticulum, Golgi apparatus, coated vesicles, and provacuoles (Maeshima, 2001). V-ATPase is a multi- (up to 10) subunit enzyme, whose structure is closely related to the F-type ATPases and consists of head complex V_1, involved in ATP hydrolysis, and transmembrane proton-conducting channel V_0. In contrast to chloroplast and mitochondrial F-type ATPases, V-ATPase is insensitive to azide and oligomycin and can be specifically inhibited by submicromolar concentrations of concanamycin A and bafiolomycin (Ratajczak, 2000). V-ATPase is inhibited by nitrate and stimulated by Cl^- at their physiological range of concentrations, and stimulated by magnesium as a constituent of divalent cation-ATP complex with an apparent K_m between 0.2-0.8 mM (Lüttge & Ratajczak, 1997). It has been shown that the mutation of the *det3* encoding C subunit of V-ATPase in *Arabidopsis* results in the organ-specific alteration of growth due to restricted cell wall expansion, light-specific morphogenesis in the dark, and failure to close stomata in response to high external Ca^{2+} and H_2O_2 (Schumacher et al., 1999). It appears that the failure to close stomata is caused by a disruption of the specific Ca^{2+} oscillator depending on proton gradient generated by V-ATPase (Harper, 2001). Notably, various isoforms of C-subunit (e.g., four isoforms in *Arabidopsis*) show tissue-specific and developmentally regulated expression (Ratajczak, 2000). V-ATPase is not just a constitutive “house-keeping” enzyme, it is also an “eco-enzyme”, because under conditions of environmental stress, especially under salt stress, the subunit’s expression is enhanced and the holoenzyme structure is modified (Barkla & Pantoja, 1996; Ratajczak, 2000). Interestingly, in cultured carrot cells, salt stress is

followed by 20% decrease in ATP-hydrolisis activity of the V-ATPase, whereas the H^+ pumping rate is increased by 60% (Löv & Rausch, 1996). This implies an increased V-ATPase coupling rate, likely due to structural modification of the channel (V_o) domain (Ratajczak, 2000). The coupling rate, i.e., the H^+/ATP ratio, depends also on the pH gradient, changing from 3.2 to 1.75 upon an increase of)pH from ~3 to 4.75 units. This permits the acidification of the vacuolar lumen to pH<4 against a steep transmembrane gradient for protons (Davies et al., 1994). The expression of the V-ATPase subunit is up-regulated via promoters by phytohormones: ABA, ethylene, and possibly also jasmonic acid (Ratajczak, 2000).

PPase. Vacuolar H^+-translocating PPase is formed by a single polypeptide with a molecular mass about 80 kD. V-PPases are found in *Archaea*, *Eubacteria* (e.g., photosynthetic bacteria such as *R. rubrum*), protists (e.g., *Trypanasoma* and *Plasmodium*), green algae, and higher plants (Drozdowicz & Rea, 2001). Compared to V-ATPase, there is no obvious clue as to the exclusive physiological role of PPase. In contrast to V-ATPase, whose expression is approximately constant at different developmental stages, V-PPase is predominantly expressed in young tissues, e.g., hypocotyls (Maeshima, 2000). This is understandable, taking into account that the substrate for V-PPase, inorganic pyrophosphate, is a byproduct of the synthesis of RNAs, proteins, and cellulose. Another situation, when the PPase activity increases in order to compensate decreased activity of H^+-ATPase, takes place under cold stress and anoxia (Rea & Poole, 1993). It has been proposed that PPase might operate as an active K^+/H^+ symporter (1.3 H^+:1.7 K^+:1 PP_i), thus promoting K^+ accumulation into the vacuole against the electrochemical gradient for K^+ (Davies, 1997). However, experiments with purified enzyme reconstituted into liposomes did not show PPase -dependent K^+ accumulation, albeit these data did not abolish the stimulatory effect of K^+ on PPase (Sato et al., 1994). However, later on, a high K_m value for the K^+ stimulation of the PPase activity was proved to be an experimental artifact, due to the competitive inhibition by organic buffers. The true K_m, about a few millimoles defined for K^+, was well below the physiological range for this ion, which makes the regulation of PPase activity by K^+ variation unlikely (Gordon-Weeks et al., 1997). Nevertheless, there are some indications that under salt stress V-PPase is inhibited due to competition between K^+ and Na^+ for the regulatory site (Blumwald et al., 2000). V-PPase requires cytosolic Mg^{2+} in a submillimolar range as a co-factor and is inhibited by Ca^{2+}, either as a free ion, or as a substitute for Mg^{2+} in its complex with pyrophosphate (Maeshima, 2000). In *Arabidopsis* and other organisms, another class of H^+-translocating PPases was found recently, whose activity was completely independent of K^+ (Drozdowicz & Rea, 2001). This (type II) PPase may reside, however, in a membrane other than the tonoplast (Maeshima, 2001).

CREATION OF TRANSTONOPLAST GRADIENTS FOR IONS OTHER THAN H^+

Calcium. The tonoplast contains two transport pathways for active Ca^{2+} accumulation into the vacuole: Ca^{2+}/H^+ (with H^+ to Ca^{2+} stoichiometry $\geq$3) antiporters (Blumwald & Gelli, 1997; Sanders et al., 1999) and plasma membrane-type (P-type) primary Ca^{2+}-pumps (Malmstrom et al., 1997). Seven different genes encoding antiporters belonging to the CAX

family (former nomenclature: CAlcium/ H^+ eXchangers, now CAtion/H^+ eXchangers) and six isoforms for Ca^{2+} pumps belonging to the ACA-family have been identified in the genome of *Arabidopsis thaliana* (Maeshima, 2001; Mäser et al., 2001). The tonoplast Ca^{2+}-pump has a higher affinity for Ca^{2+} (K_m=0.2-1.0 µM) but a lower turnover rate in comparison with the dominant Ca^{2+}/H^+ antiporter CAX1 (K_m=10-15 µM). It was speculated that they operate in a different range of free cytosolic Ca^{2+} concentrations, with the antiporter rapidly lowering excessive Ca^{2+} to the operation range of the Ca^{2+}-pump (Maeshima, 2001). However, a recent study by Geisler and co-workers (2000) revealed that the tonoplast Ca^{2+}-pump *ACA4* is located in small vacuoles rather than in the membrane of the large central vacuole. These data imply a functional difference of these compartments, in particular, a distinct role of the small vacuoles in the formation of Ca^{2+} oscillating patterns (Harper, 2001).

The particular importance of Ca^{2+}/H^+ antiporter was strengthened by experiments on transgenic tobacco plants expressing *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1 (Hirschi, 1999). It appears that the transporter was regulated improperly in the transgenic system (over-expressed), thus decreasing availability of Ca^{2+} in any other compartment but in the vacuole. This resulted in plants with necrotic and chlorotic lesions, dead terminal buds, and undersized roots. And *vice versa*, plants with an underexpressed CAX1 gene were more sensitive to high Ca^{2+} levels (Hirschi, 2001). The closely related Ca^{2+}/H^+ antiporter CAX2 has a broader substrate selectivity (Mn^{2+} and Cd^{2+}) and a higher transport capacity; its expression in tobacco can increase the tolerance to Mn^{2+} (Hirschi et al., 2000).

Magnesium. The activity of the Mg^{2+}/H^+ antiporter has been detected in the lutoid membrane of *Hevea brasiliensis* and tonoplast vesicles from Zea mays roots (Amalou et al., 1992; Pfeifer & Hager, 1993). The antiporter can also transport Zn^{2+} and Cd^{2+} but not Ca^{2+}. Thus, Mg^{2+} appears to be taken up separately from the Ca^{2+}/H^+ antiport pathway, as it was possible to isolate Mg^{2+}/H^+ antiport from Ca^{2+}/H^+ one during a solubilization/ reconstitution procedure. The antiporter has been reported to be electroneutral, i.e., exchanging 1 Mg^{2+} for $2H^+$ (Amalou et al., 1992). Recently, the *Arabidopsis* Mg^{2+}/H^+ exchanger AtMHX (closely related to the CAX-antiporter family and to Na^+/Ca^{2+} exchangers from animal cells) has been cloned and expressed in tobacco (Shaul et al., 1999). This study established that AtMHX is localized in the vacuolar membrane (likely in the membrane of central vacuole due to the co-localization with the (γ-TIP isoform of aquaporin) but not in the plasmalemma or in the endoplasmic reticulum. Furthermore, it is expressed mainly in roots and shoots and to a lesser extent in leaves. Surprisingly, the exchanger was electrogenic; in the presence of a divalent cation (2 mM Mg or 0.2 mM Zn^{2+} or Fe^{2+}) at the cytosolic side, and while the opposite side was acidified, it carried outward currents at tonoplast physiological potentials. Whereas Zn^{2+} or Fe^{2+} have ionic radii similar to Mg^{2+}, the divalent ions of different size (Co^{2+}, Ni^{2+} , Cu^2, and, especially important, Ca^{2+}) alone were inefficient as current inducers, though cytosolic but not vacuolar Ca^{2+} stimulated antiporter-mediated Mg^{2+} uptake. Ectopic overexpression of AtMHX although not changing the total Mg^{2+} or Zn^{2+} content, did result in the increased sensitivity of transgenic plants to high levels of Mg^{2+} or Zn^{2+} in the growth medium (Shaul et al., 1999).

Sodium. The ectopic expression of the *Arabidopsis vacuolar* Na^+/H^+ antiporter AtNHX1 dramatically increased the salt tolerance of these plants (Apse et al., 1999).

Whereas control plants were stunted and chlorotic when watered with a 200 mM NaCl solution, transgenic plants were unaffected, and this tolerance was paralleled by higher activity of the vacuolar Na^+/H^+ antiport. Constitutive vacuolar Na^+/H^+ antiport activity was recorded both in salt-tolerant and salt-sensitive plants, though galophyte plants commonly displayed an increase of the Na^+/H^+ antiport. This may be due to the addition of new antiporter molecules and/or, in some cases, due to the up-regulation of the existing antiporters (Blumwald et al., 2000). Bearing in mind that Na^+ is not an important nutrient in plant cells, and in contrast to animal cells, plant antiporters use electrochemical gradients for H^+ instead of those for Na^+, it was surprising to find about 40 genes encoding homologues of Na^+/H^+ antiporters in *Arabidopsis* (Mäser et al., 2001). One might suspect some novel functions assigned to these tentative Na^+ transporters in plant cells.

Potassium. Although K^+ is a major plant nutrient, the exact mechanisms of the creation of transtonoplast gradients for this ion are still uncertain. A systematic microelectrode study of vacuolar and cytosolic K^+ activity in parallel with transtonoplast electric potential measurements on barley root cells grown in different external K^+ concentrations did show a substantial change in the magnitude and direction of the transtonoplast concentration gradient for K^+, but no significant variation of the electric potential, as a function of K^+ status, was observed (Walker et al., 1996). A K^+-driving force as large as 20-27 mV from vacuole to cytosol at K^+ replete conditions, and from cytosol to vacuole at severe K^+ deficiency, was revealed. The authors concluded that at K^+ replete conditions, K^+ needed to be accumulated into the vacuole actively, via an unresolved mechanism, either by PPase or by H^+/K^+ antiport. Curiously, at K^+ starvation the situation was reversed, and K^+ release into the cytosol turned to be active, condition that could be theoretically achieved by a K^+/H^+ symport (Fernando et al., 1992; Walker et al., 1996). Neither of these hypothetical mechanisms of active K^+ transport has been established to date. However, analysis of the *Arabidopsis* genome revealed six putative K^+/H^+ antiporter homologues (KEA1 through KEA6) belonging to the cation/proton antiporter family 2, CPA2 (Mäser et al., 2001). Some of these antiporters could in principle sequester K^+ into in an acidic compartment, e.g., into the vacuolar lumen. Elucidation of the subcellular localization of KEAs may provide an initial test for this hypothesis.

Anions. Information on the mechanism of transport of anions across the tonoplast is scarce. It is well known that the tonoplast is highly permeable to Cl^- and NO_3^-, and in CAM plants also to malate (Martinoia et al., 1986, Cheffings et al., 1997). The membrane potential difference across the membrane of vacuolar vesicles is dissipated by inorganic anions with a relative potency: $SCN^->NO_3^-=Cl^->SO_4^{2-}-=HPO_4^{2-}$ (Kästner & Sze, 1987). However, this technique reveals the integral permeability of the tonoplast, without separation between different conductance pathways. Uptake of Cl^-, SO_4^{2-}, and HPO_4^{2-} displayed a saturable kinetics with respect to the substrate concentration and is characterized by a K_m value in the range of few millimoles (Martinoia et al., 2000). Under salt stress, the accumulation of Cl^- and loss of NO_3^- was observed in the epidermis of barley leaves (Leigh, 1997), and under increased light intensity NO_3^- is accumulated in lettuce vacuoles in exchange to other anions (Blom-Zandstra & Lampe, 1985). CAM plants, in contrast to other plants, display a higher tonoplast permeability for malate as compared to Cl^- (Cheffings et al., 1997). These observations corroborate the view that anions are not

transported by unique mechanism and that there might be separate transport routes for Cl^-, NO_3^- and malate across the tonoplast. Accumulation of Cl^- and NO_3^- in the vacuole does not always meet predictions for passive diffusion across the tonoplast, and active (H^+-coupled) mechanisms of anion accumulation in the vacuole might be invoked, depending on the developmental and nutritional status (Zhen & Leigh, 1990; Blumwald & Gelli, 1997).

PASSIVE PATHWAYS FOR ION EXCHANGE ACROSS THE VACUOLAR MEMBRANE: CHANNELS

Ion channels recording. The study of ion currents in biological membranes was revolutionized thanks to the invention of the patch-clamp technique (Hamill et al., 1981). This method is based on the formation of a high resistance (GOhm range) contact between a glass electrode with a tip opening of a few microns and a bare lipid bilayer (cell-attached configuration). The contact is also mechanically stable, so it is possible to isolate the membrane patch included into the pipette (inside-out configuration), which normally contains few ion channel copies. Furthermore, it is possible to study them under fixed voltage conditions and controlled solution content at both membrane sides, in the patch pipette and in the bath. An additional trick is to destroy the membrane under the pipette in the cell-attached configuration by a strong suction and/or voltage pulse, while preserving, however, the GOhm seal. Thus, a low-resistance (few MOhm) access to the cell's or organelle's interior can be achieved, allowing for the detection of ion currents in whole cell (or whole vacuole) configuration. Finally, the excision of the pipette will lead to the excised membrane patch or small vesicle with the right side oriented membrane (outside-out configuration). The patch-clamp registration scheme is a variation of a voltage-clamp method with only two electrodes in use: one is the reference electrode and the other is utilized both for fixing the voltage and measuring the compensatory current which is equal in absolute value to the current passing across the patch membrane. Employing this technique, currents down to 10^{-13} A (~10^5 elementary charges per second) can be easily detected as compared to the current range of 10^{-12}-10^{-11} A (1-10 pA) typical for single ion channels. However, the resistance of the membrane under study must be significantly higher than the patch-electrode resistance, otherwise voltage-clamp conditions will not be met. The typical MOhm resistance of the patch-pipette and the operational voltage range (~0.1 V) results in an upper current detection limit of a few tens of nA (10^{-8} A). This condition is normally fulfilled for small membrane patches but not always for the whole cell (or organelle) configuration. In particular, this applies to large plant vacuoles (several tens of microns in diameter), where major membrane ionic currents may reach or even exceed the aforementioned upper limit. Therefore, it is possible using a patch-pipette to separate smaller membrane vesicles from the large vacuole, whose higher membrane resistance allows a correct voltage-clamp detection.

SV and FV channels. Due to its large size and clean membrane surface, the vacuole is a relatively simple object for patch-clamp studies. The first ion channel to be characterized in the vacuolar membrane was called the SV (Slow Vacuolar) channel of red beet vacuoles (Hedrich et al., 1986). The SV current was both voltage- (activated by cytosol positive voltage) and time-dependent (required hundreds of milliseconds to

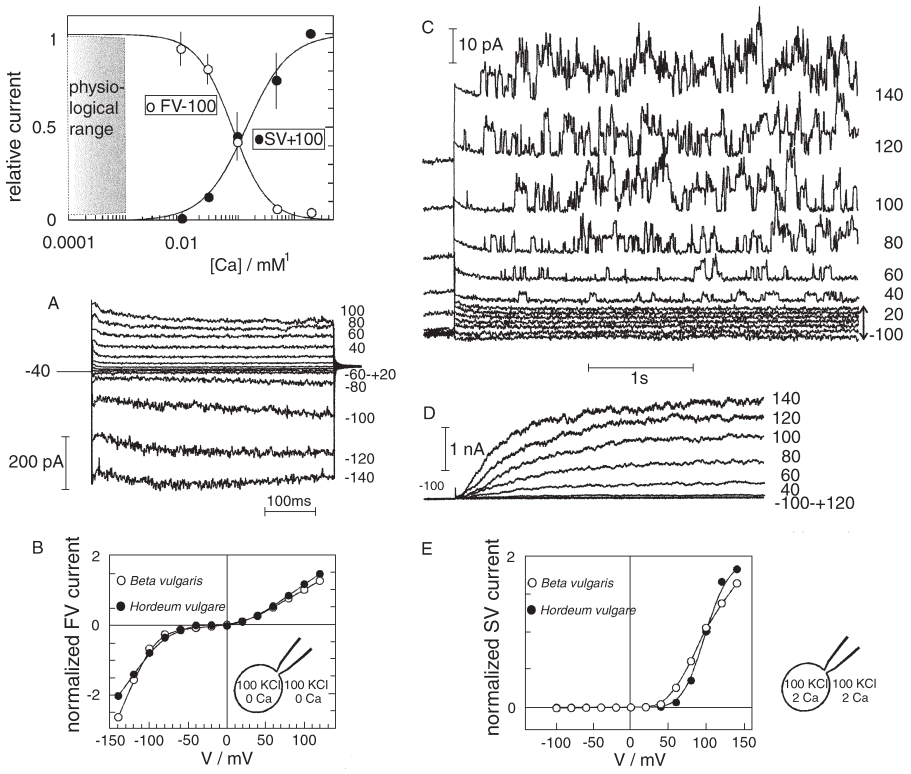

Fig. 3. Two major ionic currents of higher plant vacuoles. All records shown were obtained in symmetrical 100 mM KCl, pH 7.5. Original data are from red beet (*Beta vulgaris*) vacuolar vesicles, diameter $d \leq 5$ µm. A. At low (nM) free Ca^{2+} on both membrane sides, the membrane conductance is dominated by the FV (Fast Vacuolar) current, which is activated instantaneously by the application of positive and negative voltage steps from a holding potential of –40 mV, where the FV channel activity is minimal. B. Similar voltage dependence of the normalized FV current in vacuoles of red beet taproots (*Beta*) and of barley mesophyll (*Hordeum*). C. High (2 mM) cytosolic Ca^{2+} abolishes the FV current and activates the SV (Slow Vacuolar) channels. Free vacuolar Ca^{2+} was also increased to 2 mM, so under these conditions only positive membrane voltage steps ($\geq$40 mV) evoke SV channels activity in a tiny (d<1 µm) tonoplast patch. D. The record from a larger vacuolar vesicle under conditions identical to C reveals delayed activation of macroscopic SV current by positive voltage steps. E. The SV current shows saturable activation by membrane voltage, with a midpoint about +90 mV both in red beet and in barley mesophyll vacuoles. Insert. Shows opposite dependence of FV (at –100 mV) and SV (at +100 mV) currents in red beet vacuoles on cytosolic free Ca^{2+}, with a midpoint for the inhibition of the FV and the activation of the SV at Ca^{2+} ~100 µM.

Table 1. Characteristics of cation channels in higher plant vacuoles.

Parameter	FV channel	SV channel
Conductance		
K^+	21 pS (100 mM) K_m=30 mM (*Hordeum*[a]); 7 pS (200 mM) (*Vicia*[b]); 21 pS (100 mM) (*Beta*[C])	K_m=64 mM; G_{max}=385 pS (*Beta*[h]) K_m~550 mM; G_{max}~2000 pS (*Vicia*[i])
Na^+	15 pS (100 mM) (*Hordeum*[d])	K_m=103 mM; G_{max}=300 pS (*Beta*[h])
Mg^{2+} & Ca^{2+}	unmeasurably (<5% of K^+) low (*Hordeum*[a,e])	K_m=0.04 & 0.08 mM; G_{max}=18 & 13 pS (*Beta*[h]) (Ca^{2+}) K_m~6.5 mM; G_{max}~35 pS (*Vicia*[i])
Selectivity	NH_4^+>K^+~Rb^+~Cs^+>Na^+>Li^+(*Hordeum*[d]) K^+>>Cl^- (*Hordeum*[a], *Vicia*[b])	Na^+>K^+>Rb^+>Cs^+ (*Allium cepa* guard cell[l]) K>>Cl^-; Ba^{2+}>Ca^{2+}>Mg^{2+} (*Beta*[h,k], *Vicia*[i])
Blockers		
Physiological	vacuolar Mg^{2+} & Ca^{2+} (V-dependent) K_d(0)~40 µM & 200 µM[e]	vacuolar and cytosolic Mg^{2+} & Ca^{2+} (V-dependent) K_d(0)~1-3 mM (*Beta*[k]) Vacuolar / cytosolic polyamines: Spm^{4+}> Spd^{3+}>Put^{2+} (V-dependent), K_d(0): 60/30; 400; 8,000/2,000 µM (*Beta*[l])
Non-physiological	not determined	cytosolic application:DIDS or SITC (1 µM); Zn^{2+} (5 µM); A-9-C (100 µM); charibdotoxin (20 nM); tubocurarine (60 µM); quinacrine (15 µM); ruthenium red (<0.1µM; 36 µM); TEA^+ (10 mM); $Tris^+$ (60 mM) ([m-p])
Regulation		
Voltage (V)	activation by positive and negative V, V_{min} 20-40 mV negative to reversal V Gating charge(z) ~1.2 elem. charges[a,b,c]	activation by positive V, midpoint is a function of cytosolic Ca^{2+} and Mg^{2+} and of vacuolar Ca^{2+}. At symmetrical 1-2 mM Ca^{2+} V_m~90 mV; z=+1.4, +1.7 (*Beta*[k], *Hordeum*[q]) V_m ~60 mV z=+3.9 (*Vicia*[r])
pH	5-fold inhibition by pH change from 7.4 to 6.4[b]	(-) modulation by cyt. and vac. protons; pK= 6.9 and 5.0 (*Beta*[r]); vac. pH regulation insignificant at vac. Ca^{2+}>50 µM (*Hordeum*[q])
Ca^{2+} & Mg^{2+}	Cyt Ca^{2+}- inhibition, K_i=: 6 mM (*Hordeum*[a]); ~0.1 µM (*Vicia*[i]); 80 µM (*Beta*[c]); cyt. Mg^{2+}-stabilization of closed state; K_i (0)=10 µM[e]	High cytosolic Ca^{2+} is absolute requisite for activation; K_d~100 µM at +100 mV (*Beta*[k]; *Hordeum*[q]; *Vicia*[s]). Rise in vacuolar Ca^{2+} opposes activation by cyt. Ca^{2+}; cyt. Mg^{2+} is backing the effect of cyt. Ca^{2+}, vac. Mg^{2+} practically has no effect ([k,q,s])
Other	Cytosolic polyamines- inhibition: Spm^{4+}>Spd^{3+}>>Put^{2+}, K_i: 3-10; 75-185; ~ 5,000 µM (*Beta*[c]; *Hordeum*[g])	(+) modulation by cytosolic Cl^- ([t]), calmodulin ([s,u]), reducing agents ([v]), (+/-) modulation by phosphorylation status ([w,x]), (-) modulation by 14-3-3 proteins ([y])

seconds for its activation). Subsequently, SV channel activity was recorded in a wide variety of tissues, in all flowering plants, lower terrestrial plants such as ferns and liverworts, and even in a sea grass *Posidonia oceanica* (Hedrich et al., 1988; Schulz-Lessdorf & Hedrich, 1995; Carpaneto et al., 1997; Trebacz & Schönknecht, 2000). SV channel activity has also been found in specialized vacuoles such as the protein storage vacuole of the cereal aleuron tissue (Bethke & Jones, 1994). Another ubiquitous tonoplast current, which is differed from the SV current by its instantaneous activation, is mediated by the so called FV (Fast Vacuolar) channel (Hedrich & Neher, 1987). It differs from the SV channel also by its bimodal voltage dependence (activation at large positive and negative potentials) and the opposite Ca^{2+} dependence (Fig. 3). Single channels responsible for macroscopic SV and FV currents have been identified, and the SV channel has a ~10-fold higher conductance in comparison with the FV channel at identical ion conditions (Table 1). Based on single channel conductance values, at least two isoforms of the FV channel as well as of SV channel, likely exist in different tissues (Schulz-Lessdorf & Hedrich, 1995; Table 1 of this paper). In particular, the SV channel from guard cells is characterized by the highest unitary conductance along with a lower affinity for permeant ions than SV channels of other origin.

The FV channel conducts monovalent cations with a small preference $NH_4^+ > K^+ \sim Rb^+ \sim Cs^+ > Na^+ > Li^+$ (Brüggemann et al., 1999a). It was shown that the FV channel is highly selective for K^+ over Cl^-, and that it is inhibited by micromolar Mg^{2+} or Ca^{2+} from either membrane side (Tikhonova et al., 1997; Allen et al., 1998a; Brüggemann et al., 1999b). The ionic selectivity of the SV channel was a matter of hot debate. Whereas Hedrich and co-workers (Hedrich et al., 1986; Hedrich & Kurkdjian, 1988; Schulz-Lessdorf & Hedrich, 1995) claimed that the SV channel conducts both cations and anions, reports from other groups (Ward & Schroeder, 1994; Allen & Sanders, 1996; Pottosin et al., 1997; Allen et al., 1998b) supported the view that the anion permeability of the SV channel is negligible. Finally, it has been shown that Cl^- permeability of the SV channel is at most 1% that of K^+, and that the SV channel conducts the following physiologically abundant cations at high rate: Na^+, K^+, Mg^{2+}, and Ca^{2+} (Pottosin et al., 2001). Besides the aforementioned cation species, the SV channel also conducts Ba^{2+} (Pantoja et al., 1992a) and all alkali cations with a small preference (Amodeo et al., 1994). Both FV and SV

Table 1 footnote:
(a) Tikhonova et al., 1997; (b) Allen et al., 1998a; (c) Dobrovinskaya et al., 1999[a]; (d) Brüggemann et al., 1999[a] ; (e) Brüggemann et al., 1999[b]; (f) Allen & Sanders (1996); (g) Brüggemann et al., 1998; (h) Pottosin et al., 2001; (i) Allen et al., 1998b; (j) Amodeo et al., 1994; (k) Pottosin et al., 2000b; (l) Dobrovinskaya et al., 1999a; (m) Hedrich & Kurkdjian, 1988; (n) Weiser & Bentrup, 1993; (o) Dobrovinskaya et al., 1999b; (p) Pottosin et al., 1999; (q) Pottosin et al., 1997; (r) Schulz-Lessdorf & Hedrich, 1995; (s) Pei et al, 1999; (t) Pantoja et al. 1992a; (u) Bethke & Jones, 1994; (v) Carpaneto et al., 1999; (w) Allen & Sanders, 1995, (x) Bethke & Jones, 1997; (y) van den Wijngaard et al. (2001). *Hordeum*- barley (*Hordeum vulgare*) mesophyll vacuoles; *Vicia* – broad bean (*Vicia faba*) guard cell vacuoles; *Beta*- sugar beet (*Beta vulgaris*) taproot vacuoles. Positive and negative modulation indicated by (+) and (-), respectively. If not specified concentrations of effectors given are those to produce half-effect at 0 mV. Abbreviations: V- voltage, cyt.- cytosolic, vac.- vacuolar, V_{min} – voltage value for minimal activity of FV channel. Direct effect at the pore (block) is separated from inhibition with unknown mechanism of action, which in defined cases can be identified with down-regulation.

channels are therefore strictly selective for cations over anions, but in contrast to the SV channel, the FV channel does not show a measurable permeability for divalent cations.

VK channels. In the guard cell vacuolar membrane, a separate, highly K^+ selective channel with a unitary conductance of 70 pS in symmetrical 100 mM KCl was characterized (Ward & Schroeder, 1994). The selectivity sequence of this channel, termed VK (for Vacuolar K^+), $K^+ > Rb^+ > NH_4^+ >> Li^+$, Na^+ or Cs^+ , clearly differed it from the relatively non-selective FV and SV channels. The activity of VK was independent of membrane voltage and required cytosolic Ca^{2+} in the low micromolar range (Ward & Schroeder, 1994; Allen et al., 1998a). We (Pottosin et al., in press) have identified in the taproot of the halophyte plant *Beta vulgaris* the 41 pS K^+-selective channel with a negligible Na^+ permeability. Besides lower unitary conductance, the channel has quite similar functional characteristics to those of the VK channel of guard cells (Ward & Schroeder, 1994), and it was identical to the *B. vulgaris* vacuolar 30-40 pS cation channel, loosely defined as "FV-like" in previous studies (Hedrich & Neher, 1987; Gambale et al., 1996). So far, the VK channel is the only strictly K^+-selective channel to be identified in the vacuolar membrane. Such a property might be important for plants under salt stress. To cope both with hypertonic stress and Na^+ toxicity, salt-tolerant plants import Na^+, as a cheap osmoticum, into the vacuole (Blumwald et al., 2000). This is achieved by an enhanced activity of vacuolar Na^+/H^+ antiporter energized by H^+-pump (mainly by V-ATPase). However, the overall system turns out to be electrogenic, i.e., a net positive charge is transported into the vacuole. This needs to be balanced, otherwise the long-term transport of Na^+ into the vacuole cannot be maintained. Balancing the charge movement by a cation leak via non-selective FV and SV channels does not make sense, as these channels are highly permeable for Na^+, and futile Na^+ cycling across the tonoplast will arise. On the contrary, a K^+ leak through a K^+-selective (VK?) channel can help solve the problem[2]. Recently, one potassium channel homologue (KCO1) has been localized in the vacuolar membrane of *Arabidopsis* (Schönknecht et al., 2002). Future studies will hopefully elucidate its relation to known functional vacuolar cation channels, as well as its physiological role.

VVCa. Another Ca^{2+}-permeable channel, with voltage and Ca^{2+}-dependence opposite to those of the SV channel, was reported by studies of vacuoles from red beet and Vicia guard cells (Johannes et al., 1992; Allen & Sanders, 1994b; Johannes & Sanders, 1995). This channel was termed VVCa in subsequent review papers (Allen & Sanders, 1997; Sanders et al., 1999). A quantitative comparison of the conductance, selectivity, and absolute values of parameters of Ca^{2+} and voltage-gating of the VVCa and SV channels from the same preparation revealed their complete equality (Pottosin et al., 2001). This result was very striking, bearing in mind that SV channels from storage and guard cell vacuoles differ substantially in some characteristics (Table 1). Therefore, we (Pottosin et al., 2001) have concluded that the VVCa channel is actually the same as the SV channel, recorded on the inverted tonoplast patches. Such a technical error could

[2] The algebraic sum H^+-pump plus Na^+/H^+ antiporter plus K^+ channel is equivalent to Na^+ / K^+-ATPase of animal cells, a constitutively active enzyme consuming a major portion of cellular ATP to maintain appropriate Na^+ / K^+ balance under conditions of a permanent "salt stress", i.e., high (>100 mM) Na^+ in the extracellular medium.

occur while working with excised patches, but not with whole vacuoles. Consequently, the VVCa-mediated current has never been demonstrated at the whole vacuole level, albeit some inwardly rectifying current carried by Ca^{2+}, with characteristics distinct from VVCa, was detected in red beet vacuoles (Gelli & Blumwald, 1993). However, the existence of Ca^{2+} permeable channels open at physiological (cytosolic-side negative) potentials and resting cytosolic Ca^{2+} conditions is highly unlikely on physiological grounds, because a continuous Ca^{2+} release to the cytosol from the vacuolar store will be fatal to the cell.

Tonoplast ligand-gated Ca^{2+} release channels? In animal cells two principle families of intracellular ligand-gated Ca^{2+} release channels are recognized. They are channels activated by inositol 1,4,5-triphosphate (IP_3), a product of phospholipase C activity, and ryanodine receptor (RyR) channels, activated by cyclic adenosinephospate ribose (cADPR), a NAD^+ metabolite, respectively (Ehrlich et al., 1994; Guse, 1999). Experiments with caged IP_3 demonstrated the liberation of Ca^{2+} from internal stores in *Vicia faba* L. guard cells (Blatt et al., 1990). IP_3-induced Ca^{2+} release was detected *in vitro*, using Ca^{2+}-selective electrodes and fluorescence Ca^{2+} measurements on intact vacuoles and on tonoplast vesicles from *Chenopodium album* root cells (Lommel & Felle, 1997). *In vivo* Ca^{2+} measurements in the vacuolar microdomain of *Arabidopsis* seedlings have shown a significant contribution of IP_3-induced Ca^{2+} release from the vacuole during hyperosmotic (drought) stress, whereas, touch, cold and oxidative stress caused a reduced Ca^{2+} peak in this domain as compared to the Ca^{2+} rise in the cytosol (Knight et al., 1997). Therefore, the search for ligand-gated intracellular Ca^{2+}-permeable channels in the tonoplast of plant cells has been initiated. Alexandre and co-workers (1990) working on red beet vacuoles reported large (~500 pA at -100 mV) inwardly rectifying currents, mediated by 30 pS Ca^{2+}-permeable channels, specifically activated by submicromolar IP_3 and not by other phosphoinositides. Unlike animal IP_3-gated channels, these putative channels were not inhibited by high (1 mM) cytosolic Ca^{2+}. However, these results have not been reproduced by other groups (Chasan & Schroeder, 1992; Gelli & Blumwald, 1993). It has been claimed then (Allen & Sanders, 1994a) that the origin of the discrepancy was that Alexandre and co-workers (1990) used a hypertonic treatment of vacuoles, which favors the IP_3-induced current. Using a hypertonic treatment, Allen and Sanders (1994a) repeatedly recorded an increase in the whole vacuole current in the presence of 1 µM IP_3. However, this was in on the background of a large, unspecific leak, and comparable with the current supposedly induced by IP_3. No unambiguous single IP_3-gated channels could be demonstrated in this work. The situation has become even more complicated in the light of recent studies on purified microsomal fractions obtained from cauliflower and *Chenopodium rubrum* L. leaves (Muir & Sanders, 1997; Martinec et al., 2000). The first study, based on the IP_3-induced Ca^{2+} release assay and cross-reaction with antibodies raised against mammalian type 1 IP_3 receptor, found IP_3 receptors mainly in fractions enriched with plasma membranes in close association with endoplasmic reticulum (ER), whereas the second study localized IP_3-binding sites exclusively in ER fractions, but not in vacuolar ones.

In guard cells, the cADPR-linked signal transduction pathway plays an important role in ABA-induced response. ABA causes an increase of the intracellular cADPR level, and ABA-induced gene expression can be stimulated by microinjection of cADPR, being prevented by cADPR antagonist. Although in the absence of ABA both high cytosolic Ca^{2+} and IP_3 microinjection could mimic the effect, the natural ABA-induced response was

insensitive to heparin, an IP_3 receptor channel blocker (Wu et al., 1998). In *Vicia faba* guard cells, Ca^{2+}-induced Ca^{2+} release (CICR) caused by ABA was stimulated rather than inhibited by the IP_3 channel blockers neomycin sulfate and heparin, whereas high (10 µM) ryanodine concentrations antagonized the response (Grabov & Blatt, 1999). Because of an unspecific stimulation of the ryanodine receptor channel by polyanions, e.g., heparin, and its inhibition by high ($\geq$10 µM) ryanodine concentrations (Ehrlich et al., 1994), it may be concluded that ABA-induced CICR is mediated by the ryanodine/cADPR receptor channel. The direct injection of cADPR in guard cells of *Commelina communis* results in a Ca^{2+} rise, more commonly in a sustained form rather than in the wave or transient fashion, typical for IP_3 response observed in other cells, and ABA-induced Ca^{2+} release could be antagonized by 8-NH_2-cADPR and the byproduct of APP-ribosyl cyclase nicotinamide (Leckie et al., 1998a). Thus, cADPR-mediated release of Ca^{2+} (but not IP_3-mediated release) from intracellular stores is involved in the ABA-induced signal transduction pathway. However, the cellular localization of cADPR/ryanodine receptor could not be specified in these studies.

Allen and coworkers (1995) found that IP_3 and cADPR could induce Ca^{2+} release from red beet microsomes in an additive manner, which implies that in their microsomal preparation there were separate vesicle fractions containing different Ca^{2+} release pathways. Searching for cADPR receptor channels, the patch-clamp technique was applied to intact vacuoles and a mildly inward rectifying ionic current, seen as a small increment on the background of an unspecific leak, was attributed to the cADPR action (K_m ~ 25 nM). This current showed low sensitivity to ruthenium red (30-100 µM) and to ryanodine (10 µM), a blocker and a modulator of the animal RyR channel, respectively[3]. In guard cell vacuoles, attempts have been also made to identify the cADPR-activated current by means of the patch-clamp technique (Leckie et al., 1998a). In contrast to previous observations in red beet vacuoles, the "cADPR-induced" currents were inhibited by cytosolic Ca^{2+} with K_m ~100 nM. This value corresponds to the Ca^{2+} sensitivity of the FV current in this preparation (Allen & Sanders, 1996). Taken together with the FV-like current-voltage relationship of the current increment posterior to the cADPR application, it is likely that Leckie et al. (1998a) actually recorded a "run-up" of the FV channel activity (Allen et al., 1998a). Animal RyR channels are large (several hundred pS) conductance ones. They are activated by micromolar (and inhibited by hundred micromolar) cytosolic Ca^{2+} concentrations which underlies their function in CICR (Smith et al., 1985; Ehrlich et al., 1994). In studies on plant cells, no such channels have been detected to date.

Although the existence of IP_3- and cADPR-gated channels in the vacuolar membrane has gained broad acceptance (Muir et al., 1997; Leckie et al., 1998b; Thuleau et al., 1998; Sanders et al., 1999), we think that this notion deserves further proof. Surprisingly, no homologues for animal IP_3 or RyR channels were found in the *Arabidopsis* genome. Does this mean that plant ligand-gated channels have unique sequences? What are, then, their functional properties in comparison with their animal counterparts? Are ligand-gated Ca^{2+} release channels located in the vacuoles (cADPR-activated channel?), in the ER (IP_3-gated

[3] RyR channels from animal cells are irreversibly modified (gated open) by ~0.1 µM and subsequently blocked by 10 µM of ryanodine (Ehrlich et al., 1994). RyR channel is completely blocked by 1 µM of RR (Smith et al., 1985). For a comparison, the Ca^{2+} -permeable SV channel from the same vacuolar preparation (red beet) is inhibited by submicromolar RR (Pottosin et al., 1999).

channel) or in some other intracellular stores? Answering these questions will bring substantial progress towards our understanding of intracellular Ca^{2+} signaling in plants.

Tonoplast anion channels. Preliminary patch-clamp studies of anion channels in vacuolar membranes of different origin revealed a broad range of unitary conductance values, from 7 pS up to 125 pS (Iwasaki et al., 1992; Plant et al., 1994; Pei et al., 1996). Tentative tonoplast channels appear to conduct a wide spectrum of inorganic (Cl^-, NO_3^-, HPO_4^{2-}) and organic (acetate, oxaloacetate, succinate, fumarate, and malate) anions (Iwasaki et al., 1992; Plant et al., 1994; Cerana et al., 1995). It is not clear how many anion channel types are at work. However, there are several lines of evidence for the involvement of multiple channel types. Plant and co-workers (1994) observed that high vacuolar Cl^- down-regulates inward anion currents. The residual current as compared to the initial one was characterized by a relatively low permeability for Cl^- and malate as compared to acetate and NO_3^-. In the presence of malate, some fraction of the whole vacuole current carried by anions became time-dependent, with a characteristic time in the range of seconds (Pantoja et al., 1992b; Cerana et al., 1995; Pei et al., 1996; Cheffings et al., 1997). Whether malate modifies existing channels or induces novel, slow type channels, remains unclear, although several authors use separate names, VCL and VMAL, to designate vacuolar Cl^- and malate-permeable channels, respectively. Mechanisms of the tonoplast's permeability for Cl^- and $malate^{2-}$ differ, in fact, between C_3 and CAM plants. The tonoplast of CAM plants generally displays a larger passive permeability to malate relative to Cl^-, and the rate of malate transport is correlated with the degree of CAM activity (reviewed by Cheffings et al., 1997).

REGULATION OF THE VACUOLAR ION CHANNELS' FUNCTION

To date only three cation channels (SV, FV and VK) and an anion channel species (VCL of guard cells) are unambiguously identified in the tonoplast and explored to such an extent that allows the elucidation of their regulation by a variety of physiologically relevant factors.

Voltage. Among the aforementioned vacuolar channels, only vacuolar potassium (VK) channels are insensitive to a variation of the voltage difference across the tonoplast (Ward & Schroeder, 1994; Allen et al., 1998a).

The SV channel is an outward rectifier, i.e., it is activated by positive voltage steps (Fig. 3 C-E) favoring monovalent cation influx into the vacuole. The voltage dependence of its opening probability is further shifted by monovalent cations (e.g., K^+), and by Ca^{2+} and Mg^{2+}, whereas K^+ fluxes through the open channel are blocked in a voltage-dependent manner by divalent cations and polyamines (see below).

The unblocked FV channel has a carrier-like, bipolar κ-shape voltage dependence (Fig. 3 A-B), with a minimal activity around the vacuolar resting potential (-40 mV) and increased activity at higher potentials of either direction. The position of the minimum follows a shift of equilibrium potential for permeable cation (K^+), see regulation by permeable ions. Thus, the FV channel, as proposed by Tikhonova and co-workers (1997), might operate as functional K^+ channel, clamping the tonoplast voltage close to E_K+.

Further, monovalent cation fluxes via FV channels are strongly rectified by the vacuolar divalent cations Ca^{2+} and Mg^{2+} (see below).

The common property of anion currents reported by different authors is their inward rectification (Iwasaki et al., 1992; Pantoja et al., 1992b, Plant et al., 1994; Cerana et al., 1995; Pei et al., 1996; Cheffings et al., 1997). This means that tentative anion channels are suited for anion uptake into the vacuole but not for the anion release. Stomatal closure, for instance, requires a sustained anion efflux amounting to ~100 pA ($6x10^8$ ions/s). Such a flux may not be achieved by, so far unique, guard cell anion channel (activated by the calmodulin-like domain protein kinase, CDPK) described by Pei and co-workers (1996). Consequently, it was proposed that guard cell anion channels promote the stomatal opening instead. Similarly, in CAM plants a role for anion channels in the nocturnal malate accumulation was postulated (Cheffings et al., 1997), whereas the mechanism of malate efflux from the vacuole in light remains elusive.

Permeable ions. The voltage difference between the cytosol and the vacuole appears not to change drastically, being between +5 and -20 mV as reported by microelectrode measurements. Therefore, it is not voltage dependence itself but its modulation by physiological factors that may play an important role in the vacuolar channels' gating. In contrast to animal cells, where drastic changes of membrane voltage, known as action potentials, are caused by tiny amounts of ions flowing via membrane channels, plant ion channels can mediate large changes of intracellular ion contents (see for an example "osmotic motors" above). It is unclear whether ion channels sense these changes and/or are regulated by their own substrates, as it is true for carriers, where ion translocation and gating are intrinsically coupled. Variability of the vacuolar ion content might be especially important, as cytosolic ion concentrations generally need to be tightly controlled.

One example of such a mechanism is the regulation of the SV channel by the cytosolic and vacuolar Ca^{2+}, when the open probability of the channel became the function of electrochemical potential difference for Ca^{2+}, $)\mu Ca^{2+}$ (Pottosin et al., 1997). Due to the unique role of Ca^{2+} in signaling, this mechanism) will be considered separately below. Apart from the modulation by Ca^{2+}, which was highly specific, SV channel voltage gating is affected by other cations from the vacuolar side. Thus, the increase of vacuolar K^+ (10-400 mM range) in the presence of submillimolar vacuolar Ca^{2+} caused a negative shift of the voltage dependence (Pottosin & Martínez, unpublished). This effect was interpreted as a consequence of a dilution of the local Ca^{2+} concentration due to the screening of the negative surface charge by K^+ and other monovalent ions.

The gating of FV channels senses the variations in the permeable cation species and concentration. Inward (cytosol-directed) currents via FV channels at bi-ionic conditions (KCl inside the vacuole and XCl in the cytosol) are decreased strictly in accordance with the relative permeability of the cation X (Brüggemann et al., 1999a), which was caused by the decrease of the open channel probability (Fig. 4). Variation in the cytosolic K^+ on the other hand equally shifts the reversal potential and the position of the minimum of the FV current voltage dependence in vacuoles of *Hordeum vulgare* mesophyll and *Vicia faba* guard cells (Tikhonova et al. 1997; Allen et al., 1998a). This observation was extended by testing the effects of cytosolic and vacuolar K^+ on the FV channel in *B. vulgaris* taproot vacuoles. Remarkably, the position of the minimum is always set ~50 mV below the

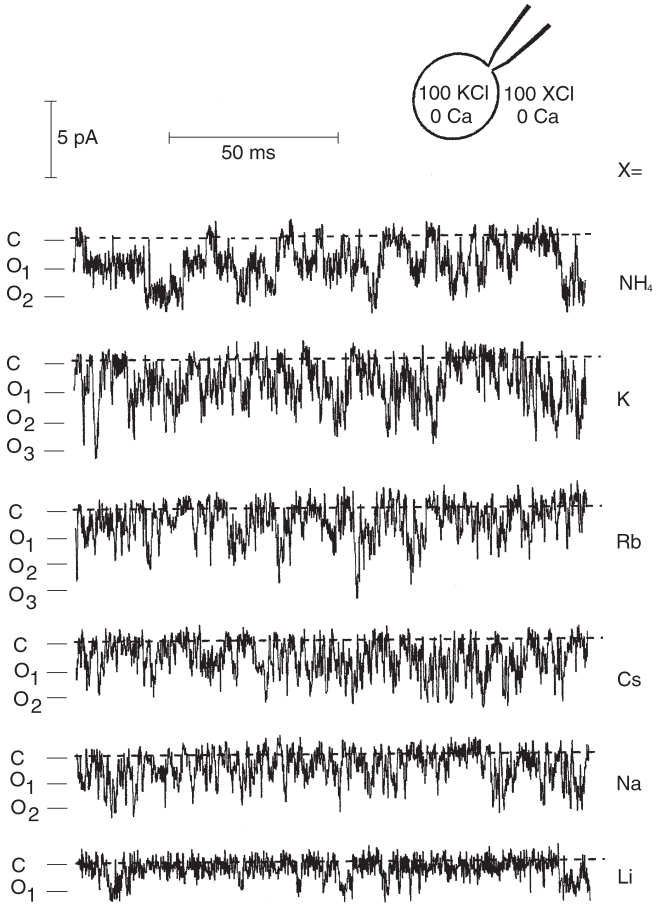

Fig. 4. Gating of the FV channel in barley mesophyll vacuoles is dependent on the permeable cation species. Channel recording at –80 mV from a single cytosolic side-out patch in bi-ionic conditions with 100 mM KCl at the vacuolar side and 100 mM XCl at the cytosolic side, where X is a variable, monovalent cation species. Free Ca^{2+} at both membrane sides is set to the nanomolar level. Indexes at the left hand side correspond to no (C), one, two or three (O_1, O_2, O_3) FV channels open. Ionic currents under these conditions are carried by K^+ in all cases. The sequence from top to bottom corresponds to the relative permeability sequence of the FV channel, and the decrease in relative activity parallels the decrease in relative permeability (Tikhonova, Pottosin & Schönknecht, unpublished data).

equilibrium potential for K^+ (Martínez & Pottosin, unpublished). In the physiologically attainable tonoplast potential range, the FV open probability was mainly dependent on vacuolar K^+: the larger the vacuolar K^+ concentration, the higher the channel activity. Down-regulation of the FV channel by decreased vacuolar K^+ could be important at K^+ starvation, in order to prevent passive K^+ re-uptake into the vacuole when the vacuole to cytosol electrochemical gradient for K^+ is reverted from the cytosol-directed to the vacuole-directed one (Walker et al., 1996). Noteworthy, both vacuolar cation channels (FV and SV) display the valve-like behavior: they are down-regulation by a decrease in vacuolar K^+. Previously, MacRobbie (1998) discovered that the ion channel-mediated loss of the radioactive analogue of K^+ ($^{86}Rb^+$) during ABA-induced stomatal closure is regulated in a feedback manner by the $^{86}Rb^+$ content in the vacuole. She hypothesized that some yet unidentified stretch-activated channels might be involved. Our finding that vacuolar K^+ content affects the open probability of the two principle vacuolar cation channels, FV and SV, might provide a more direct explanation for the observed feedback control.

Anion currents in *Arabidopsis* vacuoles can be modulated by cytosolic malate concentrations, with a threshold for activation shifting more positive at increased malate, but in such a way that always favors the malate influx (Cerana et al., 1995). Increase of the vacuolar Cl^- in the presence of Cl^- or malate at the cytosolic side also increased the voltage threshold; curiously, an opposite effect was observed when nitrate was a sole anion in the cytosolic solution (Plant et al., 1994). Further studies need to be conducted in order to resolve the impact of all these factors. In any case, it is important to learn that, as FV and SV channels, tentative tonoplast anion channels seem to be modulated by permeable ions, which potentially allows a feedback control of the channel function.

Calcium. Cytosolic Ca^{2+} is required for the activation of the VK and anion channels in guard cell vacuoles (the latter via phosphorylation by Ca^{2+} dependent kinase, CDPK) (Ward & Schroeder, 1994; Allen & Sanders, 1996; Pei et al., 1996). Cytosolic Ca^{2+} inhibits FV channels in a voltage-independent manner (Allen & Sanders, 1996; Tikhonova et al., 1997; Dobrovinskaya et al., 1999a). However, only guard cell FV channels were reported to sense cytosolic Ca^{2+} changes in the physiological range (Allen & Sanders, 1996), whereas FV channels of different origin were inhibited by many-fold higher Ca^{2+} activity (Fig. 3, Table 1). For the SV channel, cytosolic Ca^{2+} is a positive modulator of its voltage dependence (Reifarth et al., 1994; Schulz-Lessdorf & Hedrich, 1995; Pottosin et al., 1997), although in the virtual absence of cytosolic Ca^{2+}, voltage-dependent SV currents may be recorded, albeit above the physiological voltage range (Carpaneto et al., 2001).

Due to its activation by a rise in cytosolic Ca^{2+}, Ca^{2+}-permeability and localization in the membrane of an inexhaustible Ca^{2+}-store, the SV channel was considered as a prime candidate for the Ca^{2+}-induced Ca^{2+} release (CICR) in guard cells (Ward & Schroeder, 1994). Indeed, due to a huge cytosol-directed Ca^{2+} gradient (Fig. 1), the SV channel, when open, will mediate Ca^{2+} release from the vacuole. However, it has been found that the SV channel is down-regulated by vacuolar Ca^{2+} (Pottosin et al., 1997). Whereas an increase of cytosolic Ca^{2+} shifted the voltage threshold more negative (Fig. 5A-B), an equivalent increase of vacuolar Ca^{2+} caused the opposite shift. Therefore, at a given Ca^{2+} gradient (defined by equilibrium potential for Ca^{2+}, E_{Ca}) no matter what the absolute Ca^{2+} concentrations at both membrane sides were, the voltage dependence will be the same. Moreover, the voltage activation threshold and E_{Ca} were related linearly with

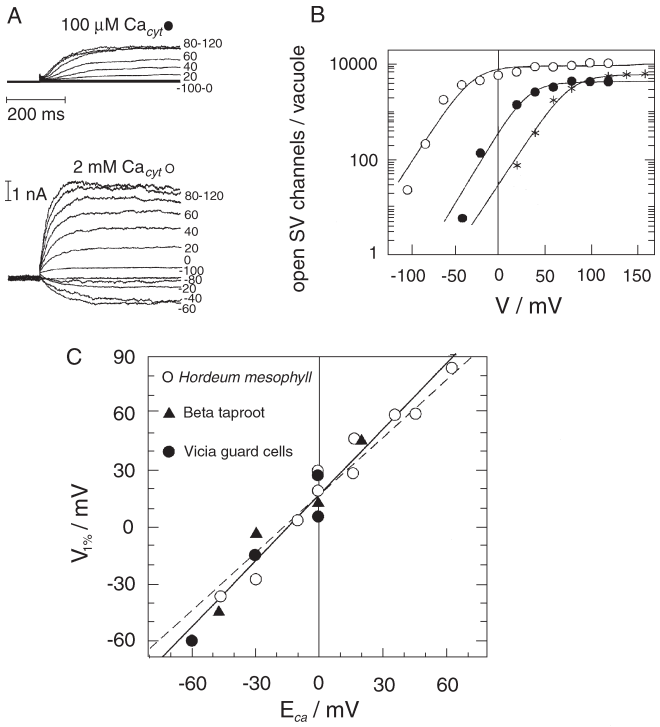

Fig. 5. Modulation of the red beet SV channel by cytosolic and vacuolar calcium. A. Activation of the SV currents in a small (<5 pF) vacuolar vesicle at two different levels of free cytosolic Ca^{2+}. The internal vacuolar solution was 100 mM KCl with nominally zero (~5 nM) free Ca^{2+}. The external solution contained 100 mM KCl and free Ca^{2+} as indicated. From the holding level of –100 mV (cytosol negative), voltage was stepped in 20 mV steps to voltages indicated at the right hand side of each current trace. An increase in cytosolic Ca^{2+} from 100 µM to 2 mM causes a shift of activation threshold to negative potentials and an inward (cytosol-directed) cation current as low as –80 mV may be recorded. B. Voltage dependence of the channel gating was calculated from the data in A and is presented as the mean number of the SV channels per typical vacuole (20 µm in diameter) as a function of transmembrane potential. Hollow circles indicate zero Ca^{2+} in the vacuole and 2 mM Ca^{2+} in the cytosol, filled circles- zero Ca^{2+} in the vacuole and 100 µM Ca^{2+} in the cytosol. For comparison, the data from the experiment in Fig. 3D are replotted (2 mM Ca^{2+} at both membrane sides, asterisks). Solid lines are modified Boltzmann functions fitted to the data. C. Potential activating 1% of the available SV channel's pool is linearly related to the Ca^{2+} equilibrium potential (E_{Ca}=29.5 mV*log $[Ca^{2+}]_{vac}$ / $[Ca^{2+}]_{cyt}$) for three different plant preparations. Potential, activating 1% of SV channels was evaluated by fitting of the voltage dependence at each Ca^{2+} gradient by Boltzmann function. Data for *Hordeum* and *Vicia* are taken from Pottosin et al. (1997), while data for *Beta* from Pottosin et al. (2000b) A dashed line corresponds to the slope factor 1.0.

a slope factor ~1 (Fig. 5C). Thus, at a zero Ca^{2+} concentration gradient, 1% (~ 10^2) of the channels will be open at the potential 17 mV above the E_{Ca}, thus mediating net Ca^{2+} uptake, not a release. Extrapolation of the relation to physiological Ca^{2+} gradient range (10^{-6} M Ca^{2+} in the cytosol, 10^{-3} M Ca^{2+} in the vacuole, membrane potential ~0 mV) resulted in less than 1 open SV channel per vacuole, which apparently leaves insufficient room for its participation in CICR. This conclusion has been confronted by Bewell and co-workers (1999), who have reported substantial $^{45}Ca^{2+}$ release from isolated vacuolar vesicles under the conditions favoring SV channel opening. Unfortunately, due to poor Ca^{2+} buffering, the principal result of this work should be questioned. It appears that Ca^{2+} contamination of the experimental medium could reach several tens of µM. In parallel with the tracer release from pre-loaded vesicles a larger, albeit invisible influx of unlabelled Ca^{2+}, driven by artificially adjusted cytosol-positive diffusion potential, could take place. Hence, in reality it would be a net Ca^{2+} uptake rather than a release. Therefore, the cytosolic Ca^{2+} and voltage may not be efficient in opening of a critical (for the CICR) number of SV channels, and there must be additional "helper" factors at work. Calmodulin could be one of intracellular agents, sensitizing the SV channel to the cytosolic Ca^{2+}, albeit the sensitization effect was moderate, 3-fold at 2.5 µM Ca^{2+} (Bethke & Jones, 1994).

pH. Lowering the cytosolic pH inhibited both SV and FV channels but activated VK channels (Ward & Schroeder, 1994; Schulz-Lessdorf & Hedrich, 1995; Allen et al., 1998a). These changes were moderate, about 2-times for all three channels in the physiological pH range (7.0-7.5). Nevertheless, opposite pH effects on VK and FV channels along with their opposite dependence on cytosolic Ca^{2+} may have an important consequence for the switching of K^+ fluxes between these two channels. It is known that in cold-acclimated plants ABA-induced stomatal closure is not accompanied by a rise in cytosolic Ca^{2+} (Allan et al., 1994). Under such circumstances, vacuolar K^+ release could be dominated by FV channels, activated by cytosol alkalinization (Fig. 2), whereas the highest contribution of VK channels is expected under elevated cytosolic Ca^{2+} conditions (Allen et al., 1998a).

SV channels are inhibited also by vacuolar protons with an apparent pK of 5.0, close to the normal vacuolar pH (Schulz-Lessdorf & Hedrich, 1995). However, due to the competitive effect of vacuolar Ca^{2+}, the effect of pH change from 7.5 to 5.5 is negligible at free vacuolar Ca^{2+} level $\geq$ 50 µM (Pottosin et al., 1997). One may expect that in vacuoles of acidic fruits (pH < 3), regulation by vacuolar protons could have larger impact. In CAM plants diurnal changes of vacuolar pH between 5.5 and 3.3 are observed, with minimal pH correlating to a maximum malate level. For CAM plants grown in a low Ca^{2+} medium, the pH-minimum also is paralleled with the decrease of the free vacuolar Ca^{2+} (Meyer & Popp, 1997). However, in vacuoles of CAM plants the free Ca^{2+} level is always high, from several mM (plants in low Ca^{2+} medium) to 100 mM. Thus, the inhibitory effect of Ca^{2+} would always override that of H^+.

Magnesium. Mg^{2+} content in plant cells, in contrast to total Ca^{2+}, is a function of the ambient concentration, and excess Mg^{2+} is deposited in vacuoles. In barley leaves total Mg^{2+} ranges from 2 mM to 17 mM depending on the cell type (Dietz et al., 1992) which is comparable to 17-20 mM for a typical mammalian cell (Romani & Scarpa, 2000). Free Mg^{2+} in animal cells (0.5-1 mM) is comparable to 0.4 mM free Mg^{2+} measured in mung bean root cells (Yazaki et al., 1988). Compared to Ca^{2+}, Mg^{2+} therefore is poorly chelated:

up to 90% in cytosol (mainly by ATP) and by >50% in the vacuole (mainly by phosphate) and there is no large vacuole to cytosol gradient of free Mg^{2+}. Work on vacuoles revealed that Mg^{2+} effects on the FV are comparable to those of Ca^{2+}, whereas modulation of the SV requires much higher concentrations of vacuolar and cytosolic Mg^{2+} as compared to Ca^{2+}. Cytosolic Mg^{2+} at micromolar concentrations stabilized the closed state of the FV channel in vacuoles of barley mesophyll and *Vicia faba* guard cells (Brüggemann et al., 1999b; Pei et al., 1999). Although cytosolic Ca^{2+} has a similar and even slightly higher potency in the same preparation (Tikhonova et al., 1997), this is due to a much higher free cytosolic concentration that is mainly Mg^{2+}, which controls the FV channel activity. Vacuolar Mg^{2+}, similar to vacuolar Ca^{2+} but with a somewhat higher potency, inhibited the inward current through a FV channel (Table 1). On the contrary, vacuolar Mg^{2+} was 100-fold less effective, compared to vacuolar Ca^{2+}, in the down-regulation of SV channels from barley mesophyll (Pottosin et al., 1997, 2000b). On the other hand, cytosolic Mg^{2+} in the low millimolar range, albeit by itself unable to activate SV currents, promotes SV channel opening at micromolar cytosolic Ca^{2+} in guard cells (Pei et al., 1999). Recent studies on beet vacuoles demonstrated that Mg^{2+} activates the SV channels via a negative shift of the voltage threshold. The magnitude of the shift depended on ionic conditions and Mg^{2+} concentration; within the physiological Mg^{2+} range, as compared to zero Mg^{2+}, the shift of voltage dependence was in the range 20-30 mV (Carpaneto et al., 2001; Pottosin et al., 2001).

The opposite effect of cytosolic Mg^{2+} on the SV and FV channels may be a dynamic factor, affecting the balance of ionic currents across the tonoplast, if the intracellular Mg^{2+} is changed in response to an external stimulus. In the animal physiology the role of Mg^{2+} compared to Ca^{2+} as a signaling ion was denied for a long time due to its high and apparently stable internal concentration (0.5-1 mM), low specificity binding and poor compartmentalization. However, recent findings on stimuli-induced free Mg^{2+} changes may revert this view (Romani & Scarpa, 2000). Less is known about Mg^{2+} distribution, free concentration and dynamics in plant cells, although Mg^{2+} is an important regulator or cofactor of many cellular enzymes. The interest in the role of Mg^{2+} in plant cell signaling is strengthened by the finding that the ABA-insensitive phenotype is linked to alterations of *abi1* and *abi2* genes, these encoding closely related isoforms of PP2C, Mg^{2+}-dependent phosphatase. The *abi1* gene product positively regulates the outward rectifier K_{out} and possibly the anion channel of plasma membrane of guard cells (Luan, 1998); both channels are major mediators of salt release in response to ABA. In contrast to their animal counterparts, Ca^{2+}-permeable channels of the plant plasma membrane show a substantial permeability to Mg^{2+} (White, 2000). In particular, the recently discovered hyperpolarization-activated Ca^{2+}-permeable (Ca^{2+}-influx) channel of guard cell plasma membrane, which was specifically activated by ABA (Hamilton et al., 2000), does not differentiate between Ca^{2+} and Mg^{2+} (Pei et al., 2000). Possible consequences could be summarized as follows: Mg^{2+} entering the cytosol via Ca^{2+}-influx channel would stimulate solute efflux across the plasma membrane via increased activity of PP2C, positively modulating the plasma membrane K_{out} and anion channels and supporting the activating effect of Ca^{2+} on the SV channel. This would in turn promote a vacuolar Ca^{2+} release and the maintenance of high cytosolic Ca^{2+} required for the vacuolar K^+ release through VK channels and Ca^{2+}-dependent activation of VCL.

Polyamines. Diamine putrescine (Put^{2+}), polyamines spermidine (Spd^{3+}), and spermine (Spm^{4+}) are ubiquitous in higher plants. They are present both in the cytosol and in the vacuole at concentrations ranging from a few µM to hundreds of µM (spermine) up to 10 mM for putrescine in K^+ deficient plants (Cohen, 1998). At this range of concentration, polyamines affect both FV and SV channels of plant vacuoles (Brüggemann et al., 1998; Dobrovinskaya et al., 1999 a,b; Table 1). Increased cytosolic polyamine concentration decreased the FV channel open probability with a relative potency Spm^{4+} (Kd~5µM) > Spd^{3+} (Kd~100 µM)>>Put^{2+} (Kd~5,000 µM), both in barley mesophyll and red beet storage vacuoles. Therefore, at their resting concentrations, the activity of FV channels is suppressed more than 2- times by Spm^{4+} and Spd^{3+}. Cytosolic and vacuolar polyamines had no effect on the open probability of the SV channel and act as open channel blockers (Fig. 6)[4], with a relative inhibition potency increasing by about 7-fold with the addition of one amine group Put^{2+}<Spd^{3+}<Spm^{4+} (Dobrovinskaya et al., 1999 a, 1999b). Despite the mechanism of action, the appearance of the polyamine effect on the macroscopic (whole vacuole) SV current was as if these substances affected the voltage gating. The physiological action of the polyamine-induced inward rectification (preferential inhibition of the outward, cytosol-directed current, Fig. 6B) may be to reduce the effect of the feed-forward channel activation by released Ca^{2+} with the respective membrane depolarization. A superposition of voltage dependence of the polyamine block on the opposite voltage dependence of the channel gating (Fig. 5B) could confine the channel opening to a narrow voltage range. The voltage-dependent block by polyamines increased at moderate positive potentials at the side of polyamines application, and relieved at larger potentials, suggesting polyamine permeability of the channel (Fig.6). Previously, polyamines have been shown to be transported through the vacuolar membrane of *Arabidopsis thaliana* by cationic channels, which in whole vacuole configuration mediated macroscopic currents with very similar characteristics (time course, voltage dependence) to those of SV current (Colombo et al., 1992). Therefore, polyamines may not only control the cation flow through the SV channel but, in turn, the redistribution of polyamines between cytosolic and vacuolar pools is likely mediated by SV channels. Effects of polyamines on vacuolar cation channels are different from their effects on plant plasma membrane transporters: inward rectifier K_{in} of *Vicia faba* guard cells and H^+-ATPase in rice coleoptile. The first is inhibited and the second stimulated by high (millimolar) concentrations, with only a weak selectivity among polyamines Spm^{4+}=Spd^{3+}>Put^{2+} (Liu et al., 2000; Reggiani et al., 1992). Thus, the increase of putrescine to a millimolar level seems to have a general effect on FV, SV, K_{in} and plasma membrane H^+-ATPase, whereas high spermine and spermidine levels will in first turn effect vacuolar cation channels, and among them preferentially the FV channel. Enhanced cytosolic Spm^{4+} (100 µM) caused a 3 to 10-fold larger decrease in the SV current as compared to the FV channel, whereas high putrescine level (3 mM) inhibited FV and outward SV currents by an average of only 30% and by 70%, respectively (Brüggemann et al., 1998; Dobrovinskaya et al., 1999a,b). In other words, the diamine to polyamine ratio may efficiently control the relation between the FV and SV currents of the

[4] Polyamines in a similar fashion, but at somewhat higher concentrations, are known to block other intracellular Ca^{2+} release channels, such as the cardiac ryanodine receptor (RyR) channel (Uehara et al., 1996). Submillimolar polyamine levels inhibit cADPR-induced as well as ryanodine- and caffeine-induced Ca^{2+} release in sea urchine eggs (Chini et al., 1995).

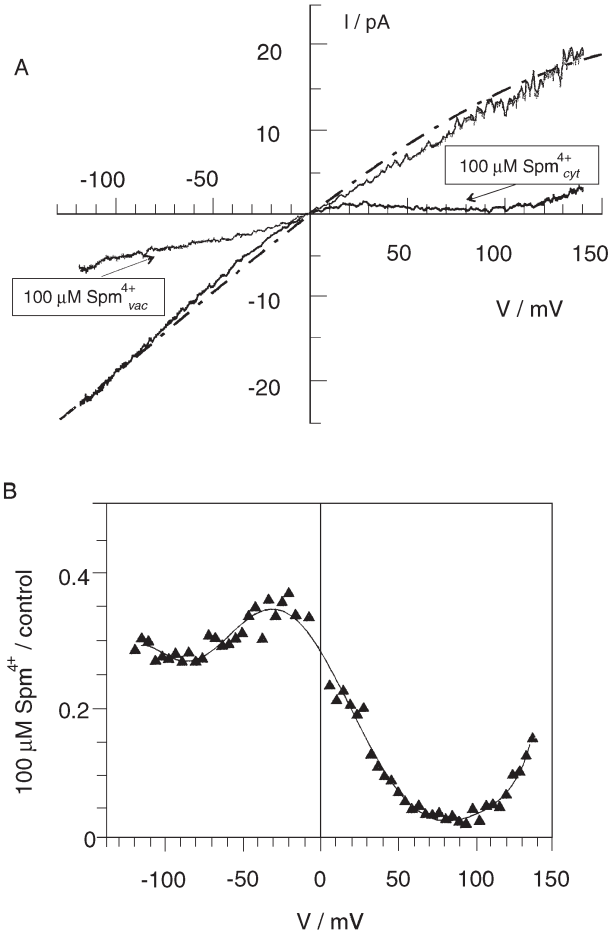

Fig. 6. Cytosolic and vacuolar spermine blocks the red beet SV channel in a voltage-dependent manner. A. Single channel current-voltage relationship in control conditions (symmetrical 100 mM KCl, dashed-dotted line) and in the presence of 100 µM spermine at the cytosolic or at the vacuolar side. A strong voltage dependence of the block is manifested by the fact that cytosolic and vacuolar Spm^{4+} mainly suppress outward or inward currents, respectively. B. Simultaneous effect of the vacuolar and cytosolic spermine on relative current/conductance of the SV channel. The voltage dependence of the effect is dominated by cytosolic Spm^{4+} and may be compared with the opposite gating by membrane voltage (Fig. 5B).

vacuolar membrane. Taking into account the selectivity properties of the vacuolar channels and the ionic gradients, the SV channel may be considered as the Ca^{2+} release channel which, when fully activated, may be also suited for K^+ or Na^+ uptake, whereas the FV channel likely mediates passive exchange of monovalent cations across the tonoplast.

The synthesis of polyamines is known to be induced in plants by various stresses and upon induction of growth and development, but the pattern seems to be stimulus-dependent (Evans & Malmberg, 1989; Galston & Sawney, 1990; Cohen, 1998). A huge accumulation of putrescine (up to 10 mM) without marked changes of higher polyamines levels under K^+ deficiency is an established and universal example (Murty et al., 1971; Crocomo & Basso, 1974; Sarjala, 1996; Watson & Malmberg, 1996). On the contrary, high external K^+ (10-15 mM) depresses putrescine synthesis and stimulates its conversion to higher polyamines (Aurisano et al., 1993; Reggiani et al., 1993). Putrescine feeding under K^+ replete conditions may evoke symptoms similar to K^+ deficiency (Sung et al., 1994). At moderate K^+ starvation, the putrescine accumulation would first switch down the SV channel without marked effect on the FV channel, thus vacuolar and cytosolic K^+ pools would be temporarily in equilibrium (Walker et al., 1996). However, at severe K^+ starvation, an increased putrescine level will also inhibit the FV channel. At these conditions, a driving force for K^+ is directed from cytosol to the vacuole, hence K^+ efflux from the vacuole must be active. On thermodynamic grounds, the active efflux of K^+ can be mediated by a H^+/K^+ symport (Walker et al., 1996) giving rise to cytosolic acidification[5]. This, along with a decreased vacuolar K^+ level (see "Permeable ions"), will further decrease the activity of vacuolar channels.

Putrescine, although at a lower yet millimolar level, is accumulated under anoxia (Reggiani et al., 1992) and osmotic stress (Flores & Galston, 1982; Aziz & Lahrer, 1995). The increase of putrescine titer is common under hypertonic conditions of protoplasts preparation, although it seems to be restricted to monocots (e.g., cereals), whereas various dicots, which are able to regenerate whole plants from leaf protoplasts, respond by a decrease in putrescine and an increase in spermidine and spermine (Tiburcio et al., 1986a,b). Both osmotic and salt stress may cause similar polyamine accumulation, though spermine accumulation, if occurring is characteristic for the salt stress (Erdei et al., 1990,1996). High spermidine and spermine levels in oat (*Avena sativa*) leaves and protoplasts delay osmotic stress-induced senescence (Tiburcio et al., 1994). Resistance to salt stress seems to be correlated with a pattern of polyamine synthesis. For example, salt-tolerant but not salt-sensitive rice (*Oryza sativa*) lines are effective in maintaining a high spermidine and spermine *versus* putrescine ratio (Krishnamurthy & Bhagwat, 1989). Similarly, in seedlings of wheat (*Triticum aestivum*) salt stress causes a slight decrease in putrescine content, an increase of spermidine, and a even larger increase of spermine. However, growth of wheat seedlings was negatively correlated with higher polyamine concentrations (Reggiani et al., 1994). In this case the accumulation of spermine, up to 500 µM in stems of the most salt sensitive wheat line, was obviously already beyond the range of positive regulation. Such a huge concentration will inevitably inhibit not only the

[5] At extreme K^+ deficiency, K^+ uptake across the plasma membrane needs to be maintained by high-affinity K^+ transport, whereas K_{in} channels, due to the outward K^+ gradient, will operate as K^+ leak. Thus, their inhibition by high putrescine (Liu et al., 2000) may be very useful, especially keeping in mind the activation of the K_{in} current by cytosol acidification (Grabov & Blatt, 1997).

FV channel but also the SV channel (Table 1), would therefore reduce or prevent Ca^{2+} release from the vacuole. It is known that salt tolerance is critically dependent on the function of the so-called *SOS* cascade, where one of the key components (protein kinase *SOS2*) is activated in a Ca^{2+}-dependent fashion (Halfter et al., 2000). Ca^{2+} release from the vacuole may not only be involved as an immediate reaction to salt or hypertonic stress (Knight et al., 1997), but also may contribute to the activation of *SOS* pathway at later periods. On the other hand, down-regulation of the FV channel alone by moderate spermine increase (and by Na^+, Fig. 5) during salt stress might help prevent the passive leaking of Na^+ sequestered in the vacuole. At the same time, the SV current in the proximity of its reversal potential would not be significantly affected. Therefore, it might even contribute to Na^+ uptake into the vacuole in exchange for Ca^{2+} release.

Overall, there is a correlation between the level of polyamines and plant development. Generally high spermine and/or spermidine levels are found in young, intensively grown tissues, such as embryos axes or root apices, whereas putrescine is accumulated at later stages; in dying tissues it may amount up to 20% of total nitrogen content (Lin, 1984; Shen & Glaston, 1985; Flores, 1991; Cohen, 1998). Inducible overexpression of arginine decarboxylase in transgenic tobacco plants results in increased putrescine content and phenotypic alterations such as short internodes, thin stems and leaves, leaf chlorosis, and reduced root growth (Masgrau et al., 1997). Plant regeneration from callus culture of rice may be improved by blocking putrescine synthesis and/or applying spermidine treatment, thus causing an increased Spd^{3+}/Put^{2+} ratio (Bajaj & Rajam, 1996). Although the toxic effect of high putrescine levels on vegetative growth and its reversal by restoring a higher polyamine to putrescine ratio is commonly observed, the mechanism of this relationship is not established. The phenotypic alterations in transgenic tobacco due to an overexpression of arginine decarboxylase and a respective increase of putrescine level are very similar to those of vacuolar Ca^{2+}/H^+ antiporter overexpression, when Ca^{2+} becomes unavailable to all but the vacuolar lumen of the cell. (Masgrau et al., 1997; Hirshi, 1999). Because the SV channel is the only firmly established Ca^{2+}-permeable channel of the vacuolar membrane, one might relate putrescine toxicity to SV channel inhibition. This would make the vacuolar Ca^{2+} pool unavailable, thus mimicking symptoms of external Ca^{2+} deficiency. However, different intracellular Ca^{2+} pools may be at work. The apical gradient of a Ca^{2+} increase alone is sufficient to establish and maintain the site of tip growth during the polarized growth of pollen tubes and root hairs. Internal Ca^{2+} stores localized in this region are likely IP_3-sensitive ones of endoplasmic reticulum origin, whereas the vacuole located at the rear part of the cell seems to take no part in localized Ca^{2+} changes (Trewavas & Malhó, 1997; Yang, 1998). The situation is different in meristematic cells, where clusters of provacuoles could come into the contact with endoplasmic reticulum (Marty, 1999). During the induction phase of the growth of transformed of roots *Catharanthus roseus*, an increase in the level of polyamines and of phospholipase C (PLC) activity are positively correlated (de los Santos Briones et al., 1997). Spermine, but not putrescine or spermidine, caused up to a 4-fold stimulation of membrane-bound PLC at concentrations between 10 and 100 µM as compared to a 50 to 160 µM Spm^{4+} increase during the induction phase, whereas higher concentrations of Spm^{4+} produced a strong inhibition (Echevarria Machado & Hernández Sotomayor, personal communication). These initial studies provide a link between increased polyamine levels in intensively growing tissues and Ca^{2+} mobilization from intracellular IP_3-

sensitive stores. In this regard, the investigation of the effects of polyamines, especially of spermine, on the dynamics of intracellular Ca^{2+} and the interaction between different Ca^{2+}-release pathways in plants is of great interest. Particularly, the polyamine stimulation of the PLC and the parallel block of the SV channels may, depending on the localization of IP_3 receptors, initiate a cross-talk via an increase in cytosolic Ca^{2+} between these two Ca^{2+}-permeable channels, or, alternatively, a spatial redistribution of the Ca^{2+} signal to the vicinity of IP_3-sensitive stores.

CONCLUSIONS

Four distinct ion channels SV, FV, VK and VCL/ VMal, two types of H^+ pumps, and several metal/H^+ antiporters have been characterized in the tonoplast of higher plants (Fig. 7). Thus, the basis for the generation of gradients for H^+ and Ca^{2+}, and mechanisms of uptake of polyvalent cations (including heavy metals) and sodium into the vacuole are understood. Potassium and anions can be actively accumulated into the vacuole, but corresponding transport mechanisms are still elusive. At replete conditions, concentration gradients for K^+ and anions combined with the membrane potential difference favor passive release of these ions from the vacuole to the cytosol (Fig. 1). However, this process is handicapped, owing the voltage dependence of the major ion currents (Fig. 7). The SV and FV channels are outward rectifiers, i.e., are able to mediate mainly cation uptake into the vacuole. This is due to intrinsic gating and inhibition of inward (cytosol-directed) currents by divalent cations from the vacuolar side. Nevertheless, a residual inward current through FV channels is comparable by absolute magnitude to that generated by H^+ pumps. Hence, H^+ pumping into the vacuole could be electrically balanced. The VCL channel favors uptake of anions into the vacuole, whereas outward currents carried by this channel are tiny. Large ion release by vacuolar channels can only take place when the activation of both cation and anion channels shifts towards the narrow voltage region roughly fitting the physiological one, where the condition of net salt (K^+ and anions) release is met (Fig. 7). Such a transition, allowing a massive volume reduction, likely occurs in motor cells, such as guard cells, but the specific mechanisms of up-regulating vacuolar channel activity in the physiological voltage range are unknown. Cation channels are efficiently down-regulated by polyamines, whose concentration tends to increase upon various stresses and growth induction. The cation (SV and FV) and anion channels are gated by permeable ions in a valve-like fashion, which might underlie their role in cell volume regulation.

PERSPECTIVES

Membrane voltage is an important factor which links the activity of membrane transporters, pumps, and channels, as well as defines the direction and magnitude of the vacuolar ion transport. Membrane potential values reported by microelectrode studies did not fit well into the very negative values expected on the ground of energetic considerations for currents generated by H^+ pumps nor did they fit well into the range of potentials delimited by known ion channels. Instead, they lie in between. The compromise

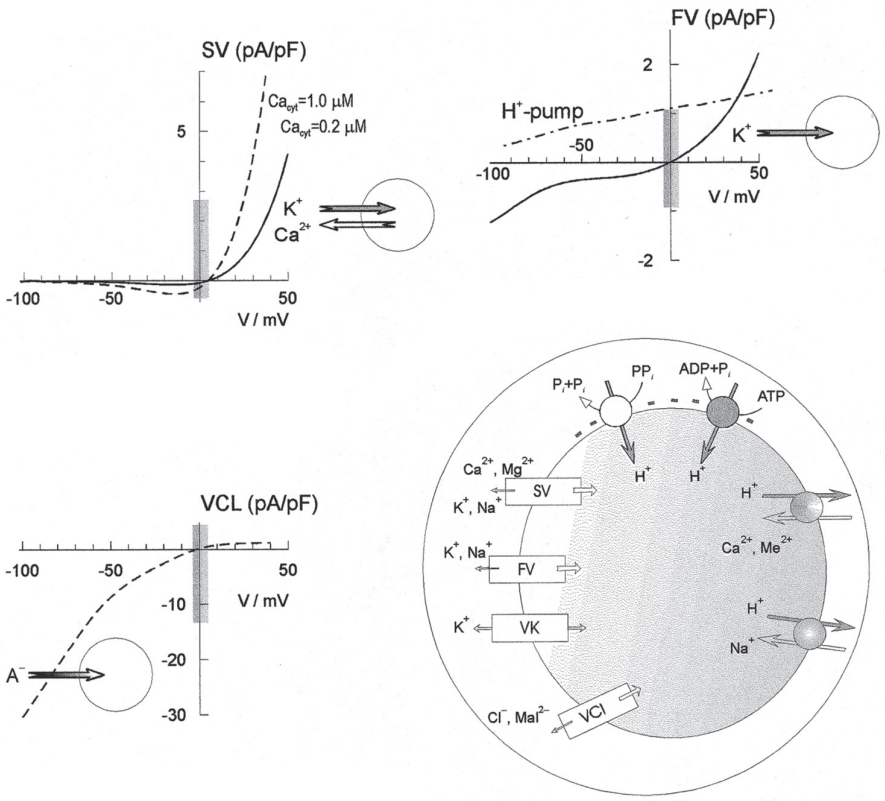

Fig. 7. Summary of the vacuolar ion transporters. Ion channels (SV, FV, VK and VCL/VMal) have been characterized only functionally. H^+-translocating pumps (V-type ATPase and inorganic pyrophosphatase) and several metal/H^+ antiporters (including CAX family members CAX1 and CAX2 transporting Ca^{2+}, Mn^{2+} and Ca^{2+}, closely related AtMHX , transporting Mg^{2+} and Zn^{2+}, and Na^+/H^+ antiporters) have been characterized by means of molecular genetics and by functional assays. Steady-state I/V relations for the SV, FV and anion currents were constructed, assuming 0.5 mM Ca^{2+} in the vacuole and 0.5 mM Mg^{2+} at both sides. The shaded bar indicates the region of net salt (K^+ plus anion) release. See text (Conclusions) for comments.

between active and passive conductance of the vacuolar membrane might be a subject of variation, but the range of possible potential displacements and their dynamics are, in fact, unknown. Are there, for instance, changes in the membrane potential of guard cell vacuoles during stomatal movements? Spectroscopic measurements with potential-sensitive dyes might serve as a useful complement to conventional microelectrode techniques for resolution of these issues.

All plant cells are not alike. There is, for instance, a remarkable difference in solute accumulation between symplastically connected cells of different types within a leaf. Such a difference could only be related to different transport capabilities of respective vacuoles (Karley et al., 2000). Does this mean that there is differential expression or that there are different ways of regulation of tonoplast transporters? In a single plant cell several types of vacuoles are found. Electrophysiologists mainly have studied large central vacuoles. Even though there are at least two distinct types of central vacuoles: acidic, lytic vacuoles containing vegetative-specific γ-TIP aquaporin, and storage vacuoles containing a- or δ-TIPs, respectively (Marty, 1999). Besides, depending on the developmental phase a plant cell can contain a variety of vacuolar "generations", including tiny provacuoles. Small vacuoles likely form a distinct Ca^{2+} store (Harper, 2001). The vacuole as a dynamic compartment is a subject of biogenesis, differentiation, and remodeling, both morphologically and biochemically. Just how these changes are related to changes in tonoplast transporter repertoires is unknown. Systematic study of vacuolar ion transport in relation to the biogenesis and specific function of vacuoles is awaited.

There are few direct measurements on free Ca^{2+} content in plant vacuoles, and almost nothing is known about free Mg^{2+} in plant cells. Given the strong impact of these ions on the function of vacuolar channels, there is a clear need to fill this gap. Free Ca^{2+} in vacuoles is believed to be in the millimolar range. However, there are indications for vacuoles of barley aleuron tissue free Ca^{2+} might be much lower, down to the nanomolar range (R. Jones, personal communication). Based on the voltage dependence of ion channels in vacuole-attached patches from barley mesophyll, the vacuolar Ca^{2+} level is in the low micromolar range in young plants (Tikhonova, Pottosin & Schönknecht, unpublished result). *In vivo* monitoring of the vacuolar free Ca^{2+} and of its impact on the vacuolar ion transport may open a new perspective for the understanding of ionic homeostasis in the vacuolate plant cell. The vacuolar SV and FV channels are strongly affected by physiological concentrations of polyamines, and therefore they may be prime targets for polyamines in plant cells. Thus, the uneven distribution of polyamines in plant tissues and specific changes of their levels during different stresses and developmental signaling will definitely have an important impact on the vacuolar ion transport and Ca^{2+} mobilization. A causal relationship between polyamine metabolism, Ca^{2+} and ionic homeostasis, and their integration for long term physiological responses could be therefore elucidated.

There has been a great variety of ion channel currents reported for the vacuolar membrane. Apparently, the actual number of *bona fide* vacuolar channels is smaller. In some cases the difference in current characteristics simply reflects different experimental conditions, and there is perhaps a number of sheer artificial currents, not convincingly supported at the whole vacuole nor at the single channel levels. More experimental evidence and stricter criteria need to be applied to resolve this issue. Nevertheless, some ion channel types await their discovery. Particularly, the search of the ion channel

responsible for anion release from the vacuole should be continued, and the presence of tonoplast ligand (IP_3, cADPR)-gated channels needs to be unequivocally demonstrated. As demonstrated by the analysis of the *Arabidopsis* genome database, there are apparently no homologues of animal Ca^{2+} and Ca^{2+}-permeable channels, except those belonging to cyclic nucleotide-gated and glutamate receptor channels' families (Mäser et al., 2001). How this could be reconciled with a variety of Ca^{2+}-permeable channels already functionally detected in membranes delineating different compartments of the plant cell? The molecular identities of vacuolar ion channels, even those best characterized by electrophysiological techniques, the FV and the SV channels, are unknown. This abscence of knowledge, handicaps the understanding of their physiological function. However, a recent study by Schönknecht and co-workers (2002) reported the first vacuolar cation channel homologue and provides an initial clue for its relation to known vacuolar currents.

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