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Characterization of an anion-permeable channel from sugar beet vacuoles: effect of inhibitors

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The vacuole occupies 25–95% of the plant cell volume and plays an essential role in maintaining cytoplasmic homeostasis of nutrients and ions. Recent patch-clamp studies identified ion channels and electrogenic pumps as pathways for the movement of ions and metabolites across the vacuolar membrane (tonoplast). At high cytoplasmic Ca^{2+} ($>10^{-6}$ M) and negative potentials (inside the vacuole) non-selective channels of the 'slow-vacuolar (SV)-type' were activated resulting in anion release or cation influx. In the present study these vacuolar channels were characterized pharmacologically by ion channel inhibitors. The cation-transport inhibitors Ba^{2+}, TEA^+ and amiloride caused only partial and reversible block of the 'SV-type' channels, whereas anion-transport inhibitors strongly affected the vacuolar channels. Pyridoxalphosphate and the dimethylamine-carboxylate derivatives anthracene-9-carboxylic acid and C 144 reversibly blocked the channels up to 70% and $ZnCl_2$ up to 95%. DIDS and SITS inhibited this channel irreversibly up to 95%. The block developed under a variety of experimental conditions using solutions containing combinations of permeant cations and anions. The DIDS binding site is located on the cytoplasmic surface of the tonoplast, as intravacuolar DIDS did not block the channels. DIDS concentrations in the micromolar range, efficient in blocking 70–80% of the 'SV-type' channels did not significantly affect ATP-induced or pyrophosphate-induced proton-pumps. Stilbene derivatives may therefore be useful tools for studies on the substrate binding site on this vacuolar channel and for channel isolation.

Key words: patch clamp/ion channels/inhibitors/vacuoles/sugar beet

Introduction

Mature plant cells are characterized by the presence of a large central vacuole. Besides storing solutes which are significant in cell metabolism, the vacuole plays a fundamental role in cell volume increase, osmo/turgor-regulation and salt tolerance (Matile, 1978).

Many inorganic and organic ion fluxes across the vacuolar membrane (tonoplast) are tightly coupled to modulation of cell metabolism or the cell cycle (Kaiser *et al.*, 1982; Gerhardt and Heldt, 1987).

As shown in our previous work, vacuolar ion channels may mediate fluxes of ions and charged metabolites enabling direct electrical and metabolic communication between the cytoplasm and the vacuolar compartment (Hedrich *et al.*, 1986; Coyaud *et al.*, 1987).

Two types of Ca^{2+} and voltage-dependent tonoplast channels have been described, the 'SV (slow activating vacuolar)-type' and 'FV (fast activating vacuolar)-type' channels (Hedrich and Neher, 1987).

At high cytoplasmic Ca^{2+} ($>10^{-6}$ M), the ionic conductance of the vacuolar membrane is entirely accounted for by 'SV-type' channels, which have been characterized in considerable detail in recent patch-clamp studies (Hedrich and Neher, 1987).

These channels were found in various plant species and plant tissues and are thus considered an ubiquitous mechanism for solute transport across the tonoplast (Hedrich *et al.*, 1988).

'SV-type' channels are strongly voltage-dependent (negative potentials inside the vacuole cause channel opening) and Ca^{2+}-dependent. Increasing cytoplasmic Ca^{2+}-concentration activates 'SV-type' channels and markedly shifts the 'activation potential' towards less negative potentials (Hedrich and Neher, 1987). This channel shows a low selectivity, conducting anions and cations (Hedrich *et al.*, 1986; Coyaud *et al.*, 1987).

To some extent 'SV'-channels are comparable with the Ca^{2+}- and voltage-activated anion transporters in excitable vacuoles of giant algae, which are supposed to mediate anion fluxes to balance the osmotic pressure (Tazawa *et al.*, 1987).

The purpose of the study reported here was to investigate the action of cation- and anion-transport inhibitors on the voltage- and Ca^{2+}-activated SV-type channels of sugar beet vacuoles. Compounds selected for specific cation- or anion-channel inhibition may become useful tools for further studies of the control of transport events across the tonoplast, for characterization of the substrate binding sites, as well as for the isolation of vacuolar channels.

Results

This study is concerned with the inhibition of 'SV-type' channels by various cation- and anion-transport inhibitors to obtain a pharmacological characterization of the substrate binding site of the non-selective vacuolar channel. All inhibitors used were tested at concentrations that were found to completely block ion channels in other organisms.

Effect of cation-transport inhibitors

Halophytes such as sugar beets achieve osmotic balance by accumulating large amounts of sodium in addition to potassium salts inside the vacuole. Based on previous results showing that 'SV-type' channels conduct K^+ and Na^+ equally well, the inhibitory effects of K^+- and Na^+-channel blockers on vacuolar currents were tested (listed in Table I).

Table I. Effect of cation transport inhibitors on whole-vacuolar SV-type currents

Inhibitor	Inhibitor type (as characterized in other organisms)	Concentration (M)	Inhibition of current (%)	Reversibility (+/−)
TEA^+	Tetraethylammonium and BA^{2+} ions, blockers of many K^+ channels, e.g. inward	10^{-2}	20−50	+
Ba^{2+}	and outward rectifiers in animal, yeast and plant cells	10^{-2}	50−70	+
Amiloride	Diuretic drug, blocks Na^+/H^+ antiport in various animal membranes and sugar beet vacuoles	2×10^{-3}	<20	+

Table II. Effect of anion transport inhibitors on whole-vacuolar SV-type currents

Inhibitor[a]	Inhibitor type (as characterized in other organisms)	Concentration (M)	Inhibition of current (%)	Reversibility[a] (+/−)
DIDS	Stilbene derivatives (DIDS and SITS), selective inhibitors of anion permeability in red blood cells and torpedo electroplax and phosphate-translocator of the	10^{-5}	90	−
SITS	chloroplast (23)	10^{-5}	80−90	−
A-9-C	Dimethylamine-carboxylate derivatives (A-9-C and C 144), Cl^- channel blockers	10^{-4}	55	+/−
C 144	in chloride transporting epithelia, e.g. rat diaphragm	10^{-5}	70	+/−
PPL	Pyridoxalphosphate, blocks anion-transport in red blood cells and phosphate-translocator of the chloroplasts	10^{-3}	50	+
EA	Ethacrinic acid, diuretic drug blocks anion channels in the plasma membrane and vacuolar membrane of giant algae	2×10^{-4}	35	+
Zn^{2+}	Zinc ions, block anion channels in vertebrate muscle	10^{-4}	95	+

[a]DIDS = 4,4-diisothiocyano-2,2-stilbene disulfonate; SITS = 4-acetamido-4-isothiocyano-2,2-stilbene disulfonate; A-9-C = anthracene-9-carboxylate; C 144 = 5-nitro-2-(3-phenylpropylamino)benzoate.
[b]+ = reversible; − = irreversible; +/− = partially reversible.

After gaining access to the interior of sugar beet vacuoles, we set the membrane potential to +20 mV (corresponding to the physiological voltage drop across the tonoplast). When the membrane potential was stepped for 5 s to negative values, inward currents were elicited, showing 'slow' activation extending over seconds. These inward currents, which we call the 'slow vacuolar type (SV)', were activated by increased cytoplasmic Ca^{2+} concentration, as described previously (Hedrich and Neher, 1987). In order to fully activate SV-type currents, experiments were conducted in the presence of 10^{-4} M Ca^{2+} on the cytoplasmic side of the tonoplast.

With 200 mM potassium or sodium salts on either side of the tonoplast, the nonselective SV-type channels (Hedrich *et al.*, 1986; Coyaud *et al.*, 1987) would allow cation influx as well as anion efflux at hyperpolarized membrane voltages. Repetitive pulses to −60 or −80 mV were used to elicit inwardly rectifying SV-currents. When the amplitude of the vacuolar currents was stable, inhibitors were tested for the reduction or blockage of the currents.

Addition of 10 mM Ba^{2+} or TEA^+ ions to the external medium suppressed the vacuolar currents up to 70% of the initial value (Table I). Upon the removal of Ba^{2+} or TEA^+ by bath perfusion inward currents regained approximately their initial values. The reversible effects of these K^+ channel blockers are similar to their previously described actions on plant and yeast cells (Gustin *et al.*, 1986; Moran *et al.*, 1986; Iijima and Hagiwara, 1987; Schroeder *et al.*, 1987). α-, β-Bungarotoxin and tetrodotoxin, toxic components from animal venoms, whose target sites are AchR, K^+ or Na^+ channels (for review, see Hille, 1984; Miller, 1986), did not cause any significant reduction in SV-type currents (not shown).

In order to distinguish between the Na^+-permeable SV-channels and the possible presence of an amiloride-sensitive Na^+ transport system in beet vacuoles (Blumwald *et al.*, 1987), the effect of amiloride on currents through SV-channels was tested in symmetric NaCl solutions. Table I shows that 2 mM amiloride, which causes 90% inhibition of a Na^+/H^+ antiporter in beet roots (Blumwald *et al.*, 1987), inhibited the vacuolar current by <20%.

The results indicate the presence of an amiloride-insensitive, Na^+-permeable ion channel allowing Na^+ movement along its concentration gradient (Table I and Coyaud *et al.*, 1987). Accumulation of Na^+ above its electrochemical gradient inside the vacuole may be mediated by an electroneutral Na^+/H^+ antiporter, driven by the proton gradient across the tonoplast as suggested by others (Blumwald *et al.*, 1987).

Effect of anion-transport inhibitors

Based on previous experiments showing that the tonoplast (Martinoia *et al.*, 1986, 1987) and especially SV-type ion channels (Hedrich *et al.*, 1986; Coyaud *et al.*, 1987) conduct anions such as nitrate, malate or chloride, we have screened anion-transport inhibitors for their inhibition of vacuolar currents (Table II).

Figure 1(B−D) shows that increasing DIDS concentrations potently reduced the whole-vacuolar SV-currents at hyperpolarizing voltage pulses. In the presence of 10 μM

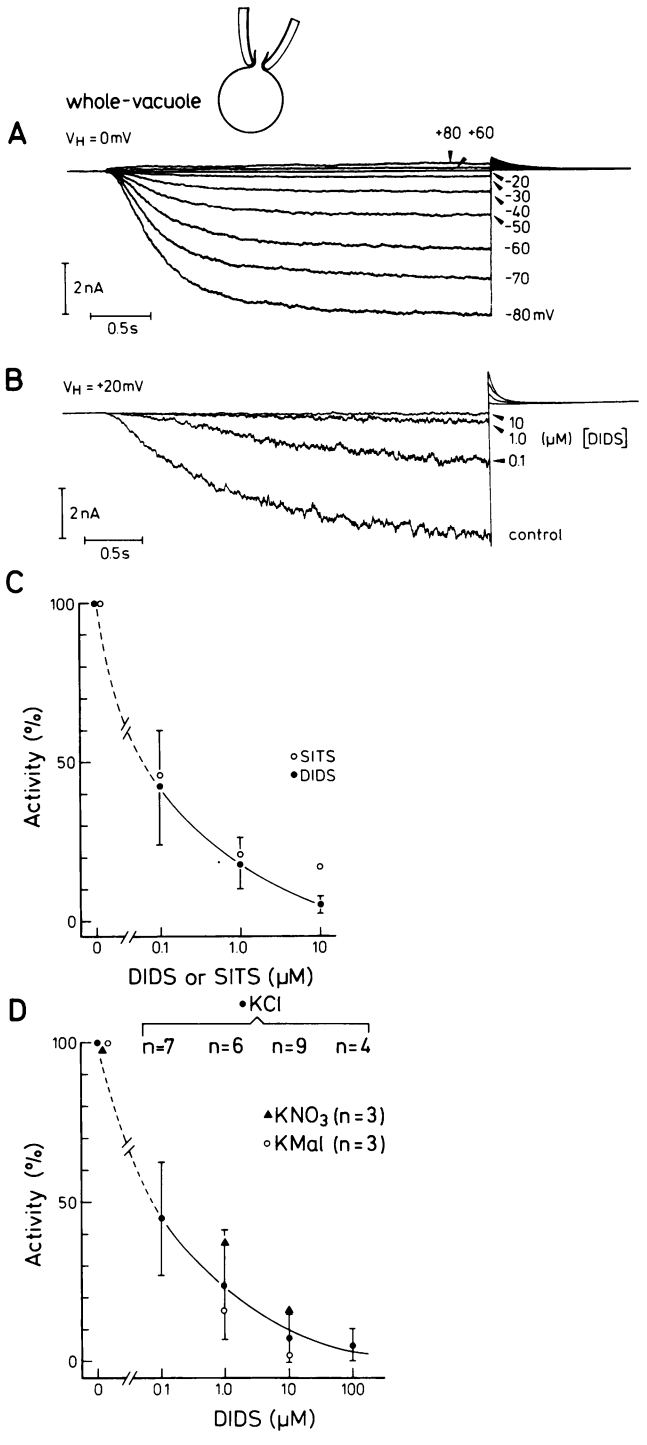

Fig. 1. Concentration-dependent irreversible inhibition of voltage-dependent SV-type currents by stilbene derivatives. (**A**) Whole-vacuolar currents elicted in response to a series of hyperpolarizing and depolarizing voltage steps from a holding potential of 0 mV. (**B**) Whole-vacuolar currents elicited in response to voltage pulses to −80 mV from a holding potential of +20 mV, recorded in various DIDS concentrations. Four current traces recorded at DIDS concentrations of 0.1, 1 and 10 μM and in the absence of DIDS in the extravacuolar medium were superimposed. (**C**) and (**D**) Current−DIDS relationship of the steady-state currents resulting from voltage pulses to −80 mV as shown in (A). (C) Results from three experiments where all DIDS concentrations were tested on the same vacuole; (D) results from 32 individual experiments. Results obtained in symmetric KNO_3 or K_2mal represent the mean of three experiments. Currents were normalized by setting steady-state currents measured before DIDS treatment to 100%. The curves were fitted by eye.

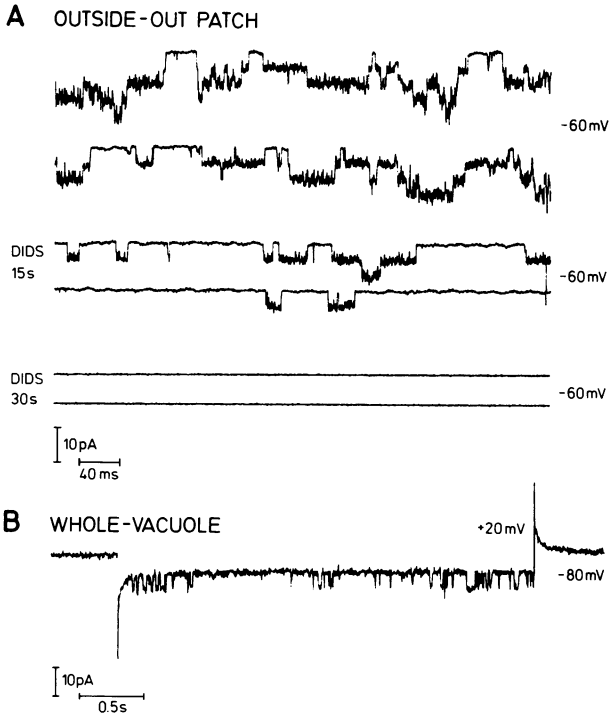

Fig. 2. Irreversible inhibition of single SV-type channels by DIDS. (**A**) Single-channel currents recorded from an outside-out patch in the absence of DIDS (upper trace) and after 15 and 30 s perfusion of the cytoplasmic side of the membrane with 10 μM DIDS (lower trace). The patch potential was held at −60 mV. (**B**) Single-channel currents recorded in a whole-vacuole incubated in 10 μM DIDS. The vacuole was held at +20 mV and during a 5 s pulse stepped to −80 mV (same voltage protocol as in Figure 1B).

DIDS (on the cytoplasmic side of the tonoplast) the inhibition of the current was nearly complete. At all concentrations tested, the block of the non-selective SV-channels was irreversible. Replacement of K^+ by Na^+, or replacement of Cl^- by nitrate or malate, did not change the blocking characteristics of DIDS (Figure 1D). The irreversible effect of this anion-transport inhibitor is similar to previously described actions on red blood cells and Torpedo electroplax (Cabantchik and Rothstein, 1984; Schumaker and Sze, 1987).

To exclude channel 'run-down' (caused by whole-vacuole perfusion) which may have occurred simultaneously with DIDS action, channel activity was also investigated in vacuoles pre-incubated in 10 μM DIDS for ~15 min before recording. In these experiments, initial SV-currents were comparable to currents recorded in non-pretreated vacuoles after application of DIDS.

In vacuole-attached membrane patches (recording from an electrically isolated patch on the intact vacuole, Hamill *et al.*, 1981) almost no channel openings were observed when the vacuoles were pre-incubated with DIDS or when DIDS was present in the pipette solution (facing the cytoplasmic side of the intact vacuole).

Figure 2 shows the reduction and finally the complete block of single-SV-channel activity after application of 10 μM DIDS to the cytoplasmic side of excised outside-out patches, indicating that DIDS action is due to a reduction of the open probability time rather than a reduction of the single-channel conductance. SITS, another stilbene derivative, caused similar inhibition of the vacuolar current (Figure 1C).

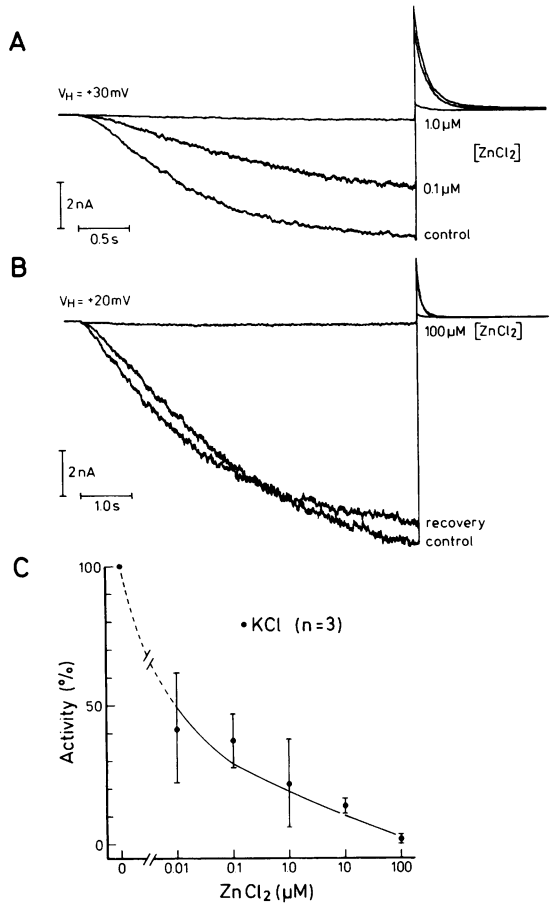

Fig. 3. Reversible inhibition of SV-type currents by zinc ions. (**A**) Whole-vacuolar currents in response to voltage pulses to −80 mV from a holding of +20 mV recorded in various $ZnCl_2$ concentrations. Three current traces recorded in the presence of 0.1 and 1 μM $ZnCl_2$ in the extravacuolar solution and the absence of zinc ions were superimposed. (**B**) Inhibition of SV-type currents by 100 μM $ZnCl_2$ and recovery from the inhibition caused by zinc ions. Currents were elicited by 10 s pulses to −70 mV from a holding potential of +30 mV. (**C**) Current−[$ZnCl_2$] relationship of the steady-state currents resulting from voltage pulses to −80 mV as in (A). Vacuoles were exposed for 2 min to each zinc concentration. Results from 3−4 vacuoles are shown. Currents were normalized by setting steady-state currents before zinc treatment to 100%. The curve was fitted by eye.

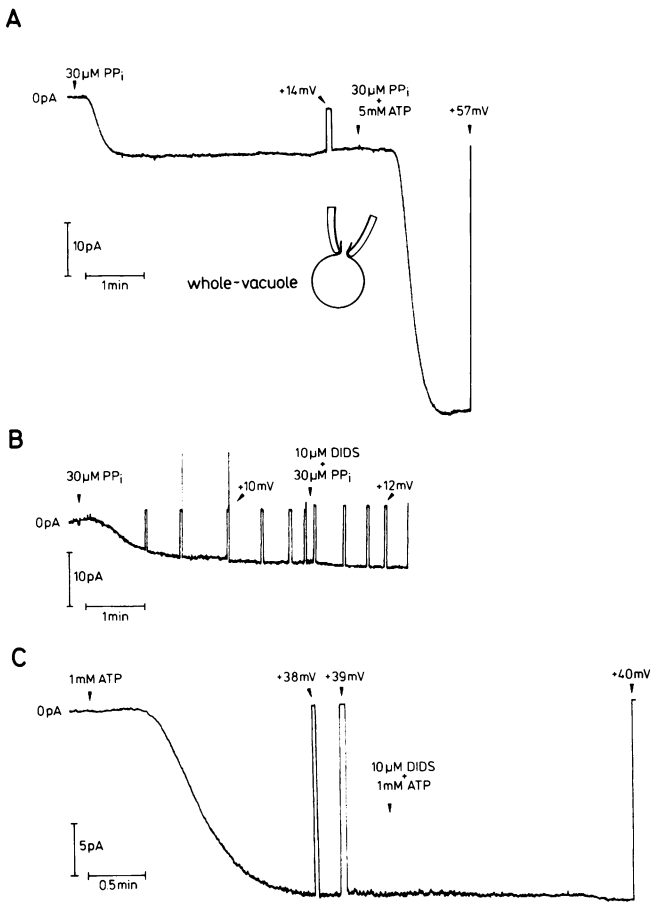

Fig. 4. Proton-translocating phosphorylases on the tonoplast are not affected by DIDS. (**A**) Voltage-clamp recording of pump currents from a whole vacuole. The membrane potential was clamped at 0 mV; application of 30 μM pyrophosphate (PP_i) to the extravacuolar solution generated inward currents of up to 15 pA which increased to ~60 pA when 5 mM ATP was added. Inwardly (into the vacuolar space) directed electrogenic currents depolarized the tonoplast to +12 or +57 mV, respectively. Membrane potentials were measured by briefly switching to the current clamp mode (measuring the zero current potential) during the PP_i or ATP induced current increase. (**B**) and (**C**) Currents and tonoplast potentials measured during the exposure to 30 μM PP_i (B) or 5 mM ATP (C) before and after the addition of 10 μM DIDS. Application of 10 μM DIDS did not inhibit electrogenic pumping of inward directed PP_iase (B) or ATPase (C).

In some whole-vacuole experiments, such as that shown in Figure 2(B), DIDS reduced the total vacuolar conductance to such an extent that the activity of unblocked SV-type channels could be resolved with high resolution.

To determine whether the DIDS action exhibits side-specificity, 100 μM DIDS was added to the pipette (internal) solution. In no case was a current reduction observed. This side-specific effect of DIDS may point to an 'anion binding site' at the cytoplasmic mouth of the channel.

The addition of pyridoxalphosphate, ethacrinic acid, anthracene-9-carboxylic acid and compound C 144, at concentrations which completely block anion transport in other systems (Cabantchik *et al.*, 1975; Flügge and Heldt, 1977; Palade and Barch, 1977; Lunevsky *et al.*, 1983; Miller and White, 1984; Wangemann *et al.*, 1986), reduced SV-currents only to 50−70% in a more or less reversible manner (Table II).

Zinc ions, which are known to inhibit Cl^- transport in giant algae (Hille, 1984), were a potent blocker of SV-type channels in sugar beet taproot vacuoles. Similar to DIDS, micromolar concentrations of $ZnCl_2$ reduced the inward currents only from the cytoplasmic side of the channel and in a concentration-dependent manner (Figure 3A and C). Two minutes exposure (bath perfusion) of the vacuoles to 0.1 μM $ZnCl_2$ caused ~50% reduction and 10 μM a complete block of the current.

Lower zinc concentrations showed the same blocking efficiency when the incubation time was prolonged. In contrast to DIDS, the blocking effect of zinc ions could be reversed by washing (Figure 3B).

Low concentrations of DIDS do not affect proton pump activity

In erythrocytes, it has been shown that the irreversible inhibition of anion transport caused by DIDS is due to a stable covalent interaction of the isothiocyanate with

amino-groups of lysine residues of the transport protein (Cabantchik and Rothstein, 1974).

We therefore investigated the effects of DIDS (10 μM) on active ion transporters of the tonoplast, namely the two proton-translocating phosphorylases (for review, see Rea and Sanders, 1987). Both phosphorylases, the ATPase and inorganic pyrophosphatase (PP_iase), are functionally and physiologically distinct enzymes that catalyse electrogenic H^+-translocation. While the electrogenic H^+-ATPase of the tonoplast has been characterized in our previous patch-clamp studies (Hedrich *et al.*, 1986, 1988; Coyaud *et al.*, 1987), Figure 4(B) directly demonstrates for the first time, using patch-clamp techniques, an electrogenic proton current energized by PP_i-hydrolysis. Clamping the tonoplast voltage to 0 mV (voltage-clamp) in the presence of symmetrical ion concentrations on either side of the membrane results in no net ion flux and thus enabled direct measurements of pump currents.

Upon the addition of 10–30 μM PP_i (Figure 4A and B) or 5 mM ATP, inwardly directed proton currents (into the vacuolar lumen) were activated, causing a 10–40 mV depolarization (current clamp) of the tonoplast. Induction of the ATPase or PP_iase on the same vacuole one after the other (Figure 4A) unequivocally indicates that both enzymes reside in the same membrane (which was not quite clear from experiments performed on tonoplast vesicles; for review, see Rea and Sanders, 1987). Application of 10 μM DIDS (causing ~90% inhibition of SV-currents) reduced neither H^+-ATPase nor H^+-PP_iase activity significantly (Figure 4B and C).

Discussion

The data presented show that among the cation transport blockers screened here only Ba^{2+} and TEA^+ ions, when present at 10 mM, cause up to 70% inhibition of the non-selective SV-channel.

Screening of the anion transport blockers revealed that the stilbene derivatives, DIDS and SITS, act as irreversible inhibitors of the channel (half-maximal inhibition per time interval, K_i = 0.1 μM) and zinc ions as a reversible blocker (K_i = 0.1–1.0 μM). In addition, stilbene derivatives and Zn^{2+} act only from the cytoplasmic face of the tonoplast.

Kinetic analyses on red blood cells have indicated that DIDS and anions compete for a common binding site. Thus, the K_i for DIDS revealed in this study may represent the affinity to the anion binding site of the channel [in analogy to the anion transporting 'Band 3 Protein' in red blood cells (Cabantchik and Rothstein, 1974)] in the presence of 200 mM potassium or sodium salts. In the absence of anions or reduced anion levels the affinity for DIDS may be much lower.

DIDS: a tool for channel protein isolation?

Covalent irreversible inhibitors such as DIDS and SITS may be useful tools in further studies on the control of transport events across the tonoplast and a potential label for counting, identifying and isolation of the structural proteins of the SV-type channel from the sugar beet vacuoles.

The problem is to know whether labelled DIDS (Cabantchik and Rothstein, 1974) can be used to purify SV-channels in the same way as α-bungarotoxin, TTX and other toxins from animal venoms were used for the isolation of AchR- or Na^+-channels (Miller, 1986). A difficulty for visualizing this purification may arise from the small number of SV-channels (~1000/vacuole; cf. red cells ~100 000/cell) and the possible unspecific binding to membrane proteins other than channels or H^+ pumps. Experiments concerning the isolation of SV-channels are currently being conducted.

Materials and methods

Vacuole isolation

Vacuoles were isolated from 4–5-month-old sugar beet taproots. Beets were grown in the field (Kleinwandzlebener Saatzucht, Einbeck, FRG) and in the greenhouse. A slice of storage tissue was cut off with a razor blade and the surface rinsed with some drops of bathing medium (solution used on the cytoplasmic side of the organelle) to collect the extruded vacuoles directly into the recording chamber. Vacuoles sedimented and adhered to the glass bottom of the chamber. Debris was removed by bath perfusion (1 ml/min, bath volume ≃ 100 μl). Patch-clamp recordings could be obtained within 5 min after vacuole isolation.

Whole-vacuole recordings

The whole-vacuole configuration of the patch-clamp technique (Hamill *et al.*, 1981, analogous to whole-cell) was established after patch pipettes were sealed against an isolated vacuole and the underlying membrane was broken by alternate +/−0.6 V pulses of 1–3 ms duration. After access to the vacuole interior was obtained, the pipette solution equilibrated with the intravacuolar space (see Figure 1A inset) so that ion concentrations on both membrane sides were well defined. Low access resistance to the vacuole was obtained when 2–6 MΩ pipettes (in symmetric 200 mM KCl) were used. The access resistance did not change during bath perfusion or inhibitor treatment.

Single-channel currents

Single-channel activity was recorded in isolated membrane patches and in whole-vacuoles (in DIDS-treated vacuoles only) at a given holding potential or in response to voltage pulses.

Electrogenic pump currents

Inwardly directed pump currents were recorded after addition of 5 mM Mg-ATP or 5–100 μM Mg-PP_i. Since ions in the pipette and in the bath solution were maintained at equimolar concentrations, holding the voltage clamp at 0 mV (resting potential of the vacuole) ensured isolation of whole-vacuole pump currents.

Control of membrane potential, measurement of currents and compensation of cell capacitive transients were performed with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, FRG). Command voltage pulses were given by a modified microcomputer ('Stühmer' from List Electronic). Data were recorded on video tape after pulse-code modulation. For off-line analysis data were low-pass-filtered (2 kHz; 8-pole Bessel), digitized and processed on a PDP-11/73 computer.

Solutions

Vacuoles were exposed to solutions containing either KCl, K_2malate, KNO_3 or NaCl at a concentration of 200 mM. All bathing solutions included 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 5 mM Tris–MES (pH 7.5). For monitoring pump currents or the effect of inhibitors, 1–5 mM Mg-ATP, 30 μM Mg-PP_i or channel blockers were applied to the cytoplasmic side of the vacuolar membrane by bath perfusion. The interior of the vacuoles was equilibrated with 200 mM concentrations of KCl, K_2malate, KNO_3 or NaCl. In addition, all intravacuolar solutions included 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM Tris–MES (pH 5.5).

The osmolarity of the taproot sap was measured with a vapour-pressure osmometer (Wescor 5100 C), so that solutions used for the experiments could be adjusted accordingly with D-sorbitol.

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