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Vacuolar Chloride Transport in *Mesembryanthemum crystallinum* L. Measured Using the Fluorescent Dye Lucigenin

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Abstract. To study vacuolar chloride (Cl^-) transport in the halophilic plant *Mesembryanthemum crystallinum* L., Cl^- uptake into isolated tonoplast vesicles was measured using the Cl^--sensitive fluorescent dye lucigenin (*N,N′*-dimethyl-9,9′-bisacridinium dinitrate). Lucigenin was used at excitation and emission wavelengths of 433 nm and 506 nm, respectively, and showed a high sensitivity towards Cl^-, with a Stern-Volmer constant of 173 M^{-1} in standard assay buffer. While lucigenin fluorescence was strongly quenched by all halides, it was only weakly quenched, if at all, by other anions. However, the fluorescence intensity and Cl^--sensitivity of lucigenin was shown to be strongly affected by alkaline pH and was dependent on the conjugate base used as the buffering ion. Chloride transport into tonoplast vesicles of *M. crystallinum* loaded with 10 mM lucigenin showed saturation-type kinetics with an apparent K_m of 17.2 mM and a V_{max} of 4.8 mM min^{-1}. Vacuolar Cl^- transport was not affected by sulfate, malate, or nitrate. In the presence of 250 μM *p*-chloromercuribenzene sulfonate, a known anion-transport inhibitor, vacuolar Cl^- transport was actually significantly increased by 24%. To determine absolute fluxes of Cl^- using this method, the average surface to volume ratio of the tonoplast vesicles was measured by electron microscopy to be 1.13×10^7 m^{-1}. After correcting for a 4.4-fold lower apparent Stern-Volmer constant for intravesicular lucigenin, a maximum rate of Cl^- transport of 31 nmol m^{-2} sec^{-1} was calculated, in good agreement with values obtained for the plant vacuolar membrane using other techniques.

Key words: Plants — Salt — Chloride transport — Vacuole — Lucigenin

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Introduction

As a response to high soil salinity, the halophilic plant *Mesembryanthemum crystallinum* L. (the common ice plant) can accumulate high concentrations of more than 700 mM NaCl in its leaf cells (Lüttge, Fischer & Steudle, 1978). Vacuolar accumulation of NaCl allows the potentially cytotoxic Cl^- ions to be sequestered away from the sites of metabolism in the cytoplasm, while at the same time maintaining a low (negative) intracellular water potential. Although *M. crystallinum* is a well-understood model for the physiological analysis of the effects of environmental stress on plants, such as water deficits and salinity (Ratajczak, Richter & Lüttge, 1994; Cushman & Bohnert, 1996; Löw et al., 1996; Tsiantis, Bartholomew & Smith, 1996), relatively little work has been done so far on the transport of NaCl into the vacuole. There is evidence for the existence of a tonoplast Na^+/H^+ antiporter in this species, as in other plants, which can drive secondary active transport of Na^+ into the vacuole in response to the H^+ gradient set up across this membrane by the primary vacuolar-type H^+-ATPase (Barkla et al., 1995). However, it is not yet known whether Cl^- transport into the vacuoles requires energization or occurs down the electrochemical gradient for Cl^-, as no attempts have yet been made to measure directly the Cl^- fluxes across the vacuolar membrane of this species.

To study vacuolar Cl^- transport in *M. crystallinum*, we have followed an approach first developed by Pope & Leigh (1988), who monitored changes in Cl^- concentrations using the Cl^--sensitive fluorescent dye 6-methoxy-*N*-(3-sulphopropyl)quinolinium (SPQ). Although SPQ proved to be a useful tool in studying Cl^- transport systems in animals and plants (Illsley & Verkman, 1987; Chao, Widdicombe & Verkman, 1990; Pope & Leigh, 1990), its short excitation wavelength and rather low fluorescence intensity has led to the search for Cl^--

sensitive fluorescent dyes with improved properties. Biwersi, Tulk & Verkman (1994) reported that lucigenin (*N*,*N*′-dimethyl-9,9′-bisacridinium dinitrate), previously used as a peroxide-sensitive probe, was very sensitive to Cl^- and other halides, while having a long-wavelength range and improved optical characteristics compared with quinolinium compounds. The present study describes the first use of lucigenin for determining absolute Cl^- fluxes across biological membranes.

In this work, we have tested the sensitivity and specificity of lucigenin for Cl^- and a range of other solutes, and have also investigated the effect of pH and buffer composition on the collisional fluorescence quenching of lucigenin by Cl^- ions. Lucigenin was subsequently used to study the kinetics and magnitude of Cl^- fluxes into tonoplast vesicles of *M. crystallinum.* A number of known inhibitors of Cl^- transport were also tested for their interference with the dye and with vacuolar Cl^- uptake. Finally, by determining the surface area to volume ratio of the vesicles, we have been able to calculate absolute values for Cl^- flux into the tonoplast vesicles of this species.

Materials and Methods

Plant Material

Seeds of *M. crystallinum* L. (from the source described in Barkla et al., 1995) were germinated in a propagation tray and after about 3 weeks transferred to single pots of 10 cm diameter containing general-purpose compost (E.A. Goundrey & Son, Dunns Tew, Oxford, UK). Plants were regularly watered with tap water and were maintained in a glasshouse with natural illumination supplemented by sodium-vapor lamps providing a 12 hr photoperiod. Air temperatures ranged between 25°C and 35°C in the light period and were on average 15°C during the dark period.

Isolation of Tonoplast Vesicles

For isolation of the tonoplast vesicles a method similar to that of Barkla et al. (1995) was used. Leaves of *M. crystallinum* were harvested and sliced into small pieces after removal of major veins. The leaf material (between 40 and 80 g, depending on leaf size and quality) was homogenized using a commercial blender in 200 ml of ice-cold homogenization buffer containing 400 mM mannitol, 0.5% (w/v) PVP-40, 0.1% (w/v) BSA, 10 mM EDTA, 5 mM $MgSO_4$, 0.5 mM butylated hydroxytoluene, 26 mM potassium metabisulfite, 10 mM Tricine-BTP (pH 7.4) and 2 mM DTT.

All subsequent steps were performed at 4°C. The homogenate was filtered through two layers of cheesecloth and centrifuged at 13,000 × *g* for 15 min using a Sorvall SS-34 fixed-angle rotor. The supernatant was subsequently centrifuged at 80,000 × *g* for 50 min (Sorvall TFT50.38 fixed-angle rotor). The microsomal pellet thereby obtained was resuspended using a paintbrush in 1,100 mM glycerol, 1 mM EDTA, 10 mM Tricine-BTP (pH 7.4) and 2 mM DTT (subsequently referred to as resupension buffer). The resuspended material was then layered on top of 23% (w/v) sucrose cushion made up in the same buffer and centrifuged at 100,000 × *g* for 2 hr using a Beckman SW-28 swinging-bucket rotor in a Beckman L8-M ultracentrifuge. Tonoplast membrane at the 0/23% interface was removed with a Pasteur pipette, diluted in resuspension buffer, and sedimented at 100,000 × *g* for 1 hr (Sorvall TFT50.38 fixed-angle rotor). The final pellet was resuspended in 400 μL of resuspension medium and aliquots of 100 μL were frozen immediately in liquid nitrogen and stored at −80°C.

Using the hydrolytic activity of the vacuolar H^+-ATPase as a marker for the origin of the membrane fraction as described by Barkla et al. (1995), we found that between 65 and 70% of the total ATPase activity was azide resistant and nitrate sensitive, and that the vesicles had a distribution of about 50% right-side out (*data not shown*). This was in good agreement with previous studies (Struve & Lüttge, 1987; Barkla et al., 1995) and showed that this membrane fraction was highly tonoplast-enriched.

Vesicle Loading

For loading the membrane vesicles with a particular experimental solution, a method according to Pope & Leigh (1988) was used with slight modification. Unless otherwise stated, all steps were performed on ice and with ice-cold media. Aliquots of tonoplast vesicles were thawed on ice and pelleted at 370,000 × *g* for 15 min (4°C, Beckman TLA-100.3 fixed-angle rotor in Beckman TL-100 ultracentrifuge). The supernatant was discarded and the pellet was resuspended in a medium containing 1 mM EDTA, 10 mM Tricine-BTP (pH 7.4), 1 mM DTT and either 20 mM SPQ (6-methoxy-*N*-(3-sulfo-propyl)-quinolinium) or 10 mM lucigenin (*N*,*N*′-dimethyl-9,9′-bisacridinium dinitrate). Sufficient mannitol was added to maintain a total osmolality of 500 mosmol kg^{-1}, measured using a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). To load vesicles with different potassium concentrations as indicated for the various experiments, K_2SO_4 was added in appropriate amounts. The vesicle suspension was incubated for 30 min at 37°C to allow equilibration of the vesicle interior with the outside medium (Pope & Leigh, 1990). Loading vesicles overnight at 4°C did not produce any difference in dye loading or Cl^- transport. After keeping the vesicles on ice for 10 min external dye was removed by washing three times with dye-free washing medium (same as above but without fluorescent dye). The vesicles were pelleted at 370,000 × *g* for 15 min (4°C, TLA-100.3 fixed-angle) and the supernatant removed. After resuspending the vesicles in washing medium, the washing step was repeated. More than three subsequent washing steps produced no further decrease in the external dye concentration. After storing the vesicles on ice for more than 3 hr, an increase in external dye could be detected again, so loaded vesicles were used within 3 hr of the last wash.

Transport Measurements

A fluorescence plate reader (Perkin Elmer, Beaconsfield, Buckinghamshire, UK) was used for a number of experiments to determine the Stern-Volmer constants of various substances and the effect of pH changes on lucigenin fluorescence in different buffers (96-well plates, maximum assay volume 300 μL). Stern-Volmer constants were determined using either aqueous solutions or a buffer containing 500 mM mannitol, 10 mM Tricine-BTP (pH 7.4), 1 mM DTT. The effect of pH on lucigenin fluorescence was studied using various buffer systems adjusted to pH 7.4 and pH 8.0, respectively, using two different buffer ions to ensure a constant ratio of buffering ions. The pH was then altered using sulfuric acid or potassium hydroxide to adjust the pH of the test buffer. Measurements performed at room temperature were between 21 and 23°C.

Cl^- transport measurements were performed at 25°C in a stirred

0.5 ml cuvette. Fluorescence intensities were measured using a Perkin-Elmer Luminescence Spectrometer LS50B. For measurements of SPQ fluorescence, an excitation wavelength of 350 nm and an emission wavelength of 430 nm were used (slit width of 5 nm for both in the vesicle studies). Lucigenin fluorescence measurements were performed at wavelengths of 433 nm and 506 nm for excitation and emission, respectively (slit width 5 nm for both). Unless otherwise stated, assays were performed using 10 mM Tricine-BTP (pH 7.4), 5 μm valinomycin (from 1 mM stock in 100% ethanol) and sufficient mannitol to maintain iso-osmolality with the vesicles (500 ± 5 mosmol kg^{-1}). KCl or BTP-Cl (pH 7.4) were used to vary the Cl^- concentration. When KCl was used, an appropriate amount of K_2SO_4 was added to maintain a constant K^+ concentration. The total concentration of K^+ was kept at 50 mM inside and outside the vesicles to maintain the membrane potential clamped at 0 mV in the presence of valinomycin. All solutions were filtered using disposable filter units (0.45 μM, Sigma) to reduce noise in the recordings, and care was taken that prior to starting transport experiments the temperature of the cuvette and the medium were equilibrated to 25°C to avoid a drift in the fluorescence signal intensity.

Cl^- transport was initiated by adding 20 μg protein of vesicles (determined as in Barkla et al., 1995) loaded with the fluorescent dye to the assay medium. An IBM compatible 486 microcomputer was used to record the time course of the fluorescence quenching using FL Data Manager software (Perkin Elmer). A falling exponential was then fitted to the first part of the curve using the simplex algorithm using the program Clampfit (part of the pClamp6.02 soft-ware package, Axon Instruments, Foster City, CA). The parameters obtained were used to calculate the initial rate of change of intravesicular Cl^- concentration ($d[Cl^-]/dt$ for $t = 0$) following the procedure of Pope & Leigh (1988). Michaelis-Menten kinetics were fitted using the least-squares algorithm of Marquardt (Fig.P 6.0a: Fig.P Software Corporation, Durham, NC).

Calculation of the Initial Cl^- Transport Rates

Initial rates of Cl^- transport were calculated using the approach of Pope & Leigh (1988), who gave the following equation to describe the initial Cl^- flux:

$$d[Cl^-]_i/dt\ (t = 0) = -1/K_{SV} \cdot 1/F_{in} \cdot dF/dt(t = 0);\ \text{mM min}^{-1} \quad (1)$$

with the luminal Cl^- concentration $[Cl^-]_i$, the Stern-Volmer constant (K_{SV}), the internal fluorescence (F_{in}) in the absence of luminal Cl^-, and the initial change in fluorescence (dF/dt at $t = 0$, in min^{-1}), which was obtained by fitting a single exponential to the first part of the traces. Addition of vesicles to the assay medium determined the time point zero ($t = 0$). The internal fluorescence could be calculated from

$$F_{in} = F_0 - (F_0 - F) \cdot (1 + K_{SV} \cdot [Cl^-]_o)/(K_{SV} \cdot [Cl^-]_o) \quad (2)$$

with F_0 being the normalized total fluorescence (in percent) in the absence of external Cl^- $[Cl^-]_o$, and F the normalized fluorescence at $t = 0$.

All results presented in this work are the average of several measurements and are quoted where appropriate with the standard error of mean. Statistical significance was evaluated by the Student's *t* test.

Electron Microscopy

About 100 μg of tonoplast vesicles were pelleted at 100,000 × *g* for 15 min (TLA 100.3 fixed-angle rotor) and then resuspended and fixed in 5% (v/v) glutaraldehyde in resuspension medium. The vesicles were then pelleted again for 15 min at 100,000 × *g* and resuspended in a 2% (v/v) solution of aqueous osmium tetroxide in resuspension buffer. The sample was left to blacken in a fume hood for 1 hr at room temperature. After the osmium coating, the vesicles were centrifuged at 7,000 × *g* in a bench-top centrifuge for 5 min and the pellet resuspended in distilled water and left for 10 min at room temperature. The suspension was then centrifuged and washed three times by resuspension and pelleting in distilled water. To dehydrate the vesicles, a similar cycle was repeated six times with increasing ethanol concentrations (70, 80, 90%, and 3 changes at 100%). The dehydrated vesicles were embedded in equal volumes of Spurr resin (Spurr, 1969) and 100% ethanol overnight at room temperature. They were then transferred to 100% Spurr resin the next morning, and after 4 hr transferred to a further solution of 100% Spurr resin. The following morning the vesicles were transferred to a final 100% Spurr resin and incubated at 60°C until the resin had hardened. The samples were sectioned to a final thickness of 50 to 60 nm. The sections were placed on a copper grid and stained with a 2% (w/v) solution of uranyl acetate and lead citrate. Finally, the sections were viewed and photographed under a JEOL 1010 electron microscope at 80 kV (Japanese Electronics Optics, Welwyn Garden City, UK).

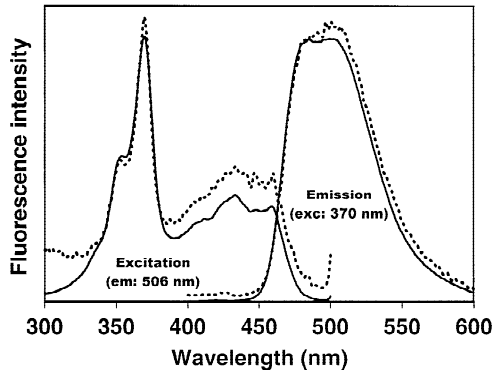

Fig. 1. Excitation spectra (shorter wavelengths) and emission spectra (longer wavelengths) of lucigenin fluorescence. The excitation wavelength for the emission scan was 370 nm, and the emission wavelength for the excitation scan was 506 nm. The continuous lines are a solution of 0.05 mM lucigenin in standard Tricine-BTP buffer at pH 7.4 (*see* Materials and Methods); the dotted lines represent similar scans of tonoplast vesicles loaded with 10 mM lucigenin.

Results

The Fluorescent Probe Lucigenin

To study vacuolar Cl^- transport in *Mesembryanthemum crystallinum*, we used the fluorescent probe lucigenin (*N*,*N*′-dimethyl-9,9′-bisacridinium dinitrate), which has been shown in a previous study by Biwersi et al. (1994) to be sensitive to Cl^-. Figure 1 shows the excitation and emission spectra of lucigenin. Maximum excitation occurred at 368 nm, with two further peaks in the longer wavelength range at 433 and 458 nm. Maximum emission was observed at 475 and 506 nm. To assess the properties of lucigenin, a range of experiments were per-

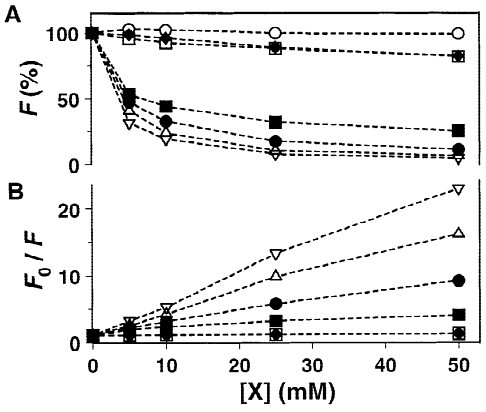

Fig. 2. Fluorescence quenching of lucigenin by various substances. (*A*) Fluorescence quenching of 0.1 mM lucigenin by increasing concentrations of potassium chloride (●), potassium sulfate (□), potassium nitrate (○), potassium bromide (△), potassium iodide (▽), sodium citrate (■) and malic acid (♦). (*B*) The corresponding Stern-Volmer plots F_0/F. All measurements were performed in assay medium as described in the Materials and Methods at room temperature using a 96-well plate reader. Total assay volume was 300 μl. All points are the means of duplicate measurements.

formed to determine the specificity, sensitivity and other properties of lucigenin.

As shown for various salts in Fig. 2*A*, lucigenin fluorescence decreased hyperbolically with increasing concentration of Cl^- (or other halides). The relative changes in fluorescence were the same for all lucigenin concentrations tested (*data not shown*). The hyperbolic relationship could be expressed as a Stern-Volmer plot (maximum fluorescence F_0 divided by the quenched fluorescence F). This showed an almost linear increase with increasing Cl^- concentration (Fig. 2*B*), following the Stern-Volmer relationship $F_0/F = 1 + K_{SV}[Cl^-]$ (where K_{SV} = Stern-Volmer constant, and $[Cl^-]$ = concentration of Cl^-). At Cl^- concentrations above 100 mM the increase became slightly steeper (*data not shown*). The Stern-Volmer constant for Cl^--dependent lucigenin fluorescence quenching was 173 M^{-1} (25°C, pH 7.4).

To determine the specificity of the lucigenin fluorescence quenching, various ions were tested at a range of concentrations up to 50 mM (Fig. 2*A*) and the corresponding Stern-Volmer plots derived (Fig. 2*B*). The results for all solutes tested are summarized in Table 1. Besides Cl^-, lucigenin was even more sensitive towards the other halides bromide and iodide, as shown previously (Biwersi et al., 1994). BTP-Cl (pH 7.4) showed a slightly higher Stern-Volmer constant of 217 M^{-1}, due to additional quenching by BTP. Citrate, DTT and BTP showed a smaller amount of fluorescence quenching, while nitrate, sulfate and many of the organic anions caused only a limited quenching. Mannitol and sorbitol did not show any quenching of lucigenin fluorescence. These results thus demonstrate a high degree of specificity of the lucigenin fluorescence quenching for halides.

Table 1. Effect of various solutes on lucigenin fluorescence

Addition	Stern-Volmer quench constant (M^{-1})	Solute concentration giving 50% fluorescence quench (mM)
KI	495.9	2
KBr	357.0	3
BTP-Cl, pH 7.4[a]	216.5	5
KCl	191.9	5
KCl[a]	173.0	6
DTT[b]	91.5	11
Na_3-citrate	79.4	13
BTP[b]	51.6	19
K-acetate	31.0	32
K-gluconate	22.6	44
Maleic acid	22.5	44
Oxalic acid	21.1	47
DMSO	19.1	52
Tricine-BTP (pH 7.4)	18.2	55
K-aspartate	11.7	85
Malic acid[b]	6.3	159
K_2SO_4	5.2	192
$(NH_4)_2SO_4$	4.7	213
Malic acid	4.5	222
Tricine[b]	4.4	227
Iminodiacetic acid	3.0	333
KNO_3	0.5	2000
Succinic acid	0.3	3333
Sorbitol[b]	0	–
Mannitol[b]	0	–

Unless indicated otherwise, measurements were made in assay medium (*see* Materials and Methods) at room temperature using a 96-well plate reader. The slope of the F_0/F plots gives the Stern-Volmer quench constant.

[a] Measurements were performed at 25°C in a 500 μl cell.

[b] Measurements were performed in water.

Biwersi et al. (1994) observed no change in lucigenin fluorescence when altering the pH to values between 4 and 9 in aqueous solution. However, depending on the buffer used, we found that quenching of lucigenin fluorescence by Cl^- could be affected by pH. Initially, a 10 mM Tricine buffer was used that was adjusted to pH 8.0 with BTP. As is apparent from Fig. 3*A*, in Tricine-BTP buffer above pH 7 the fluorescence showed a steady decline and above pH 11 fell to below 30% of the maximum intensity. In the presence of 45 mM Cl^-, the fluorescence signal was generally much lower due to the Cl^- quenching, and the pH-dependent decrease was much less pronounced. Hence, the Stern-Volmer constant K_{SV} for Cl^--dependent quenching, as obtained from the ratio of F_0/F, also showed a nonlinear pH dependence above pH 7.0 (Fig. 3*B*). This decrease was steepest around the pK_a value for Tricine of 8.1.

To find a more suitable pH-buffer system, several biological buffers were tested, but all of these showed a

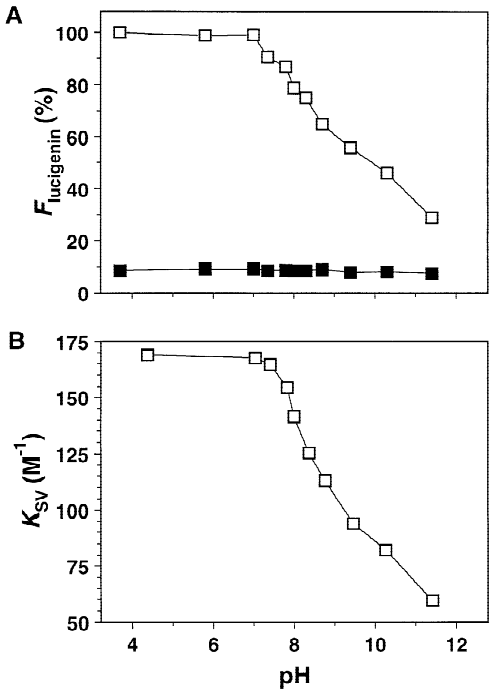

Fig. 3. Effect of pH on lucigenin fluorescence. (*A*) The fluorescence intensity of 0.05 mM lucigenin was measured as a function of pH. The assay medium contained 10 mM Tricine, which was initially adjusted to pH 8.0 with BTP. Subsequently the pH was varied using sulfuric acid or KOH. Measurements were performed either in the absence (□) or the presence of 45 mM Cl^- (■) using a 96-well plate reader (excitation wavelength 433 nm; emission wavelength 506 nm). (*B*) Stern-Volmer constants (K_{SV}) for Cl^--dependent quenching of lucigenin fluorescence calculated from similar measurements to those in (*A*).

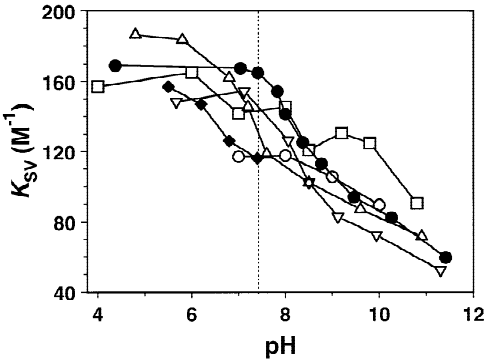

Fig. 4. Effect of pH on the Stern-Volmer constant. The Stern-Volmer constants for Cl^--dependent quenching of lucigenin fluorescence were measured in various buffer systems. The pH was first adjusted to a defined pH and then varied with sulfuric acid or KOH. Measurements were performed as described in Fig. 3 (all points are means of duplicate measurements). The following buffer systems were tested (all 10 mM, with initial pH values given): Tricine-BTP, pH 8.0 (●); Hepes-Tris, pH 8.0 (○); Tricine-Tris, pH 8.0 (▽); MOPS-BTP, pH 8.0 (△); MES-BTP, pH 6.2 (♦); phosphate buffer, pH 8.0 (□).

more or less pronounced pH effect with regard to Cl^- quenching (Fig. 4). Hepes reduced fluorescence quenching at all pH values tested, indicating that there is also a pH-independent component of the fluorescence quenching caused by this buffer ion.

In light of these results, all subsequent experiments were standardized by using Tricine-BTP (pH 7.4). This pH was within the effective pH-buffering range of this buffer combination ($pK_a = 8.1$) and is close to the pH range for maximum Cl^--dependent lucigenin fluorescence quenching (Fig. 4).

Determination of Initial Cl^- Transport Rates

The initial rate of Cl^- transport into tonoplast vesicles of *M. crystallinum* was determined for Cl^- concentrations ranging from 0 to 150 mM. In the absence of Cl^-, the fluorescence of the intravesicular lucigenin remained stable, whereas in the presence of Cl^- a decrease in fluorescence with time could be detected (Fig. 5*A*). From this time course of fluorescence decrease, the initial rate of Cl^- transport could be derived as described in the Materials and Methods. After adding Triton X-100 to disrupt the integrity of the membrane vesicles, a sharp increase in fluorescence could be observed at lower Cl^- concentrations. This might suggest that the vesicles contained an endogenous quencher, which is released and diluted after rupture of the vesicles, as was the case in studies using the fluorescent probe SPQ (6-methoxy-*N*-(3-sulfopropyl)quinolinium) with red-beet tonoplast vesicles (Pope & Leigh, 1988).

A plot of initial Cl^- transport rates against the corresponding Cl^- concentrations showed that Cl^- transport exhibited saturation-type kinetics (Fig. 5*B*). Fitting the data to a Michaelis-Menten function gave an apparent K_m value of 17.2 ± 2.4 mM and a maximum transport rate of 4.8 ± 0.7 mM min^{-1} ($n = 6$ replicate samples from 3 independent membrane preparations).

For comparison, similar experiments were performed with vesicles loaded with 20 mM SPQ according to the method of Pope & Leigh (1988). As shown in Fig. 6, recordings of SPQ fluorescence were noisier, probably due to the lower excitation and emission wavelength range of SPQ. Furthermore, time-dependent changes in SPQ fluorescence were more difficult to resolve at lower Cl^- concentrations. These factors led to higher variability in the data due to a lower signal to noise ratio when using SPQ compared with lucigenin. However, similar results for the concentration dependence of Cl^- uptake were obtained with both dyes.

To determine the selectivity of this transport system, Cl^--transport measurements were performed in the presence of 50 mM BTP-malate or 50 mM sodium nitrate. As shown in Fig. 7, there was no change in Cl^- transport activity in the presence of malate. Nitrate, however, seemed to reduce the rate of Cl^- transport at all Cl^-

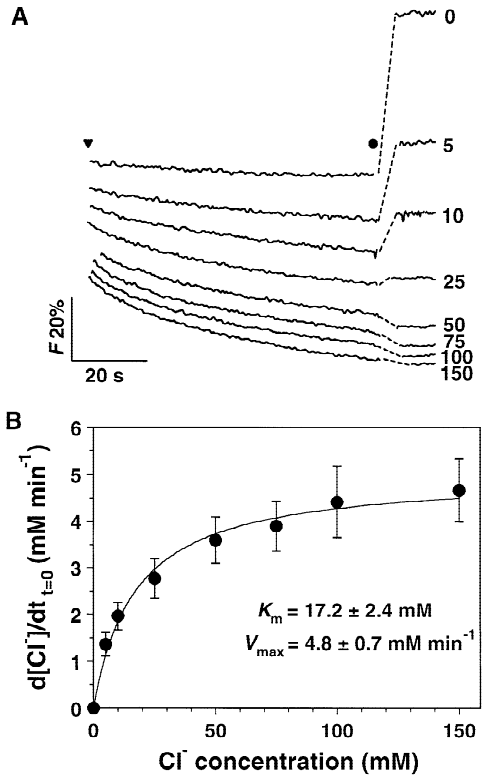

Fig. 5. Measurement of initial rates of Cl^- transport with lucigenin. (*A*) Time course of Cl^--dependent lucigenin fluorescence quenching. The arrowhead marks the addition of 20 μg (protein) of tonoplast vesicles loaded with 10 mM lucigenin to an assay medium (described in the Materials and Methods) containing Cl^- as indicated for each trace (the first seconds of the recording mixing have been omitted). The filled circle marks the addition of Triton X-100 to a final concentration of 0.03% (w/v). The dashed lines replace the traces during stirring. (*B*) Concentration dependence of the initial rate of Cl^--transport. Vesicles were loaded with 10 mM lucigenin and added to media with increasing Cl^- concentrations at pH 7.4 ($n = 6$; error bars are standard errors of the mean). The curve represents a best-fit to Michaelis-Menten kinetics with parameters as indicated.

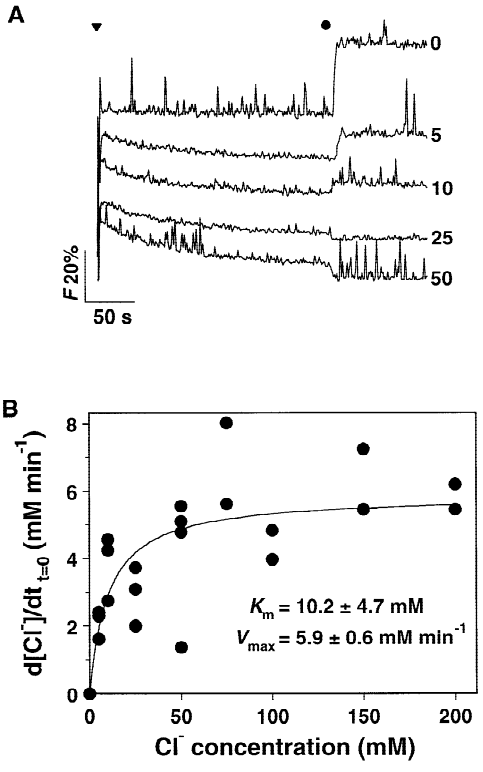

Fig. 6. Measurement of initial rates of Cl^- transport with SPQ. (*A*) Time course of SPQ fluorescence. Thirty μg (protein) of vesicles loaded with 20 mM SPQ and 100 mM K_2SO_4 were added to assay medium containing various concentrations of KCl (as indicated in mM) and sufficient K_2SO_4 to give a total K^+ concentration of 200 mM (5 μM valinomycin was present in addition). The filled circle marks the addition of 0.03% (v/v) Triton X-100 to disrupt the vesicles. (*B*) Concentration dependence of the initial rate of Cl^- transport with lucigenin. The values are individual measurements with vesicles from two separate preparations loaded with 20 mM SPQ. The curve indicates a best fit to Michaelis-Menten kinetics with parameters as indicated. A Stern-Volmer constant of 82 M^{-1} determined for these conditions was used to calculate the initial rate of Cl^- transport.

concentrations by about the same amount. However, this apparent reduction was due to a nonspecific effect that was observed even in the absence of any Cl^-, causing the fluorescence to increase slightly after addition of vesicles (*see* inset in Fig. 7). This nitrate-dependent increase in fluorescence intensity was superimposed on the Cl^--dependent fluorescence quenching of lucigenin and hence caused an offset when calculating the initial Cl^- transport rate. As the dotted line in Fig. 7 shows, subtracting the constant offset of −1.29 mM min^{-1} demonstrated that there was no direct effect of nitrate on Cl^- transport. Also, changing the background concentration of K_2SO_4 from 50 mM to 100 mM did not affect the initial Cl^- transport rate (*data not shown*).

Inhibitor Sensitivity of Cl^- Transport

Before testing the effect of known anion-transport inhibitors on Cl^- transport using the lucigenin-quenching technique, we determined the direct effect of these reagents on lucigenin fluorescence (Table 2). Bumetamide (3-[aminosulfonyl]-5-[butylamino]-4-phenoxybenzoic acid), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), DNDS (4,4′-dinitrostilbene-2,2′-disulfonic acid) and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) themselves showed a detectable fluorescence signal at the wavelengths tested. However, the fluorescence intensity at the concentrations of these reagents used (100 μM) was less than 3% of the fluorescence signal of lucigenin-loaded vesicles. All substances tested interfered with lucigenin fluorescence and, at a concentration of 100 μM, DIDS, DIOA (R(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid), NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) and SITS caused a reduction of lucigenin fluorescence of between 10 and 50%. The strongest quencher was NPPB, with a Stern-

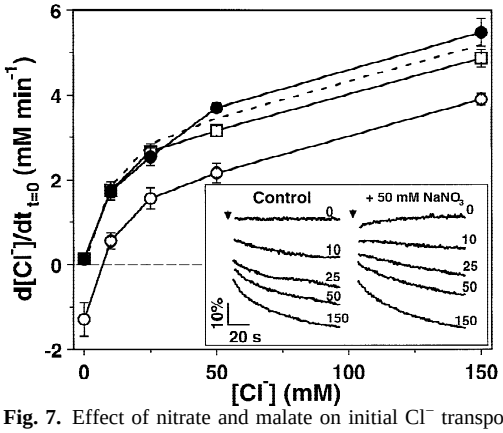

Fig. 7. Effect of nitrate and malate on initial Cl^- transport. Measurements were made either in the absence (●) or presence of 50 mM sodium nitrate (○) or 50 mM BTP-malate (□) in the assay medium (n = 6 from 2 preparations; standard errors of the mean are indicated by error bars where larger than symbols). The dashed line represents the measurements in the presence of nitrate, corrected for a constant offset of −1.29 mM min^{-1}. The inset shows original recordings from control experiments with 50 mM sodium nitrate in the assay medium. The arrowhead marks the addition of vesicles, and the Cl^- concentration in mM is indicated for each trace.

Table 2. Effect of inhibitors on lucigenin fluorescence

Inhibitor	Quenching constant for lucigenin (M^{-1})	Lucigenin fluorescence at 100 μM inhibitor concentration (%)	Fluorescence (exc. 433 nm; em. 506 nm)
9-AC[a]	140	99	–
Bumetamide	260	98	+
DMSO	19	100	–
DIDS	2200	82	++
DIOA[a]	1240	89	–
DNDS	206	98	+
Flufenamic acid	390	96	–
Furosemide	208	98	–
N-EM	10	100	–
Niflumic acid	470	96	
NPPB[c]	11400	47	–
*p*CMBS[b]	156	99	–
SITS[b]	2500	80	+

Aliquots of inhibitor stock solutions in 0.2 M KOH were added to assay medium, pH 7.4. Since most inhibitors were used at 100 μM final concentration, the fluorescence of lucigenin at that inhibitor concentration is given. To determine the fluorescence signal of the inhibitors, wavelength scans for emission (excitation at 433 nm) and excitation (emission at 506 nm) were performed (both slits at 5 nm).

[a] Stock solution in 100% ethanol.

[b] Stock solution in assay buffer, pH 7.4.

[c] Stock solution in 20% DMSO, 50% ethanol.

Volmer constant of 11,400 M^{-1}. NPPB and flufenamic acid also interfered with vesicle integrity, leading to rapid leakage of the dye. Furthermore it was found that DIDS was able to permeate the vesicle membrane and slowly quench intravesicular lucigenin fluorescence (*data not shown*).

In transport experiments, 100 μM niflumic acid, 9-AC (anthracene-9-carboxylic acid) and SITS did not show any effect, while 100 μM DIDS, flufenamic acid and 28 mM DMSO led to an apparent reduction in Cl^- transport (*data not shown*). However, with the last three substances it was not possible to separate their effect on vacuolar Cl^- transport from those on the lucigenin-based assay system. It is noteworthy however that *p*CMBS (*p*-chloromercuribenzene sulfonate), which does not interfere with the dye, was able to stimulate the vacuolar Cl^- transport significantly by 24% (n = 8; $P < 0.01$) at a concentration of 250 μM.

Calculating the Absolute Cl^- Flux

The fluorescent dyes used in this study report only the time-dependent changes in the luminal Cl^- concentration of the vesicles. To calculate the actual Cl^- flux across the membrane, the average volume and membrane area of the vesicle preparation had to be determined. The size of the vesicles was therefore measured using transmission electron microscopy. From these electron micrographs, the diameter of 86 vesicles was measured. Since for each individual vesicle a micrograph is a geometric section through a random plane, the measured diameter was corrected according to the following formula (Elias, Henning & Schwartz, 1971): actual diameter = observed diameter × $4/\pi$.

The size distribution was skewed towards the larger diameters, with a mode between 250 and 300 nm, probably because the smaller vesicles escaped detection. The average vesicle size was 0.336 ± 0.019 μm, with the largest being over 0.860 μm in diameter. The total area of 86 measured vesicles was 3.92×10^{-11} m^2 and the total volume was 3.46×10^{-18} m^3, giving a corresponding average area to volume ratio of 1.13×10^7 m^{-1}.

The actual Cl^- sensitivity of the dye when loaded into the vesicles was estimated according to Fig. 8. The external fluorescence as a percentage of the total was calculated to be 58% before and about 3% just after the addition of 100 mM Cl^-, according to the Stern-Volmer equation $F = F_0/(1 + K_{SV}[Cl^-])$, with the external K_{SV} being 173 M^{-1}. The internal fluorescence in the presence of Cl^- could therefore be calculated to decrease from initially 42% of the total down to 8% after about 20 min of Cl^- transport. Assuming that the internal Cl^- concentration was essentially identical to the external Cl^- concentration of 100 mM after that time, this decrease would be equivalent to an internal apparent K_{SV} for Cl^- of only 40 M^{-1}. This is about 4.4-fold lower than the K_{SV} measured in assay buffer. Correcting the apparent maximum rate of change of Cl^- concentration of 4.8 mM min^{-1} for the lower apparent K_{SV} in vesicles gives an actual rate of change of 21.1 mM min^{-1}. With the known vesicle area

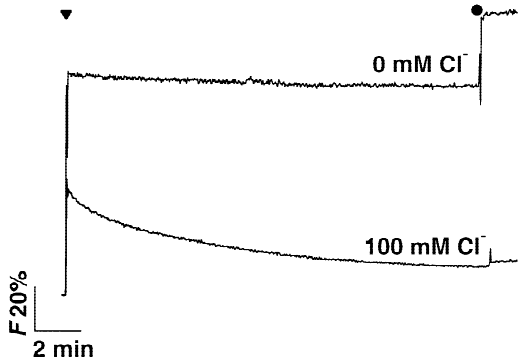

Fig. 8. Time course of fluorescence intensity of lucigenin-loaded vesicles (one of three replicate experiments). Twenty μg (protein) of lucigenin-loaded vesicles were added to medium without Cl^- or with 100 mM Cl^- (indicated by the arrowhead). The filled circle marks the addition of 0.03% Triton X-100 (v/v, final concentration).

to volume ratio, this corresponds to an initial rate of Cl^- influx into the tonoplast vesicles of 31 nmol m^{-2} sec^{-1}.

Discussion

This study describes the first use of lucigenin to measure Cl^- transport in a plant membrane system, or indeed in biological membranes generally. The dye proved to be a useful tool for detecting changes in Cl^- concentration, showing a high fluorescence intensity and a very high sensitivity for halides. Similar experiments had been performed previously by other researchers using the Cl^--sensitive dye SPQ (Illsley & Verkman, 1987; Pope & Leigh, 1988; Chao et al., 1990; Pope & Leigh, 1990; Pope et al., 1990), but lucigenin possesses superior optical properties. It gives a much greater emission intensity when excited with UV light than SPQ (Biwersi et al., 1994), which means that a small amount of dye loaded vesicles is sufficient to give a detectable signal. Lucigenin was also much more sensitive to Cl^-, with a Stern-Volmer constant double that of SPQ under similar conditions. Biwersi et al. (1994) reported a Stern-Volmer constant of 390 M^{-1} for the quenching of lucigenin fluorescence by Cl^- in aqueous solution, while under the conditions used in this study the constant was reduced to 173 M^{-1} for KCl. This was caused by the presence of other fluorescence-quenching compounds, such as DTT and BTP, in the assay media used for these plant membrane vesicles. Nevertheless, this value is much higher than the Stern-Volmer constant measured for SPQ. Hence, even small changes in Cl^- concentration can lead to easily detectable changes in lucigenin fluorescence. Furthermore, because lucigenin can be used with much longer excitation and emission wavelengths, the assay system is less susceptible to signal noise caused by the absorption of short-wavelength light.

As with SPQ (Vasseur, Frangne & Alvarado, 1993), the fluorescence intensity and Cl^--dependent quenching of lucigenin fluorescence was affected by pH. Although lucigenin fluorescence is pH-independent in aqueous solution (Biwersi et al., 1994), it is presumably the buffer-ion which interferes with the fluorescence. The effect of pH on the quenching of lucigenin fluorescence follows closely the titration curves for the buffers tested. This indicates that the protonation of the buffer ion might play a role (Vasseur, Frangne & Alvarado, 1993). Hence, care has to be taken to perform Cl^--transport studies using lucigenin in a pH range over which the fluorescence remains constant. Moreover, all calibration experiments and the determination of Stern-Volmer constants have to be performed under the same conditions as the transport experiments. On the other hand, the pH-dependent fluorescence quenching by the buffer-ion could be a useful tool for assaying proton transport into vesicles, as changes in fluorescence can reflect changes in protonation of the buffer ion (Garlid et al., 1996). Furthermore, the pH sensitivity can be adjusted by carefully choosing a buffering ion with the appropriate pK_a.

Lucigenin showed a similar behaviour to SPQ in terms of water solubility and vesicle-loading properties, so the experimental design of transport studies was very similar to the experiments performed by Pope & Leigh (1990). As in the case of the red beet vesicles studied by Pope & Leigh (1988, 1990) using SPQ, there appeared to be an endogenous quencher present inside the tonoplast vesicles of *Mesembryanthemum crystallinum*, which was released and diluted by disrupting the vesicle membrane. This meant that the volume of the vesicle had to be kept constant. Any shrinkage of the vesicles would lead to an increase in the endogenous quencher concentration and hence to a decrease in fluorescence (Pope & Leigh, 1988).

This study has shown that the initial Cl^- transport rate clearly followed saturation-type kinetics, making a protein-mediated Cl^- transport process into tonoplast vesicles of *M. crystallinum* very likely (Stein, 1990). In *M. crystallinum*, the apparent K_m of about 17 mM for the initial rate of vacuolar Cl^- transport is somewhat higher than the apparent K_m of 6.5 mM for tonoplast Cl^- transport in red beet (Pope & Leigh, 1988). In corn root tonoplast vesicles, Cl^--stimulated proton translocation also showed a lower apparent K_m of 4.3 mM (Bennett & Spanswick, 1983). Similar experiments in oat root tonoplast vesicles gave an apparent K_m of 2.3 mM (Pope & Leigh, 1987) and 2.6 mM (Kaestner & Sze, 1987). These data suggest that vacuolar Cl^- transport in the extreme halophyte *M. crystallinum* saturates at somewhat higher Cl^- concentrations than in glycophytes or in the halotolerant red beet. Whether this reflects an adaptation to higher cytosolic Cl^- concentrations in the halophyte *M. crystallinum* remains uncertain, since no detailed data

concerning the subcellular compartmentation of Cl^- are available for this species.

The absolute Cl^- flux of 31 nmol m^{-2} sec^{-1} calculated on the basis of our results obtained in *M. crystallinum* vesicles is close to the values measured for intact barley mesophyll vacuoles. Recalculating the data from Martinoia et al. (1986) on $^{36}Cl^-$ uptake into barley vacuoles gives a value of 33 nmol m^{-2} sec^{-1} at 50 mM Cl^-. Similarly, electrophysiological studies comparing vacuoles from salt-tolerant and nontolerant species have shown that these do not differ significantly in terms of current densities (Maathuis, Flowers & Yeo, 1992).

Testing various known inhibitors of anion transport using a lucigenin-based assay proved problematic. It was not possible to separate the effects of vesicle lysis (flufenamic acid) or the permeation of a quenching inhibitor such as DIDS from a genuine inhibition of Cl^- transport. SITS, niflumic acid and 9-AC, although all known anion-transport inhibitors (Hedrich & Kurkdjian, 1988; Lambert, Bradley & Mircheff, 1991; Chao & Mochizuki, 1992; Marten et al., 1993; Meyer & Korbmacher, 1996), did not show any effect on the initial Cl^- transport rate at the concentration tested (100 μM).

The significant increase in the rate of Cl^- transport observed in the presence of 250 μM *p*CMBS (*p*-chloromercuribenzene sulfonate) is notable, since Martinoia et al. (1986) reported a similar increase in Cl^- flux for barley vacuoles after incubation with *p*CMBS. Furthermore, *p*CMB (*p*-chloromercuribenzoic acid), a homologue of *p*CMBS, has been shown to activate Cl^-/OH^- exchange activity in mammalian cells (Karniski, 1989). In contrast, vacuolar malate transport in barley has been shown to be inhibited by *p*CMBS (Martinoia et al., 1991), as is dicarboxylate transport in tonoplast vesicles of *Kalanchoë daigremontiana* (Bettey & Smith, 1993). Finding the optimum concentration range for the activation of vacuolar Cl^- transport might therefore provide a useful tool for discerning between these different vacuolar anion-transport processes in plants. However, given the strong interference of some of the tested transport inhibitors with the assay system, further experimentation using other techniques, such as patch clamping vacuoles of *M. crystallinum*, is needed to allow for a more complete pharmacological characterization of the vacuolar Cl^- transport.

In conclusion, the Cl^--sensitive fluorescent dye lucigenin proved to be a useful tool for studying vacuolar Cl^- transport processes in plant membrane vesicles. While the maximum vacuolar Cl^- influx of 31 nmol m^{-2} sec^{-1} obtained for the halophyte *M. crystallinum* was not different from vacuolar Cl^- fluxes measured in glycophytic plants, the apparent K_m value was in general much higher when compared with nonsalt-tolerant plants. This would suggest an adaptation of *Mesembryanthemum crystallinum* towards elevated Cl^- concentrations under salt stress not so much by increased vacuolar transport activity, but rather by a reduced sensitivity towards Cl^-.

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