

Fast-activating Channel Controls Cation Fluxes across the Native Chloroplast Envelope

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Abstract. A prerequisite for photosynthetic CO₂ fixation is the maintenance of alkaline pH in the stroma. This is achieved by H⁺ pumping from the stroma to the cytosol, electrically balanced by an influx of cations through some unidentified non-selective envelope channels. In this study, the patch-clamp technique was applied to isolated *Pisum sativum* L. (pea) chloroplasts, and a fast-activating chloroplast cation (FACC) channel was discovered in the native envelope. This channel opens within a few milliseconds upon voltage steps to large positive or negative potentials. Remarkably, the single-channel conductance increased fivefold, from ~40 pS to ~200 pS (symmetric 250 mM KCl), upon a potential change from zero to \pm 200 mV. The FACC channel conducts all physiologically essential inorganic cations (K⁺, Na⁺, Ca²⁺, Mg²⁺) with little preference. An increase of stromal pH from 7.3 to 8.0, mimicking dark-light transition, caused about a 2-fold decrease of the FACC channel activity within a physiologically relevant potential range. The FACC channel was completely and irreversibly blocked by Gd³⁺. Based on the estimated transport capacity of the whole chloroplast population of FACC channels together with the envelope H⁺-ATPases, these channels can mediate electroneutral K⁺/H⁺ exchange across the envelope, enabling stroma alkalinization, thereby allowing an optimal photosynthetic performance.

Key words: Chloroplast — Membrane — Ion fluxes — Patch clamp — Adaptation — Photosynthesis

Introduction

The photosynthetic efficiency, estimated as CO₂ fixation, is strictly controlled by stromal pH, being optimal at pH ~8 and negligible at pH < 7.3. Onset of illu-

mination induces stromal alkalinization by almost 1 pH unit, primarily due to H⁺ import into thylakoids (Werdan, Heldt & Milovancev, 1975). However, light onset also triggers uptake of protons from the cytosol to the stroma, down the electrochemical gradient for the ion (Thaler, Simonis & Schönknecht, 1991). To maintain the ΔpH of about one pH unit between the stroma and cytosol in the light, an additional, active mechanism of H⁺ extrusion from stroma to the cytosol is required. It has long been known that H⁺ extrusion from the stroma to the cytosol is linked to K⁺ (Na⁺) counterflux. A decrease in external monovalent cation concentration favors their efflux from stroma and causes stroma acidification and photosynthetic inhibition, which can be reverted by repletion of the incubation medium with Na⁺ or K⁺ (Demmig & Gessler, 1983; Wu, Peters & Berkowitz, 1991; Heiber et al., 1995). The transenvelope K⁺/H⁺ exchange is electroneutral (Wu & Berkowitz, 1992a). According to the current model, the K⁺/H⁺ exchange mechanism across the inner chloroplast envelope consists of functionally coupled H⁺-ATPase and putative cation channel(s) (Wu & Berkowitz, 1992a, b; Berkowitz & Peters, 1993; see Neuhaus & Wagner, 2000, for review). H⁺-pumping activity of the dominant inner envelope ATPase has been directly demonstrated (Shingles & McCarty, 1994). Several attempts towards the characterization of the envelope channel activity *in vitro* have been undertaken, either via reconstitution of the detergent-treated envelope membranes into liposomes (Wang, Berkowitz & Peters, 1993) or through their fusion into planar lipid bilayers (Mi, Berkowitz & Peters, 1994; Heiber et al., 1995). The latter studies revealed some multistate large-conductance and poorly regulated cation channels. However, a disadvantage of the reconstitution technique is that incorporation of a new material into a lipid bilayer is likely to induce non-specific defects, whose fluctuations resemble current transitions generated by ion channels. Also, true channel proteins, once they are removed from their

Table 1. The composition of basic solutions (in mM) used for the selectivity experiments

Solution	KCl	CaCl ₂	MgCl ₂	Sorbitol	pH = 7.3
“250 KCl”	247	2	2	247	10 HEPES-KOH
“100 KCl”	97	2	2	502	10 HEPES-KOH
“25 KCl”	22	1	1	611	10 HEPES-KOH
“10 KCl”	7	0	1	631	10 HEPES-KOH
“250 NaCl”	247	2	2	239	10 HEPES-NaOH
“150 CaCl ₂ ”	0	150	0	205	5 HEPES-NaOH
“150 MgCl ₂ ”	0	0	150	246	5 HEPES-NaOH

natural environment, could be (almost certainly are) functionally altered. Therefore, preference should be given to *in situ* studies on native envelope membranes by application of the patch-clamp technique to intact chloroplasts. This is an extremely challenging task because typical chloroplasts of higher plants are small in size (3–5 µm), and have proved to be a complicated object for patch-clamp studies. Nonetheless, high-resistance seals have been obtained with the envelope membranes of intact *Pisum sativum* L. (pea) chloroplasts, and an anion channel involved in peptide transport has been characterized in these envelope patches (van den Wijngaard & Vredenberg, 1997, 1999; van den Wijngaard et al., 2000). In this study, by applying the patch-clamp technique directly to isolated pea chloroplasts, a novel non-selective cation channel with very rapid kinetics has been identified in the envelope. This fast-activating chloroplast cation (FACC) channel displayed a marked dependence on voltage and stromal pH, which can account for changes of transenvelope electric potential difference upon dark-light transition.

Materials and Methods

PREPARATION OF PEA CHLOROPLASTS

Pisum sativum L. (cv Los Molinos, Tepoztlan, Mexico) plants were grown from seeds at 24–26°C, 12 h day length (200 µmol m⁻² s⁻¹ of white fluorescence light). All measurements were performed on the 2nd or 3rd leaf taken from 3–4 week old plants. Chloroplasts were isolated by dissecting excised leaves with a razor blade in a solution containing (in mM): 100 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES-KOH (pH 7.3) adjusted with sorbitol to 740 mOsm. A small (20–50 µl) aliquot of chloroplast suspension was added to the experimental chamber (approximately 300 µl volume); after chloroplast sedimentation the bath was rinsed with 5–10 volumes of fresh solution (as above), or with isoosmotic 25 mM KCl solution.

EXPERIMENTAL MEDIA

All experimental solutions were adjusted to 720–740 mOsm (measured with Osmomat 030 cryoscopic osmometer; Gonotec, Germany) by adding the required amount of sorbitol. All chemicals were of analytical grade (Sigma Chemical Co, St Louis, MO). The ionic composition of basic solutions used for the selectivity series is given in Table 1. Additional solutions for the selectivity series were:

“50 K₂SO₄” (49 mM K₂SO₄, 10 mM HEPES-KOH, pH 7.3) and “100 NMDG” (100 mM *N*-methyl D-glucamine, titrated by HCl, plus 2 mM HEPES-KOH, pH 7.3).

To test the pH effects, 1N KOH stock was added to “250 KCl” solution to adjust solution pH to 8.0; for the pH 6.3, 10 mM MES-KOH was used instead of HEPES-KOH. A “250 mM KCl” solution with free Ca²⁺ = 200 nM was prepared by adding 3.25 mM EGTA to the solution shown in Table 1 (free Mg²⁺ was ~1 mM) and subsequent pH adjustment to 7.3 by addition of KOH. To test the effects of ATP, the solution was composed of 237 mM KCl, 5 mM of ATP (dissodium salt), 3.2 mM EGTA, 2 mM CaCl₂ and 4 mM MgCl₂ (free Ca²⁺ = 200 nM, free Mg²⁺ = 0.34 mM), 10 mM-KOH (pH 7.3). Free divalent (Ca²⁺, Mg²⁺) concentrations were calculated using Winmaxc32 v2.50 software (Chris Patton, Stanford University), based on total concentrations of divalent cations and chelators (EGTA, ATP) at given pH and corrected for ionic strength and temperature.

PATCH-CLAMP EXPERIMENTS AND ANALYSES

Inside (stromal side)-out patches (Hamill et al., 1981) were obtained on freshly isolated pea chloroplasts. Patch membrane capacitance was evaluated from amplitude and characteristic time of the artifact of membrane charging by rectangular voltage pulses of several tens of mV from zero clamped voltage. Patch pipettes were pulled from Kwik-Fil borosilicate glass with filament (1B150F-4 World Precision Instruments, Sarasota, FL) in three steps on a Brown/Flaming model P-97 puller (Sutter Instruments Co, Novato, CA), and fire-polished (LPZ 101 microforge, List Medical, Germany). Pipettes were filled with “250 KCl” solution; when the patch pipette was immersed in “100 KCl” solution, electrode resistance values were 12–17 MΩ. Current measurements were performed using an Axopatch 200A Integrating Patch-Clamp amplifier (Axon Instruments, Foster City, CA). A reference AgCl electrode was connected to the bath via a 3% agar bridge filled with 100 mM KCl. In this study, the envelope is treated as a single barrier and, therefore, the membrane potentials are reported as cytosol minus stroma, with positive currents corresponding to cation uptake into the chloroplast. Correction was made for junction potentials, generated between the reference electrode and bath containing one of the following solutions: “250 NaCl”, “150 CaCl₂”, “150 MgCl₂” (Table 1), “50 K₂SO₄” or “100 NMDG” solutions. Junction potentials were measured as described by Ward and Schroeder (1994). Using high-saline (3 M KCl) reference electrode and patch pipettes filled with a solution identical to the normally used reference electrode solution (100 mM KCl), junction potentials were estimated in the track mode of the patch-clamp preamplifier.

Records were filtered at 5 kHz by a 4-pole low-pass Bessel filter (80 dB per decade), digitized using a DigiData 1200 Interface (Axon Instruments), and recorded directly onto a hard disk of an

IBM-compatible PC at 4–15 kHz sampling rate. The command voltage protocols were applied and the analyses were carried out using the pClamp 6.0 software package (Axon Instruments). Single-channel currents were evaluated at fixed potentials (by use of all-amplitude histograms, where possible). Alternatively, fast voltage ramps (7.5 ms, ± 180 mV) were applied to obtain single-channel current-voltage relations. Due to superlinear current-voltage (I/V) relations, single-channel conductance was expressed in differential form as $G(V) = dI/dV$, rather than as a more traditional chord conductance. To obtain the open probability (P_o) value at a given potential, the time-averaged single-channel current was divided by a single-channel current amplitude at this potential, and by a maximal number of simultaneously open FACC channels observed in a given patch. Voltage dependence of the FACC channel was fitted to a three-state model with two open states connected via a closed state, as described by Pottosin and Martínez-Estévez (2003) with the modification:

$$P_o = 1 - [1 + \exp(z_1 F(V_1 - V)/RT) + \exp(z_2 F(V - V_2)/RT)]^{-1} \quad (1)$$

where $z_1(V_1)$ and $z_2(V_2)$ are gating charge (midpoint potential) for negative and positive branches of voltage dependence; V is voltage, F , R , T have their usual meanings.

Results

IDENTIFICATION OF THE FACC CHANNEL: VOLTAGE-DEPENDENT ACTIVATION, CONDUCTANCE AND SELECTIVITY

High-resistance seals (4–20 G Ω ; about 9 G Ω on average) were routinely obtained with isolated pea chloroplasts. With small pea chloroplasts it was difficult to maintain the attached configuration; normally the chloroplast soon ruptured, and the patch pipette, containing an envelope patch in the inside-out configuration, spontaneously excised. Methodological measurements of membrane capacitance in excised patches revealed a surprisingly high mean value, 0.35 ± 0.05 pF ($n = 16$). As discussed in detail in the Discussion section, the most probable configuration of such patches were two envelope membranes, one highly permeable (low resistance), and one of high resistance, which were connected in series. At a physiological voltage range (around 100 mV, cytosol positive) the dominant ion-channel type found in 22 out of >100 patches examined displayed fast activation kinetics. About 75% of excised patches did not display any channel activity and in 7 patches, a distinct, strongly voltage-dependent channel whose activity at physiological (cytosol-positive) potentials was negligible, was recorded. Up to 7 individual fast-activating channels were found in one patch, with an average of ~ 2 copies per successful patch. The activity of these channels in inside-out envelope patches was very stable over time, allowing long-lasting recordings with multiple solution changes. An example of typical activity, recorded on a stromal inside-out patch bathed in symmetric 250 mM KCl is

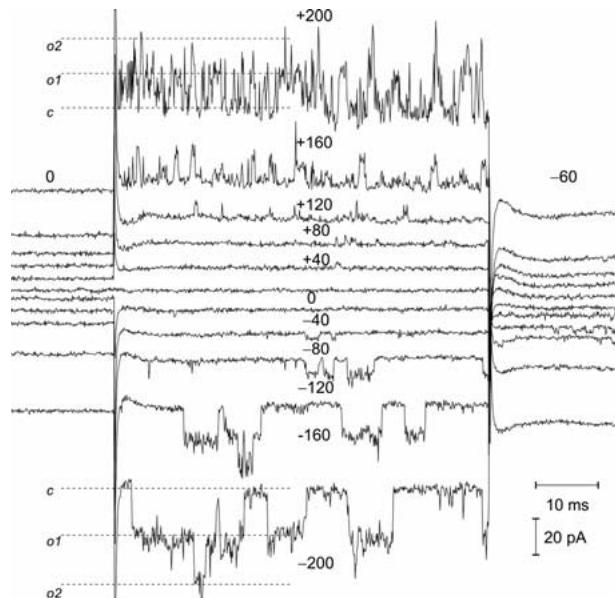


Fig. 1. Rapid and voltage-dependent activation of the chloroplast envelope channel. Original single-channel traces (filtered at 5 KHz and sampled at 10 KHz) of an inside-out envelope patch in symmetrical 250 mM KCl solution (supplemented with 2 mM CaCl₂ and 2 mM MgCl₂, pH 7.3). Voltage was stepped from 0 mV to indicated potentials, and switched to -60 mV at the end of a test pulse. Simultaneous openings of up to two identical FACC channels (denoted as *o1* and *o2*, whereas *c* indicates closed state leak current) can be seen at high membrane potentials of either sign.

presented in Fig. 1. The channels' open probability increased with the increase of potential of either sign; at potentials within the ± 80 mV range, channel openings were very rare. The channels displayed very fast, flickery openings at cytosol-positive potentials and longer openings at negative voltages. The transitions from low to higher open probability upon voltage steps to large positive or negative potentials occurred within a few milliseconds. The steady-state voltage dependence of the open probability is presented in the next section, where its modulation by pH and Ca²⁺ will be analyzed.

With 250 mM KCl in the pipette and 100, 25 or 10 mM KCl in the bath (stromal side) the amplitude of currents at negative potentials decreased much more strongly than compared to those at positive potentials, referring to the I/V curve in symmetric 250 mM KCl (Fig. 2A, B). Therefore, the current was mainly carried by K⁺ ions. In line with this, the single-channel currents' reversal potentials for 250/100, 250/25 and 250/10 KCl gradients were close to equilibrium potentials for K⁺ (E_K) under these conditions (Table 2). For comparison, E_{Cl} values under these conditions were +20.6, +53.3 and +79.4 mV, respectively. A calculation based on reversal potential values measured for 250/25 and 250/10 KCl gradients using the Goldmann-Hodgkin-Katz equation sug-

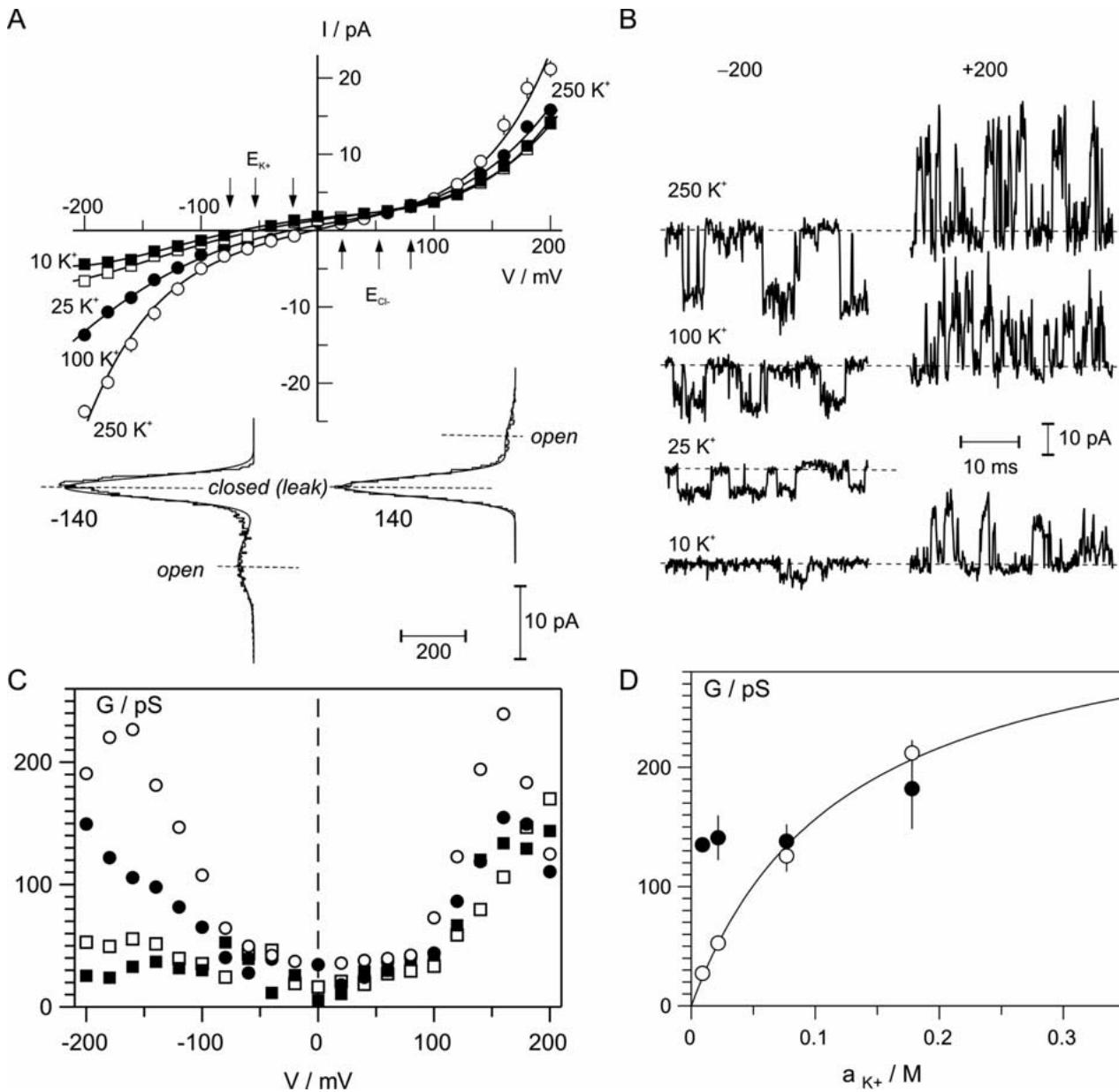


Fig. 2. The fast-activating chloroplast envelope channel is selective for K^+ over Cl^- . (A) Unitary current-voltage (I/V) relations. Data points presented in the I/V plot are mean \pm SD ($n = 5$ to 9 separate inside-out envelope patches). Solid lines are 4-order polynomials. The patch pipette was filled with 250 mM KCl solution, and bath contained 250, 100, 25 or 10 mM KCl (see Materials and Methods for detailed composition of solutions). Arrows indicate Nernst potentials for K^+ and Cl^- for the three KCl-gradients tested (activity values instead of concentration have been used for this calculation). All-point amplitude histograms for a patch, containing 3 FACC channels and bathed in symmetric 250 mM KCl, are presented in the inset. Records of ~ 1 s stretches were filtered at 5 kHz and sampled at 10 kHz. Histograms were fitted by two Gaussians, yielding values for the open-channel currents of 7.3 pA (+140 mV) and of -11.3 pA (-140 mV). Bath and pipette solution were always iso-osmotic (adjusted by sorbitol). (B) Examples of single-channel recordings (5 kHz filtering, 10 kHz sampling) at -200 and +200 mV. From symmetric 250 mM KCl, the solution at the stromal side of an excised envelope patch has been changed to 100, then 25, and finally to 10 mM KCl. The dashed lines indicate the leak current level (channel's closed state). (C) The single-channel conductance (G / pS) as a function of membrane voltage. Symbols are as in panel A. Conductance has been calculated as a first derivative of the mean I/V curves presented in panel A (e.g., as $G(V) = dI/dV$). (D) Single-channel conductance ($G(V) = dI/dV$) as a function of bath K^+ activity. Each point is a mean conductance for either -160 to -200 mV (denoted as ○) or +160 to +200 mV (denoted as ●) range where ionic current through the channel is dominated by K^+ flux into and K^+ efflux from the pipette, respectively. Solid line is the best fit of (○) to the Michaelis-Menten equation, with $G_{\text{max}} = 350 \pm 45$ pS and $K_m = 123 \pm 27$ mM.

gested that the channel permeability for Cl^- was only 2.7–2.9% of that for K^+ . Also, substitution of KCl in the bath to K_2SO_4 (50 mM) yielded a reversal po-

tential that was close to $E_K = -32$ mV (Table 2). Curiously, the single-channel conductance (calculated in differential form as $G(V) = dI/dV$, due to a non-

Table 2. Reversal potentials (V_r) of single-FACC-channel currents in different ionic conditions

Pipette (mm)	Bath (mm)	V_r (mV)	E_K (mV)	Number of patches
250 KCl	250 KCl	-1.5 ± 1.7	0	9
250 KCl	100 KCl	-22.4 ± 2.3	-21.3	6
250 KCl	25 KCl	-48.6 ± 3.5	-54.0	9
250 KCl	10 KCl	-65.6 ± 1.9	-76.2	5
250 KCl	50 K ₂ SO ₄	-31.2 ± 3	-32.0	4
250 KCl	100 NMDG-Cl	-70.5 ± 1.2	—	2
250 KCl	250 NaCl	-3.3 ± 2.4	—	6
250 KCl	150 CaCl ₂	$+1.5 \pm 1.2$	—	5
250 KCl	150 MgCl ₂	$+1.6 \pm 1.6$	—	4

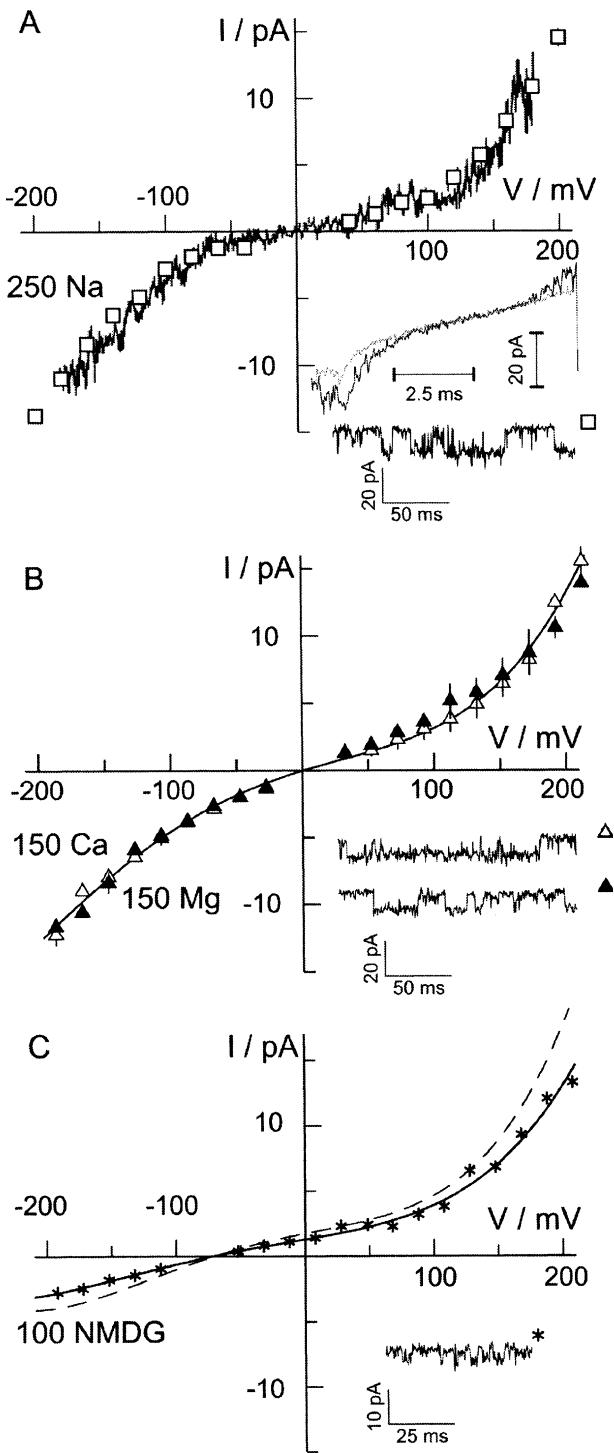
linear shape of the current-voltage relation) greatly increased with an increase of voltage of either sign. For symmetric 250 mM KCl it was 40–50 pS at potentials within the ± 100 mV range, whereas at potentials close to ± 200 mV it was ~ 200 pS (Fig. 2C). At large negative potentials, where the channel conductance was dominated by K⁺ influx from the bath, the single-channel conductance as a function of K⁺ activity in the bath could be fitted to a Michaelis-Menten equation, with an apparent K_m ~ 120 mM and limiting conductance of 350 pS. At the same time, at large positive potentials, where the current was dominated by K⁺ efflux from the pipette (250 mM KCl in the pipette), the mean conductance was in the range 135–180 pS for the bath [KCl] ranging from 10 to 250 mM, i.e., it was almost independent of KCl variation in the bath (Fig. 2D). Taken together, these findings suggest that the dominant channel in the envelope of pea chloroplast was selective for K⁺ over anions.

As the next step, other physiologically important cations were tested. K⁺ in the bath was replaced by Na⁺, Ca²⁺ or Mg²⁺. Based on the reversal potentials measured in these bi-ionic conditions, the envelope channel was equally permeable to K⁺ and Na⁺ (Fig. 3A; Table 2). However, currents carried by 250 mM Na⁺ were smaller than those for 250 mM K⁺, and roughly equivalent to those carried by 100 mM K⁺. Qualitatively, the rectification of the single-channel current is conserved under bi-ionic conditions, and the current-voltage relationship obtained by an alternative technique (application of fast (7.5 ms) voltage ramps) yielded the same result (Fig. 3A). Currents carried by 150 mM of Ca²⁺ or Mg²⁺ (~ 150 mM of elementary charges, $a_{Ca^{2+}} \sim a_{Mg^{2+}} \sim 75$ mM) were comparable to those earned by 250 mM Na⁺ ($a_{Na^+} = 180$ mM). Ca²⁺ and Mg²⁺ have a similar relative permeability (Fig. 3B), which was ~ 0.5 of that for K⁺, as judged by the reversal potential of about zero (Table 2) and by applying a special case of the Goldmann-Hodgkin-Katz equation (Eq. 13-11 in Hille, 1992). A small permeability for Cl⁻ was ignored, and only K⁺ and divalent cation

activities were taken into account for this calculation. Therefore, the chloroplast envelope channel is readily permeable for both mono- and divalent cations. From this moment we will term this channel a FACC (Fast-Activating Chloroplast Cation) channel. As Mg²⁺ is a strongly hydrated ion, a large current carried by Mg²⁺ implies that the caliber of the pore is large enough to carry Mg²⁺ with its first hydration shell, i.e., it must be $> 7\text{ \AA}$ nm in diameter (Hille, 1992). Therefore, it was tempting to test the permeability of even larger cations such as N-methyl-D-glucamine, NMDG, whose diameter is $\sim 9\text{ \AA}$ (Eikhorst et al., 2002). Although bath substitution to 100 mM NMDG-Cl eventually led to the induction of large-amplitude channel-like mediated currents and apparent loss of the seal, in the first moments after solution exchange the NMDG-induced currents were still absent and the registration of the FACC channel activity was possible in two patches out of 5 tested. In Fig. 3C, the I/V relationship of a single FACC channel obtained on one successful patch bathed in 250 mM KCl at cytosolic and 100 mM NMDG-Cl at the stromal side is shown. NMDG⁺ carried small, yet easily detectable cytosol-directed currents (inset), comparable to that carried by 10 mM K⁺ (registered previously on the same patch). The reversal potential for 250 KCl/100 NMDG-Cl (Table 2) was close to that measured in 250/10 KCl gradient, thus the relative permeability of NMDG⁺ was about 10% of that for K⁺.

EFFECTS OF CALCIUM AND STROMAL pH ON THE FACC CHANNEL ACTIVITY

For the sake of the gigaohm seal stability, the amount of Ca²⁺ in both bath and pipette was normally kept at 2 mM in our experiments. This is much higher than physiological free Ca²⁺ concentrations (~ 200 nM). A reduction of stromal Ca²⁺ to 200 nM (3 mM EGTA added to the bath) merely caused a 2-fold increase of FACC channel open probability at -200 mV without any effect at physiological cytosol-positive potentials (Fig. 4B). We then attempted to decrease Ca²⁺ in the



patch pipette (cytosolic side) to 200 nM. Surprisingly, this did not affect greatly the percentage of successful gigaohm seals, although the average seal quality was lower (apparent seal resistance $5.4 \pm 0.9 \text{ G}\Omega$, $n = 6$). Resulting inside-out patches were also much less stable. When voltage steps as large as $\pm 200 \text{ mV}$ were applied, this often led to the loss of a seal. Decreasing the duration of voltage steps allowed recording on some occasions. A 10,000-fold reduc-

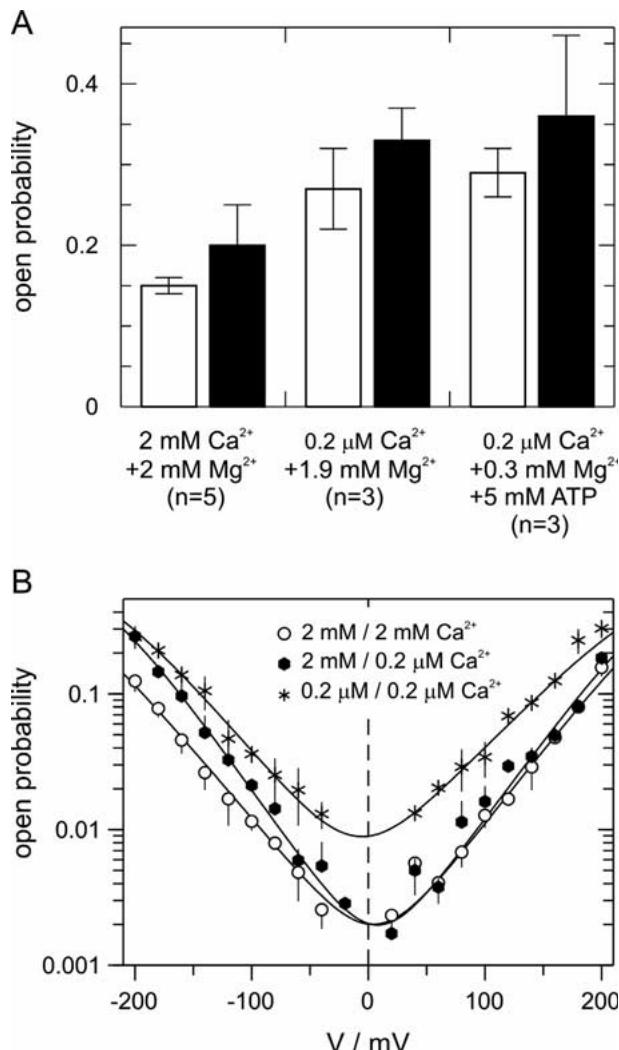


Fig. 4. Regulation of the fast-activating chloroplast cation channel by divalent cations. FACC channel activity was analyzed on separate inside-out patches bathed in symmetric 250 mM KCl, pH 7.3 solutions. (A) For the first batch of samples (control, $n = 5$ patches) free Ca^{2+} and Mg^{2+} concentrations in pipette and bath were 2 mM each. In the second batch of samples ($n = 3$ patches), free Ca^{2+} was reduced to 200 nM by introduction of 3 mM EGTA both in bath and in the pipette. In the third batch ($n = 3$ patches), 5 mM ATP has been added, free Ca^{2+} was 200 nM and free Mg^{2+} was reduced to 0.3 mM, symmetrically, at both patch sides. Open probability (mean \pm SD) has been evaluated at -200 mV and $+200 \text{ mV}$ (white and black bars, respectively). (B) Voltage dependence of FACC channel open probability at different levels of stromal and cytosolic Ca^{2+} . In a direct experiment (mean \pm SD, $n = 3$ patches), free Ca^{2+} was decreased from 2 mM (empty symbols) to 200 nM (filled symbols); 2 mM Ca^{2+} was present in the patch pipette. Experimental data for 200 nM Ca^{2+} at both membrane sides (mean \pm SD, $n = 3$ separate patches) are also plotted (asterisks). Data were fitted to Eq. 1 (see Methods), with parameter values for symmetric 2 mM and 200 nM, respectively, as follows: $z_1 = 0.61 \pm 0.02$ and 0.61 ± 0.03 ; $z_2 = 0.65 \pm 0.02$ and 0.53 ± 0.02 ; and midpoint potentials: $V_1 = -286 \pm 7$ and $-238 \pm 4 \text{ mV}$, and $V_2 = +277 \pm 5$, and $255 \pm 2 \text{ mV}$. For a 2 mM/200 nM Ca^{2+} gradient, fitted parameters values were: $z_1 = 0.71 \pm 0.05$, $z_2 = 0.69 \pm 0.03$, $V_1 = -240 \pm 8$, and $V_2 = +264 \pm 7$.

tion of free Ca^{2+} on both membrane sides neither prevented the channel activity nor did it strongly affect the single-channel current amplitude. Analysis on three successful patches bathed in 200 nm Ca^{2+} and 1.9 mM Mg^{2+} showed an increase ~ 2 times the mean open probability at -200 and $+200$ mV when compared to 2 mM free Ca^{2+} plus 2 mM Mg^{2+} (Fig. 4A). A more detailed analysis of the voltage dependence (Fig. 4B) revealed a somewhat higher increase of open probability, ~ 3 times, at smaller potentials within a physiological range, around 100 mV (cytosol more positive). Introduction of 5 mM ATP in bath and pipette solutions (another 3 inside-out envelope patches), with a further reduction of free Mg^{2+} concentration to 0.3 mM, did not result in a significant change in the FACC channel open probability (Fig. 4A).

Mimicking stromal pH changes during dark-light transition, we studied the pH effect on the FACC channel activity. An increase of stromal pH from 7.3 to 8.0 decreased the open channel probability in a physiologically relevant range (70–120 mV) by $\sim 40\%$. Experimental points were fitted to a 3-state model, similar to the gating model previously developed for the fast vacuolar (FV) channel (Tikhonova et al., 1997; Brüggemann, Pottosin & Schönknecht, 1999a, b; Pottosin & Martínez-Estevez, 2003). The analysis of pH effects shows that alkalinization of stroma results in a small (by ~ 0.18 elementary charges) but significant (according to *F*-test) increase of effective gating charge, making the channel more sensitive to

the applied voltage. Consequently, the open channel probability substantially decreases at low potentials, whereas at high potentials the difference between the voltage dependence measured at pH 7.3 and 8.0 tends to diminish; even the open probability at pH 8.0 exceeds that at pH 7.3 ($+200$ mV). Stromal acidification by 1 pH unit (from pH 7.3 to 6.3) also caused a decrease in FACC channel open probability. Therefore, a pH around 7.3 seems to be an optimum for the FACC channel operation. Variation of pH within the 6.3–8.0 range did not affect the single-channel current-voltage relation (Fig. 5).

PHARMACOLOGY OF THE FACC CHANNEL

Several cation channel blockers were tested. Due to the inside-out orientation of isolated envelope patches, their application was possible from the stromal side only. Addition of 10 mM TEA^+ was ineffective (*result not shown*). Addition of 1 mM Cd^{2+} caused only a slight (~ 2 -fold) decrease of single-channel current at large negative potentials, but did not affect the FACC channel open probability. The immediate effect of Gd^{3+} (100 μM) was very similar to that of Cd^{2+} , i.e., a weakly voltage-dependent block of the open-channel current, implying that both blockers can reside within the FACC channel pore. Longer incubation in Gd^{3+} resulted in a complete and irreversible loss of the FACC channel activity (Fig. 6). This may be interpreted as evidence that Gd^{3+} slowly permeated through the patch, perhaps via the channel pore itself and exerts its inhibitory effect from the cytosolic side.

Fig. 3. The fast-activating chloroplast channel weakly discriminates between different cations. Single-channel I/V curves (mean \pm SD, 4–5 separate patches, except a single experiment with NMDG) have been obtained in bi-ionic conditions, with 250 mM KCl in the pipette and cation concentrations (in mM) in the bath as indicated on the graph. Solid lines are 4th order polynomials. For comparison purposes, the I/V plot for 100 mM NMDG-Cl in the bath (panel C) is supplemented by the I/V curve for a 10/250 mM KCl gradient, measured in the same patch (dashed line; points are omitted for the sake of clarity). Inset in panel A shows a continuous I/V relation obtained by application of fast voltage ramps (7.5 ms, ± 180 mV). Here, the trace with a lower conductance is a leak current, and the second trace is a leak plus the current, mediated by a single FACC-channel, open most of the time. Traces are sampled at 20 KHz and filtered at 5 KHz. Selecting the traces with an open FACC channel, a leak-current response was subtracted from them, yielding a unitary I/V relation. The noisy curve in the graph is the average of 11 individual I/V curves (mean \pm SD). Steady-state single-channel recordings (dominated by the influx of the tested cation) are also shown as insets in the appropriate panel. These were obtained at the largest negative potential tested for each condition (-199 mV for Na^+ , -187 mV for Mg^{2+} and Ca^{2+} , and -191 mV for NMDG; the potential of -200 mV applied across the patch has been corrected for the liquid junction potential that arose at the contact surface between bath and reference electrode). Note the different scaling bar for the single-channel currents carried by NMDG $^+$.

Discussion

The outer chloroplast envelope is highly permeable to various substances, particularly inorganic ions, due to the presence of high-conductance and poorly selective pores (Flüegge & Benz, 1984). In contrast, the higher-resistance inner membrane serves as a barrier between the two metabolic compartments, the stroma and cytosol. The two envelope membranes are aligned parallel at their whole continuity, with an intermembrane distance of 2–3 membrane thicknesses or 10–20 nm (Staehelin, 1986). The outer envelope membrane of pea chloroplast contains at least 3 types of large (~ 0.5 nS) conductance porins, two weakly cation-selective, OEP24 and OEP16, and an anion-selective one, OEP21 (Pohlmeyer et al., 1998; Böltner et al., 1999; Steinkamp et al., 2000). As we did not observe the porin activity in excised patches, it is unlikely that the patches consisted of the outer membrane alone. Due to a close alignment of the outer and inner envelope bilayers it seems more likely that the two membranes are pulled into the patch pipette together. Therefore, the patches may contain a sandwich-like structure of the inner and outer

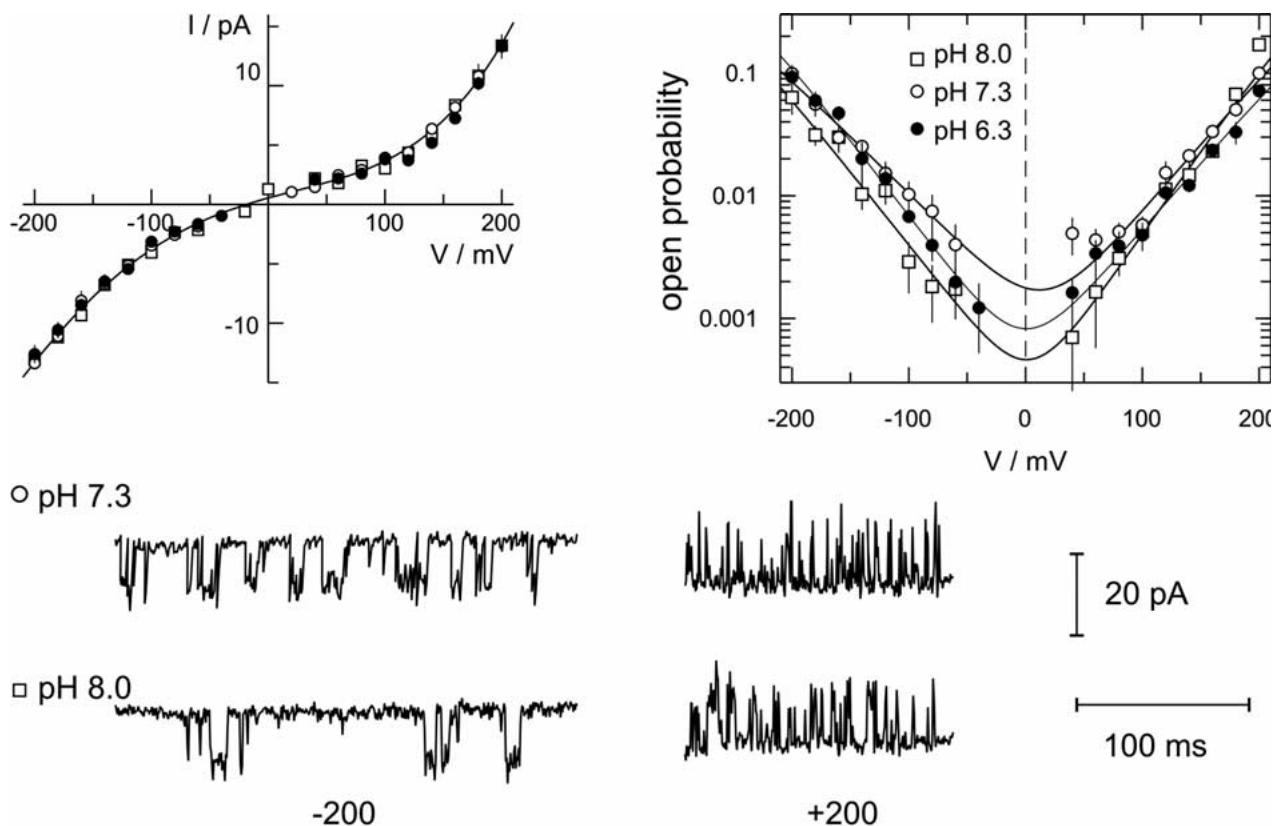


Fig. 5. pH-Regulation of the fast-activating chloroplast cation channel. Single-channel I/V curves and voltage dependence of the FACC channel open probability are shown for stromal pH 6.3 (□), 7.3 (●) and 8.0 (○); pipette and bath were 250 and 100 mM KCl, respectively. Data are mean \pm SEM ($n = 5$ to 8). Experimental points were fitted to Eq. 1, with parameter values for gating charge $z_1 = 0.74 \pm 0.04$, 0.56 ± 0.06 and 0.74 ± 0.07 ; $z_2 = 0.64 \pm 0.04$, 0.65 ± 0.03 and 0.80 ± 0.09 ; and midpoint potentials: $V_1 = -274 \pm 8$, -300 ± 32 and -294 ± 15 mV, and $V_2 = +309 \pm 10$, $+292 \pm 7$ mV and 270 ± 13 mV for pH 6.3, 7.3 and 8.0, respectively.

membranes in a tight contact (van den Wijngaard and Vredenberg, 1997). Alternatively, they may contain the inner envelope membrane alone (van den Wijngaard et al., 1999). However, the reasons why the outer membrane is necessarily and selectively disrupted are unclear. Van den Wijngaard et al. (1999) came to this conclusion, using identical patch-pipettes and measuring approximately the same capacitance of inside-out patches from vacuoles (single membrane-bound organelle) and pea chloroplasts. However, a portion of a membrane pulled into the tip (and the patch capacitance) is defined not solely by the tip geometry, but also by the membrane elasticity and availability. Consequently, it is not surprising that with a large and elastic vacuole the pulled membrane fragment was larger as compared to that pulled from a small rigid chloroplast. In our case, the pipette apertures were substantially smaller than the chloroplast diameters (~ 1 and ~ 4 μm , respectively). As a result, $< 50\%$ of the chloroplast surface was pulled into the pipette. For the average chloroplast diameter of ~ 4 μm , the total surface area is ~ 50 μm^2 . At the same time the average capacitance of the envelope inside-out patches in our experiments

was 0.35 pF, which corresponds to ~ 35 μm^2 (or 70%) of the chloroplast surface area, based on specific capacitance for biological membranes of ~ 1 $\mu\text{F}/\text{cm}^2$ (Hille, 1992). The most logical explanation for this discrepancy is that the patch is composed of a double envelope membrane. The presence of a highly permeable outer envelope seems not to interfere significantly with the voltage-clamp and current recording across the inner membrane in series. Thus, the voltage was clamped¹, and the measured specific current was delimited by a highly resistant inner envelope membrane patch.

¹Along with capacitance (impedance) determinations, we have also measured access resistance and found it to be 1.15 ± 0.17 G Ω ($n = 16$) as compared to ~ 9 G Ω apparent seal resistance. In accord with our working model, access resistance is mainly defined by the outer envelope membrane, and apparent seal resistance is a parallel combination of a shunt conductance between patch and pipette, a leak through the intermembrane space, and resistance of the inner envelope membrane. Therefore, the maximal inaccuracy in our measurements under voltage-clamp conditions has not exceeded 10% of the command-potential value.

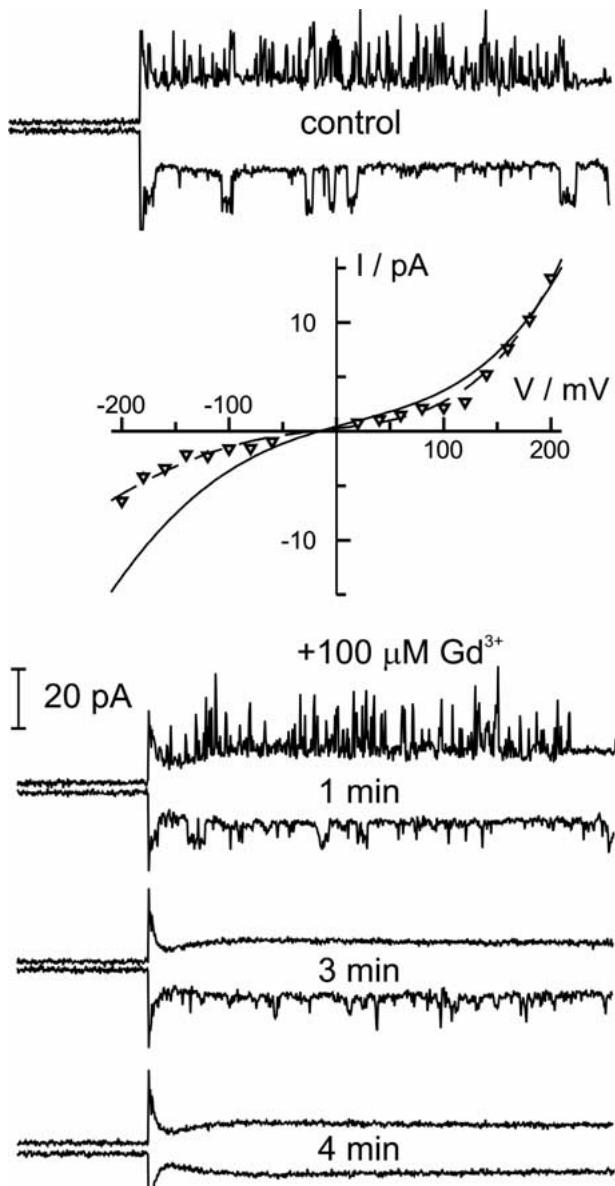


Fig. 6. Inhibition of the fast-activating Chloroplast Cahon Channel by Gd^{3+} . Single-channel currents registered on an inside (stromal side)-out envelope patch are shown. Test pulses from zero to $\pm 200\text{mV}$ before and at 1, 3, and 4 minutes after addition of Gd^{3+} (100 μM) to the bath. Single-channel I/V curve at 1 min after addition of Gd^{3+} (∇); the I/V curve in control (250/100 mM KCl) is represented by a 4th order polynomial, fitted to the data (points are omitted for the sake of clarity).

The FACC channel described in this study displays a characteristic voltage-dependent pattern: the open probability increased 30 to 50-fold (Fig. 4B, 5) and the single-channel conductance increased \sim 5-fold (Fig. 2C) upon transition from 0 to $\pm 200\text{ mV}$. This is in striking contrast with the reported properties of cation channels, whose activity was detected after reconstitution of envelope membranes into planar lipid bilayers (Mi et al., 1994; Heiber et al., 1995), which displayed linear I/V relationships with a

unitary conductance of 100–160 pS and were practically voltage-independent. Thus, it appears that we are dealing with a completely new type of chloroplast channel, not reported so far in the literature.

The present data could not completely rule out the possibility that the FACC channel activity is comprised of two channels with opposite voltage dependence. However, in all 22 successful patches, the maximal number of channels open at -200 and $+200\text{ mV}$ coincided. Bearing in mind the relatively small number of channels present in the patch, it is statistically unlikely that 1:1 stoichiometry would strictly hold for independent channels (the upper limit for a probability of a random coupling is 0.5²² or $2.38 \cdot 10^{-7}$). Therefore, we are dealing with either one channel protein with two distinct open conformations, or two intrinsically coupled channels. It should be noted that both the currents, activated at positive and negative potentials, were eliminated by 100 μM Gd^{3+} (Fig. 6). This favors the first alternative.

The description of the voltage-dependent activation of FACC channels requires a minimal model with the two distinct open states, favored by large negative and positive voltages, respectively, interconnected via a closed state, whose occupancy is maximal at voltages around zero. We are aware of only one similar pattern of bipolar voltage dependence: the FV channel of the tonoplast (Tikhonova et al., 1997; Brüggemann et al., 1999; Pottosin & Martínez-Estevez, 2003, and references therein). The FV channel conducts monovalent cations with little preference and is also rapidly activated by voltage steps to large potentials of either sign. In contrast to FV, the FACC channel readily conducts divalent cations (Fig. 3B) and is not very sensitive to Ca^{2+} from either side (Fig. 4B). Interestingly, both FV and FACC channels are permeable to NMDG^+ (Pottosin & Martínez-Estevez, 2003; Fig 3C, this paper). *N*-methyl-*D*-glucamine is a large organic cation with an approximate diameter of 9 Å. Most of the cationic channels in biological membranes are not permeable to NMDG^+ due to a smaller caliber of their pores. A notable exception are P2X channels of purinergic receptors (Eikhorst et al., 2002). At the same time, the FACC channel displayed a high degree of K^+ over Cl^- selectivity, \sim 30 (Fig. 2A). High cation to anion selectivity does not necessarily require a narrow pore. On the contrary, studies on mutant (cation-selective) glycine receptor channels have shown a positive correlation of the cationic selectivity with an increase in the pore size: an almost ideal selectivity for Na^+ over Cl^- was predicted for a pore diameter of 11.5 Å (Keramidas et al., 2004).

The physiological function of the FACC channel would depend on the direction of electrochemical gradients for the most abundant ions, primarily K^+ , across the envelope. Most papers report a transenvelope voltage difference of about 70 to 110 mV,

cytosol more positive than stroma, and K^+ concentration in the stroma of 110 to 170 mM as compared with ~100 mM in the cytosol (Demmig & Gummel, 1983; Wu et al., 1991; Igamberdiev & Kleczkowski, 2003). Thus, K^+ will be taken up passively by chloroplasts (electric driving force ~80 mV), provided a suitable transporter for this ion is situated in the inner envelope. Tracer measurements on isolated intact chloroplasts revealed a transport capacity for this uptake of 150 to 500 nmol K^+ / mgChl $^{-1}$ min $^{-1}$ (Demmig & Gummel, 1983; Wu et al., 1991). This flux perfectly balances the current generated by the envelope H^+ pumps, so that the overall H^+ / K^+ exchange across the envelope is electroneutral (Berkowitz & Peters, 1993). Given the chloroplast volume of ~30 μm^3 and specific chloroplast volume of 30 $\mu\text{l}/\text{mgChl}$ (Demmig & Gummel, 1983), K^+ influx will generate a current of only ~0.25–0.80 pA per single chloroplast. For comparison, given the electrical driving force for K^+ transport across the inner envelope is ~80 mV (cytosol more positive), and the open probability of a 100 pS channel described by Heiber et al. (1995) is ~40%, the average K^+ current mediated by this channel will be 3.2 pA. In other words, the presence of a *single* channel of this type in the chloroplast envelope implies at least a 4-fold larger K^+ uptake than is dictated by the measured tracer fluxes and is necessary for H^+ current compensation. Under such conditions, the transmembrane potential difference would collapse down to ~25 mV or even lower, impairing chloroplast function. In contrast, the mean current generated under the same conditions by a single FACC channel would only be 0.1 pA, assuming unitary conductance of 30 pS (Fig. 2C) and open probability of 0.03 as an upper limit (Fig. 4B). Therefore, few (up to 10) fast-activating cation channels per chloroplast, which seems to be a realistic number, could electrically balance the current generated by the whole chloroplast population of H^+ -ATPases; a high (~90 mV, cytosol positive) transenvelope voltage difference would then be maintained across the envelope. This voltage gradient is necessary to fuel the ATP/ADP translocator in the inner envelope membrane and to maintain a cytosol-directed gradient of Mg^{2+} in the light (Igamberdiev & Kleczkowski, 2003). Accumulation of Mg^{2+} in the stroma under illumination is required for the activation of key stromal enzymes (e.g., Rubisco), and Mg^{2+} depletion results in photosynthetic inhibition (Portis & Heldt, 1976). It is well known that the onset of illumination increases the voltage gradient across the chloroplast envelope by ~10 mV (Demmig & Gummel, 1983; Wu et al., 1991). An explanation for this phenomenon might be a decrease in the FACC channel activity. Indeed, in the light, stromal pH increases by almost 1 pH unit, from 7.3 to 8.0 (Werdan et al., 1975). Our data (Fig. 5) suggest that this pH change causes a reduction in the FACC channel open

probability by ~40%, within a physiological range of potentials. The pH-induced positive shift of membrane potential difference by 10 mV will increase by ~30% the open probability of FACC channel, and by ~10% the single-channel current.

The existence of a significant driving force for a K^+ uptake into stroma on one hand, and K^+ -permeable FACC channel in the envelope on the other, raises the problem of volume regulation. This situation might be compared with that in mitochondria, where maintenance of a huge (~180 mV) electric potential difference between matrix and cytosol would (due to an extremely high (150 M) value of equilibrium K^+ concentration in the matrix) inevitably cause osmotic swelling and rupture of mitochondria. In reality, K^+ distribution is far from equilibrium, and reflects a kinetic steady state between K^+ influx through K^+ -permeable channels and its efflux via K^+ (Na^+)/ H^+ antiporters (Bernardi, 1999). Recently, a novel K^+ (Na^+)/ H^+ antiporter has been characterized in the envelope membranes of *Arabidopsis thaliana* chloroplasts (Song et al., 2004). Due to the direction of ionic (K^+ and H^+) gradients across the envelope this apparently neutral antiporter could only transport H^+ into the stroma and extrude K^+ from chloroplasts, thus partly compensating expected K^+ accumulation via FACC channels. Once again, tiny currents generated by FACC channels are compatible with the transport capacity of antiporters and pumps. In contrast, a current generated by a big channel (such as those described by Mi et al. (1994) and Heiber et al. (1995)) would certainly outweigh the transport of ions by all other transporters. It could be compared with the opening of the so-called mitochondrial transition pore (MTP), a relatively rare event, taking place, for instance, in apoptosis development (Bernardi, 1999).

The inner envelope cation channel, which serves as a counterpart for the H^+ pump in K^+ / H^+ exchange between stroma and cytosol is not strictly K^+ -selective. Rather, it also conducts Na^+ . Channel-mediated Na^+ tracer fluxes were about one half of those for K^+ (Demmig & Gummel, 1983; Wang et al., 1993), which agrees with the relative conductance of Na^+ in the FACC channel (Figs. 2A and 3A). The FACC channel also readily conducts Ca^{2+} and Mg^{2+} (Fig. 3B) but due to low physiological concentrations of these ions, they could comprise only a tiny fraction of the FACC channel-mediated current. This in case of Ca^{2+} would definitely be physiologically insignificant, as maximal stromal Ca^{2+} concentration is 5–10 μM (Sai & Johnson, 2002). The envelope of intact chloroplasts seems to be hardly permeable for Mg^{2+} (Demmig & Gummel, 1983), whereas Ca^{2+} transport across the envelope is mediated by a highly specific Ca^{2+} uniporter (Roh et al., 1998).

A direct role of the FACC channel in Ca^{2+} signaling is not likely. As shown by the data in Fig. 4B,

a stromal Ca^{2+} increase to 2 mM had no effect on the FACC channel; a cytosolic Ca^{2+} increase to the same level apparently reduces the FACC channel open probability 2- to 3-fold, yet this Ca^{2+} variation was well beyond the physiological range. Previous studies (Demmig & Gimmler, 1983; Wu et al., 1991) have shown that increases in cytosolic Mg^{2+} from 1 to 5 mM reduces by ~35% both K^+ uptake by chloroplasts and photosynthetic rate. In this work we have varied free Mg^{2+} between 0.3 and 1.9 mM, and the increase of FACC channel activity was insignificant (Fig. 4A). It should be noted, however, that the range of free cytosolic Mg^{2+} variation in leaves seems to be much smaller, 0.4 mM in the dark to 0.2 mM in the light (Igamberdiev & Kleczkowski, 2001), therefore, regulation of FACC channel by Mg^{2+} is physiologically irrelevant.

In conclusion, the fast-activating channel, characterized in the envelope of intact pea chloroplasts, appears to be a plausible companion to the envelope pump, with these two systems *in assembly* mediating an electroneutral H^+/K^+ exchange across the envelope. The latter underlies maintenance of alkaline pH in the stroma, a pre-requisite for optimal photosynthesis. Potentially, the FACC channel could also have a role in volume regulation.

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