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Ion channels in the thylakoid membrane (a patch-clamp study)

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Thylakoid membranes were fused with azolectin liposomes and enlarged to giant thylakoid/liposome vesicles with diameters between 20 μm and 50 μm by a modified dehydration/rehydration procedure. Ion transport across the membrane of these giant thylakoid/liposome vesicles was investigated by the patch-clamp technique. Single-channel currents were observed and four different specific ion conductances could be distinguished. Our results indicate that these conductances can be attributed to three types of ion channel: a putative potassium channel with a conductance of 110 pS (in 100 mM KCl) a chloride channel with a conductance of 220 pS (in 100 mM KCl) and a moderate selectivity of about 4:1 for anions over cations. The third type of putative ion channel we observed was permeable to Mg^{2+} and Ca^{2+} ions with conductances of 20 pS for Ca^{2+} and 35 pS for Mg^{2+}, respectively.

Introduction

Light-induced electron transport in the photosynthetic membranes of chloroplasts and bacteria gives rise to proton pumping across the respective membranes. The bulk-to-bulk electrochemical potential difference of the protons generated by the light-driven redox pumps is subsequently used for ATP synthesis in the H^+ ATPase [1]. In thylakoids, a proton gradient of 3–4 units can be established under saturating steady-state illumination [2,3], while the transmembrane electric potential between the two aqueous phases seems to be small (approx. 10 mV positive in the lumen) under these conditions [4,5]. The accumulation of protons in the thylakoid lumen appears to be electrically balanced by the counterflow of K^+ and Mg^{2+} [6–8] and uptake of Cl^- [9,10]. Significant increase in stromal Mg^{2+} and K^+ concentrations [7,11–13] as well as Cl^- uptake [7,9] upon illumination of chloroplasts have been reported. Several lines of evidence suggested Mg^{2+} being the major physiological counterion for H^+ pumping across the thylakoid membranes [6,8,14].

Ion-channel proteins which facilitate ion movements across the thylakoid membrane have not yet been identified. However, electrophysiologically a voltage-dependent Cl^- channel has been measured in patch-clamp studies on swollen chloroplasts of *Peperomia metallica* which revealed a unit conductance of 110 pS (Ω^{-1}) in 110 mM KCl [15]. Potassium-specific and voltage-dependent channels with unit conductances of 14 pS at 500/300 mM KCl and 120 pS at 1000/300 mM KCl have been observed in planar lipid bilayers with fused thylakoid membranes [16]. In addition, the purified reconstituted chloroplast ATPase has been investigated by electrophysiological methods [17,18]. The reconstituted CF_0 CF_1 in bilayers formed on the tip of a micropipette ('dip stick' bilayer) revealed ion channels with a unit conductance of about 3 pS [18] which possibly were carried by H^+. With the same technique ion channels with different unit conductances between 20 and 60 pS permeable to monovalent cations were observed in bilayers containing mainly the channel part CF_0 of the chloroplast ATPase [18]. In patch-clamp measurements on giant liposomes containing reconstituted CF_0CF_1, voltage-activated K^+-selective channels with different unit conductances (10–100 pS in 100 mM KCl) were observed. These channels have been attributed to partially disintegrated CF_0CF_1 [17].

In this study we report on further characterisation of ion fluxes across the thylakoid membrane using patch-clamp measurements on swollen *giant* thylakoid vesicles and on spinach thylakoid vesicles fused to giant vesicles with azolectin liposomes.

Materials and Methods

Preparation of thylakoids

Preparation of thylakoids was carried out essentially as described in Ref. 19 and chlorophyll was determined as described in Ref. 20. In order to remove possible

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Abbreviations: CF_0CF_1, chloroplast ATPase; CF_0, membrane part of the chloroplast ATPase; CF_1, soluble part of the chloroplast ATPase; PMS, *N*-methylphenazonium methosulfate. Enzyme: chloroplast ATPase (EC 3.6.1.34)

contaminations of thylakoids by envelope membranes a five-step sucrose density gradient centrifugation was subsequently performed. The five-step gradient (800/1000/1200/1400/1600 mM sucrose) was contained in a buffer with 10 mM NaCl, 5 mM $MgCl_2$, 100 mM Tricine (pH 7.8), and centrifugation was carried out at $70000 \times g$ for 60 min with an SW 28 swing-out rotor in a Beckman ultracentrifuge. The dark green band of 1200 mM sucrose contained the purified thylakoid fractions as judged by assay of photophosphorylation and measurement of linear electron transport from $H_2O \rightarrow$ *Methylviologen*. Aliquots of the thylakoids were rapidly frozen in liquid nitrogen and stored at −80°C prior to use. After thawing they still revealed typically about 80% of the rate of photophosphorylation of freshly prepared thylakoids. Swollen ('giant') thylakoid vesicles were prepared according to the following protocol (all steps were carried out at 4°C). 2 ml of the thylakoid solution in the above described buffer (2–4 mg/ml Chl) were incubated with 2–3 ml of 700 mM sucrose, 10 mM NaCl, 10 mM tricine (pH 7) at 4°C for 60 min and subsequently diluted 1000 fold with a buffer containing 30 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM Hepes-Tris (pH 7.0). After stirring for 15 min in the dark thylakoids were transferred into the patch-clamp bath chamber where giant thylakoid-vesicles with typical diameters of 10 to 30 μm could be observed.

Fusion of thylakoids with liposomes and preparation of giant vesicles for patch-clamp experiments

Small liposomes were obtained by dissolving 100 mg/ml of azolectin lipid (Sigma, type IV) in 10 mM Hepes-KOH (pH 6.5) using a Branson sonifier equipped with a microtip. Liposomes were then freeze-thawed once and mixed with thylakoid solution to yield final chlorophyll concentrations of 200 μM to 10 μM. With respect to the occurrence of only a few ion-channels in the membrane patches the best results were obtained at final chlorophyll concentrations of 50–100 mM. Then the thylakoid/liposome mixtures were frozen at −80°C and thawed at 4°C. Giant-vesicles from the fused thylakoids suitable for patch-clamp measurements were obtained by a modified dehydration-rehydration procedure according to Keller et al. [21]. After thawing, 5–7 μl of the mixture were spread on a glasslide and dehydrated at 4°C for 45–60 min in a 500 μl exsiccator filled to 1/3 volume with dry $CaCl_2$. Rehydration was performed in a petri-dish, the bottom of which was covered with water-saturated paper. 10 μl of the electrolyte solution to be used in the patch-clamp measurements were added to the partially dried sample on the slide. After 1 h, giant liposomes with incorporated thylakoid membranes could already be observed. These giant thylakoid/liposome vesicles typically had diameters between 20 μm and 50 μm. Sometimes single vesicles up to 100 μm in diameter could be observed. They were stable and could be used for patch-clamping for up to 3 days; however, our measurements were performed with freshly prepared vesicles.

Giant liposomes without fused thylakoid membranes when used as controls under the applied experimental conditions of the patch-clamp experiments did not show any single-channel activity.

Patch-clamp measurements

The experiments described below were performed with unilamellar giant thylakoid/liposome-vesicles. (Some experiments have been carried out with swollen giant thylakoids, but no reproducible GΩ-seals could be obtained, therefore the results obtained with these swollen giant thylakoids have a very preliminary character.) Giant thylakoid/liposome vesicles were placed in a tissue bath, 0.5 ml final volume, and mounted on an Olympus IMT-2 inverted microscope. The vesicles were then viewed using phase contrast optics. Single-channel current recordings using the patch-clamp technique were performed as described by Hamill et al. [22]. The bath (agarelectrode) was grounded and membrane voltages across excised inside-out patches are referred to the pipette. GΩ-seals ranging from 5 to 100 GΩ could be achieved by slight suction, once the pipette tip was brought in contact to the giant thylakoid/liposome-vesicle membrane. Sealing was apparently effected by the chlorophyll content of the vesicles, i.e., the lower the chlorophyll concentration, the higher the probability of GΩ-seal formation. Current recordings were digitised at 36 kHz sampling rate using a VR 10 (Instrumental corp.) digitiser and stored on VHS video tapes. For analysis, current recordings were filtered with an 8-pole bessel filter, typically at 1(0.5) kHz, digitised at a sampling interval of 1(2) ms in an Axolab 1100 A/D converter (Axon Instr.) and stored in a PC. Analysis of single-channel currents was performed with the Fetchan/Pstat analysis programs (Axon Instr.).

Materials

Ca^{2+} and Mg^{2+} gluconate (2,3,4,5,6,-pentahydroxycapronic acid $\times$ 1/2 Ca^{2+} (Mg^{2+}) salts; analytical grade) were purchased from Sigma (Germany) and concentrations given for these salts in solution refer to the cations.

Results

Swollen 'giant' thylakoid vesicles prepared as described in the materials and methods section revealed typical diameters between 10 and approx. 30 μm when diluted into a suspension medium with an ionic strength of 30 mM. It was, however, not possible to form

GΩ-seals between the pipette and the thylakoid membrane in a reproducible manner. We only obtained very few GΩ-seals in the range between 1 and 10 GΩ where we were able to resolve single-channel currents. These currents corresponded to a conductance of 30 pS to 70 pS at an ionic strength of 30 mM monovalent cations at pH 7. Variation of experimentally accessible parameters (type of glass, geometry of the patch pipette, plant species for thylakoid preparation, pulsed suction of the patch pipette, partial proteolytic digestion of the thylakoid membrane) to improve the GΩ seal formation were unsuccessful. In order to 'dilute' the high protein density in the thylakoid membrane which probably was the major obstacle in obtaining a high-resistance seal, we fused thylakoid vesicles with azolectin liposomes. This fused vesicles were enlarged to giant vesicles by a modified procedure of the dehydration/rehydration technique [21]. These vesicles typically revealed diameters of 20 to 100 μm. After optimal adjustment of the chlorophyll/lipid ratio during fusion (see Materials and Methods) GΩ seals up to 100 GΩ between the patch pipette and the membrane of giant thylakoid/liposome vesicles were formed readily in a highly reproducible manner.

Membrane voltages and currents in giant thylakoid/liposome vesicles were measured after formation of a high resistance (GΩ) seal and excising of the patch. At single-channel current resolution we observed at least three classes of different ion-channel conductances which we will describe in the following in respect to open channel current amplitude, ion-specificity and gating behaviour.

Fig. 1A (left) shows single-channel currents recorded from an inside out (excised) patch bathed in symmetrically 100 mM KCl solutions at a holding potential of −25 mV. The corresponding all points amplitude histogram of the single-channel currents is shown in the right part of the figure. Single-channel openings are displayed as downward current deflections. The figure shows that the excised patch contained at least two active ion-channels (one opens at the top of the other) both of them showing fast opening and closing transitions. Both ion channels revealed the same open-channel current amplitudes and apparently the same open probability with a unitary conductance of approx. 110 pS (see below).

Fig. 1B shows ionic currents recorded from an inside out patch excised from giant thylakoid/liposome vesicles bathed in asymmetric KCl solutions in response to a series of voltage sweeps. This excised patch contained only one copy of the 110 pS channel and was therefore used to determine the zero-current potential of this particular ion conductance. The membrane voltage was changed with a sweep-rate of 100 mHz between −20 mV and 20 mV and the time axis of the records converted to the respective voltage. The channel openings displayed a unit conductance of approx. 110 pS and the reversal potential of the channels as obtained from extrapolation of the current-voltage relationship of the closed and open state (filled arrows) was very close to the Nernst potential for K^+ (−27 mV). This shows that the 110pS channel observed in giant thylakoid/liposome vesicles is a specific K^+ channel. The plot of the single-channel currents versus applied voltage for the '110 pS channel' revealed a linear relationship between +40 mV and −40 mV (data not shown).

Fig. 2A shows a typical example for another type of ion-channel conductance observed in the giant thylakoid/liposome vesicles. The top of the figure shows single-channel recordings from an excised patch bathed symmetrically in 100 mM KCl solutions at a holding potential of 30 mV. In the lower part the corresponding all point amplitude histogram of the single-channel currents is shown. Channel openings are displayed as upward current deflections. This type of ion-channel displayed longer lived openings and closures and the shown current trace in Fig. 1B revealed an open probability (P_O) of about 50% with a unitary conductance of approx. 220 pS. In the closed state of the 220 pS channel, short-lived (~ 1 ms) ion-channel openings with lower amplitude occurred, this is also manifested in the peak shoulder associated with the 'closed peak' in the amplitude histogram. Fig. 2B shows ionic currents recorded from an inside out patch excised from giant thylakoid/liposome vesicles bathed in asymmetric KCl solutions in response to a series of voltage sweeps. As judged from single-channel recordings at different holding potentials this excised patch contained one active copy of the 110 pS K^+-channel (see above) and additionally one active copy of the 220 pS channel (see Fig. 2A). In Fig. 2B the membrane voltage was changed with a sweep-rate of 100 mHz between −70 mV and 70 mV. The 110 pS K^+ channel remained open during the time interval shown in Fig. 2B and from extrapolation of the current-voltage relationship of the closed and open state the zero-current potential of this particular ion conductance was determined to be the Nernst potential for K^+ (−27 mV, open arrow). While the 220 pS ion channel opened at a membrane voltage of ~ 55 mV and the extrapolated zero-current potential (filled arrow) of this ion channel was ~ 17 ± 3 mV. This shows that the 220 pS conductance channel is preferentially permeable to Cl^- with a selectivity of about 4 for anions over cations as calculated from the Goldman-Hodgkin-Katz equation.

It is worth noting that a plot of the single-channel currents versus applied voltage for the '220 pS channel' also revealed a linear relationship between +40 mV and −40 mV (data not shown).

Fig. 3A (left) shows single-channel currents recorded from an inside out patch excised from giant thyl-

akoid/liposome vesicles. The patch is bathed symmetrically in 100 mM $CaCl_2$ solutions with a holding potential of 30 mV. The corresponding all points amplitude histogram of the single-channel currents is shown in the right part of the figure. Single-channel openings are displayed as upward current deflections. The figure shows single-channel openings with a mean open channel current amplitude of 0.6 pA (see amplitude histogram) which corresponds to a unit conductance of 20 pS. In order to determine the ion specificity of this particular ion channel conductance measurements in asymmetric Ca^{2+}-solutions were performed. They are documented in Fig. 3B, which shows currents recorded from the inside out patch bathed in asymmetric Ca^{2+}-gluconate solutions in response to a series of voltage sweeps (50 mHz) from −100 to +100 mV. Extrapolation of the current-voltage relationship of the closed and open channel revealed a zero-current potential of −13 mV, the Nernst potential for Ca^{2+} ions. Therefore, the ion channel with a unit conductance of 20 pS observed in giant thylakoid/liposome vesicles is selective for Ca^{2+} ions. Additionally, the Ca^{2+} channel revealed significantly smaller open channel current amplitudes which corresponded to a conductance of approx. 10 pS.

In further experiments we tested whether the giant thylakoid/liposome vesicles also contain ion-channel conductances permeable for Mg^{2+} ions. Fig. 4A shows

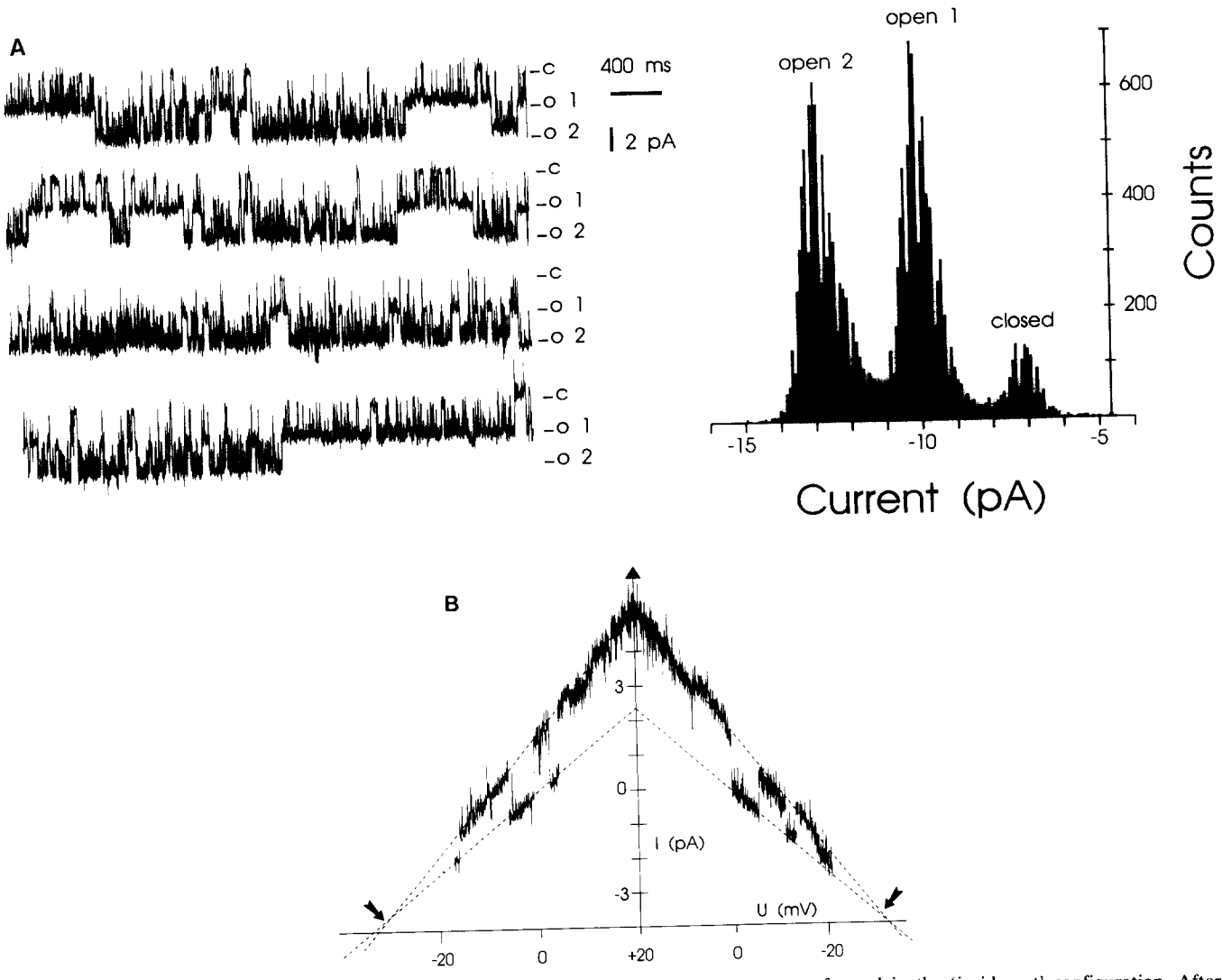

Fig. 1. Single-channel currents mediated by K^+-permeable channels. Measurements were performed in the 'inside-out' configuration. After gigaseal formation membrane patches were excised from giant thylakoid/liposome vesicles (see materials and methods section). (A) (left) Current-traces at a holding potential of −25 mV. Measurements were carried out in a buffer solution containing 100 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0) either in the pipette and in the bath. Fig. 1B shows a current-trace under asymmetrical salt concentrations on both sides of the membrane. The pipette contained 100 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0) whereas the bath solution contained 30 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0). The holding potential was changed at a sweep rate of 100 mHz between −20 and +20 mV. Current and voltage were monitored and the time axis was converted to the respective voltage.

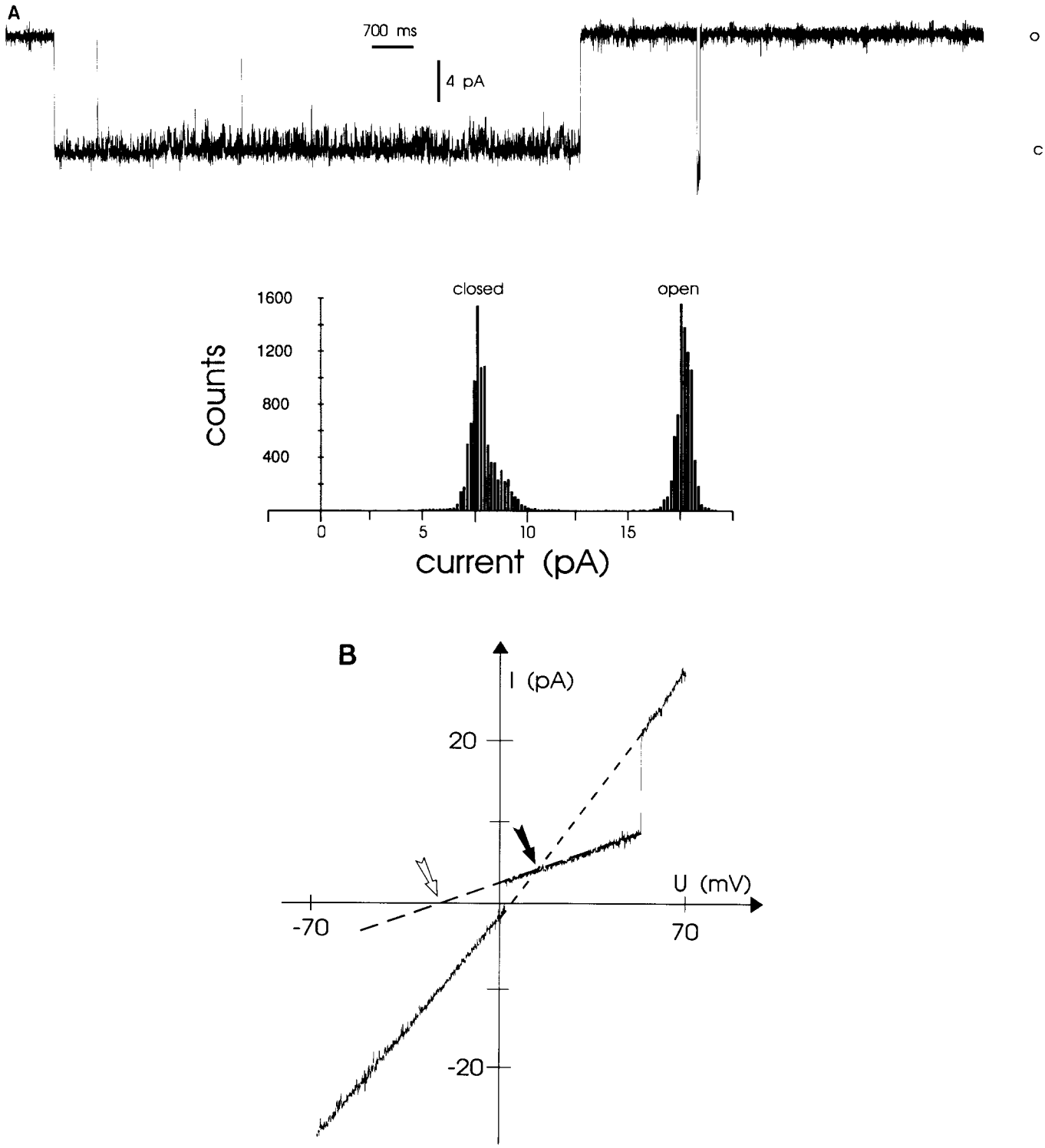

Fig. 2. Single-channel currents mediated by Cl^--permeable channels. Measurements were performed in the inside-out configuration. After gigaseal formation membrane patches were excised from giant thylakoid/liposome vesicles (see materials and methods section). In (A) (top) a single-channel current-trace at a holding potential of +30 mV is shown, the corresponding amplitude histogram being shown at the bottom. The buffer solution contained 100 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes/Tris pH 7.0 symmetrically on both sides of the membrane patch. (B) A current-trace under asymmetrical salt concentrations on both sides of the membrane. The pipette contained 100 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0), whereas the bath solution contained 30 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0). The holding potential was changed at a sweep rate of 100 mHz between −70 and +70 mV. Current and voltage were monitored and the time axis was converted to the respective voltage.

single-channel currents recorded from an inside out patch excised from giant thylakoid/liposome vesicles. The patch is bathed in 40 mM Mg^{2+}-gluconate on the bath side and 80 mM Mg^{2+}-gluconate solution on the pipette side at a holding potential of −70 mV. The corresponding all points amplitude histogram of the single-channel currents is shown in the right part of the figure. Single-channel openings are displayed as downward current deflections. Different conductance levels of 12 pS and 38 pS were observed. Fig. 4B shows the current recordings from an inside out patch bathed in asymmetric Mg^{2+}-gluconate solutions in response to a series of voltage sweeps. Single-channel openings are displayed as downward current deflections. Extrapolation of the current-voltage relationship of the closed and open channel revealed a zero-current potential of −9 mV, close to the Nernst potential for Mg^{2+}. This shows that the ion channel with a unit conductance of 38 pS observed in giant thylakoid/liposome vesicles is permeable to Mg^{2+} ions. Again, as observed for the Ca^{2+}-channel currents, significantly smaller open channel currents were observed with Mg^{2+} currents which corresponded to a unit conductance of about 12 pS.

Moreover, first experiments with asymmetric (Ca^{2+}/

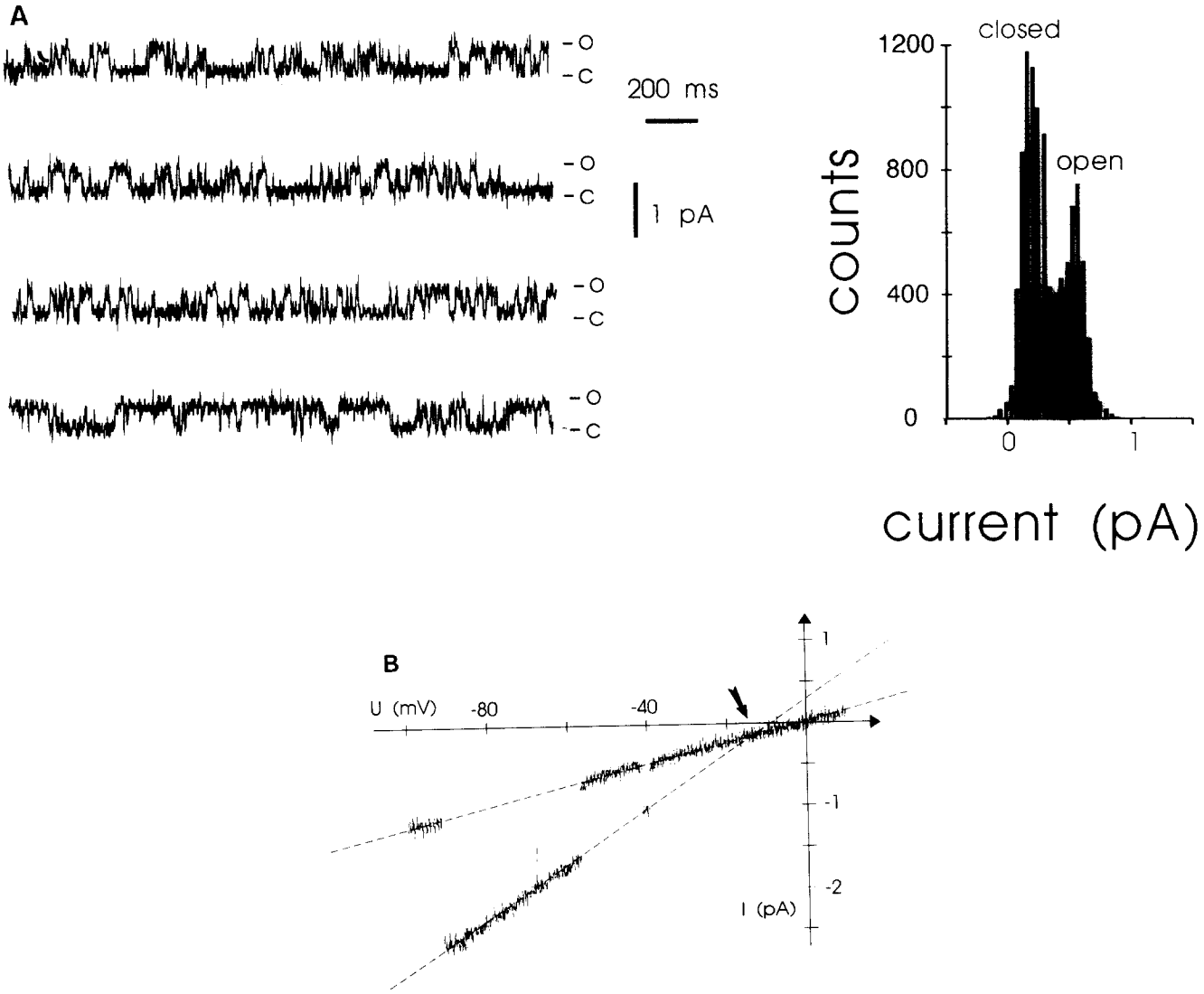

Fig. 3. Single-channel currents mediated by Ca^{2+}-permeable channels. Measurements were performed in the inside-out configuration. After gigaseal formation membrane patches were excised from giant thylakoid/liposome vesicles (see materials and methods section). In (A) the current-trace (left) at a holding potential of +30 mV and the corresponding amplitude histogram (right) are shown. The electrolyte solution contained 100 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0) symmetrically on either side of the membrane. (B) A current-trace under asymmetrical salt conditions on both sides of the membrane. The pipette contained 60 mM Ca-gluconate, 2 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0), whereas the bath solution contained 30 mM Ca-gluconate, 1 mM $CaCl_2$, 5 mM Hepes/Tris (pH 7.0). The holding potential was changed at a sweep rate of 50 mHz between −100 and +100 mV. Current and voltage were monitored and the time axis was converted to the respective voltage.

Mg^{2+}) buffers on both sides of the membrane indicate that the Mg^{2+} and Ca^{2+} ion pathways in the thylakoid membrane are provided by the same channel protein.

In Table I the results of our patch-clamp measurements on giant thylakoid/liposome vesicles are summarised. This table shows that we were able to detect three different single-channel ion conductances in the membrane of giant thylakoid/liposome vesicles, which

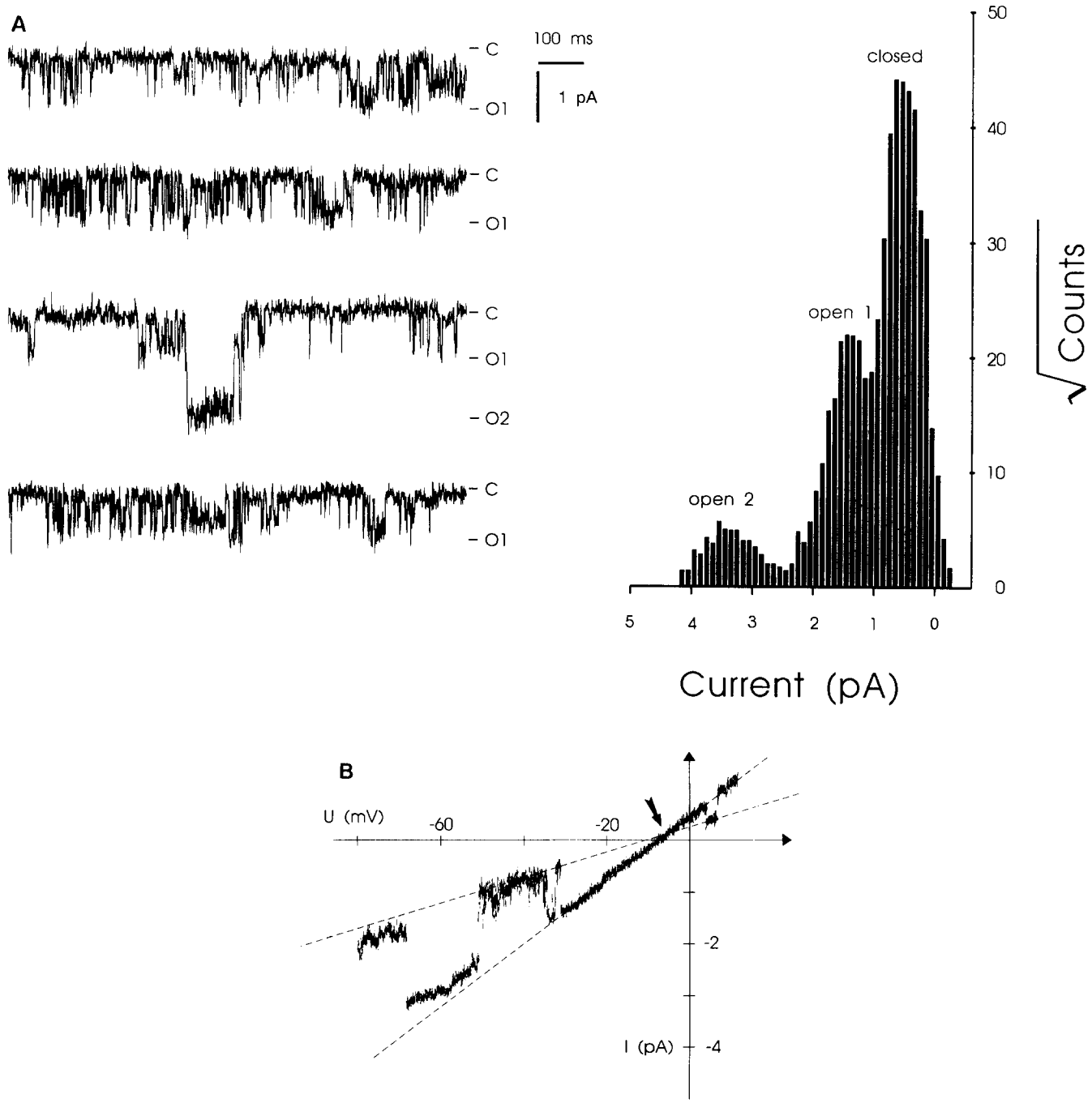

Fig. 4. Single-channel currents mediated by Mg^{2+}-permeable channels. Measurements were performed in the inside-out configuration. After gigaseal formation membrane patches were excised from giant thylakoid/liposome vesicles (see Materials and Methods section). In (A) current-traces at a constant holding potential of −70 mV (left) with the corresponding amplitude histogram (right) are shown. In the amplitude histogram the square root of the counts were plotted in order to improve the visual resolution of the 'open 2' peak in respect to the other one. The buffer solutions contained 80 mM Mg-gluconate, 2 mM $CaCl_2$, 10 mM Hepes/Tris (pH 7.0) in the pipette and 40 mM Mg-gluconate, 1 mM $CaCl_2$, 5 mM Hepes/Tris (pH 7.0) in the bath. (B) A current-trace under the same asymmetrical salt concentrations on both sides of the membrane as in (A). The holding potential was changed at a sweep rate of 50 mHz between −80 and +80 mV. Current and voltage were monitored and the time axis was converted to the respective voltage.

TABLE I

Summary of ion channels observed in giant thylakoid / liposome vesicles

A summary of different ion channels observed in giant thylakoid/liposome vesicles with respect to their unit conductance and ion-selectivity is presented. The unit conductances listed in the table are slope conductances obtained from the linear part of the current-voltage relationship for the single channel conductance and n is the number of independent measurements. The selectivity was estimated by determination of the zero current potential with asymmetric ion concentrations on both sides of the membrane (for details see Results section).

Number	Unit conductance (pS)	Selectivity
1	$220 \pm 19\%$ ($n = 7$)	Cl^-
2	$110 \pm 17\%$ ($n = 12$)	K^+
3	$35 \pm 11\%$ ($n = 5$)	Mg^{2+}
	$20 \pm 15\%$ ($n = 5$)	Ca^{2+}

could be discriminated on the basis of their unit conductance and ion-specificity.

Discussion

Using the patch-clamp technique we have identified three different types of voltage-dependent ion pathway in the membrane of giant thylakoid/liposome vesicles. A putative potassium channel with a conductance of about 110 pS (in 100 mM KCl), a chloride channel with a conductance of approx. 220 pS (in 100 mM KCl); the selectivity for Cl^- over K^+ was calculated from the Goldman-Hodgkin-Katz equation to be about 4. The third type of putative ion channel observed was permeable to Ca^{2+} and Mg^{2+} ions with conductances of 20 pS for Ca^{2+} and 35 pS for Mg^{2+}, respectively. Our results presented in the previous section indicate that these specific ion pathways are constituted by at least three different proteins.

We started our investigation with patch-clamping of swollen 'giant' thylakoid vesicles, which had been shown to be still competent in photophosphorylation [23]. However, this approach was not successful, since we were not able to obtain high resistance ($G\Omega$) seals in a reproducible manner. Although we systematically varied the experimental conditions to improve the reproducibility of the $G\Omega$ seal formation, so far the only possibility to make the thylakoid membrane accessible to patch-clamp measurements with a signal to noise ratio suitable to resolve single-channel currents, was the fusion of thylakoid vesicles with azolectin liposomes. One of the major reasons for this difficulty is probably the unusual high protein/lipid ratio in the thylakoid membrane. Depending on the source, about 60–80% (by wt.) of the thylakoid membrane of higher plants are proteins (for a review see Ref. 24). Secondly, the lipid composition of the thylakoid membrane which contains mainly galactolipids [24] is probably another reason for the difficulties in obtaining a high-resistance seal. These conclusions are supported by the fact that after fusion of the thylakoid membrane with azolectin liposomes and enlargement of these fused vesicles to giant thylakoid/liposome vesicles $G\Omega$-seals were obtained easily and were reproducible. It has been shown previously that during the preparation of giant liposomes by partial dehydration/rehydration technique the characteristic properties of ion channel proteins were retained [21,25]. In these cases intrinsic membrane ion channel proteins were used. In contrast to these results it has been concluded that the dehydration/rehydration technique destroyed the reconstituted chloroplast ATPase (CF_0CF_1), which gave rise to the formation of various cation channels by the subunits of the membrane part CF_0 in giant liposomes [17]. In the latter case a protein complex with a large extrinsic water-soluble part (about 80% by wt.) is concerned and it seems likely that this protein complex is less stable during the dehydration/rehydration procedure. We therefore expect the applied preparation procedure to be a suitable method to study reconstituted ion channel proteins as long as intrinsic membrane proteins are concerned [21,25].

Voltage-sensitive Cl^--selective ion channels have been found previously by patch-clamping the membrane of osmotically swollen giant thylakoids of *Peperomia metallica* [15]. These channels had a conductance of approx. 110 pS (in 110 mM KCl) and in asymmetric KCl solutions across the membrane the zero current potential was the Nernst potential for Cl^- [15]. We also found a voltage-dependent Cl^--selective channel in the membrane of giant thylakoid/liposome vesicles. This channel however revealed a higher unit conductance, approx. 220 pS in 100 mM KCl, and a lower selectivity (approx. 4:1) for anions over cations. Although these differences between the two 'chloride channels' are significant, both ion channels are still comparable considering their possible physiological function. The difference could be due to the different lipid environments. It has been shown previously that differences in the lipid environment may alter single-channel conductances [26].

By fusing spinach thylakoid vesicles into planar lipid bilayers voltage-dependent channels with potassium selectivity have been detected previously [16]. These channels revealed a conductance of 14 pS ('low' conductance channel) in 500:300 KCl and a second 'high' conductance channel with a conductance of 120 pS in 1000:300 mM KCl. This high conductance potassium channel is comparable to the one we observed (110 pS in 100 mM KCl), provided saturation of the channel current is achieved at 100 mM KCl. One may argue that the observed cation channel in the giant thyl-

akoid/liposome vesicles could be attributed to the disrupted membrane part of the chloroplast ATPase (CF_0) as observed previously [17]. However, this is very unlikely, since we never detected the large variety of conductance levels typically observed for disrupted CF_0 or the reconstituted subunit III [17,18,27].

In contrast to our results, in neither of the above reports [15,16] on ion channels in the thylakoid membrane have any channels permeable to divalent cations (Mg^{2+}, Ca^{2+}) been reported. Since the Ca^{2+} concentrations in chloroplasts have been shown to be rather low under physiological conditions [28,29] and higher Ca^{2+} levels have been shown to inhibit stromal enzymes [28] Ca^{2+} fluxes should be less important for counterbalancing proton pumping in thylakoids. However, considering previous reports on the efflux of Mg^{2+} from the thylakoid lumen counterbalancing proton pumping [6,8,11–13] and the importance of the Mg^{2+} concentration for the regulation of the stromal enzymes [30,31], the observed channel currents carried by Mg^{2+} and Ca^{2+} are very interesting observations. They are the first evidence for the existence of an Mg^{2+} channel in the thylakoid membrane, which could provide an important direct link between the light and dark reactions of photosynthesis.

We have demonstrated the existence of channel mediated ionic pathways for Mg^{2+}, Ca^{2+}, K^+ and Cl^- across the thylakoid membrane. From this we would expect that, except for Ca^{2+}, all of them participate in counterbalancing proton accumulation in the thylakoid lumen. Several lines of evidence coming from flux measurements [6,8,11–13] and analysis of ionic composition [12,28,29] of chloroplasts have led to the conclusion that in situ proton uptake of thylakoids in the light is predominantly balanced by the efflux of Mg^{2+} [8]. In this report we provide additional evidence for this conclusion by demonstrating the existence of an ion-channel-mediated pathway for Mg^{2+} in the thylakoid membrane.

However, K^+ efflux mediated by the putative potassium channel with an conductance of 110 pS would also allow the balancing of the proton charges accumulated in the thylakoid lumen. The uptake of Cl^- as counter-ion for proton pumping should be less important, since the influx of higher concentrations of Cl^- through the putative Cl^- channel would increase the internal osmolarity of the thylakoid vesicles and increase the volume of the lumen but swelling of thylakoids in intact chloroplasts in the light is normally not observed [32].

Calculations of the H^+ flux during light-induced electron flow to establish the steady-state pH gradient lacks from the unknown values for the final pH in the thylakoid lumen, its buffer capacity and volume [5,8,16]. However, considering a maximal pump rate of 1000 H^+/s per electron transport chain under cyclic (PMS-mediated) electron flow [33,34] and 10^6 electron transport chains per thylakoid vesicle (for a spherical vesicle with a radius of 10 μm [35], 2.2 nm^2/chlorophyll and 500 chlorophyll/electron transport chain [24]) one obtains a flux of 10^9 H^+/s as the upper limit. In order to keep the membrane potential in the range of 10 mV positive inside this flux has to be balanced by counter-flowing ions as long as no protons are used in photophosphorylation. At 10 mV a 100 pS channel would allow a current of 1 pA, equivalent to approx. 10^6 monovalent ions/s. Provided this 100 pS channel has an open probability of $P_O = 1$, 1000 copies of this channel would be necessary per thylakoid vesicle to account for the required counterflux of ions. This calculation may easily be extended for a more realistic open probability (e.g. $P_O = 0.1$) and divalent cations to be transported which will end at approx. 5000 copies of a respective Mg^{2+} channel/thylakoid vesicle equivalent to a surface density of about 2 copies/μm^2 as an upper estimate.

The above rough estimation shows that the transport capacity of the three observed putative ion channels in the thylakoid membrane meets the requirements to balance the accumulated proton charges. Light driven proton pumping in thylakoids can be subject to fast changes in the rate upon changes of light intensity. To keep the membrane potential at constant low values (about 10 mV positive in the lumen) during these changes in proton pumping an efficient regulation is required. The above channels would enable rapid and efficient regulation of the thylakoid membrane potential upon changes in the rate of proton pumping.

To date, ion-channel proteins have not been identified in the thylakoid membrane and they are not considered in the classical picture of the energy coupling membrane of thylakoids, although they could play a significant physiological role. In mitochondria a porin like channel the so called VDAC (voltage-dependent anion channel) channel protein of the outer membrane has been identified and characterised in detail. Additional high conductances have also been identified in the inner mitochondrial membrane. However, so far no possible physiological function has been ascribed to these latter putative ion channels (see Ref. 36 for a mini-review).

Our further work is intended to identify the protein components which constitute ion channels in the thylakoid membrane and to investigate the ion specificity and regulation of these channels in more detail.

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