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# Single channel recording in the chloroplast envelope

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The patch-clamp technique was applied to study ion channels in the intact chloroplast envelope. Three channel types were characterized: two cation-selective, with a conductance (in 100 mM KCl) of 517 and 1016 pS, respectively, and one anion-selective with a conductance of 159 pS. All three channels showed voltage-dependent closures at both positive and negative membrane potentials.

Chloroplast; *Nitellopsis obtusa*; Ion channel; Patch-clamp

## 1. INTRODUCTION

Chloroplasts as well as mitochondria are surrounded by two envelope membranes, which are usually in close proximity. The barrier function is inherent in the inner membrane, which contains transporters for a selected number of low molecular weight substrates; some of the organelle proteins, however, must be imported from the cytoplasm, which calls for the specific transfer mechanism, probably involving contact sites between the two membranes [1,2]. The outer membrane of both organelles is permeable to the substances with a molecular mass of up to several kilodaltons due to the presence of specific pore-forming proteins. Properties of these channels were mostly investigated after reconstitution in artificial membranes [3,4]. More recently the patch-clamp technique was applied to study the ion transfer properties of both mitochondrial membranes and of contact sites [5–10]. This paper demonstrates the feasibility of applying this method to make a tight seal recording with intact chloroplasts, and reports the properties of single channel currents observed in these experiments.

## 2. MATERIALS AND METHODS

Leaf cells (3–5 cm long) of a Charophyte *Nitellopsis obtusa* were allowed to stand overnight in APW (in mM: 1 NaCl, 0.1 KCl, 0.5 $CaCl_2$, 1 TES-NaOH (pH 7.2)). Prior to the experiment both cell ends were cut off and cell sap, containing large (around 10 μm in diameter) chloroplasts, was released into the bath solution, containing (in mM): 100 KCl, 1 $CaCl_2$, 5 TES-KOH (pH 7.2). A small portion of this mixture was transferred into a chamber filled with the same solution. A standard patch-clamp technique was used further for detection of single channel currents in the chloroplast envelope. Pipettes with a tip diameter of 4–5 μm were pulled from soft glass in two steps. Electrodes were further intensively fire-polished, and those with the narrowest cross-section of around 1 μm (10–15 MΩ resistance with 0.1 M KCl inside) were selected and filled with the same solution as in the bath. To study the channels selectivity, concentration of KCl in the bath was either raised (to 500 mM) or lowered (to 20 mM). 185 mM mannitol was added to the latter solution to adjust osmolality. Records were filtered at 1 kHz, stored on magnetic tape (TEAC MRC-10e) and analyzed with an IBM AT-compatible computer using Strathclyde Electrophysiological Data Analysis software (courtesy of J. Dempster, University of Strathclyde, Scotland). The sign of the membrane potential given throughout refers to that of the chloroplast exterior.

## 3. RESULTS

Patch-clamping of the intact chloroplast envelope revealed a broad set of conductance steps ranging from a few tens of pS up to around 1 nS in 100 mM KCl. Patch excision apparently caused no changes in the channel's behaviour, thus, the major part of the experiments was done in the inside-out patch configuration. It appears that some patches contained more than one channel type, and it was not always possible to distinguish between different channels in this case. Thus, only the patches containing single channels of the same type are considered here.

Fig. 1 shows voltage dependence and selectivity of the most frequently detected channel. It has a conductance of 517 ± 8 pS ($n = 28$) in symmetrical 100 mM KCl. The channel was mainly in the maximally open state in the potential range of ±16 mV (Fig. 1A). At larger potentials of either sign, the channel is forced to flicker between close and open states or to switch to the lower conductance states (Fig. 1A, insert). Finally, it closed completely when the potentials of about ±50 mV were applied. In contrast to the slow voltage-dependent channel closure, its reopening while returning to zero voltage was rapid, thus, allowing repetitive voltage stimulation. The voltage dependence was asymmetric in

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*Abbreviations:* TES, *N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid; $E_r$, reversal potential.

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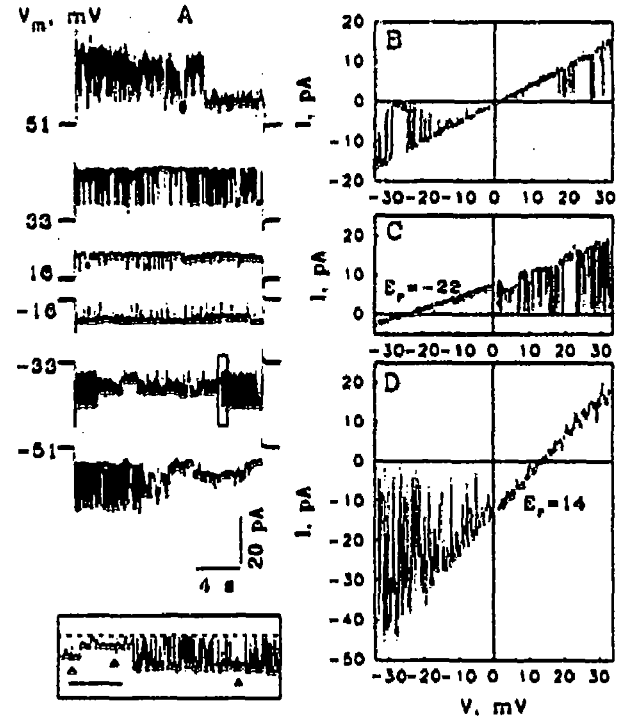

Fig. 1. Examples of voltage-dependent kinetics and open state current–voltage (*I/V*) relationships of the large conductance cation channel in the chloroplast envelope. (A) Single channel kinetics in response to 17 s voltage steps from 0 mV to different potentials as indicated. The insert shows part of the record at −33 mV at higher time resolution (bar = 200 mS); subconductance states of 105, 250 and 380 pS are marked by triangles. Symmetrical 100 mM KCl. (B–D) *I/V* relationships of the same channel in symmetrical 100 mM KCl (B) and when the concentration of KCl in the bath was either lowered to 20 mM (C) or raised to 500 mM (D). The applied voltage was changed linearly (13.3 mV/s) from −33 to 33 mV. The leak current was estimated in the neighboring records with a low channel activity and further subtracted.

the sense that channels closed less frequently at positive potentials than at negative ones of the same magnitude. The ion selectivity was determined by measuring the reversal potentials for single channel currents in asymmetrical KCl solutions (Fig. 1B). In 100/20 mM and 100/500 mM KCl solutions the reversal potentials, averaged for [illegible] patches, were −22.2 ± 0.9 mV and 13.0 ± 1.1 mV, respectively. The latter value could be an underestimate, however, due to possible contribution of the streaming potential since the large (≈0.8 OsM) osmotic gradient was present across the patch membrane [11]. Thus, the value obtained with the 20 mM KCl solution was taken, giving a permeability ratio $P_{K^+/Cl^-}$ = 4.6 ± 0.3.

An anion-selective channel with a conductance of 159 ± 5 pS ($n$ = 16) in 100 mM KCl was the other frequently detected channel type. Usually, 2 to 4 individual channels were present in the patch (Fig. 2A–D). Relative permeability $P_{Cl^-/K^+}$ = 12 ± 3 ($n$ = 6) was calculated from single channel current reversal potentials ($E_r$ = −29.5 ± 1.4 mV) measured in asymmetrical 100/500 mM KCl solution; the channel substrate showed the same selectivity (Fig. 2c). At zero voltage and under symmetrical 100 mM KCl conditions, the channel activity was about half-maximal as estimated from initial responses to small voltage steps (as ±16 mV in Fig. 2A). Stepping the membrane potential to negative values caused channel activation followed by inactivation (Fig. 2A). The steady-state channel activity, however, was larger at negative potentials than at positive ones of the same magnitude. Thus, the asymmetry of voltage dependence was opposite to that found with the cation-selective channel above. The channel activity increased (in 6 patches out of 6 examined) when the KCl concentration in the bath was raised to 500 mM (Fig. 2B,C). Therefore, this channel could be involved in osmoregulation. A similar role is suspected for the 100 pS anion channel from the inner mitochondrial membrane [5].

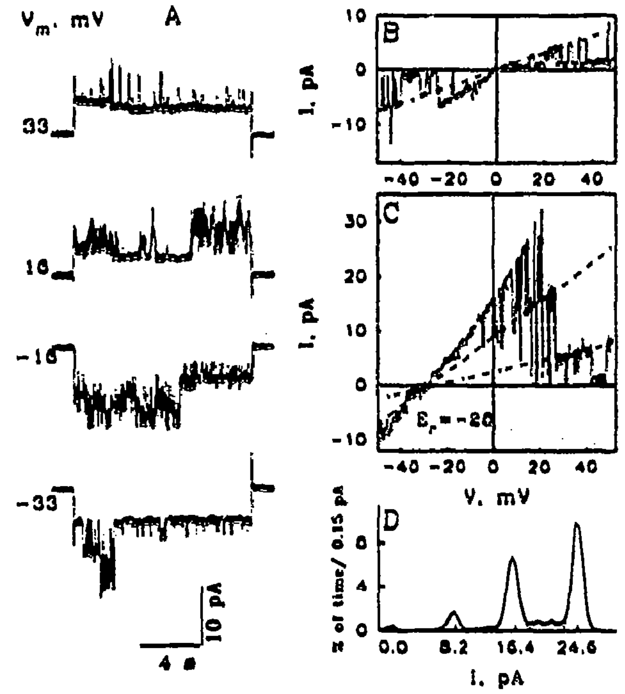

Fig. 2. Voltage-dependent behaviour, conductance and selectivity of the chloroplast anion channel. (A) The potential was repetitively stepped from 0 mV to various potentials as indicated for 12 s and the activity of up to 4 individual channels could be detected. Symmetrical 100 mM KCl. (B,C) *I/V* relationships measured in the patch containing 2 channels. Symmetrical 100 mM KCl (B) and 500 mM KCl in the bath (C). Dashed lines indicate the levels with one of two channels opened and in (C) also the substate level showing similar selectivity. Leakage was subtracted from the records as in Fig. 1. The potential was continuously increased in the form of ramp wave (40 mV/s). (D) Amplitude distributions of single channel currents measured over a 30 s interval at 0 mV in asymmetrical 100/500 mM KCl. The data are from different patches and indicate the presence of three independent anion channels with the same conductance and selectivity. The open channel probability was 0.8 as calculated from binomial distribution fitted to the data.

In addition, channels of very large conductance (maxi-channels, 1016 ± 27 pS in symmetrical 100 mM

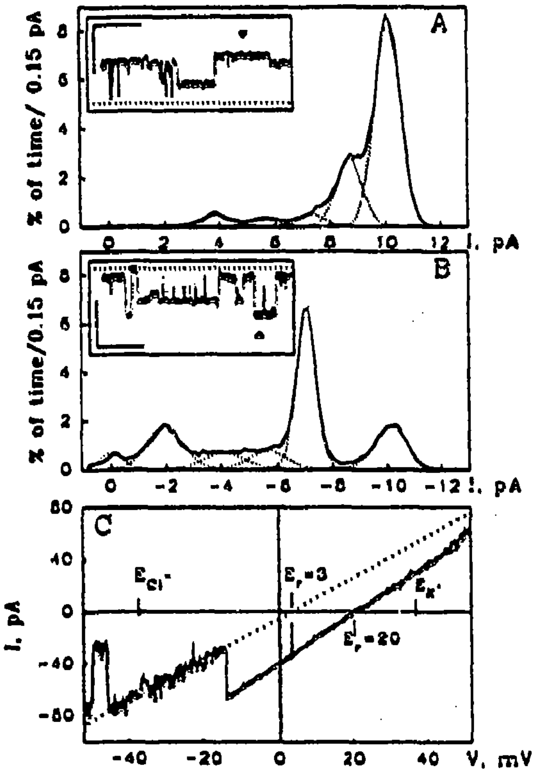

Fig. 3. Representative recordings of the maxi-conductance channel. (A,B) Amplitude histograms of single channel currents measured at 10 mV (A) and −10 mV (B) in symmetrical 100 mM KCl. Records of approx. 1 min duration at each potential were analyzed. Amplitude distributions were fitted by a sum of gaussians. Mean peak positions are correspondent to the conductance states of 1020, 890, 720, 570, 400 and 180 pS. Time-averaged conductance of the channel at 10 and −10 mV in this experiment was 90 and 61% of maximal, respectively. Inserts show examples of current recordings at corresponding potentials; triangles indicate the channel in the fully open state. Calibration: 10 pA (vertical bar), 400 ms (horizontal bar). (C) Example showing different selectivity of the main and intermediate conductance states (500 mM KCl in the bath and 100 mM KCl in the pipette). Slope conductance in the main and intermediate states was 2100 and 1600 pS, respectively. The voltage protocol was the same as in Fig. 2B. Leak conductance (not subtracted here) was ≤120 pS as estimated from the channel closures at the potentials above the equilibrium points for $K^+$ and $Cl^-$ (±36.9 mV). Linear extrapolation of the *I/V* curve of the channel substate to the positive potential range gives the intercept with the *I/V* curve of the main open state at 85 mV.

KCl, $n = 9$) were recorded. Similar to the 517 pS channel described above, maxi-channels showed voltage-dependent closure to lower conductance states at large (>30 mV) positive or negative potentials. This behaviour was partially evident even at small steps from zero voltage (Fig. 3A,B, inserts). By careful inspection of amplitude distributions (Fig. 3A,B) it can be seen that a 1 nS conductance state is likely to be attributed to the single channel event rather than to unresolved multiple transitions of several independent channels. First, although the transitions between open and completely closed states were not frequently seen, the large conductance steps (≥700 pS) were often recorded. On the other hand, the lower conductance events down to 180 pS have been observed. If one attributes these events to the transitions of independent channels, their possible juxtapositions would lead to a broad distribution of amplitude peaks, which is inconsistent with a clear lack of an expected conductance level of around 900 pS at −10 mV (Fig. 3B). In addition, it was found that the ion selectivity is changed from cation to non-selective when the channel is closed to a subconductance state (Fig. 3C). Alternatively, one may attribute the observed decrease in slope and selectivity to the closure of an individual high selective channel. This assumption, however, could not be valid, since the reversal of this channel would occur at round 85 mV, which is well above the equilibrium potential range (±36.9 mV) for the ions present. Similar changes in selectivity upon channel transitions to subconductance states have been previously demonstrated with the mitochondrial outer membrane channel [4].

## 4. DISCUSSION

This study showed that high resistance seals (usually, in the range of 3–10 GΩ) are attainable with intact chloroplasts. Moreover, there were usually one or a few channels to be detected both in the chloroplast-attached and inside-out patches. This result is quite different from that obtained with intact mitochondria, where the seal resistances were rather low due to the presence of a few thousands of large conductance pores per 1 μm² area of the outer membrane [6]. While comparing reconstitution procedures with the chloroplast and mitochondrial porins, a substantially larger concentration of the outer membranes was required in the former case to produce the same channel activity in lipid bilayers in the latter one [3,12]. Providing it is a consequence of lower concentration of pores in the chloroplast outer membrane as compared with mitochondria, the concentration of porin within the membrane of a few hundreds per 1 μm² can be calculated. This value, however, is too large to account for the gigaohm (GΩ) resistance seals obtained here, providing the patches consist of the outer membrane alone. Alternatively, a sandwich-like structure of the patch formed by two adjacent envelope membranes is conceivable. The overall specific conductance of the patch in this case would be limited by the conductance of the less permeable inner membrane and of the contact sites between membranes. This proposal is consistent with the fact that, in contrast to mitochondria, it has not been possible so far to remove the chloroplast outer envelope membrane without lysis of the organelle [1].

Are there any other possible recording configurations which may account for some of the results obtained here? Recently, the patch-clamp technique was applied to study photocurrents in isolated chloroplasts [13] and it was proposed that the electrode had a low resistance access to the thylakoid lumen in these experiments (the envelope membranes were suggested to be leaky). However, in contrast to the present study, the seal resistances in the previous report did not exceed 100 MΩ. Thus, it seems unlikely that after obtaining a tight seal contact with the chloroplast envelope here, the membrane had to be ruptured and the pipette resealed with the thylakoid membrane, although this possibility cannot be definitely ruled out at present. Moreover, Schonknecht et al. (1988) reported the 110 pS channel, presumably originated from the thylakoid membrane, which had similar selectivity and voltage-dependent properties to these of the anion channel described here. However, the method used by these authors did not completely exclude the contamination of the preparation by other chloroplast membranes. Accordingly, the presence of the anion channel was not confirmed by reconstitution of isolated thylakoid membranes in lipid bilayers [15].

Two cation-selective channels described here had conductance values close to that of the 720 pS channel from the chloroplast outer envelope [3], although due to incomplete characterization of the latter channel it may not be possible to draw a straightforward conclusion on their possible relation. In mitochondria a number of large channels, with a conductance ranging from 250 to 900 pS (in 100 mM KCl) has been previously described, and these channels were proposed to be located in the inner membrane or in the contact sites [5–9]. In addition, the 450 pS channel with a clear porin activity, also resembling this of the channels described in this study, has been measured in the outer mitochondrial membrane by patch-clamp technique [10]. Further, porin is thought to play an important role in the communication between two mitochondrial membranes [2]. Using this analogy, I would tentatively suggest that the large conductance channels found here have a similar function in the junctions between the chloroplast membranes.

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