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One of the chloroplast envelope ion channels is probably related to the mitochondrial VDAC

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The voltage dependence of large conductance channels in the intact chloroplast envelope of *Nitellopsis obtusa* was examined using the patch-clamp technique. The channel switched to the lower conducting substates with amplitudes of around 45 and 20% of that in the open state when potentials larger than 30 mV were applied. The steepness of the voltage dependence approximately corresponds to 4 elementary charges being transferred across the entire voltage drop to close the channel both at positive and negative potentials. The transition to the closed substates could also be induced by König's polyanion, a well known modulator of the mitochondrial outer membrane channel.

Chloroplast; Ion channel; Voltage dependence; Patch clamp

1. INTRODUCTION

The outher membranes of chloroplasts and mitochondria are freely permeable to substances with a molecular mass of up to several kDa due to the presence of large hydrophilic pores in these membranes [1–4]. In mitochondria this pore is formed by a voltage-dependent anion channel (VDAC). The extensive study of this channel over the last 15 years revealed a comprehensive picture about its voltage-gating, selectivity, and molecular design [2–11]. Similar large conductance channels were found upon reconstitution of the chloroplast outer envelopes into a planar lipid bilayer [1]. Recently patch-clamp recordings from the intact chloroplast envelope have been performed, and three major channel types have been described [12]. One of these channels displayed a kinetic behaviour resembling that of VDAC, i.e. voltage-dependent closure at both positive and negative potentials. In the present paper this finding was substantiated by quantative analysis of the chloroplast ion channel voltage dependence whose parameters were found to be remarkably close to those typical for VDAC. Furthermore, it was found here that König's polyanion, a substance which is known to increase VDAC's voltage-dependence [13], affects the chloroplast ion channel gating in a similar manner.

2. MATERIALS AND METHODS

Chloroplasts were isolated from the leaf cells of *Mitellopsis obtusa* as described previously [12]. Pipette filling and bath solutions contained (in mM): 100 KCl, 1 $CaCl_2$, 5 TES-KOH (pH 7.2). In the experiments with the modulator, the bath solution contained an additional 6 mg/ml of König's polyanion, a copolymer of methacrylate, maleate and styrene (1:2:3), MW 10 kDa (generous gift of Dr. T.A. Mirzabekov). The volume of the experimental chamber was 150 μl, and the solution exchange in the bath was performed by perfusion of approximately 5 ml of medium with a flow rate of about 2 ml/s. Single-channel recordings were made using a patch-clamp amplifier (SKB 'Biopribor', Pushchino) in the inside-out mode. Records were filtered at 1 kHz, stored on magnetic tape and analysed with sampling intervals of 0.2 ms using Strathclyde SCAN and PAT programs (courtesy of J. Dempster, University of Strathclyde, UK). The voltages reported correspond to the pipette (the external side of the chloroplast envelope).

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3. RESULTS

In agreement with a previous study [12] the chloroplast envelope membrane patches generally contain different channel types, which can be discerned by their conductance, selectivity and voltage-dependent kinetics. Only patches containing one or two 520 pS (in 0.1 M KCl) channels were selected for further analysis. Stepping the membrane voltage from zero to positive or negative potentials caused channel transitions to the lower conductance substates. An example of such transitions occurring at ± 33 mV with a single channel containing patch is shown in Fig. 1. It can be seen from the figure that the channel displayed a somewhat asymmetric behavior depending on the sign of applied voltage. At +33 mV the current fluctuates between a completely open state and a lower conductance level of approximately 20% of the maximum, with rare transitions to the baseline. A similar conductance substate is also detected at −33 mV, but the transitions between completely open and closed states are also frequently detected, as well as transition to another stable substate

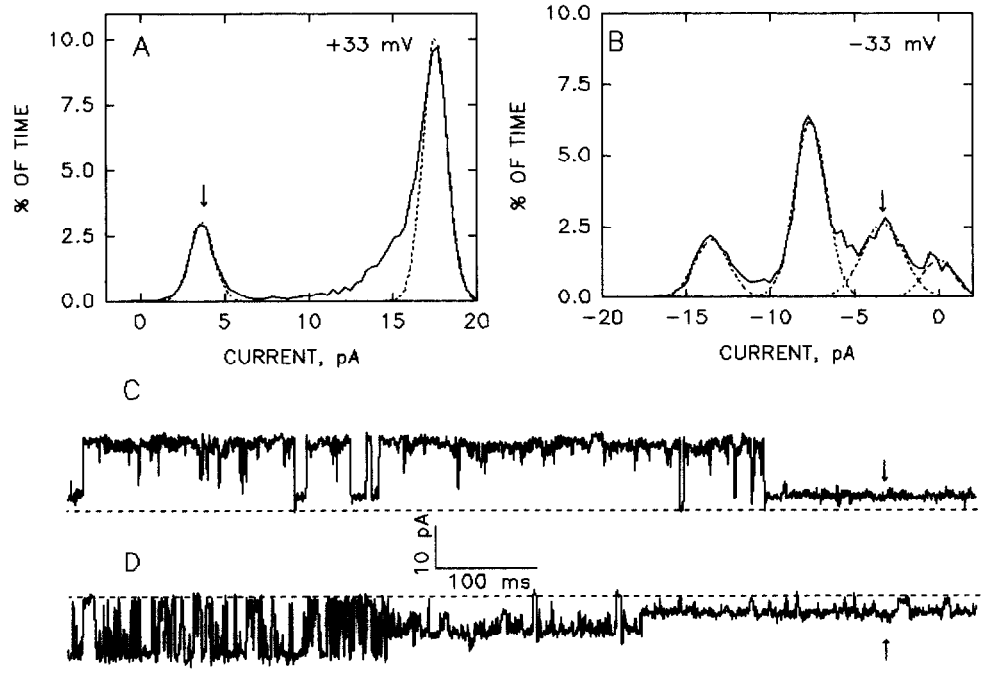

Fig. 1. Voltage induces the channel transition to lower conducting substates. (A,B) Amplitude histograms showing occupancy of current level as % of time (bin width was 0.33 pA) at +33 and −33 mV, respectively. The amplitude distributions were fitted by a sum of Gauss functions (dotted lines) with mean peak positions at: (A) 3.6 pA, 17.5 pA; (B) −13.5 pA, −7.6 pA, −3.3 pA and 0.1 pA. Both current traces were analyzed at 1 kHz with 0.2 ms sampling for 15 s. (C,D) Single-channel recordings from the same patch as in A and B. The voltage was stepped from 0 mV, and the first few seconds of the records where the channel was mainly in the open state are omitted. Arrows indicate the lowest detectable conductance substate. The patch resistance was 11 GΩ.

with an amplitude of around 40% of the maximum (see amplitude histograms, Fig. 1A,B). This substate, however, could also be detected with some samples at positive potentials (e.g. histogram in Fig. 3A). The mean conductance of the corresponding substates was determined (see Table I) by constructing amplitude histograms similar to these presented in Fig. 1 with a number of single channels containing patches at membrane potentials in the range of ± 50 mV. In addition, partly resolved flickering in the open state was observed both at positive and negative potentials, which caused an apparent decrease in the open-state amplitude. This effect is illustrated in Fig. 1A and B by the presence of a shoulder with the open-state peak at +33 mV and with the shift of corresponding peak to the right at −33 mV, respectively.

Although the channel voltage-dependent behavior found here was complex, a reasonable simplification could be derived if only transitions between the open state (O) and substates with a conductance of 20% of that at the open state (C_1, C_2) both at positive and negative potentials were assumed. The corresponding state diagram could be drawn as follows:

$$C_1 \underset{-V}{\overset{+V}{\rightleftharpoons}} O \underset{-V}{\overset{+V}{\rightleftharpoons}} C_2 \qquad (1)$$

The voltage-dependent equilibrium between C_1 or C_2 and O are given by Boltzmann's distribution as follows: $O/C_1 = \exp(z_1 eF(V-V_{o1})/RT)$, where z_1 is the effective gating charge, V the applied membrane voltage, V_{o1} is the voltage where the channel spends one-half of the

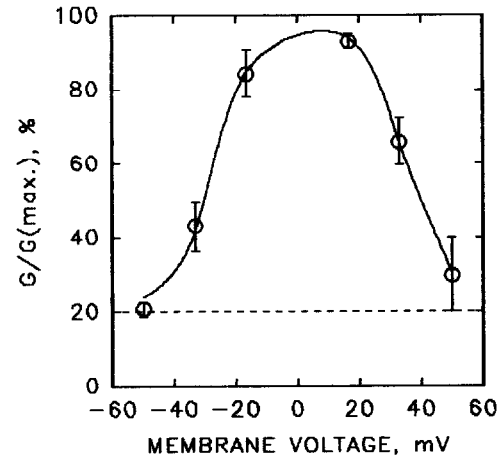

Fig. 2. The channel's conductance to voltage relation obtained with six patches containing one or two channels. The mean current was measured by averaging of 15–30 s of successful recordings at each potential, and the mean conductance (G) of the patch was calculated by dividing this value by the membrane voltage. The value of the mean conductance was then corrected by the leak conductance (the seal resistance for these patches was in the range of 3–11 GΩ), and expressed as % of maximal conductance (G_{max}) measured at 0 mV where all the channels were in the open state. The solid curve is fitted to the data according to Eq. 1 (see text), and the model parameters are given in Table I.

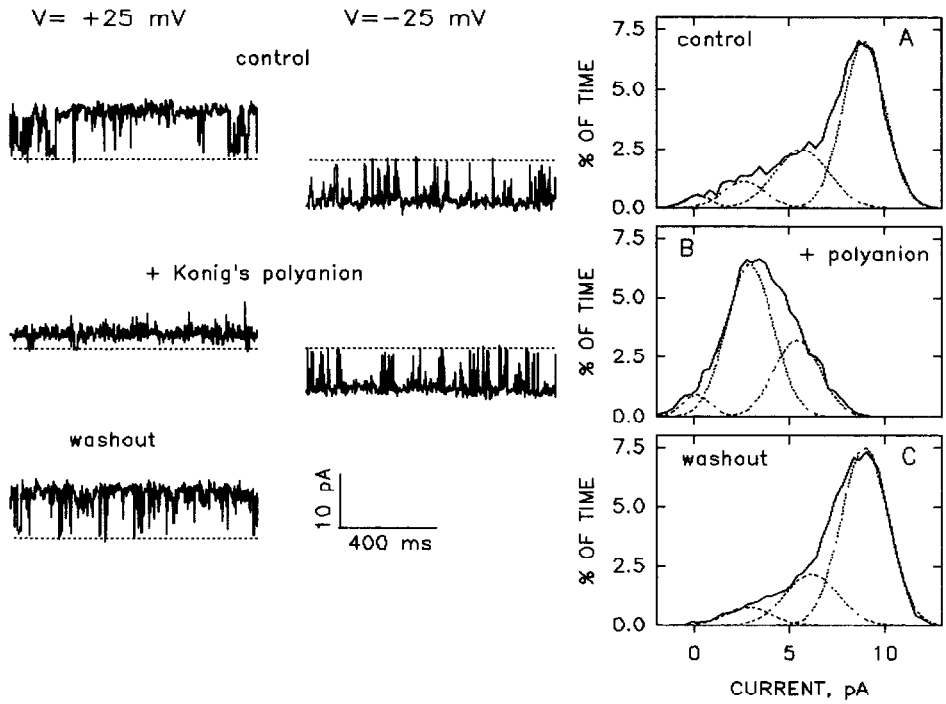

Fig. 3. König's polyanion increases the occupancy of lower conducting substates. Amplitude histograms in A, B and C were obtained by analysis of 20 s recording at +25 mV, or as in Fig. 1. The amplitude distribution was fitted by a sum of gaussians (dotted lines) with a mean peak position (in pA) and area under the peak (in brackets) as follows: (A) 0.0 (3%), 2.7 (10%), 5.7 (27%), 8.9 (60%); (B) 0.1 (6%), 2.9 (61%), 5.5 (28%); (C) 2.9 (7%), 6.1 (22%), 8.9 (71%). The current recordings in the left panel show that, in contrast to +25 mV, no effect of König's polyanion is observed at −25 mV. Polyanion (6 mg/ml in standard solution) was added to the bath, and the voltage corresponded to that within the pipette taken relative to the bath. The patch resistance was 5 GΩ.

time in the 'closed' substate, and e, F, R, T have their usual meanings. The normalized mean channel conductance averaged over 15–30 s of recording at given potentials was then fitted using the above scheme (Fig. 2). It could be seen that at leas in the range of ± 50 mV this model could satisfactory explain the observed voltage dependence. The best fitted values of $z_1(z_2)$ and V_{o1} (V_{o2}) are given in Table I.

Synthetic polyanion, which is known as König's polyanion, specifically increases the VDAC's probability of being in the low-conductance ('closed') substate when the side of polyanion application is made negative [14]. In this study König's polyanion was added to the bath (intrachloroplast membrane side). An example of its effect on the single channel kinetics is shown in Fig. 3. Without polyanion the channel is mainly in the fully open state both at +25 and −25 mV. When the polyanion was added to the bath, the channel was stabilized in the lower conductance substate(s) at +25 mV (plus in the pipette means that the bath side is made more negative) while no significant effect was observed at −25 mV. The above effect was readily reversible, and the initial channel's kinetics at +25 mV was restored during superfusion of the bath with a fresh portion of control solution containing no polyanion.

4. DISCUSSION

The results obtained in this study indicate that one type of chloroplast channel has a set of characteristics similar to those previously found in the outer mitochondrial membrane channel, VDAC (Table I). Namely, both channels can switch from open to less conducting ('closed') substates at both positive and negative potentials. The position of the switching region and the steepness of the voltage dependence are also in good agreement. An important characteristic of DVAC is its sensitivity to König's polyanion, and here it is found that this compound affects the chloroplast channel in a similar fashion.

There is, however, one difference between the two channels. Open VDAC channels have a weak anion selectivity ($Cl^-/K^+ \cong 2$) while the chloroplast channels are weakly cation-selective, with a K^+-to-Cl^- ratio of around 5 at neutral pH [12,15]. On the other hand, the 'closed' substate of VDAC is weakly cation-selective ($K^+/Cl^- \cong 3$) [3–4,11], and the lower conducting substates of the chloroplast channel are also cationic, with a minor increase in selectivity as compared to the open state (I. Pottosin, unpublished). The conductance of VDAC's 'closed' substate is averaged around 40% of

Table I

Properties of single channels studied by the patch-clamp technique in the chloroplast envelope and outer mitochondrial membranes

Preparation	Conductance (in 0.1 M KCl) (pS)		Parameters of voltage dependence (pH = 7.2)		Effect of König's polyanion	
	Open state	Lower conductance (close) state(s)	V_{o1} (V_{o2})	z_1 (z_2)	Increase of close state probability	Voltage dependence
Whole mitochondrial membranes of *Neurospora crassa* reconstituted into liposomes*	430***	133*** (31%)	−29 ± 5 (38 ± 6)	4.4 ± 0.9 3.9 ± 0.9	Yes	Yes
Intact chloroplasts of *Nitellopsis obtusa***	518 ± 8 (n = 8)	234 ± 4 (45%) 105 ± 3 (20%)	−26 ± 1 (35 ± 1) (n = 6)	3.9 ± 0.4 3.5 ± 0.4	Yes	Yes

*Data taken from [14]
**Data of this paper
***Recalculated from original values obtained in 0.15 M KCl

that in the open state in experiments with the channel reconstituted into planar lipid bilayers [3]. However, the investigation of VDAC properties in a wider range of potentials (± 100 mV) revealed two stable substates with amplitudes of 40 and 20% of the maximum respectively [10]. Therefore, the value of around 30% obtained using the patch-clamp technique by Wunder and Colombini [14] implied that these authors probably observed a mixture of the above 'closed' substates with their preparation. Similarly, at higher time resolution and with single channel containing patches, the above two 'closed' substates were demonstrated here with the chloroplast ion channel. The effect of König's polyanion on both chloroplast channels and VDAC could then be interpreted as a consequence of its predominant binding to the lower conducting substates, which will lead to a shift of the open–closed transition region to lower voltages. Hence, based on this pharmacological evidence, the molecular configurations of the 'closed' substates of these two channels appeared to be similar; this assumption is also supported by the closeness of their conductance and selectivity characteristics.

In the open state VDAC is thought to form a β-barrel with a hydrophobic side of β-sheets facing the lipid bilayer and charged residues facing the cavity of the large cylindrical pore [8,11]. Therefore, the selectivity of the open state is determined by the distribution of a large number of positive and negative charges along the pore, and the same charges are likely to contribute to the channel's voltage sensor [9–11]. At neutral pH the effective gating charge is around +4, in agreement with the net positive charge at the pore walls and anionic selectivity of the VDAC's open state [9–11]. Providing the chloroplast ion channel tertiary structure is close to that of VDAC, the open-state cationic selectivity of the former channel implied that the net charge within its pore is negative, and the voltage sensor would also be negatively charged. The latter prediction is a matter of further experimental testing; it would be expected, for instance, that, in contrast to VDAC, a decrease of pH will cause a decrease in the steepness of the chloroplast ion channel voltage dependence.

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