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A chloride-permeable channel from *Phaseolus vulgaris* roots incorporated into planar lipid bilayers

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Abstract

Ion channels are key participants in physiological processes of plant cells. Here, we report the first characterization of a high conductance, Cl^--permeable channel, present in enriched fractions of plasma membranes of bean root cells. The Cl^- channel was incorporated into planar lipid bilayers and its activity was recorded under voltage clamp conditions. The channel is voltage-dependent, excludes the passage of cations (K^+, Na^+, and Ca^{2+}), and is inhibited by micromolar concentrations of Zn^{2+}. The Cl^- conductance here characterized represents a previously undescribed channel of plant cells.

Keywords: Cl^--channels; Bean roots; Planar bilayers; Zn^{2+}-inhibition

In order to fix nitrogen, root cells of the bean *Phaseolus vulgaris* establish a symbiotic relationship with the soil bacteria *Rhizobium etli*. This biologically relevant process involves the exchange of signals between bacterial and root cells [1,2] that trigger ion fluxes (Ca^{2+}, K^+, and Cl^-) across the membrane of root hair cells [3]. The transport systems responsible for these fluxes are not yet well determined. Therefore, given that ion channels play a key role in signal transduction in all kinds of cells [4], we became interested in characterizing the ion channels present in the plasma membrane of bean root cells, in order to gain insight into the molecular events that take place during the symbiotic process.

Taking into account the fact that the cell wall hinders direct electrophysiological, patch-clamp recording, we have followed the alternative approach of incorporating the ion channels present in the plasma membrane of bean root cells into black lipid membranes (BLMs) to study them under voltage clamp conditions [5].

Here, we report the first description of a voltage-dependent, high conductance, and chloride-permeable channel present in the plasma membrane of bean root cells. The channel excludes the passage of cations (K^+, Na^+, and Ca^{2+}) and is inhibited by micromolar concentrations of Zn^{2+} ions.

Materials and methods

Enriched fractions of plasma membrane (PM) vesicles from bean roots were prepared as previously described [6]. The PM-enriched preparation shows ~38% vacuolar and ~2% mitochondrial membrane contamination, as assessed by measurements of ATPase-P [7], PPiase [8], and cytochrome *c* oxidase activities [9].

Black lipid membranes (BLMs) were formed from a 20 mg/ml solution of dyphytanoilphosphatidylcholine (DPhPC) (Avanti Polar Lipids) in *n*-decane (Merck), on a 250 μm diameter hole separating two Teflon compartments of a cell (Warner), by the brush technique of Muller and Rudin [10]. The front (*cis*) compartment, where PMs were added, was filled with (in millimolar): 300 XCl, 10 $CaCl_2$, and 10 Hepes-X, pH 7.2, where X stands for K or Na, as indicated. The rear (*trans*) compartment was filled with (in millimolar): 150 KCl, 1 $CaCl_2$, and 10 Hepes-K, pH 7.2. The osmotic gradient across the BLM and the high *cis* [Ca^{2+}] facilitate the fusion of membrane vesicles with BLM [11]. Experiments were performed at room temperature (20–24 °C). Currents were recorded under voltage-clamp conditions with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Two Ag–AgCl electrodes were used to connect the rear, *trans*, compartment to the headstage

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(CV-4B) of the amplifier and the *cis* compartment to ground, respectively. The current output of the amplifier was stored in VHS tapes with the use of a modified digital audio processor connected to a VCR [12]. Currents were low pass-filtered at 20 kHz and sampled at 50 kHz with a Digidata 1322A interface (Axon Instruments). Channel analysis was done with pClamp 8.0 software (Axon Instruments). Results are expressed as means ± SEM of the indicated number of experiments.

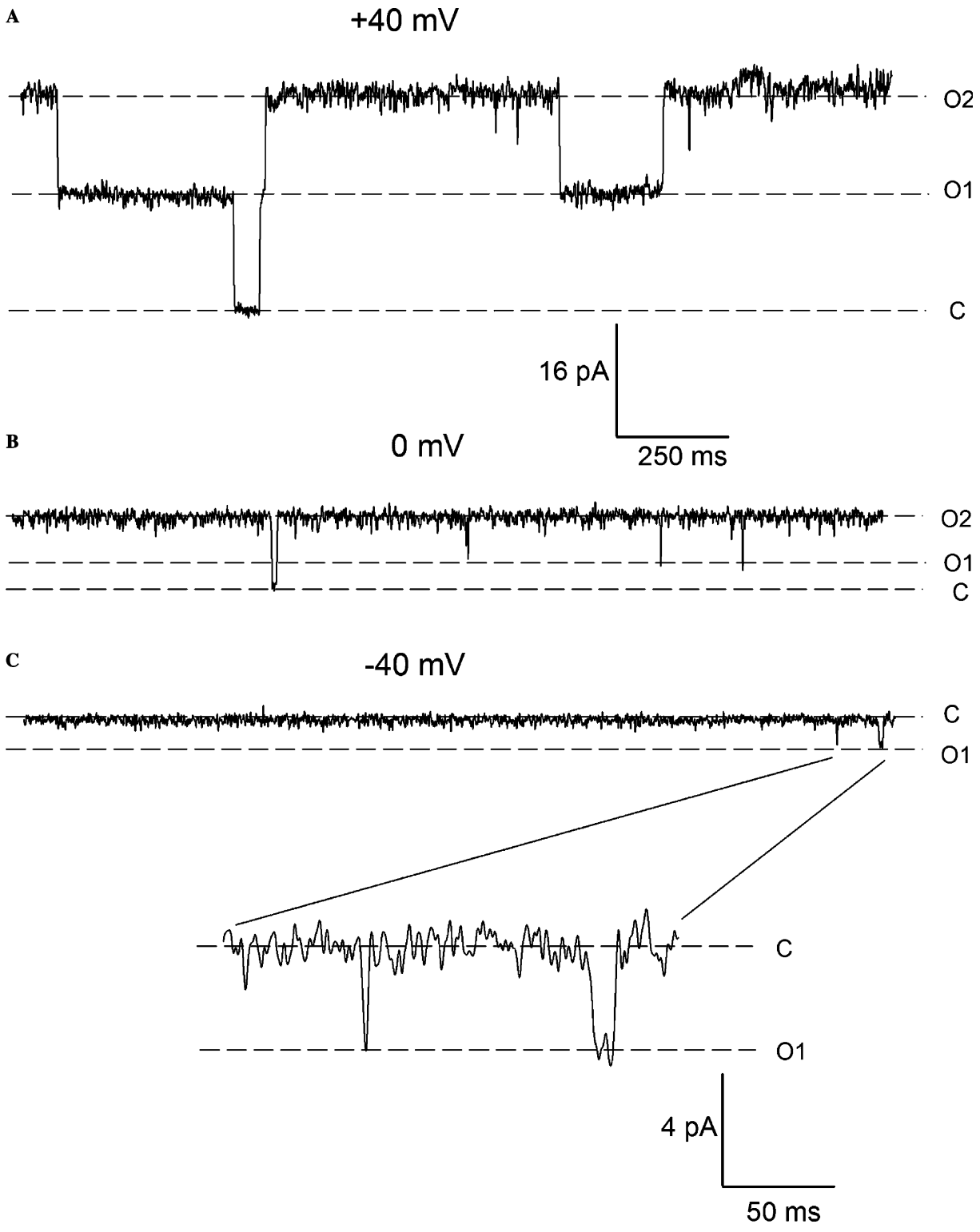

Fig. 1. Current through a Cl^--permeable channel incorporated into a DPhPC planar membrane. (A) Channel current at +40 mV (see Materials and methods). The channel fluctuates among two high conductance substates (labeled O_1, O_2) and the zero-current level (labeled C), being open most of the time. (B) Current at 0 mV. (C) Current at −40 mV. At negative potentials the channel remains closed most of the time. *cis* (mM): 300 KCl, 10 $CaCl_2$ and *trans* (mM): 150 KCl, 1 $CaCl_2$. Buffer was 10 Hepes, pH 7.2, on both sides. Traces were filtered at 200 Hz with pClamp 8.

Results and discussion

Single channel currents of a chloride-permeable bean root channel

At the beginning of every experiment a ±100 mV control pulse was routinely applied. Membranes showing instability were discarded. After applying the control pulse, root PMs were added at 5–100 μg protein/ml to the *cis* compartment and single channel currents were observed within ~0.5–60 min.

Fig. 1 shows single channel currents from a Cl^--permeable channel present in our bean root PM fraction. At *trans*-positive voltages (*cis* side is grounded, see Materials and methods), the channel presents a high probability of being open (see below), and random transitions between the closed, zero current level (labeled C), and two high conductance substates (O_1, O_2) are observed. This is clearly seen in the top panel that shows the single channel current at +40 mV. The middle panel shows the current at 0 mV. Note that the channel is still open most of the time. In the 2-s trace, only a brief transition to the zero current level (C) is observed. The channel dwells most of the time in the substate of higher conductance O_2. In contrast, at negative potentials (bottom panel, −40 mV) the channel exhibits a very low probability of being conductive. Only scant, brief, transitions from the zero current level to the lower conductance substate (O_1) are observed.

At 0 mV current is positive, which could be accounted for either by a flux of cations from the *trans* to the *cis* side of the membrane, or by the passage of anions moving passively in the opposite direction. Taking into account the composition of the solutions across the membrane (see Fig. 1 legend), the polarity of the current indicates that at 0 mV there is a flux of anions (Cl^-) from the *cis* to the *trans* side of the membrane, which shows that the incorporated channel is permeable to Cl^- ions.

The anionic selectivity of the channel is best seen in Fig. 2A that presents the unitary current vs. voltage relationship with either KCl (filled circles) or NaCl (open circles) solutions in the *cis* side of the membrane. Note that with both solutions: (a) the channel exhibits two conductance substates (O_1, O_2 in Fig. 1) and (b) the least-squares lines through the points corresponding to either the O_1 ($\gamma = 293$ pS) or the O_2 ($\gamma = 570$ pS) substates cross the voltage axis (zero-current–voltage) at negative potentials, of either −23.2 or −18.2 mV, respectively, near the Nernst potential of Cl^- ions, $E_{Cl} = -19$ mV. This demonstrates that the channel excludes the passage of the cations present in the bilayer chamber, all of which have positive Nernst potentials (Na^+, K^+, and Ca^{2+}), allowing the selective flux of chloride ions through it.

It is known that the ClC family of chloride channels characteristically presents a double-barreled structure,

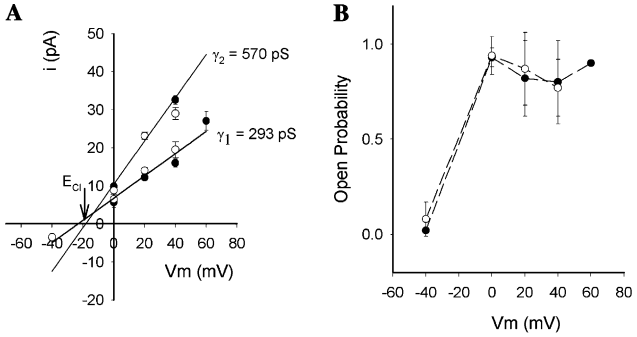

Fig. 2. Conductance and voltage dependence of the bean root Cl^- channel. (A) Single channel current vs. voltage relationship. The plot gathers observations from independent recordings obtained with either 300 mM NaCl (○, $n = 4$) or KCl (●, $n = 5$) *cis*-solutions, other conditions were like in Fig. 1. The arrow points at the theoretical Nernst potential of Cl^- ions (−19 mV). Regardless of the main cation in the *cis* side, the channel presents two substates of high 293 pS (O_1 in Fig. 1) and 570 pS (O_2) conductance. The latter values are obtained from the slope of the least squares lines ($r(O_1$ line$) = 0.98$; $r(O_2$ line$) = 0.99$) through the points. (B) Open probability (P_o) as a function of the applied potential. Just like the two conductances, P_o values are also independent of the main cation (K^+ or Na^+) present in *cis*. P_o was determined as the fraction of time that the channel is open at the indicated voltages. The points are means ± SEM of at least three experiments. The line through the points has no theoretical meaning.

with two independent pores, whose probability of opening is binomially distributed [13,14]. In the case of the bean Cl^- channel, the observations at −40 mV, where only the O_1 open level is observed, as well as the statistical analysis of the traces at positive voltages (not shown), indicate that the frequency of the two conductance levels (O_1, O_2) does not follow the binomial distribution. Therefore, our observations suggest that the channel does not belong to the ClC family of chloride channels.

The elevated Cl^- conductance of the incorporated channel (293 and 570 pS) is higher than those of other reported anionic channels of plant cells, recorded under similar [Cl^-]; e.g., the so-called R (rapid, 36–39 pS) and S (slow, 33 pS) PM channels of *Vicia faba* and *Xanthium strumarium* guard cells [15,16], the anionic PM channel (77 pS) of *Hordeum vulgare* [17], the high conductance (200 pS) PM channel of *Amaranthus tricolor* [18], the stretch-activated channel (27 pS) of *V. faba* [19], and tonoplast anion channels of *Chara* (48 pS) [20]. On the other hand, regarding the bean *P. vulgaris*, to our knowledge only two types of channels have been reported previously. Both were recorded in coat protoplasts and were found to be non-selective (therefore, they are clearly different to the Cl^- channel here studied), namely: one slowly activating, and presenting a permeability ratio PK: PCl ~2.9, the other is fast activating with PK: PCl ~2.5 [21]. Thus, the Cl^- conductance here studied represents a previously undescribed channel of plant cells.

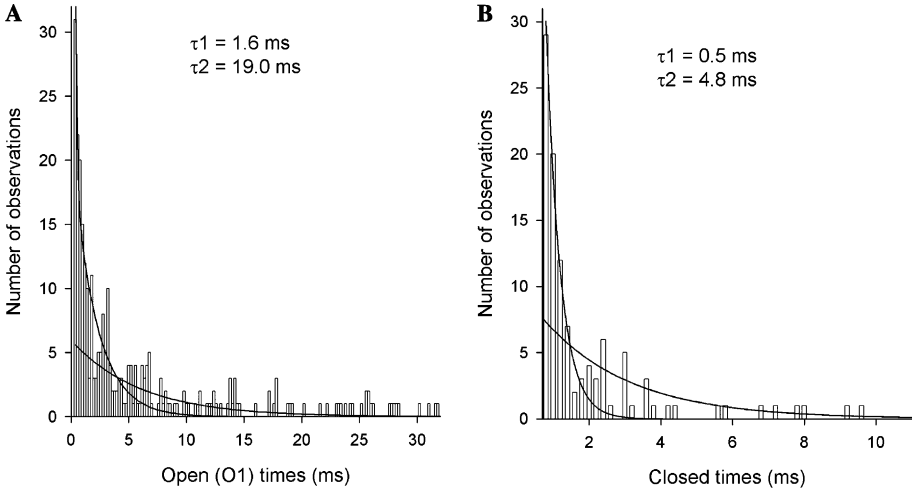

Fig. 3. Distribution of closed and open (O_1) dwell times. (A) O_1 state dwell time distribution. The distribution is well fitted by two exponentials (lines) with time constants $\tau_1 = 1.6$ ms and $\tau_2 = 19$ ms; and amplitudes $A_1 = 6$ and $A_2 = 101$ events. (B) Closed state dwell time distribution. The distribution is well fitted by the sum of two exponentials with $\tau_1 = 0.5$ ms and $\tau_2 = 4.8$ ms; and amplitudes $A_1 = 8$ and $A_2 = 43$ events. The histograms were obtained from a 1-min recording at +20 mV in 300 mM KCl (*cis*): 150 mM KCl (*trans*).

Fig. 2B presents the probability of opening as a function of the membrane potential. As already noticed in Fig. 1, the channel is markedly voltage-dependent, with high probability of opening (P_o) at 0 and *trans*-positive potentials, and a very low P_o at negative potentials, where only scant, brief, transitions to the O_1 substate are observed.

In a channel that presents several conductance substates, dwelling times as well as transitions among the states cannot be determined without ambiguity. In the case of the Cl^- channel, in three experiments, during a 1-min recording at +20 mV, we only observed fluctuations between the zero and the O_1 (293 pS) current levels. We therefore used these experiments to determine the distribution of the closed and O_1 state durations. The results in Fig. 3A illustrate that the open O_1 dwell time distribution was well fitted by two exponentials, with time constants $\tau_1 = 1.6$ ms and $\tau_2 = 19$ ms. The closed dwell time was also well fitted by the sum of two exponentials, with $\tau_1 = 0.5$ ms and $\tau_2 = 4.8$ ms. Thus, in those experiments, at the steady state conditions of planar bilayer recordings, the channel dwells in the O_1 conductance state with either short (1.4 ± 0.3 ms, $n = 3$) or long lasting (19.0 ± 0.2 ms, $n = 3$) mean times, from which it fluctuated to the zero current level either briefly (0.3 ± 0.2 ms, $n = 3$) or with longer mean closing times (4.7 ± 2.5 ms, $n = 3$).

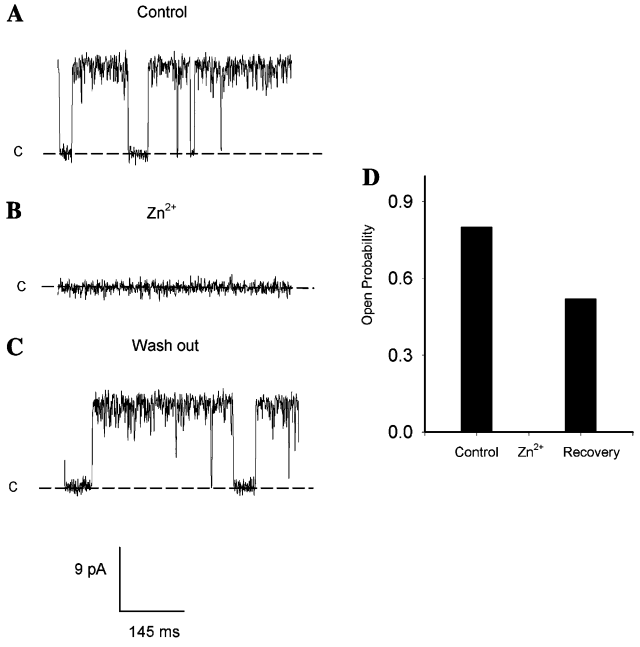

Fig. 4. Channel inhibition by Zn^{2+} ions. (A) Control currents at +20 mV. (B) Current left after the addition of 100 μM $ZnCl_2$ to both sides of the membrane. In the presence of Zn^{2+}, the channel remains non-conductive. (C) Current recovery after washing the bilayer compartments with the control solutions. (D) Open probability of the whole traces in A–C. Recording conditions were like in Fig. 1.

Channel inhibition by Zn^{2+} ions

It is known that Zn^{2+} inhibits Cl^- channels of both plant and animal cells [20,22], e.g., Zn^{2+} inhibits R but not S anionic plant channels [15,23]. Therefore, we tested the effect of this divalent cation on the bean root Cl^- channel. Fig. 4 presents the results of a representative experiment. Fig. 4A, left panel, shows a control trace at +20 mV. As expected (Fig. 2B) the channel is open most of the time. Fig. 4B shows that the subsequent addition of 100 μM Zn^{2+} to both sides of the membrane completely inhibited the current through the channel. The inhibitory effect was partly reversed by the removal of Zn^{2+} from the chamber solutions (Fig. 4C). The above

observations are summarized in Fig. 4D that presents the open (conducting) probability of the channel before, during, and after the addition of Zn^{2+}, as indicated. After Zn^{2+} removal, the channel conducted again, although with smaller P_o, maybe because of an incomplete washout of Zn^{2+}. A similar behavior was observed in two other bilayers but in those cases the membrane broke before the completion of the experiments.

We have presented the first characterization of a voltage-dependent, high conductance, and anion-selective channel from bean root cells. The channel is not likely to have a mitochondrial origin since the contamination of our preparation by mitochondrial membranes is low (~2%), and the properties of the well-known voltage-dependent anionic channel (VDAC) present in the outer mitochondrial membrane [24] are different to those of the Cl^- conductance of bean roots. Although the origin of the channel (plasma membrane or tonoplast) remains to be determined, its marked voltage-dependence as well as its elevated conductance suggest that it may play important roles in the physiology of bean root cells. The possible participation of this, not previously described, Cl^--channel in the symbiotic interaction between bean and *R. etli* cells is currently under investigation.

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