Outwardly Rectifying Anionic Channel from the Plasma Membrane of the Fungus *Phycomyces blakesleeanus* \(^\vee\)

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The molecular identities and electrophysiological and structural properties of anion-selective channels are well characterized in animal cells and, to a lesser extent, in plant cells (7, 22, 29, 31). In fungi, on the other hand, data on anion channels are scarce. Up to now, only two electrophysiological studies have demonstrated the existence of anion channels in fungal membranes. Both of these studies, however, were mainly metastudy, dealing with the development of techniques for successful patch clamping (i.e., gigaseal formation) of fungal plasma membranes. The channel activity recordings primarily illustrated the validity of the techniques. The available data show that anion channels from cell membranes of *Neurospora crassa* (37) and *Aspergillus niger* (33) are selective for CI⁻ over K⁺, with single-channel conductances of 17 pS and 43 pS, respectively, and that the one from *N. crassa* is a weak outward rectifier while the one from *A. niger* is a strong rectifier. No data on selectivity among anions, kinetic parameters, or blockers of these channels are available.

The lack of knowledge about fungal anion channels is mainly the result of technical difficulties in the formation of a high-resistance seal, a “gigaohm seal” (≥1 GΩ) between hyphal plasma membranes and patch clamp pipettes. The “gigaohm seal” is necessary for detailed analysis of ion channel properties (i.e., selectivity and gating). These difficulties are a consequence of the specific structure of the fungal cell wall. The only readily patchable fungal cell membrane is that of a yeast (*Saccharomyces cerevisiae*), the experimental model used in virtually all electrophysiological characterizations of fungal cationic channels (3). None of these experiments showed anion channel activity, indicating the absence of anionic channels. However, unicellular organization and cell walls composed of mannans and glucans clearly separate yeasts from the majority of other fungi denoted filamentous fungi. Filamentous fungi are characterized by multicellular mycelia, polarized hyphal growth, and chitin cell walls. Electrophysiological study of the only cationic channel from a filamentous fungus (*N. crassa*) that has been well characterized was conducted by cloning and expression of the channel in yeast plasma membranes (32).

Removal of the cell wall with cell wall-degrading enzymes, which gave good results in plants, failed to solve the problem in fungi, since patch clamping of the obtained protoplasts resulted only in subgigaohm seals that were not suitable for detailed examination of ionic channels (14, 25). The only exception is the report of a gigaohm seal on enzyme-derived germling protoplast from *Uromyces* (40). In another report, the problem of cell wall removal was resolved by using a wall-less slime mutant of *N. crassa* (24). Despite gigaohm seal formation due to the specific nature of the slime mutant cell membrane, only the whole-cell perforated patch clamp configuration was obtained, and therefore, characterization of ionic channels was incomplete. The only successful approach so far has been the removal of the hyphal cell wall by the laser ablation technique. This technique enabled the formation of gigaohm seals and the above-mentioned identification of anion channels in cell membranes of *N. crassa* (37) and *A. niger* (33). However, in the past 10 years, no new investigations of ion channels in fungi using this technique have been published.

Such slow progress implies a need for the development of...
new experimental approaches for ion channel investigation in filamentous fungi. Zaichkin and coworkers in 1975 (39) produced cytoplasmic droplets from sporangiophores of *Phycomyces blakesleeanus* that were capable of de novo synthesis of their membranes, formation of the cell wall, polarized growth, and regeneration of the mycelia. In such an experimental procedure, one obtains fungal protoplasts with no residues of the cell wall on their surfaces, which greatly enhances the chance for the formation of a stable gigaohm seal. In our previous study, we defined the experimental conditions under which cytoplasmic droplets from sporangiophores of *P. blakesleeanus* gave stable gigaohm patches sufficient for complete identification of ionic channels (42). In the present report, we describe an outwardly rectifying anionic channel (ORAC) as the most prominent feature of the membrane in the absence of energizing substrates.

**MATERIALS AND METHODS**

A wild-type strain of the fungus *P. blakesleeanus* (Burgeff NRRL11555–) was used in this study. The fungal cytoplasmic droplets were prepared by essentially following the method of Zaichkin et al. (39) as described in our previous publication (42). Stage IV sporangiophores were the source of the cytoplasmic droplets. The tips of the sporangiophores were immersed in a solution isosmotic following the method of Zaichkin et al. (39) as described in our previous publication. The fungal cytoplasmic droplets were prepared by essentially replacing with the bathing solution for patch clamping, containing (in mM) KCl, 140; CaCl₂, 1; MgCl₂, 2; HEPES, 10; sucrose, 230; pH 7.0 (KOH); osmolarity, 495 mosmol. The osmolarities of the solutions were measured with an automatic 1440 ZIVIC ET AL. EUKARYOT. CELL

The membrane potential of the droplet membrane with the cell membranes of intact sporangiophores, the membrane potential of the cytoplasmic droplets was recorded. The micropipette for intracellular recording were pulled from borosilicate glass capillaries (Kimax-51; Kimble Products) on a vertical puller (Stoelting; IL) and filled with 1 M KCl. The reference electrode was an Ag/AgCl wire in a small glass tube, also filled with 1 M KCl, contacting the bath solution via a piece of porous ceramic. The membrane potential was measured with a high-input-impedance amplifier (610C Electrometer; Keithley Instruments) and recorded continuously on custom-made software installed on a PC computer. The mean transmembrane potential from five experiments with SO₄⁻ was fitted by a modified Boltzmann function:

\[
\text{np}_o = \sum_{n=1}^{N} \frac{n \cdot t_n}{1 + \exp(2(F V_{1/2} - V)/RT)}
\]

where \(n\) is the number of channels that were open simultaneously, \(t_n\) is the fraction of time in which \(n\) channels were open simultaneously, and \(a\) is the maximal number of channels observed simultaneously.

The relationship of the normalized channel activity (\(\text{np}_o/\text{np}_o^{\text{max}}\)) to the voltage was fitted by a modified Boltzmann function:

\[
\text{np}_o/\text{np}_o^{\text{max}} = \frac{1}{1 + \exp(2(F V_{1/2} - V)/RT)}
\]

where \(n\) is the number of channels in the patch, \(p_n\) is the single-channel open probability, \(\text{np}_o\) is the channel activity at membrane voltage \(V\), \(\text{np}_o^{\text{max}}\) is the maximal number of \(n\) channels, \(z\) is the partial charging charge, \(V_{1/2}\) is the value of the voltage where half-maximal activation of the channel occurs, \(F\) is the Faraday’s constant, \(R\) is the gas constant, and \(T\) is the absolute temperature.

Time-averaged single-channel currents (\(I_s\)) were obtained as a product of the normalized channel activity and the amplitude of the single-channel at the given membrane voltage. The relationship of time-averaged single-channel currents to the voltage was determined by fitting the curve to a modified Boltzmann function:

\[
I_s = \frac{g_{\text{max}}(V - E_{\text{rev}})}{1 + \exp[2(F V_{1/2} - V)/RT]}
\]

where \(I_s\) represents the time-averaged single-channel current at the membrane voltage \(V\), \(g_{\text{max}}\) is the maximum conductance obtained at the voltage where maximal relative channel activity occurs, \(E_{\text{rev}}\) is the reversal potential of the current, and the other parameters have the same meaning as in equation 2.

The voltage-dependent activation and deactivation of the current after a step pulse was fitted with an exponential equation:

\[
I = I_0 (1 - \exp(-t/\tau)) + C
\]

where \(I\) is the time- and voltage-dependent current, \(I_0\) is the steady-state current after activation or deactivation, \(\tau\) is the activation or deactivation time constant, and \(C\) is the constant.

The reversal potential was determined either as the intercept of the voltage of analytical grade. All stock solutions were prepared using glass-distilled deionized water, except A9C, which was dissolved in 96% ethanol.

Anion channel activity was measured in inside-out patches. The micropipettes for patch clamp recordings were made from thick-wall borosilicate glass capillaries (0.86-mm inside diameter; Clark Electro-Medical Instruments, Berkshire, United Kingdom) pulled on a vertical puller (Stoelting Co., Wood Dale, IL). After the micropipettes were filled with an internal solution with the same composition as the bathing solution, the resistance of the microelectrodes was 10 to 20 MΩ. An Ag/AgCl reference electrode was connected to the bath via a 3 M KCl agar bridge. Channel activity was monitored in continuous recordings, as well as using ramp protocols (−120 mV to +120 mV in 240 ms and −60 mV to +60 mV in 300 ms). Single-channel currents were measured by means of an EPC9 patch clamp amplifier (Heka Elektronik GmbH, Lambrecht/Pfalz, Germany) controlled by an EPC9 SCREEEN (Heka) acquisition program installed on an Atari Mega ST-4 minicomputer. Acquisition was performed at a frequency of 3 kHz. Single-channel recordings were analyzed by the half-threshold technique (6), using TAC software (Brunxton Corporation, Seattle, WA). Additional filtering of 300 Hz was employed to improve the signal-to-noise ratio, and dwell times were corrected for the dead time as implemented in the TAC software.

Currents carried by anions moving from the outer to the inner side of the patch membrane were considered positive and are shown as upward deflections in current traces. The results are reported as means ± standard errors, with \(n\) as the number of independent patches averaged. Ionic-equilibrium potentials and permeability ratios calculated after corrections for ionic activities (as calculated by GEOCHEM-PC v.2.0 [36]) were applied.

Only patches containing the anionic rectifier channel unobstructed by the other channels were used in this study. The single-channel conductance of this channel was determined in the following way. Current/potential (I/V) data from an individual patch were obtained by measuring the amplitudes of the unitary current at different membrane voltages. Conductance was obtained as the slope of the linear fit to the I/V data. Only the patches with current values determined for at least five membrane voltages (at least two of them with a different sign from the others) were taken into account.

Channel activity was calculated according to the following equation:

\[
\text{np}_o = \sum_{n=1}^{N} \frac{n \cdot t_n}{1 + \exp(2(F V_{1/2} - V)/RT)}
\]

where \(n\) is the number of channels that were open simultaneously, \(t_n\) is the fraction of time in which \(n\) channels were open simultaneously, and \(a\) is the maximal number of channels observed simultaneously.

The relationship of the normalized channel activity (\(\text{np}_o/\text{np}_o^{\text{max}}\)) to the voltage was fitted by a modified Boltzmann function:

\[
\frac{\text{np}_o}{\text{np}_o^{\text{max}}} = \frac{1}{1 + \exp(2(F V_{1/2} - V)/RT)}
\]

where \(n\) is the number of channels in the patch, \(p_n\) is the single-channel open probability, \(\text{np}_o\) is the channel activity at membrane voltage \(V\), \(\text{np}_o^{\text{max}}\) is the maximal number of \(n\) channels, \(z\) is the partial charging charge, \(V_{1/2}\) is the value of the voltage where half-maximal activation of the channel occurs, \(F\) is the Faraday’s constant, \(R\) is the gas constant, and \(T\) is the absolute temperature.

Time-averaged single-channel currents (\(I_s\)) were obtained as a product of the normalized channel activity and the amplitude of the single-channel at the given membrane voltage. The relationship of time-averaged single-channel currents to the voltage was determined by fitting the curve to a modified Boltzmann function:

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\]

where \(I_s\) represents the time-averaged single-channel current at the membrane voltage \(V\), \(g_{\text{max}}\) is the maximum conductance obtained at the voltage where maximal relative channel activity occurs, \(E_{\text{rev}}\) is the reversal potential of the current, and the other parameters have the same meaning as in equation 2.

The voltage-dependent activation and deactivation of the current after a step pulse was fitted with an exponential equation:

\[
I = I_0 (1 - \exp(-t/\tau)) + C
\]

where \(I\) is the time- and voltage-dependent current, \(I_0\) is the steady-state current after activation or deactivation, \(\tau\) is the activation or deactivation time constant, and \(C\) is the constant.

The reversal potential was determined either as the intercept of the voltage
axis with the linear fit of the $I/V$ data or the exponential fit of averaged ramp recordings of open channels or the Boltzmann fit of time-averaged $I/V$ data. Averaged ramp recordings of open channels were fitted with an exponential equation:

$$I = I_0 + a \exp(bV)$$

(5)

All methods gave very similar results, so the intercepts with the Boltzmann fit were reported as $E_{rev}$, unless otherwise noted.

After determination of the reversal potential, permeability ratios were calculated by the Goldman-Hodgkin-Katz voltage equation (19):

$$P_m/z^m \exp(z_m E_m F/RT) - 1 = a_i \exp(z_i E_i F/RT) - a_m$$

$$P_n/z^n \exp(z_n E_n F/RT) - 1 = a_m \exp(z_m E_m F/RT) - a_n$$

(6)

where $P$ is the permeability, $z$ is the valence, $E_{rev}$ is the reversal potential, $F$ is Faraday’s constant, $R$ is the gas constant, $T$ is the absolute temperature, $a$ is the activity, $m$ and $n$ are the ion species, and $o$ and $i$ are the extracellular and intracellular sides (for a detailed derivation of this equation, see reference 23).

**RESULTS**

Ionic-channel activity in de novo-formed fungal plasma membranes was explored by a patch clamp technique in an inside-out configuration. The whole-cell, and thus outside-out, configuration of the patch clamp method was hard to obtain and was not sufficiently stable to be used in ionic-channel characterization, presumably due to the specific properties of the membranes of the cytoplasmic droplets. The inside-out configuration proved to be more reliable, since it was mechan-ically more stable and persisted long enough to be used for detailed ionic-channel characterization.

Several types of ionic currents were observed in membrane patches. Two were briefly described previously (42). The most frequently observed ionic channel, appearing in 40% of all patches ($n = 135$), was the channel reported in this study. The description of this channel was usually hindered by the presence of other channels with higher conductivities. However, in 24 independent experiments, the channel was highly active and unobstructed by the other channels, which enabled its detailed characterization.

The conductances from 10 independent patches obtained as the slopes of linear fits to the $I/V$ data were averaged and gave a mean value of $11.3 \pm 0.4$ pS (Fig. 1B). Current recordings from multichannel patches at membrane voltages, $V_m$, in the range $-80$ to $120$ mV (Fig. 1A) showed that the channel was more active at depolarizing potentials, usually with more than 10 simultaneously active channels at potentials of 80 mV and above, while at potentials below reversal potential, the channel activity virtually ceased. Minimal channel activity was observed at $-50$ mV, with a slight increase at more negative potentials.

Since multichannel patches were routinely obtained even when high-resistance microelectrodes were used, normalized channel activity, $np/np_{max}$, was analyzed in order to compare the channel activities among different patches. $np/np_{max}$ val-
ues were averaged over a number of patches (n = 4) and plotted against the voltage. The values of the parameters of the best fit to the Boltzmann function (equation 2 and Fig. 1C) were as follows: $V_{1/2} = 60 \pm 2$ mV, and $z \delta = -2.1 \pm 0.1$. The channel activity was strongly voltage dependent (with a high value of the apparent gating charge), which indicated that the amplitudes of the steady-state whole-cell currents were strongly outwardly rectified. It is considered that the time-averaged current-voltage relationship qualitatively corresponds to the $I/V$ relationship of the steady-state whole-cell currents (2, 4). Therefore, in order to show rectification, we plotted against the voltage (Fig. 2A) time-averaged single-channel currents representing the product of the relative channel activity and the amplitude of the single-channel current at a given membrane voltage. $I_{\text{ps}}/n_{\text{ps max}}$ versus $V$ showed strong outward rectification, as expected.

Open-channel ramp recordings also demonstrated strong outward rectification (Fig. 2B). The trace shown is an average of 80 ramp scans. Before the average was calculated, leak subtraction was performed, and closing events that occurred during the scans were removed. Since individual current recordings were obtained by applying rapid voltage ramps (from $-120$ to $120$ mV in $240$ ms), the existence of rectification indicated that the voltage gating of the channel was relatively fast.

In order to assess the time constants of the current activation ($\tau_a$) and inactivation ($\tau_d$), current responses to voltage pulses were measured, since measurements from fast ramp recordings give only qualitative information about the presence of rectifications. Starting from the holding potential of $-50$ mV (500 ms), where the activity of the ORAC is the lowest, to $50$ mV (500 ms) and back to the holding potential. The trace shown is the mean of 76 scans. An exponential function fitted to the current trace (equation 4; see Materials and Methods) gave the following values of the time constants of channel activation and deactivation: $\tau_a = 32$ ms ($r = 0.724$) and $\tau_d = 50$ ms ($r = 0.756$).

FIG. 2. Voltage dependence of the channel gating. (A) The time-averaged single-channel current ($I_o$) plotted against the membrane voltage ($V_m$). $I_o$ was calculated as the product of the normalized channel activity ($n_{\text{ps}}/n_{\text{ps max}}$) (Fig. 1C) and the amplitude of the single-channel current at a given membrane voltage ($I$) (Fig. 1B). The solid curve is the best fit of the Boltzmann function to the data (equation 3; see Materials and Methods) obtained for the following values of the maximal conductance: $g_{\text{max}} = 10.3 \pm 0.1 \text{ pS}$, the half-maximal activation potential $V_{1/2} = 58 \pm 1$ mV, and the apparent gating charge $z \delta = -2.4 \pm 0.2$. Quality of the fit: $r = 0.9998$. (B) Open-channel current responses obtained by applying rapid voltage ramps (from $-120$ to $120$ mV in $240$ ms). The trace shown is the mean of 80 scans; before the average was formed, leak subtraction was performed and closing events that occurred during a scan were removed. (C) Outward current as a function of the time resulting from a voltage pulse from the holding potential of $-50$ mV (500 ms), where the activity of the ORAC is the lowest, to $50$ mV (500 ms) and back to the holding potential. The trace shown is the mean of 76 scans. An exponential function fitted to the current trace (equation 4; see Materials and Methods) gave the following values of the time constants of channel activation and deactivation: $\tau_a = 32$ ms ($r = 0.724$) and $\tau_d = 50$ ms ($r = 0.756$).
Another striking feature of the outward rectifier channel examined in this study was its long open and closed times, evident in patches with only one or two channels active in the recording (Fig. 1A). However, at positive voltages, in only 1 of 24 patches was channel activity sufficiently low (only two channels were simultaneously present) to allow the analysis of open and closed times. On the other hand, at negative membrane voltages, the activity was usually very low. In order to collect enough events for analysis, very long recordings (more than 100 s) had to be analyzed. Thus, current traces obtained at −30 mV, characterized by higher stability than those at −80 mV and higher activity than those at −50 mV, were used for dwell time analysis. Time histogram analysis revealed the presence of two open states and two closed states (Fig. 3), with the following mean time constants: τ_{o1}, 6.07 ± 0.97 ms; τ_{o2}, 35.0 ± 3.9 ms; τ_{c1}, 6.5 ± 1.1 ms; and τ_{c2}, 166 ± 28 ms. Mean values were obtained for five independent patches at a holding potential of −30 mV. Long open and closed times were more abundant and occupied 63% ± 8% and 79% ± 3% of total open and closed times, respectively.

In order to determine the selectivity of the channel between anions and cations, the extracellular (pipette) concentrations of KCl were varied (Fig. 4A). Two extracellular KCl concentrations were used, 18.35 mM and 250 mM, which gave values of the equilibrium potential for Cl⁻ of 39.4 mV and −15.7 mV for K⁺, −45.4 mV and 16.3 mV, respectively (Fig. 4). The obtained average value of E_{rev} for 18.35 mM KCl in the pipette was 38.2 ± 0.3 mV (n = 3), while that for 250 mM KCl in the pipette was −15.0 ± 0.5 mV (n = 3). It is apparent that the E_{rev} values were very close to that of the equilibrium voltage for chloride (E_{Cl}), which indicates that this channel is permeable mainly for Cl⁻ and that K⁺ permeation is negligible. Additionally, it seems that Cl⁻ is one of the factors controlling the gating process. A qualitative appreciation of this fact is readily obtained from time-averaged I/V curves generated at different extracellular Cl⁻ concentrations, as shown in Fig. 4A for three separate patches. Shifting the extracellular [Cl⁻] from 256 to 131 mM shifted the I/V curves 15 mV positive, with little or no change in their shapes. Similarly, lowering the extracellular [Cl⁻] from 125 to 18.35 mM moved the I/V curve further (38.2 mV) in a positive direction. The rightward shift of gating voltages closely approximated the imposed changes of E_{Cl} (Fig. 4B), which led to the conclusion that the gating voltage is actually coupled to E_{Cl} rather than simply to the membrane voltage.

Channel selectivity among physiologically important anions was determined in bi-ionic solutions, where the concentration of KCl on one side of the membrane was replaced with an equivalent concentration of the potassium salt of an appropriate anion. For precise determination of the reversal potential, a protocol of rapid voltage ramps (from −60 to 60 mV in 500 ms) was used (Fig. 5). For leak subtraction, current traces representing the closed state were averaged and subsequently subtracted from averaged recordings of open channels. The intercept of the exponential fit (equation 5) of obtained average recordings and the voltage axis gave E_{rev}. When 125 mM KCl was used in the bathing solution and 125 mM KBr or 125 mM KNO₃ in the pipette, current-voltage relationships derived from the fits of averaged open-channel ramps intersected the voltage axis at −20 ± 2 mV (KBr) (n = 3) and −36 ± 2 mV (KNO₃) (n = 4) (Fig. 5A). When 125 mM KCl in the bathing solution was replaced with 125 mM KI or 62.5 mM K₂SO₄, the values of E_{rev} obtained were 39 ± 3 mV (KI) (n = 3) and −30 ± 1 mV (K₂SO₄) (n = 4) (Fig. 5C). The same values of E_{rev} were obtained from linear fits of the I/V data (data not shown). The average values of the permeability ratios of I⁻, NO₃⁻, Br⁻, and SO₄²⁻ in respect to Cl⁻ were 4.8 ± 0.5 (n = 3), 4.4 ± 0.3 (n = 4), 2.2 ± 0.2 (n = 3), and 0.55 ± 0.04 (n = 4), respectively. In accordance with these results, the anion selectivity sequence of the ORAC was I⁻ > NO₃⁻ > Br⁻ > Cl⁻ > SO₄²⁻ = 4.8 > 4.4 > 2.2 > 1 > 0.55, which implied that the ORAC did not discriminate strongly among anions.

We also tested the selectivity of the channel for malate. However, in 20 independent patches where 125 mM KCl in the pipette was replaced with 62.5 mM K₂-malate no anionic outward channel activity was recorded. Furthermore, the addition of 2 mM K₂-malate to the bathing solution in three independent patches reversibly inhibited 59% ± 12% of the channel activity. Close inspection of continuous current traces before and during the malate treatment (Fig. 6) revealed that its application on the intracellular side induced dramatic changes in the channel kinetics. The characteristic long opening time was drastically reduced, and the channel started to open in a
flickering fashion. In one of three patches, the number of open channels was sufficiently small (at least two channels) to allow dwell time analysis at a holding potential of \(-40\) mV. As can be seen from Fig. 6, the addition of 2 mM malate had a major effect on the open-channel times. Open-duration histograms in the control experiment, just like those previously analyzed at a holding voltage of \(-30\) mV, demonstrated the presence of two open states with mean open-time constants of similar durations (\(\tau_{o1}, 6.60\) ms, and \(\tau_{o2}, 51.49\) ms), with the longer mean open time occurring in 75.8% of the total open time. After the addition of 2 mM malate, the longer mean open time completely disappeared, so that complete open-time data could be fitted with a single probability density function with a mean open-time constant of 4.23 ms. Unlike malate, the addition of 200 \(\mu\)M A9C, a common blocker of animal anion channels, exerted no effect on our channel (data not shown).

**DISCUSSION**

The results presented here show an outwardly rectifying anion current in the membranes of cytoplasmic droplets of the fungus *P. blakesleeanus*. The data presented in this work (see Materials and Methods), together with previous investigations, showed that the droplet membrane corresponds to the hyphal plasma membrane of the fungus (39, 42).

Unfortunately, no in-depth comparison with other fungal anion channels is possible due to the lack of data. Up to now, only two electrophysiological studies have shown the existence
of anion channels in fungal cell membranes (33, 37). It can be said that the ORAC is similar to the anion channel from the *N. crassa* cell membrane in regard to its single-channel conductivity and that it is also similar to the anion channel from the *A. niger* plasma membrane in regard to its voltage-dependent gating properties. While the identities and properties of fungal anion channels are mostly unknown, it has been shown that chloride fluxes represent one of the major contributors to the plasma membrane ion transport in intact hyphae of *N. crassa* (26), underlining the importance of Cl−/H+ channels for the plasma membrane physiology of filamentous fungi.

The genomes of several fungal species have been fully or partially determined, enabling an extensive search for genes homologous to those that encode putative anion channels in animals. Thus, homologues of the CLC animal chloride channel were found in the genomes of 15 fungal species (30). So far, the protein product of *GEF1*, a CLC gene homologue in *S. cerevisiae*, is the only one that has shown expression and function in yeast endomembranes and plasma membranes when expressed in a heterologous system (*Xenopus* oocytes) (27). CLC channels represent a family of anion-selective, voltage-dependent ionic channels, with well-characterized molecular and electrophysiological properties in mammalian cells (21, 29), so they could be a good starting point in the search for anion channels with electrophysiological properties similar to those of the ORAC.

Besides the membrane voltage as a major factor determining channel activity, the main common characteristic of CLC anion channels is their permeability (P) sequence for monovalent anions: $P_{\text{Cl}} > P_{\text{Br}} > P_{\text{I}}$, which is different from those in other animal anion channels (29) and is quite opposite to the anion permeation sequence of the ORAC. The selectivity sequence of the ORAC ($P_{\text{I}} > P_{\text{NO3}} > P_{\text{SO4}} > P_{\text{Cl}}$) corresponds to Eisenman series 1, which describes a large hydrated ion channel bearing a lining of weak positive charges. For the Eisenman series 1 selectivity sequence, the ion selectivity of the channel reflects the order of free solution mobilities, and the channel does not discriminate strongly among anions (38).

Some similarity with the ORAC is apparent only in ClC-3, -4, and -5 out of six electrophysiologically characterized CLC channels. These channels share high gene homology (80%) and represent a separate branch within the CLC family (21). Strong outward rectification of their currents is a common characteristic with the ORAC. It is interesting that the homologues of the CIC chloride channel that have been found so far in fungi belong to this branch of the CLC family (11, 30).
However, it should be noted that CIC-3, -4, and -5, as well as the yeast homologue Gef 1, are mainly expressed in intracellular membranes and that recent studies have demonstrated that they function as $\text{Cl}^-/\text{H}^+$ exchangers rather than $\text{Cl}^-/\text{H}^+$ channels (20, 21).

Analysis of other major families of animal anion channels indicated that calcium-activated chloride channels (CaCC) show the greatest similarity to the ORAC. Unlike CIC, CaCC are characterized by a permeability sequence for monovalent anions virtually identical to that of the ORAC (17). CaCC are also characterized by a depolarization-activated, strongly outwardly rectified current, similar to the one we observed in the experiments with the ORAC. Furthermore, current rectification, just like that demonstrated in the experiments with the ORAC, is a consequence of apparent open-probability dependence on the voltage (9). On the other hand, the ORAC is characterized by fast activation/deactivation kinetics (~50 ms), while CaCC activate slowly at positive potentials and deactivate at negative potentials (>100 ms).

For all the reasons presented above, no extensive similarity between the electrophysiological properties of the ORAC and those of animal anion channels could be established. The situation is somewhat different when functional characteristics of the ORAC are compared to plant anion channels. Electrophysiologically, the best-studied group of plant anion channels is the rapidly activating anion efflux channels (R-type channels) (31). The ORAC shares a number of common characteristics with those channels. The unitary conductance of R-type channels is in a range of 10 to 20 pS, which is comparable to the unitary conductance of the ORAC (11 pS). Their permeability sequences for monovalent anions are virtually identical, both qualitatively and quantitatively (34). Another common characteristic is a high permeability for sulfate. In the case of the R-type channel in Arabidopsis thaliana hypocotyls, permeability for $\text{SO}_4^{2-}$ is extremely high, two times higher than that for chloride (12). Even though in the case of the ORAC the permeability for $\text{SO}_4^{2-}$ is somewhat lower (0.55 $P_{c_l}$), it is still significant, since sulfate is usually considered to be a weakly permeant or nonpermeant ion in the majority of anion channels (12). Since the significant sulfate permeability, usually not seen in other anion channels, may be a conserved feature of R-type channels (31), this similarity could have special significance in the determination of a functional homologue of the ORAC.

The gating properties of R-type channels and the ORAC are similarly regulated by anions, so that 10-fold (R-type) and 8.4-fold (ORAC) increases of extracellular $\text{Cl}^-$ activity shifted the $E_{\text{rev}}$ to $-59.8$ mV (8) and $-53.2$ mV, respectively (Fig. 4A). In both cases, the values of $V_{1/2}$ changed in a similar manner, i.e., the whole $I/V$ curves were shifted to the left without any change in their shapes. For R-type channels, these effects were interpreted by the specific position of the anion-
binding site, which is coupled to the voltage-sensing mechanism (8). A similar interpretation could be applied to the ORA, as well.

Another common characteristic of R-type channels and the ORAC is the modulation of their activities by malate. While in the case of the ORAC the effect of malate is restricted to blocking channel activity, its effects on R-type channels are much more complex (18). In R-type channels, extracellular malate blocks inward and outward currents positive to the potential at which a current has its maximal value and shifts the $I/V$ curve to the left. In the ORAC, the effect of extracellular malate could not be assessed, since no channel activity could be recorded with malate present in the pipette solution and insufficient stability of outside-out patches prevented further investigation. However, intracellular 2 mM malate potently blocked the outward current of the ORAC. Low channel activity at negative potentials in the control prevented assessment of the malate effect on inward currents. Malate is a very important cell metabolite, so its regulation of any channel could be a very important clue in the effort to determine the physiological function. This has already been shown for R-type channels, since their sensitivity to the extracellular malate could be a way in which guard cells sense change in the ambient CO$_2$ concentration and coordinate stomatal movements with this important environmental signal (18). Further similarities between R-type channels and the ORAC are fast activation/deactivation kinetics and voltage dependence of apparent open probability as one of the causes for current rectification.

In addition to these striking similarities, there are several features of R-type channels that are different from the ones we found in the ORAC. Namely, R-type channels show a 50% block with 100 µM A9C and, most importantly (28), R-type channels have a very different type of voltage dependence than the one observed with the ORAC. R-type channels are depolarization-activated inward rectifiers characterized by a U-shaped $I/V$ relationship, with the peak current activation at voltages that are more negative then the equilibrium potential for the permeant anion, thus favoring anion efflux (31).

However, outwardly rectifying depolarization-activated anion channels mediating anion influx have been reported in plants (OR-DAAC) (5, 10, 15, 35). These currents are less well characterized than R-type currents, but nevertheless, their analysis can help in further clarification of the physiological role of the ORAC. Out of four reports on this type of anion current, the one from the protoplast derived from wheat roots is the best characterized (35). This current is strongly outwardly rectified, with fast activation/deactivation kinetics, and is gated in similarly to the ORAC (Fig. 4) by extracellular Cl$^-$ (an increase in the extracellular Cl$^-$ concentration shifts the $I/V$ relationship of the current to the left). It has been proposed that in OR-DAAC channels, shifting of the $I/V$ curve to the left activates the channel, allowing sizable anion influx currents at more negative potentials, closer to the resting membrane potential. However, it differs from the ORAC in several properties: this channel is less permeable to I$^-$ than to Cl$^-$ and has very small single-channel conductance of 4.4 pS in 300 mM extracellular KCl.

Plant OR-DAAC anion channels were proposed to have an important role in the NO$_3^-$ and Cl$^-$ uptake pathway into roots under conditions of high external salinity (16, 35). *P. blakesleeanus* inhabits a variety of substrates rich in organic matter, from decaying wasp nests to peaches in a refrigerator, but most commonly it inhabits mouse, rabbit, and horse dung (1). Those substrates are characterized by both greater variation and higher anion concentrations than the soil. With that in mind, together with the enhancement of channel activity by extracellular Cl$^-$, the ORAC could provide a pathway for anion uptake in substrates with high mineral content.

Despite the fact that among physiologically important ions NO$_3^-$ is the most permeant, it is not likely that nitrate uptake is the physiological function of the ORAC. Unlike most fungi and plants, *P. blakesleeanus* cannot grow on medium with nitrate as the sole source of nitrogen, because it does not possess a nitrate reductase and thus cannot use nitrates as sources of nitrogen for organic synthesis (13). On the other hand, sulfates are essential for the growth of the fungus, which together with the relatively high sulfate permeability of the ORAC, makes them physiologically the most important ions to be absorbed, possibly via this channel, under conditions of sufficiently high extracellular concentration.

The time-averaged $I/V$ relationship of the ORAC (Fig. 2A) implies that, at membrane potentials more negative than the $E_{Cl}$, the activity of the channel is negligible. The fact that the activity of the channel was recorded in only 40% of patches but that when recorded it was usually in great numbers (at least 10 channels) suggests the possibility that the channels were colocalized in small areas of the membrane. Such a high concentration of the ORAC at low extracellular anion concentrations, i.e., when the $E_{Cl}$ is more positive than the membrane potential, could generate an inward anion current of sufficient intensity that the resulting efflux of anions could locally depolarize the inner part of the membrane.

In conclusion, the present study provides the first detailed electrophysiological characterization of an anion channel in fungi characterized by a strong voltage-dependent ($\delta > 2$) outward rectification, single-channel conductivity of 11 pS, and strong selectivity for anions over cations but weak selectivity among anions, fast depolarization-induced activation/deactivation kinetics ($<50$ ms), insensitivity to A9C ($<200$ µM), and reversible inhibition by malate (2 mM).

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