Nucleotides Provide a Voltage-sensitive Gate for the Rapid Anion Channel of Arabidopsis Hypocotyl Cells*

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The rapid anion channel of Arabidopsis hypocotyl cells is highly voltage-dependent. At hyperpolarized potentials, the channel is closed, and membrane depolarization is required for channel activation. We have previously shown that channel gating is regulated by intracellular nucleotides. In the present study, we further analyze the channel gating, and we propose a mechanism to explain its regulation by voltage. In the absence of intracellular nucleotides, closure at hyperpolarized voltages is abolished. Structure-function studies of adenyl nucleotides show that the apparent gating charge of the current increases with the negative charge carried by nucleotides. We propose that the fast anion channel is gated by the voltage-dependent entry of free nucleotides into the pore, leading to a voltage-dependent block at hyperpolarized potentials. In agreement with this mechanism, in which intracellular nucleotides need to be recruited to the channel pore, kinetic analyses of whole-cell and single-channel currents show that the rate of closure is faster when intracellular nucleotide concentration is increased, whereas the rate of channel activation is unchanged. Furthermore, decreasing the concentration of extracellular chloride enhances the intracellular nucleotide block. This result supports the hypothesis of a mechanism in which blocking nucleotides and permeant anions interact within the channel pore.

The molecular mechanisms of voltage gating are well understood for some channels. Those mechanisms can vary, but they always involve the movement of charged gating particles through the transmembrane electric field. These gating charges can be intrinsic to the channel protein, as described in potassium, sodium, and calcium channels (1). For instance, in the shaker-like potassium channels described in animal and plant membranes, mutations of basic amino acids of the S4 transmembrane domain drastically change the voltage regulation of the channel, indicating that these amino acids contribute to the gating charge (2). Alternately, voltage-dependent closure can be achieved by voltage-dependent block of the channel pore by a soluble charged molecule. This is the case for NMDA ionotropic receptors (3, 4) and inward rectifying potassium channels (5), which are gated by soluble cations. Except

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EXPERIMENTAL PROCEDURES

Plant Material, Protoplast Isolation, and Electrophysiological Investigations—Arabidopsis thaliana (ecotype Columbia) plantlets were grown, and protoplasts from hypocotyl epidermal cells (diameter, 25–75 μm; membrane capacity, 20–50 pF) were prepared as described previously (21). Patch clamp experiments were performed as described by Hamill et al. (24) using an Axon 200A amplifier (Axon Instruments, Foster City, CA) for whole-cell and single-channel recordings. During measurements, freshly isolated protoplasts were maintained in a bath medium containing 50 mM CaCl2, 0.5 mM LaCl3, and 10 mM MES-Tris (pH 6). The bath contained 50 mM CaCl2, 0.5 mM LaCl3, and 10 mM MES-Tris (pH 6), adjusted to 450 mosmol. Bath solutions with variable Cl concentrations were prepared either by mixing the 50 mM CaCl2, 0.5 mM LaCl3, and 10 mM MES-Tris (pH 6) to reduce a possible effect of MES.

The patch pipettes were filled with 25 mM Cs2SO4 for whole-cell experiments or with 140 mM Cs2SO4 for single-channel experiments, together with 2 mM MgCl2, 10 mM Hepes-Tris (pH 7.2), 5 mM EGTA, and 4.2 mM CaCl2 (free [Ca2+]i = 1 μM). Nucleotides were added in the intracellular solution as indicated in the figure legends. ATP was added to the pipette solution as MgATP salts, and the corresponding free ATP concentrations were calculated by assuming that the main species of ATP were ATP4-, ATPH3-, MgATP2-, and MgATPH with pKa = 6.63 and 4.72 for ATP and MgATP, respectively, and a dissociation constant of 10^{-7} for the MgATP complex (25). The osmolarities of bath and pipette solutions were adjusted to 450 mosmol with mannitol using a Wescor 5500 vapor pressure osmometer (Wescor, Logan, UT).

Patch clamp experiment and data acquisition were performed as described previously (23). To change the bath solution, the reference electrode was filled with 100 mM KCl prolonged by a plug (1% agarose and 3% KCl), and the bath solutions were continuously perfused at a rate of 1–2 ml/min.

RESULTS

Intracellular Nucleotides Affect the Voltage Dependence of the Rapid Anion Channel—To study the characteristics of the rapid anion channel, depolarizing voltage steps were applied to the plasma membrane (Fig. 1A). Fig. 1B shows representative steady-state I/V relationships obtained with 50 mM CaCl2 in the bath and 25 mM Cs2SO4 in the pipette solution, with 1, 3, or 10 mM MgATP corresponding to 9.8, 28.9, and 92.8 μM intracellular free ATP (ATP(i)) (25). At very negative potentials, the voltage pulses did not activate any current, indicating that voltage-dependent sulfate-permeable channels are closed. Depolarization of the membrane activated an inward current corresponding to a sulfite efflux. When the membrane was further depolarized, the inward anion current decreased and reversed, and then an outward current corresponding to a chloride influx increased. Under these conditions, when MgATP was raised from 1 to 3 and 10 mM, the peak of sulfite current shifted toward less negative potentials, indicating a shift of the activation threshold (Fig. 1B) as expected from a previous report (22). In addition, when intracellular nucleotides were removed, the channel gating at hyperpolarized voltages was strongly reduced (Fig. 2). Taken together, these initial results suggest a major role of intracellular nucleotides in the gating of the fast anion channel of Arabidopsis cells and led us to investigate in more detail the mechanisms by which nucleotides alter anion channel gating.

The Gating Charge Increases with the Negative Charge Carried by a Nucleotide Molecule—To further analyze the gating of the fast anion channel by voltage, a tail protocol (Fig. 3A) was performed. In this protocol, a depolarizing prepulse induces the activation of the fast anion current. The current deactivates rapidly when the membrane potential is stepped back to hyperpolarized values. The instantaneous amplitude at the beginning of the hyperpolarizing step (tail current amplitude) reflects the activation status of the fast anion current at the end of the preceding depolarizing step. Fig. 3B shows the plot of normalized tail current amplitudes against the prepulse potentials, illustrating the voltage-dependent activation of the fast anion channel by depolarization with either 10 mM AMP or 10 mM MgATP in the cytosol. The gating charges deduced from the fit of the data with Boltzmann functions were −3.63 ± 0.12 for free ATP (n = 7) and −1.96 ± 0.12 for AMP (n = 4), indicating a correlation between the theoretical valence of the free nucleotide and the gating charge of the fast anion current (Fig. 3C). This result shows that the charge carried by the free nucleotide accounts for the gating charge of the current. When raising the MgATP concentration from 1 to 10 mM, the activation curve...
was shifted toward less negative potentials, with no change in the apparent gating charge (Fig. 3B). Fitting with Boltzmann curves yielded half-activation potentials \((V_{1/2})\) of \(-169.4 \pm 1.1, -154.9 \pm 2.1,\) and \(-146.8 \pm 1.4\) mV and apparent gating charges \((z_g)\) of \(-3.93 \pm 0.08, -3.59 \pm 0.12,\) and \(-3.63 \pm 0.12\) \((n = 5, 4,\) and 7\) for 9.8, 28.9, and 92.8 \(\mu M\) ATP, respectively.

**Increasing the Internal ATP Concentration Accelerates Fast Anion Channel Deactivation without Altering Its Activation Kinetics.** To analyze the effect of intracellular nucleotides on the fast anion current kinetics, we studied the activation and deactivation kinetics in the whole-cell configuration. Stepping from a hyperpolarized potential, where channels are mostly closed, to depolarized potentials, where channels are mostly open, allowed us to monitor the activation kinetics. Upon depolarization of the membrane, the fast anion current activated with biexponential kinetics. The fast time constant that accounts for the activation of 80% of the current was studied. The fast activation time constant was voltage-dependent (Fig. 4A). When the activation kinetics were studied using various concentrations of MgATP, the time constants were not significantly different at a given potential. For example, at \(-93\) mV (Fig. 4B), a potential at which maximal activation is reached for 9.8, 28.9, and 92.8 \(\mu M\) ATP, the activation time constants were \(1.84 \pm 0.25, 1.62 \pm 0.20,\) and \(1.77 \pm 0.23\) ms \((n = 5, 5,\) and 7\), respectively.

Alternatively, stepping back from \(-13\) mV to hyperpolarized potentials at which the channel is closed allowed us to monitor the deactivation kinetics of the current. Under these conditions, the current decayed with biexponential kinetics, and both time constants were voltage-dependent. In contrast with what we observed for the activation kinetics, both deactivation time constants were strongly altered when ATPi was changed (Fig. 4C). For example, at \(-203\) mV, a potential at which the fast anion current is completely deactivated with 9.8, 28.9, and 92.8 \(\mu M\) ATP, the slow deactivation time constants were \(7.20 \pm 1.24, 1.27 \pm 0.25,\) and \(0.66 \pm 0.13\) ms \((n = 4, 3,\) and 7\), respectively. The relative ratio of the current corresponding to the two time constants was voltage-dependent, with slow deactivating current dominating at moderately hyperpolarized potentials (more depolarized than \(-193\) mV for \(10\) \(\mu M\) MgATP), and fast deactivating current dominating at moderately hyperpolarized potentials (more hyperpolarized than \(-193\) mV for \(10\) \(\mu M\) MgATP). Thus, the biexponential kinetics likely reflects the distribution of the channel between two different closed states in a voltage-dependent manner.

To test directly the effect of intracellular free ATP on the rate of channel closure, we recorded single-channel events with different MgATP concentrations. These outside-out signals were previously shown to carry the fast anion whole-cell cur-

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**Fig. 3.** ATP concentration affects the half-activation potential, whereas the adenosine nucleotide valence affects the apparent gating charge. A, typical tail currents recorded from a voltage protocol consisting of a holding potential of \(-213\) mV and 610-ms pulses from \(-213\) to \(-23\) mV with a 10-mV increment every 2 s. Pipette and bath compositions were the same as those described in Fig. 1A. B, representative examples of normalized gating curves with 10 \(\mu M\) intracellular AMP or 10, 3, or 1 \(\mu M\) intracellular MgATP. The tail currents were measured at the peak after a jump to \(-243\) mV for AMP or a jump to \(-213\) mV for MgATP using the tail protocol described in A. The parameters of the fits with Boltzmann curves for the data presented were: \(z_g = -2.5, -3.5, -3.7,\) and \(-3.8\) and \(V_{1/2} = -209, -147, -161,\) and \(-173\) mV for 10 \(\mu M\) AMP and 10, 3, and 1 \(\mu M\) MgATP, respectively. C, values of the apparent gating charge as a function of adenine nucleotide concentrations. The tail protocol described in A was used to measure the \(z_g\) for different nucleotides. Data were fitted with Boltzmann equations. Pipettes contained either 10 \(\mu M\) AMP, 10 \(\mu M\) KADP, or 1, 3, or 10 \(\mu M\) MgATP, and the \(V_{1/2}\) were \(-202.6 \pm 2.1, -144.0 \pm 2.6, -169.4 \pm 1.1,\) and \(-154.9 \pm 2.1,\) and \(-146.8 \pm 1.4\) mV \((n = 4–7)\), respectively. Each point is the mean \pm S.E. The bath contained 50 mM CaCl\(_2\), 0.5 mM LaCl\(_3\), and 10 mM MES-Tris (pH 6), adjusted to 400 mosmol.

**Fig. 4.** The deactivation kinetics is dependent on intracellular MgATP concentration. Pipette and bath compositions are the same as those described in Fig. 1A with the indicated internal MgATP concentrations. A, activation and deactivation kinetics were both voltage-dependent, but only the deactivation was affected by intracellular ATP. Activation kinetics (diamonds) and deactivation kinetics (circles and squares) were fitted by biexponentials. For the activation, only the time constant for the major component was plotted (diamonds). Each point represents the mean \pm S.E. \((n = 3–7)\). B, ATP dependence of the activation kinetics. The white circles correspond to data samples before and after a step from \(-213\) to \(-93\) mV, showing the time dependence of current activation. Horizontal bar, 100 ms; vertical bar, 200 pA. Lines correspond to biexponential fits, but only the prominent kinetic constants \((\mu s)\) are indicated. C, ATP dependence of the deactivation kinetics. The white circles correspond to data samples before and after a step from \(-13\) to \(-213\) or \(-93\) mV, showing the time dependence of current deactivation. The membrane potential was clamped at \(-13\) mV, and 488-ms voltage pulses from \(-213\) to \(-23\) mV with a 10-mV increment were applied every 3 s. Horizontal bar, 20 ms; vertical bar, 1 nA. Lines correspond to biexponential fits, and prominent kinetic constants \((\mu s)\) are indicated.
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FIG. 5. Intracellular MgATP alters the rate of channel closure. A, example of single-channel recordings at −205 mV with 10, 3, and 1 mM intracellular MgATP. The pipette has the same composition as that described in Fig. 2, except for the indicated MgATP concentrations. Bath is the same as that described in Fig. 2. Horizontal bar, 10 ms; vertical bar, 2 pA. B, the closing rate constant (1/open time) varies linearly with ATPi. Same data acquisition as described in A. Each point is the mean ± S.E. (n = 3–4).

Increasing the External Chloride Concentration Attenuates the Effect of Nucleotides—To further investigate the mechanism by which nucleotides alter the voltage dependence of anion channels, we tested the effect of changing the concentration of extracellular permeant anions on the voltage dependence of the fast anion current. The gradual substitution of the CaCl2 bath by a Ca(NO3)2 bath led to a shift of the peak current (Fig. 7A). Decreasing Cle by replacing CaCl2 with CaMES2 in the bath solution slowed down the activation kinetics of the fast anion current (Fig. 7, A and C). For instance, at −93 mV, the activation constants were 1.77 ± 0.22, 18.75 ± 1.90, 140.50 ± 16.80, and 322.25 ± 19.58 ms (n = 7, 4, 4, and 4) for 101.5, 10.15, 1.015, and 0 mM Cle, respectively (Fig. 7A). In contrast, deactivation constants were poorly affected by changes in the external chloride concentration (Fig. 7, B and C). Similar results were obtained when the Cle concentration was reduced by decreasing CaCl2 in the bath while maintaining a constant concentration of 1 mM MES (data not shown). Thus, the faster channel activation upon depolarization accounts largely for the effect of Cle on the gating of the fast anion channel.

The results obtained in the whole-cell configuration suggest that, at the single-channel level, the rate of opening of the channel should increase with the external chloride activity, whereas the rate of closure should not be strongly affected. We could only measure the mean open time because of the high channel density. At −205 mV, a potential for which open events are distinct, when the external chloride concentration was decreased gradually from 101.5 to 60.9, 30.45, and 0 mM, the mean open times were 4.22 ± 0.41, 3.58 ± 0.67, 3.82 ± 0.84, and 3.42 ± 0.52 ms (n = 6, 3, 2, and 3; Fig. 7D). Thus, as expected from the whole-cell data, changing the Cle concentration did not significantly affect the mean open time.

Nitrate, a More Permeant Anion, Is More Efficient than Chloride in Attenuating the Effect of Nucleotides—To confirm that the interaction between internal nucleotides and external anions is related to their ability to permeate the channel pore, we studied the effects of external nitrate that has a high permeability ratio relative to chloride (23). When the CaCl2 bath was replaced by a Ca(NO3)2 bath, we observed a shift of the voltage dependence toward more negative potentials. The half-activation potential shifted from −145.6 ± 4.0 to −169.3 ± 5.5 mV (n = 5; Fig. 8A), but the apparent gating charge did not change after replacement of chloride (zg = 3.49 ± 0.18) with nitrate (zg = −3.36 ± 0.16), indicating that nitrate is more efficient than chloride in counteracting the effect of nucleotides. The two-component deactivation kinetics was poorly affected by the nature of the extracellular anion in the bath (Fig. 8B). However, at a given potential, the activation kinetics became faster when extracellular chloride was replaced by the more permeant anion nitrate. For example, at −133 mV, the activation time constant was 14.6 ± 1.7 ms (n = 5) with the CaCl2 bath and 3.8 ± 0.2 ms (n = 5) with the Ca(NO3)2 bath (Fig. 8B).

External Nucleotides Induce a Rectification of the Inward Anion Current—Because intracellular nucleotides modify the voltage dependence of the inward current carried by the fast anion channel, we tested whether extracellular perfusion of...
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**FIG. 6.** Decreasing Cle shifts the half-activation potential of the rapid anion channel toward depolarized voltages. A, examples of steady-state I-V plots obtained from the protocol described in Fig. 1A. Pipette has the same composition as described in Fig. 1A. The bath chloride concentrations varied from 101.5 to 0 mM as indicated in the figure and were obtained by replacing CaCl₂ with CaMES₂. B, representative examples of normalized gating curves obtained using the tail protocol described in Fig. 3A and the conditions described in A. The parameters of the fits with Boltzmann curves for the data presented were: $g_a = -3.3, -3.1, -2.9,$ and $-2$ and $V_{1/2} = -146, -126, -108,$ and $-97$ mV for 101.5, 10.15, 1.015, and 0 mM extracellular chloride, respectively. C, half-activation potential ($V_{1/2}$) as a function of Cle at 1, 3, and 10 mM MgATP. The bath chloride concentrations were modified either by replacing CaCl₂ with CaMES₂ (diamonds) or by decreasing the CaCl₂ concentration while maintaining a 1 mM concentration of MES (circles). Each point represents the mean ± S.E. ($n = 3–7$). $V_{1/2}$ values were obtained by fitting with Boltzmann equations.

**Nucleotides Confer Voltage Dependence to the Channel Through an Open Channel Voltage-dependent Block Mechanism**—Our finding that the gating charge increases with the negative charge carried by a nucleotide molecule led us to propose that nucleotides act as gating charges for the fast anion channel by entering the channel pore in a voltage-dependent manner. A similar mechanism of open channel voltage-dependent block has been demonstrated for NMDA ionotropic receptors (3, 4) and inward rectifying potassium channels (Kᵣ, Ref. 5). However, these cation-selective channels are blocked by the entry of soluble cations in the pore such as Mg²⁺ in the case of NMDA receptors or Mg²⁺ and spermidine in the case of Kᵣ channels, whereas for the plant fast anion channel, the gating is achieved by the entry of an anionic nucleotide in the pore.

The voltage-dependent open channel block mechanism predicts that channel closure, which requires recruitment of a nucleotide molecule from the cytosol to the channel pore, should be affected by ATPi, whereas channel opening should not. In agreement with the proposed mechanism, we found that the deactivation kinetics of the fast anion current, which reflects the rate of channel closure, is accelerated when the concentration of intracellular nucleotides is increased, whereas the activation kinetics remains unchanged. Under our experimental conditions, biphasic deactivation kinetics could be observed, suggesting that the blocking by nucleotides can lead to two different closed states. One of them is reached by slower kinetics and predominates at moderately hyperpolarized potentials, whereas the other is reached by fast deactivation kinetics and predominates at very hyperpolarized potentials. Compared with the open channel voltage-dependent block of NMDA receptor by Mg²⁺ (3, 4), the mechanism of nucleotide voltage-dependent block of the fast anion channel is more complex. This is likely due to the fact that nucleotides are larger and more complex blocking particles than Mg²⁺ that can take several different positions in the channel pore and possibly even modify the conformation of the channel protein. Despite this complexity, it is important to note that because both deactivation constants are sensitive to ATP, both closed states correspond to conformations of the channel blocked by ATP. Our data showing that nucleotides can also induce a voltage-dependent block when perfused in the extracellular solution further support the hypothesis that nucleotides block the channel by entering the channel pore. The fact that nucleotides can act from both sides suggests that the blocking mechanism relies on the basic geometry of the pore.

In addition, we studied the effect of intracellular nucleotides on the rate of channel closure at the single-channel level. In agreement with a block of the channel by nucleotides, the rate of channel closure follows the equation:

$$\frac{1}{t_{closed}} = K_{on}[ATP] + A$$

where $K_{on}$ is the rate constant of free ATP entry into the channel pore at the voltage studied ($M^{-1}s^{-1}$) and $A$ reflects the ATP-independent closure. A linear fitting of Fig. 5B allowed us to determine a value of $7.3 \times 10^6 M^{-1}s^{-1}$ for $K_{on}$ at $-205$ mV. This constant is much faster than what is expected for simple diffusion in water and likely reflects the fact that the membrane voltage accelerates nucleotide motion inside the pore. Thus, the binding of ATP, leading to channel closure, occurs within the transmembrane electric field. When the linear relationship is extrapolated to [ATP] = 0, the rate of closure is low. On one hand, this indicates that the voltage-dependent block by intracellular nucleotides largely accounts for the closure of the channel at hyperpolarized potentials. On the other hand, this residual rate of channel closure extrapolated for 0 ATPi, together with the incomplete loss of voltage dependence when the inside of the cell was perfused with an ATP-free solution, suggests the existence of some ATP-independent voltage gating. Finally, it is important to note that this ATP-independent gating would occur at a highly hyperpolarized potential and would only account for a minor fraction of the channel voltage dependence under physiological conditions.
Sensing the Voltage—In addition to the effect of intracellular nucleotides, we also show that external permeant anions can further argue in favor of our hypothesis that nucleotides between external permeant anions and internal nucleotides can alter the gating of the fast anion channel. This dose-dependent effect does not saturate at external chloride concentrations as high as 100 mM, suggesting a low affinity mechanism associated with ion permeation in the channel. This is supported by the observation that replacing chloride with a more permeant anion, nitrate, also reduces the effect of intracellular nucleotides. Because the interaction between external permeant anions and internal nucleotides can only take place inside the channel pore, this result provides further argument in favor of our hypothesis that nucleotides gate the fast anion channel by blocking the pore in a voltage-dependent manner. Furthermore, kinetic analysis of the effect of external chloride shows that increasing external chloride or replacing chloride with nitrate, a more permeant anion, accelerates fast anion channel activation while only slightly modifi-
fying the rate of closure. According to our hypothesis, channel activation reflects the expulsion of ATP from the channel pore. This result shows that rather than competing with ATP for a common or overlapping binding site within the pore, permeant external anions are able to trigger channel relief from block by kicking the nucleotide out from the pore. In this view, a more permeant anion, such as nitrate, is more efficient in triggering the expulsion of a nucleotide molecule from the channel pore.

The analysis of the effect of external permeant anions also reveals a role for permeant anions in voltage sensing. Indeed, a reduction of the external permeant anion concentration results in a decrease of the gating charge of the current. Most of the variation of the gating charge occurs when the chloride concentration is raised from 0 to 10 mM, suggesting that this effect involves a high affinity mechanism distinct from the nucleotide expulsion described above. The effect of chloride on the gating charge suggests that the charge carried by the permeating anions contributes to the sensing of membrane voltage by the fast anion channel. This is in agreement with the results obtained by Dietrich and Hedrich (27) on the fast anion channel from V. faba guard cells. It is also reminiscent of the gating of the torpedo voltage-dependent chloride channel CLC-0. For CLC-0, channel activation is strongly facilitated by external chloride, and Pusch et al. (28) have suggested that voltage-dependent gating is conferred by the permeating charge itself, acting as the gating charge. Additional studies on the role of Cl− ions in the fast voltage-dependent gating of CLC-0 confirmed that Cl− movement within the channel is the major source of voltage dependence, whereas charge movement intrinsic to the channel protein contributes little to this process (29).

**Physiological Relevance of Nucleotide and Extracellular Anion Regulations**—The various regulations of its gating likely enable the fast anion channel to sense important cellular parameters such as the concentrations of internal nucleotides and external anions. The regulation of the channel by intracellular nucleotides could couple the metabolic status of the cell and its membrane excitability (22). A similar role has been demonstrated in the case of K-ATP channels from pancreatic β cells (30). In this case, an increase in the metabolic energy charge increases the inhibition of the channel by ATP (30). This allows membrane depolarization that favors insulin secretion. In the case of the fast anion channel, the regulation by ATP could represent a means to generate a depolarization signal in response to a decrease in the metabolic charge of the cell under stress conditions, such as hypoxia, for example (31). The regulation by extracellular permeant anions would then act as a feed forward regulatory loop to amplify and maintain a depolarization sufficient to trigger downstream signaling events, as already suggested for the fast anion channel in V. faba guard cells (15, 16, 26).

In conclusion, our results showing that intracellular nucleotides affect the voltage dependence of the fast anion channel and that the gating charge correlates with the valence of the nucleotide led us to propose that the voltage-dependent closure of the channel at hyperpolarized membrane potentials results from voltage-dependent occlusion of the channel pore by intracellular nucleotides. From our data, we also suggested that permeant anions such as chloride or nitrate can chase the nucleotide from the channel pore by a repulsion mechanism, thus favoring channel relief from block. In addition, voltage sensing by permeant anions going through the pore could also contribute to the activation process. To our knowledge, this study represents the first evidence that plant anion channels can be gated by a voltage-dependent block mechanism and also represents the first demonstration that nucleotides, at physiological concentrations, can gate a channel through a simple biophysical mechanism.

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