Distinct pH regulation of slow and rapid anion channels at the plasma membrane of Arabidopsis thaliana hypocotyl cells

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Abstract

Variations in both intracellular and extracellular pH are known to be involved in a wealth of physiological responses. Using the patch-clamp technique on Arabidopsis hypocotyl cells, it is shown that rapid-type and slow-type anion channels at the plasma membrane are both regulated by pH via distinct mechanisms. Modifications of pH modulate the voltage-dependent gating of the rapid channel. While intracellular alkalinization facilitates channel activation by shifting the voltage gate towards negative potentials, extracellular alkalinization shifts the activation threshold to more positive potentials, away from physiological resting membrane potentials. By contrast, pH modulates slow anion channel activity in a voltage-independent manner. Intracellular acidification and extracellular alkalinization increase slow anion channel currents. The possible role of these distinct modulations in physiological processes involving anion efflux and modulation of extracellular and/or intracellular pH, such as elicitor and ABA signalling, are discussed.

Key words: Anion channels, Arabidopsis thaliana, pH regulation, plant, plasma membrane.

Introduction

Patch-clamp studies have revealed that anion channels are present in plant cells from various organs (Barbier-Brygoo et al., 2000) where they play specific physiological roles. The best documented system so far is the stomatal guard cell where two anion channels from the plasma membrane have been extensively characterized. One of them, called GCAC1, shows fast activation and deactivation kinetics in the 10 ms range, and was also termed R-type (for rapid-type) channel (Keller et al., 1989; Dietrich and Hedrich, 1998). The second exhibits slow kinetics in the 10 s range and was termed S-type (for slow-type) channel (Schroeder and Keller, 1992; Schmidt and Schroeder, 1994). Abscisic acid-induced guard cell closure involves anion channel activation (Ward et al., 1995) together with apoplastic pH variations (Felle et al., 2000) and cytosolic pH alkalinization.

At the plasma membrane of Arabidopsis hypocotyl epidermal cells, R-type and S-type anion channels have been shown to coexist (Frachisse et al., 2000). These two channels show distinct properties: a high nitrate-permeation with slow activation and deactivation kinetics for the S-type channel (Frachisse et al., 2000), and rapid kinetics, strong voltage-dependence associated with sulphate and nitrate permeation for the R-type (Thomine et al., 1995, 1997; Frachisse et al., 1999). Both channels are highly regulated. The slow anion channel of hypocotyl cells is modulated by phosphorylation (Frachisse et al., 2000) while the nucleotides regulate the rapid channel through an open pore voltage-dependent manner (Colcombet et al., 2001). This block is counteracted by extracellular permeant anions at physiological concentrations. In addition, cytosolic sulphate is required to maintain maximal activity of the rapid channel (Frachisse et al., 1999), whereas a rapid run-down occurs with other permeant anions. Anion channels, including those described at the plasma membrane of epidermal cells of Arabidopsis thaliana hypocotyl, thus appear as multi-regulated transport systems (Barbier-Brygoo et al., 2000).

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In response to various stimuli, such as elicitors of defence reaction or osmotic stresses, intracellular pH (pHi) decreases as extracellular pH (pHₑ) increases. Often these responses are accompanied by anion effluxes (Mathieu et al., 1996; Pugin et al., 1997; Cazalé et al., 1998).

In this study, the regulation by pH of the R-type and S-type channels from Arabidopsis hypocotyls was investigated. Both channels are regulated by pH with distinct mechanisms and represent good candidates to play a role in pH-dependent cellular processes.

Materials and methods

Arabidopsis thaliana (Columbia) plants were grown in vitro and hypocotyl protoplasts were prepared as previously described (Elzenga, 1991; Thomine et al., 1995). Whole cell patch-clamp experiments were performed as described in Colcombet et al. (2001).

During measurements, protoplasts were maintained in an extracellular solution referred to as the ‘reference bath’ containing 50 mM CaCl₂, 5 mM MgCl₂, 0.5 mM LaCl₃, 10 mM MES, with the pH adjusted to 5.5 with TRIS. Acid loads were performed by adding 3 mM propionate/Na (pH 5.5) to the bath. Extracellular baths with different pH were obtained by adding NaOH to a solution containing 50 mM CaCl₂, 5 mM MgCl₂, 0.5 mM LaCl₃, 10 mM MES, 10 mM HEPES, with the pH adjusted to 5.5 with TRIS (pHₑ). Thus, the pH buffer capacity ranges from 5.5 to 8.2 pH units (Ferguson et al., 1980) and the maximal concentration of NaOH added was 16 mM for pH 8.2. A check was made to ensure that sodium ions do not affect anion channel activity by adding 20 mM NaCl to the extracellular bath (data not shown). The patch-clamp pipettes were filled with anion channel activity by adding 20 mM NaCl to the extracellular bath. The reference bath is buffered at pH 5.5 with 10 mM MES/TRIS. During measurements, protoplasts were maintained in an extracellular solution referred to as the ‘reference bath’ containing 50 mM CaCl₂, 5 mM MgCl₂, 0.5 mM LaCl₃, 10 mM MES, with the pH adjusted to 5.5 with TRIS. Acid loads were performed by adding 3 mM propionate/Na (pH 5.5) to the bath. Extracellular baths with different pH were obtained by adding NaOH to a solution containing 50 mM CaCl₂, 5 mM MgCl₂, 0.5 mM LaCl₃, 10 mM MES, 10 mM HEPES, with the pH adjusted to 5.5 with TRIS (pHₑ). Thus, the pH buffer capacity ranges from 5.5 to 8.2 pH units (Ferguson et al., 1980) and the maximal concentration of NaOH added was 16 mM for pH 8.2. A check was made to ensure that sodium ions do not affect anion channel activity by adding 20 mM NaCl to the extracellular bath (data not shown). The patch-clamp pipettes were filled with 2 mM MgCl₂, 10 mM or 50 mM HEPES, 5 mM EGTA, 4.2 mM CaCl₂, 5 mM MgATP, 5 mM LiGTP, together with 25 mM Cs₂SO₄ or 50 mM CsNO₃ for the recording of rapid-type and slow-type currents, respectively. Intracellular pipette solutions were adjusted to various pH with TRIS (pHi). The bath and pipette osmolarities were adjusted to 450 mOsm with mannitol. It had previously been observed that cytosolic calcium does not affect the activities of rapid and slow-type anion channels (J Colcombet et al., unpublished results).

Results

The activity of the rapid anion channel is poorly affected by intracellular pH changes

The effects of pH on the activity of the R-type channel were investigated using the patch-clamp technique in the whole-cell configuration. A sulphate-based pipette solution allowed rapid channel currents with a high stability to be recorded over several hours (Frachisse et al., 1999). To investigate the effects of intracellular pH (pHi), protoplasts were superfused with pipette solutions at pH 6.5, 7.2, and 8.0. After the activation of the R-type current and its stabilization (typically after 3 min), depolarizing voltage pulses (Fig. 1A) were performed to determine the steady-state current–voltage relationship (Fig. 1B). At hyperpolarized potentials, the voltage pulses did not activate any current, indicating that voltage-dependent sulphate-permeable channels are closed. Depolarization of the membrane activated an inward current corresponding to a sulphate efflux. Voltage steps to positive voltages elicited outward currents corresponding to chloride influx. At pHᵢ, 6.5 and 7.2, the steady-state activity of the rapid anion channel is not significantly affected, with peak potentials of −113.0±2.7 mV (n=6) and −111.2±2.2 mV (n=4), respectively. At pH 8.0, a significant shift of the voltage-dependent gating toward more negative potential was observed, leading to a shift in the peak potential to −125.1±2.2 mV (n=5). To evaluate the pH dependence of channel kinetics, current activation in response to depolarizing pulses (the protocol described in Fig. 1A)
was fitted with a mono-exponential function and the time constants were plotted against the voltage (Fig. 1C, circles). A hyperpolarizing pulse protocol (Fig. 1D) was used to analyse the deactivation kinetics which were fitted by a bi-exponential function (Fig. 1C). Both activation and deactivation are slightly, but significantly, slowed down by intracellular alkalization.

**The rapid channel gating is affected by extracellular pH changes**

The modulation of R-type channels by extracellular pH (pHe) was investigated with a highly buffered pipette solution at pH 7.2 (50 mM HEPES/TRIS). Voltage pulse protocols were performed in extracellular bath solutions with pHe ranging from 5.6 to 9.5. Alkalization of the bath led to a shift of the peak current toward less negative potentials corresponding to a shift of the voltage-dependent gating. To monitor the voltage-dependent gating (Fig. 2B), the open probability of the channel was analysed as a function of membrane potential for different values of pHe. Boltzmann fit of the channel open probabilities showed that, whereas the gating charges ($z_g$) are not clearly affected (from $2.5 \pm 0.1$ at pHe 5.6 ($n=6$) to $2.8 \pm 0.1$ at pHe 9.5 ($n=5$)), bath alkalization decreases the half-activation potentials ($V_{1/2}$) from $-129.0 \pm 1.7$ at pHe 5.6 to $-99.2 \pm 3.4$ at pHe 9.5 (Fig. 2C). In addition, the current intensity at a depolarized potential also decreased with alkalization. This indicates that extracellular alkalization may also decrease either the unitary conductance of the channel or the number of active channels following a non-voltage-dependent gating mechanism. Activating and deactivating protocols described in Fig. 1A and D were used to investigate the dependence of channel kinetics on external pH. Figure 2D shows that extracellular alkalization affected the deactivation kinetics marginally, but strongly slowed down the activation. For example, at $-81 \text{ mV}$, the activation constant increases from $1.51 \pm 0.19 \text{ ms}$ at pH 5.6 to $54.20 \pm 22 \text{ ms}$ at pH 9.5 ($n=5$).

**pHe modulates slow-type channel activity**

To study the activity of the S-type channel, the protoplast intracellular medium was superfused with a nitrate-based pipette solution. After the run-down of the rapid anion channel and the stabilization of current intensity (typically after 10 min), the slow anion channel activity was recorded (Fig. 3A). Because of the high variability in the slow anion current amplitude from one protoplast to another, it was not possible to compare currents recorded in different protoplasts superfused with pipette solutions at different pHe. For this reason, the choice was made to acidify the intracellular compartment by performing an acid load. For this purpose, after getting a stable activity of the slow anion channel in the whole cell configuration, the protoplasts were superfused with a bath solution at pH 5.5 supplemented with 3 mM sodium propionate (Fig. 3A). The effect of such an acid load on cytosolic pH cannot be precisely quantified, but referring to previous work from Mathieu et al. (1996) on tobacco cells, an estimation of the
Acidification can be given. For 3 mM of propionate in the bath solution, a concentration of 0.47 mM of the protonated form of the weak acid at bath pH of 5.5 is calculated with the Henderson–Hasselbalch equation. In this condition, a decrease of 0.4–0.6 pH units is expected. Current–voltage relationships determined before, during, and after the acid load show an increase of channel activity upon propionate addition (Fig. 3B, C) and a reversibility of this effect (Fig. 3C). The acid load also induced a decrease of the deactivation kinetics (Fig. 3).

The slow-type anion channel activity is activated by extracellular alkalinization

To investigate the pH dependence of the S-type channel, protoplasts held in the whole cell configuration with a nitrate-based pipette containing 10 mM HEPES/TRIS (pH 7.2) were perfused with alkaline bath solutions. Figure 4A and B illustrate that changing pH from 5.5 to 8.2 increased the S-type channel activity at negative voltages and slowed the deactivation down. From the example in Fig. 4, the deactivation time constant went up from 7.5±0.8 s (n=5) to 14.1±3 s (n=5) when pH was changed from 5.5 to 8.2. To characterize the pH regulation of the slow channel gating better, the open probability, expressed as the ratio (I_{rel}/I_{inst}) was plotted against membrane potentials (Fig. 4C). The Boltzmann fit of the mean open probabilities yielded V_{1/2} values of −132.5 and
To determine the pH range of the slow channel modulating is not affected by alkalinization of the bath solution. Discussion

Mechanisms underlying the pH sensitivity of the R-type anion channel

It has recently been shown (Colcombet et al., 2001) that the rapid channel of Arabidopsis hypocotyls is gated by intracellular nucleotides able to block the channel pore in a voltage-dependent manner. Increasing the nucleotide concentration shifts the activation gate towards more positive membrane potentials and accelerates deactivation. Intracellular alkalinization to pH 8 has two effects: it shifts the activation potential toward negative potentials and slows down activation and deactivation kinetics. This indicates that pH increase may interfere with the nucleotide blocking process of the R-type channel pore. This hypothesis will need to be further investigated using different concentrations of nucleotides and nucleotide analogues with distinct pKa values.

Extracellular and intracellular alkalinization have opposite effects on the R-type channel regulation. Indeed, increasing extracellular pH induces a shift of the voltage-dependent gating toward positive voltages and slows the activation kinetics down, without modifying the deactivation kinetics. A similar effect caused by extracellular alkalization has previously been described (Schroeder and Hagiwara, 1989). The pH sensitivity of the slow-type anion channel is regulated by phosphorylation processes as well (Frachisse et al., 1996; Thomine et al., 1997). The activity of the Arabidopsis slow anion channel is regulated by phosphorylation processes as well (Frachisse et al., 2000). It is shown here that both intra- and extracellular pH modulate the slow anion channel activity in Arabidopsis hypocotyls. A cytosolic acidification by about 0.5 pH units obtained by acid load (Mathieu et al., 1996) induces activation of the slow channel, leading to a doubling of the steady-state current measured at $-189 \text{ mV}$, and extracellular pH higher than 6.5 decreases the apparent extracellular chloride concentration sensed. This is in agreement with the decrease of chloride-dependent outward current observed at positive voltages upon bath alkalinization. However, the extracellular pH increase might act by a distinct mechanism, such as stabilization of the channel in the nucleotide-blocked conformation.

The rapid channel of Arabidopsis hypocotyl cells displays pH regulations distinct from those of its Vicia faba counterpart, GCAC1, possibly revealing species- or tissue-linked variations. While the cytosolic acidification from 7.8 to 5.6 did not affect GCAC1 activation and deactivation kinetics (Schulz-Lessdorf et al., 1996), the single-channel activity was increased. For GCAC1, a pH increase mainly affects the time constant of slow inactivation, while it is shown here that, for the Arabidopsis rapid channel, extracellular alkalization increases the activation time constant. This pH sensitivity represents an additional element, besides nucleotide regulation (Schulz-Lessdorf et al., 1996; Thomine et al., 1997; Colcombet et al., 2001) and pharmacology (Marten et al., 1992, 1993; Thomine et al., 1997) showing that two anion channels with similar voltage dependence may present pronounced differences in their mode of regulation.

pH sensitivity of the slow-type anion channel

The S-type channel of Vicia faba has been shown to be controlled by various cellular parameters such as calcium (Schroeder and Hagiwara, 1989), abscisic acid (Pei et al., 1997), ATP binding cassette modulators (Leonhardt et al., 1999), and protein phosphorylation (Schmidt et al., 1995; Pei et al., 1997). The activity of the Arabidopsis slow anion channel is regulated by phosphorylation processes as well (Frachisse et al., 2000). It is shown here for the first time that both intra- and extracellular pH modulate the slow anion channel activity in Arabidopsis hypocotyls. A cytosolic acidification by about 0.5 pH units obtained by acid load (Mathieu et al., 1996) induces activation of the slow channel, leading to a doubling of the steady-state current measured at $-189 \text{ mV}$, and extracellular pH higher than 6.5 decreases the apparent extracellular chloride concentration sensed. This is in agreement with the decrease of chloride-dependent outward current observed at positive voltages upon bath alkalinization. However, the extracellular pH increase might act by a distinct mechanism, such as stabilization of the channel in the nucleotide-blocked conformation.

Table 1. Divergent characteristics between rapid-type and slow-type anion channel activities at the plasma membrane of Arabidopsis epidermal cells (TS this study)

<table>
<thead>
<tr>
<th></th>
<th>R-type activity</th>
<th>S-type activity</th>
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<tbody>
<tr>
<td>Voltage-dependence</td>
<td>High ($z_v$ almost $-4$)</td>
<td>Low ($z_v &lt; -1$)</td>
</tr>
<tr>
<td>Kinetics</td>
<td>Range of milliseconds</td>
<td>Range of 10 s</td>
</tr>
<tr>
<td>Selectivities</td>
<td>$\text{NO}_3^- = \text{SO}_4^{2-} &gt; \text{Cl}^-$</td>
<td>$\text{NO}_3^- &gt; \text{Cl}^-$ $\gg \text{SO}_4^{2-}$</td>
</tr>
<tr>
<td>Nucleotide regulations</td>
<td>High: Nif</td>
<td>High: DIDS</td>
</tr>
<tr>
<td>Blocker effects</td>
<td>Intermediate: NPPB</td>
<td>Intermediate: NPPB, Nif</td>
</tr>
<tr>
<td>Activity variations during seal</td>
<td>Run-down with Cl$^-$ and NO$_3^-$-based pipettes</td>
<td>Sustained activity with SO$_2^-$-based pipette</td>
</tr>
<tr>
<td>pH$_i$ increase</td>
<td>Inhibited$^{TS}$</td>
<td>Activated$^{TS}$</td>
</tr>
<tr>
<td>pH$_i$ decrease</td>
<td>Inhibited/weak effect$^{TS}$</td>
<td>Activated$^{TS}$</td>
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$-133.5 \text{ mV}$ and $z_v$ of $-0.46$ and $-0.50$, for pH$_i$ 5.5 and pH$_i$ 8.2, respectively ($n=5$). Thus, the voltage-dependent gating is not affected by alkalinization of the bath solution. To determine the pH$_i$ range of the slow channel modulation, protoplasts were perfused with the external bath at increasing pH values. Figure 4D, which shows normalized instantaneous and relaxed currents as a function of pH$_i$, indicates that the increase of channel activity occurs at pH$_i$ higher than 6.5 ($n=5$).
also up-regulates this channel. Similar voltage-dependent open probabilities were obtained at pH 5.5 and 8.2, indicating that neither the activation potential, nor the voltage-dependent gating is affected by external pH. Such a pH-dependent activation might be explained either by a pH-dependent, voltage-independent increase of the open probability, by recruitment of silent channels, or by an increase of single channel conductance at alkaline pH. Despite the authors’ efforts, they were never able to monitor any single channel activity for the slow-type anion channel, suggesting a very low conductance for this channel. Thus, for the moment, it is not possible to reach a conclusion between those different hypotheses.

**Physiological relevance of the pH regulation**

It has previously been shown that rapid-type and slow-type hypocotyl anion channels co-reside at the plasma membrane of hypocotyl cells (Frachisse et al., 2000), but exhibit divergent properties in terms of voltage-dependence, activation and deactivation kinetics, selectivity, nucleotide regulation and pharmacological sensitivity (Thomine et al., 1995, 1997; Frachisse et al., 1999, 2000). These characteristics and regulation modes of the two channels are summarized in Table 1. The distinct pH modulation of rapid and slow channels described in this study provides a new argument in favour of the existence of two different channels, in contrast to an interconversion from a rapid mode to a slow mode for a single channel, as proposed by Dietrich and Hedrich (1994) for guard cell anion channels.

In plant cells, according to the plant species and the technique of measurement used, cytosolic pH values in resting conditions are between 6.8–7.9 (Guern, 1991). In response to elicitor application, osmotic stress or hormone treatment, the cytosolic and apoplastic pH both fluctuate (Guern, 1991; Tretyn et al., 1991; Cazalé et al., 1998; Nühs et al., 2000). For example, among the earliest responses triggered by a variety of elicitors of defence reaction, cytosolic acidification (in the range of 0.6 pH units) accompanied by an extracellular alkalinization (up to 1.5 units) is well documented in several species (Nürnberger et al., 1994; Mathieu et al., 1996; Nühse et al., 2000). These pH variations are generally concomitant with an anion efflux (Nürnberg et al., 1994; Zimmermann et al., 1998). The range of pH values which activate the slow anion channel matches the extracellular pH reached after elicitor treatment. Therefore, the slow anion channel is a potential candidate for mediating anion effluxes triggered by elicitors. In addition, alkaline environmental conditions (pH higher than 7.0) occur in one-third of the arable soils (Marschner, 1995) and might lead to alkalinization of the extracellular space. The very small apoplastic volume favours large variations of pH and of solute concentration (Grignon and Sentenac, 1991). When high proton pumping activity is necessary to compensate for extracellular alk-line conditions, activation of S-type channels at high pH could represent a mechanism to electrically balance strong outward H+ currents by inward anion currents. The pH effect on the R-type channel is more subtle but still occurs in a physiological range. This channel has been proposed to play a role in cell excitability and in long-distance electrical signalling (Thomine et al., 1995; Barbier-Brygoo et al., 2000; Colcombet et al., 2001). The pH regulation of R-type channels could represent a mechanism to modulate the propagation of electrical signals according to environmental conditions.

Since the work reporting proton-regulated anion channels in Chara inflata (Tyerman et al., 1986), the regulatory role of pH on anion channel-mediated processes has not been investigated deeply in the plant kingdom. Considering the data presented in this paper, pH represents a new element of regulation of S-type and R-type Arabidopsis anion channels. This regulation via the pH might occur in several signalling networks including anions fluxes and pH variations, such as those observed in response to pathogens or hormones. As a next step it is believed that pharmacology, combined with a refined description of these channels, should lead to the discovery of their functions.

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