Chloride fluxes in lily pollen tubes: a critical reevaluation

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Abstract

Microelectrodes, made from a Cl\textsuperscript{−}-selective liquid ion exchanger previously used to measure putative Cl\textsuperscript{−} fluxes in 

Lilium longiflorum pollen tubes, were characterized. The electrodes were poorly selective, possessing only about 10-fold selectivity for Cl\textsuperscript{−} over other anions tested. They had only 2.4-fold selectivity for Cl\textsuperscript{−} over the anionic form of the H\textsuperscript{+} buffer, MES, indicating that the electrode can indirectly detect H\textsuperscript{+} gradients. Apparent anion influx was detected along the pollen tube shafts and at the grains while apparent anion efflux was detected near the tip of the tube. During oscillating growth, the peak of the oscillating apparent anion efflux at the tip occurred, on average, 7.9 sec after the peak of the growth oscillations. Consideration of the previously characterized H\textsuperscript{+} fluxes in lily pollen grains and tubes, as well as the poor anion selectivity of the Cl\textsuperscript{−} electrodes, indicates that the putative Cl\textsuperscript{−} fluxes are in fact changes in the anionic concentration of the buffer resulting from H\textsuperscript{+} gradients and not changes in Cl\textsuperscript{−} concentration. The claim of a central role for Cl\textsuperscript{−} in lily pollen tube growth is further undermining by the fact that these tubes grow at the same rate if the Cl\textsuperscript{−} content of the growth medium is reduced to trace levels (≤31 μM), and that the grains have only small reserves of Cl\textsuperscript{−}. These results lead to the conclusion that Cl\textsuperscript{−} fluxes are not a significant component of pollen tube growth and Cl\textsuperscript{−} itself is not required for growth.

Keywords: pollen tube, growth, Cl\textsuperscript{−}, H\textsuperscript{+}, flux.

Introduction

The growing pollen tube is an extreme example of the general phenomenon of biological polarity. Growth is typically rapid, with elongation supported by the addition of new material at the apex of the tube. Careful measurements of elongation rates revealed that growth is oscillatory. In lily, the oscillations typically have a period of 30–60 sec, with growth rates oscillating between 0.1 and 0.4 μm sec\textsuperscript{−1}, and are often stable for a particular pollen tube (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997; Messerli et al., 1999, 2000; Pierson et al., 1995, 1996). Lily tubes accomplish this under an average turgor pressure of 0.21 MPa (Benkert et al., 1997). Understanding how the cellular machinery is organized in order to direct and properly insert secretory vesicles has been the focus of considerable recent research. Those efforts have established that tip-high, intracellular Ca\textsuperscript{2+} gradients are an essential component of the system, and that if the Ca\textsuperscript{2+} gradients are disrupted by any means, growth ceases (Messerli and Robinson, 1997; Miller et al., 1992; Pierson et al., 1994; Rathore et al., 1991). In addition to Ca\textsuperscript{2+} fluxes, oscillatory influxes of both H\textsuperscript{+} and K\textsuperscript{+} have been detected (Feijó et al., 1999; Messerli et al., 1999). K\textsuperscript{+} entry is the largest of these fluxes, around 700 pmol cm\textsuperscript{−2} sec\textsuperscript{−1} (Messerli et al., 1999) and presumably is necessary for maintaining the osmolarity of the tube cytoplasm during cytoplasmic expansion.

Identifying the ions that are essential for pollen tube germination and growth has been a longstanding endeavor. Calcium was identified as an essential ion by Brewbaker and Kwack (1963). They defined a medium for a wide variety of pollen tube growth studies that included H\textsubscript{3}BO\textsubscript{3}, Ca(NO\textsubscript{3})\textsubscript{2}, MgSO\textsubscript{4}, and KNO\textsubscript{3}. Later, Weisenseel and Jaffe (1976) determined that no inorganic anions were necessary for growth and found that specific concentration ranges of Ca\textsuperscript{2+}, K\textsuperscript{+}, and H\textsuperscript{+} were necessary for germination and growth of Lilium longiflorum pollen tubes. More recently, Cl\textsuperscript{−} has been brought back into consideration as an important anion...
involved in the growth of tobacco and lily pollen tubes. Zonia et al. (2002) reported a large oscillatory efflux of Cl\textsuperscript{−} at the tips of tobacco pollen tubes and steady Cl\textsuperscript{−} influx along the shaft immediately behind the tip. The magnitude of the peak Cl\textsuperscript{−} efflux from tobacco ranged between 1000 and 60 000 pmol cm\textsuperscript{−2} sec\textsuperscript{−1} (Figures 1a and 7a, Zonia et al., 2002). Importantly, they found that Cl\textsuperscript{−} efflux oscillations from tobacco pollen were in phase with growth oscillations, thus identifying Cl\textsuperscript{−} efflux as the first oscillating variable to be temporally coincident with growth. They also measured large, oscillating Cl\textsuperscript{−} efflux from the tips of lily pollen tubes, but the phase relationship with growth was not reported.

Massive Cl\textsuperscript{−} efflux at this point in the pollen tube growth cycle would be expected to rapidly decrease turgor pressure during the critical point at which the cell wall at the pollen tube tip is weakest. The uncompensated efflux of Cl\textsuperscript{−} would also be expected to have profound effects on the membrane potential. These results were collected almost entirely with measurements made by a Cl\textsuperscript{−}-selective liquid-ion exchanger microelectrode and therefore the interpretation of the results is limited by the physical properties of that measuring system.

In view of the importance of Cl\textsuperscript{−} flux to the understanding of ion dynamics in pollen tube growth, we have investigated the nature of Cl\textsuperscript{−} movements and Cl\textsuperscript{−} concentrations in lily pollen tubes and grains. We have determined the characteristics of the Cl\textsuperscript{−} anion exchanger electrode in the growth medium used to perform the Cl\textsuperscript{−} flux studies, especially with regard to its selectivity for Cl\textsuperscript{−} over other anions. This was carried out both statically and dynamically in order to better match the actual conditions of self-referencing measurements. We have used the electrode to map apparent Cl\textsuperscript{−} gradients near lily pollen tubes and grains and have also determined the phase relationship between oscillating growth and apparent Cl\textsuperscript{−} efflux at the tip.

**Results**

**Anion exchanger selectivity**

The selectivity of the chloride-selective liquid ion-exchanger (LIX) 24899 for Cl\textsuperscript{−} over all other anions in the growth medium was determined using the separate solutions method. This included H\textsubscript{2}BO\textsubscript{3}, HCO\textsubscript{3}, the anionic form of the H\textsuperscript{+} buffer, MES, as well as other potentially relevant anions. The electrodes showed about a 10-fold selectivity for Cl\textsuperscript{−} over H\textsubscript{2}BO\textsubscript{3} and HCO\textsubscript{3} anions (Table 1). We found that the selectivity for Cl\textsuperscript{−} over MES varied with pH increasing from 2.5 to 6.3 to 63-fold when the pH of the MES solution was changed from 8.2 to 6.2 to 4.5, respectively. MES exists primarily as a zwitterion at pH below its pKa, 6.15 (25\(^\circ\)C), but exists primarily as an anion at pH above its pKa (Good et al., 1966). Under these conditions, the concentration of the anionic form of MES at pH 8.2, 6.2, and 4.5 is 99.1, 50.0, and 2.2 mM, respectively, out of the 100 mM MES used for this measurement. Using these values we generated a linear model of activity versus selectivity for Cl\textsuperscript{−} over MES,\( y = 17.6x − 1.7 \), and calculated that the LIX is only 2.4 times more selective for
Absence of pollen tube Cl\textsuperscript{−} fluxes

Table 1 Reported selectivity coefficients for the anion exchanger 24899

<table>
<thead>
<tr>
<th>Interfering anion</th>
<th>Native LIX\textsuperscript{a}</th>
<th>Equilibrated LIX\textsuperscript{a}</th>
<th>Fluka\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsubscript{2}BO\textsubscript{3} (pH 11.2)</td>
<td>−1.3</td>
<td>−1.3</td>
<td>−1.4</td>
</tr>
<tr>
<td>HCO\textsubscript{3} (pH 8.2)</td>
<td>−0.9</td>
<td>−1.0</td>
<td>−1.4</td>
</tr>
<tr>
<td>MES (pH 4.5)</td>
<td>−0.2</td>
<td>−1.8</td>
<td>−1.8</td>
</tr>
<tr>
<td>MES (pH 6.2)</td>
<td>−0.6</td>
<td>−0.8</td>
<td>−0.8</td>
</tr>
<tr>
<td>MES (pH 8.2)</td>
<td>−0.2</td>
<td>−0.4</td>
<td>−0.4</td>
</tr>
<tr>
<td>Methylsulfonate (pH 5.8)</td>
<td>−0.1</td>
<td>−0.1</td>
<td>−0.7</td>
</tr>
<tr>
<td>Acetate (pH 6.8)</td>
<td>−1.3</td>
<td>−1.3</td>
<td>−0.5</td>
</tr>
<tr>
<td>Propionate (pH 8.7)</td>
<td>−1.0</td>
<td>−0.3</td>
<td>−0.3</td>
</tr>
<tr>
<td>Gluconate (pH 6.5)</td>
<td>−1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H\textsubscript{2}PO\textsubscript{4} (pH 5.0)</td>
<td>−1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{3}\textsuperscript{−}</td>
<td>+1.9</td>
<td></td>
<td>+2.2</td>
</tr>
</tbody>
</table>

Values are listed as the log\textsubscript{10} of the potentiometric selectivity coefficient, (K\textsubscript{B/C0}) where 'B' is the interfering anion. Equilibrated LIX refers to LIX that was rinsed with growth medium before use while native LIX was not equilibrated with growth medium.

\textsuperscript{a}Values reported in this article.

\textsuperscript{b}Values reported by Fluka Chemical Co. (1991).

Cl\textsuperscript{−} than the anionic form of MES (MES\textsuperscript{−}). The negative charge on MES comes from a sulfonic acid group on the molecule. While Fluka lists a fivefold selectivity of this LIX for Cl\textsuperscript{−} over methylsulfonate, we found a much smaller value of only 1.3-fold (Table 1).

We tested the selectivity for Cl\textsuperscript{−} over other possible interfering anions and list the results in Table 1. The pH values listed next to the anion in column 1 are the values acquired from our solutions. Column 2 shows the selectivities from electrodes made with equilibrated LIX, column 3 shows selectivities determined from electrodes made with the medium-equilibrated LIX and column 4 shows the selectivities listed by Fluka. Medium-equilibrated LIX was necessary to reduce toxicity to cells (see below). The LIX only has a 10-fold selectivity for Cl\textsuperscript{−} over acetate, propionate, gluconate and monovalent phosphate. Surprisingly, the LIX is 100 times more selective for NO\textsubscript{3}\textsuperscript{−} than Cl\textsuperscript{−}.

The Cl\textsuperscript{−} channel inhibitors, furosemide, bumetanide and SITS, act as interferents (Chao and Armstrong, 1987) similar to some of the anions listed above. We screened four different anion channel blockers, DIDS, niflumic acid, NPPB and tamoxifen to determine whether they acted as interferents as well. Figure 1 shows the influence of different concentrations of the Cl\textsuperscript{−} channel blockers on the Cl\textsuperscript{−}/LIX response to increasing concentrations of Cl\textsuperscript{−}. DIDS, at 10 μM, significantly decreased the response of the Cl\textsuperscript{−}/LIX to Cl\textsuperscript{−} changes by 31% between 0.1 and 1.0 mM Cl\textsuperscript{−}. At 100 μM, DIDS significantly decreased the response between 0.1 and 1.0 mM Cl\textsuperscript{−} by 63% and 1.0–10.0 mM Cl\textsuperscript{−} by 12%. DIDS did not affect the response to Cl\textsuperscript{−} between 10 and 100 mM Cl\textsuperscript{−} at any concentration studied. niflumic acid, NPPB and tamoxifen did not appear to interfere with Cl\textsuperscript{−} detection. While NPPB and tamoxifen do show points that are significantly different from the Cl\textsuperscript{−} standard solutions, the differences are small. At 1 μM NPPB, the Cl\textsuperscript{−} response was decreased from −60.7 to −57 mV between 10 and 100 mM Cl\textsuperscript{−} (P < 0.05). Likewise, 50 μM tamoxifen, in Cl\textsuperscript{−} solutions, decreased the response from −54.2 to −52.0 mV (P < 0.05) between 1.0 and 10.0 mM Cl\textsuperscript{−} but did not affect the response at lower Cl\textsuperscript{−}. Cl\textsuperscript{−} measurements in the presence of 10 μM NPPB showed no significant differences from the Cl\textsuperscript{−} standards.

Dynamic response to Cl\textsuperscript{−} in the presence of interfering anions

In the presence of interfering anions, the electrodes may display slower responses to dynamic changes in Cl\textsuperscript{−} giving rise to selectivities much greater than found with the separate solutions determination discussed above. Under our conditions of self-referencing, the electrode has approximately 0.25 sec to reach equilibrium at each pole of excursion before data are collected. A time response to reach 95% of its final value ≥0.25 sec would lead us to underestimate the differential anion activity and flux. We examined both the effects of interfering anions on changes to the Cl\textsuperscript{−} response and the time response for measuring dynamic changes of Cl\textsuperscript{−}. A flow system incorporating a three-barreled pipette was used to pass variations of culture medium over the surface of the Cl\textsuperscript{−}-selective LIX. The three-barreled pipette was moved intermittently to expose the stationary electrode to different concentrations of Cl\textsuperscript{−}. Figure 2(a) shows a recording where the LIX was exposed to culture medium with 50 mM MES and either 0.1, 1, or 10 mM KCl while Figure 2(b,c) shows magnifications of changes from 10 to 1 mM Cl\textsuperscript{−} and 1–10 mM Cl\textsuperscript{−}, respectively. The steady-state changes indicate that the average potential difference between 0.1 and 1 mM Cl\textsuperscript{−} and 1 and 10 mM Cl\textsuperscript{−} measured by four electrodes is −25.7 and −41.5 mV, respectively. Without buffer, the values are slightly greater, −26.6 and −46.1 mV. For comparison, measurements acquired in static solutions of only 0.1, 1, and 10 mM KCl with 5% mannitol yielded potential differences of −40 ± 2.2 and −55.8 ± 0.6 mV for 0.1–1 and 1–10 mM Cl\textsuperscript{−}, respectively. In the flow system, potential differences are decreased further from the expected Nernstian value of 59 mV for a 10-fold change by increasing buffer concentration, as summarized in Table 2. Likewise, the presence of another interfering anion, 1 mM NO\textsubscript{3}\textsuperscript{−}, decreases the response of the electrode to changes in Cl\textsuperscript{−}. This indicates that other anions in the culture medium reduce the electrodes response to 10-fold changes in Cl\textsuperscript{−}.

The time response of the electrode to changes in anionic concentration is given as the time it takes to reach 95% (t\textsubscript{95%}) of the average steady-state. Figure 2(b,c) displays arrows at the measured potential just before the potential changes in response to Cl\textsuperscript{−} and at 95% of the average steady-state change. The average t\textsubscript{95%} for different conditions is given in

Figure 2. Potentiometric response of the Cl\textsuperscript{−}-selective electrode to step changes in Cl\textsuperscript{−} in the growth medium.
(a) Intermittent exposure to 10, 1, and 0.1 mM KCl in growth medium causes the electrode to step between −52, −7.5 and +21 mV, respectively.
(b) Magnification of the change from 10 to 1 mM KCl shows a rapid 95% time response of about 10 msec.
(c) Magnification of a change from 1 to 0.1 mM KCl also shows a rapid 95% response time of just over 15 msec.

Table 2. Physical parameters of Cl\textsuperscript{−} (24899) and H\textsuperscript{+} (95293) electrodes

<table>
<thead>
<tr>
<th></th>
<th>Potential response to Δ[Cl\textsuperscript{−}] (mV)</th>
<th>Average time response to Δ[Cl\textsuperscript{−}] (msec)</th>
<th>Potential response to Δ[H\textsuperscript{+}] (mV)</th>
<th>Time response to pH change (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1–1.0 mM Cl</td>
<td>1.0–10 mM Cl</td>
<td>pH 5.5–6.5</td>
<td></td>
</tr>
<tr>
<td>LIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl\textsuperscript{−} LIX (24899)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No buffer\textsuperscript{a}</td>
<td>−26.6 ± 1.5 (4)</td>
<td>−46.1 ± 0.8</td>
<td>24.3 ± 1.6</td>
<td>−0.3 ± 0.0 (4)\textsuperscript{b} ND</td>
</tr>
<tr>
<td>0.05 mM MES\textsuperscript{a}</td>
<td>−25.7 ± 1.6 (3)</td>
<td>−41.5 ± 1.3</td>
<td>32.0 ± 4.5</td>
<td>−1.1 ± 0.2 (4)                  216.7 ± 0.2</td>
</tr>
<tr>
<td>0.5 mM MES\textsuperscript{a}</td>
<td>−17.0 ± 1.4 (4)</td>
<td>−37.0 ± 2.4</td>
<td>64.9 ± 9.4</td>
<td>−1.9 ± 0.0 (4)                  87.3 ± 16.1</td>
</tr>
<tr>
<td>5 mM MES\textsuperscript{a}</td>
<td>−10.2 ± 0.3 (4)</td>
<td>−33.5 ± 1.1</td>
<td>36.9 ± 12.9</td>
<td>−12.8 ± 1.2 (4)                24.7 ± 6.4</td>
</tr>
<tr>
<td>0.05 mM MES, 5 mM BIS-TRIS\textsuperscript{c}</td>
<td>−9.2 ± 0.2 (4)</td>
<td>−32.6 ± 1.1</td>
<td>41.6 ± 0.7</td>
<td>−4.9 ± 0.0 (4)                  112.8 ± 29.4</td>
</tr>
<tr>
<td>0.05 mM MES Cl\textsuperscript{−} free\textsuperscript{d}</td>
<td>−0.71 ± 0.21 (4)</td>
<td>−3.5 ± 0.2</td>
<td>ND</td>
<td>456.5 ± 102.2</td>
</tr>
<tr>
<td>H\textsuperscript{+} LIX (95293)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 mM MES\textsuperscript{a}</td>
<td>−22.3 ± 2.2 (4)</td>
<td>−1423.7 ± 107.3</td>
<td>155.6 ± 12.6</td>
<td></td>
</tr>
<tr>
<td>LCDM\textsuperscript{a}</td>
<td>−61.7 ± 0.2 (4)</td>
<td>−155.6 ± 12.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM MES\textsuperscript{f}</td>
<td>+0.5 ± 0.0 (4)</td>
<td>−10.7 ± 0.2</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Medium consisted of (in mM) 0.05 Ca-gluconate, 1.6 H\textsubscript{3}BO\textsubscript{3}, 5% sucrose MES buffer. KCl was varied from 0.1, 1.0, and 10 mM to determine the Cl\textsuperscript{−} LIX response to Cl\textsuperscript{−} under different H\textsuperscript{+} buffer concentrations. The standard medium with 1 mM KCl was used while testing the response to H\textsuperscript{+} changes.

\textsuperscript{b}Medium consisted of 1 mM KCl, 5% sucrose in solution. KOH was added to achieve final pH values of 5.5 and 6.5.

\textsuperscript{c}Standard medium with MES replaced with 5 mM BIS-TRIS. Sulfuric acid was used to set the pH to 5.5 and KOH was added to the pH 5.5 medium to create the pH 6.5 medium.

\textsuperscript{d}Cl\textsuperscript{−} free medium was made by replacing Cl\textsuperscript{−} with gluconate. KOH was used to set the pH of the medium.

\textsuperscript{e}Low-calcium Dickinson’s medium (LCDM) consisted of (in mM) 1 KNO\textsubscript{3}, 0.13 Ca(NO\textsubscript{3})\textsubscript{2}, 0.16 H\textsubscript{3}BO\textsubscript{3}, 5 mM MES and 10% sucrose set to pH 5.5.

\textsuperscript{f}Medium consisted of 5 mM MES, 5% sucrose set to pH 5.5 with KOH, with different concentrations of KCl.
Dynamic response to pH

As the electrode is capable of sensing the deprotonated state of the H⁺-buffer, it is also capable of indirectly sensing changes in H⁺. As large H⁺ fluxes occur near the tips of pollen tubes (Feijó et al., 1999; Messerli et al., 1999), it is important to characterize this sensitivity to determine to what extent it is responsible for the observed signals. Dynamic changes in pH were determined for the Cl⁻-selective LIX similar to the dynamic changes to Cl⁻ between pH 5.5 and 6.5 when 5 mM MES was used as the buffer, a buffer that does not interfere with cellular the H⁺-gradient (Feijó et al., 1999). The results are shown at the bottom of Table 2. The H⁺ LIX detected only a −22.3 mV difference between pH 5.5 and 6.5 when using the culture medium of Feijó et al. (1999) and Zonia et al. (2002) (in mM): 0.050 MES, 1 KCl, 0.05 CaCl₂, 1.6 H₂BO₃ and 5% sucrose. The time response of the electrode was very long, 1.4 sec. There was an undershoot that sometimes occurred at the beginning of medium exchange that lasted between 0 and 200 msec. We could not determine whether this was a mechanical artifact produced by flow or was due to a rapid chemical change at the surface of the electrode. For comparison, we also characterized the H⁺ LIX to pH changes in Low Calcium Dickinson’s medium that we have used previously (Messerli et al., 1999), consisting of (in mM): 5 MES, 1 KNO₃, 0.13 Cal(NO₃)₂, 0.16 H₂BO₃ and 10% sucrose. The electrode measured a slightly superNerstian response with a −61.7 mV response between pH 5.5 and 6.5 with an average t95% of 155.6 msec. We also checked to see whether the H⁺ LIX could sense changes in Cl⁻. We used a simplified medium consisting of only 5 mM MES, and 5% sucrose set to pH 5.5 with KOH containing 0.1, 1.0, and 10 mM KCl. The LIX measured +0.5 mV between 0.1 and 1 mM KCl and +3.5 mV between 1 and 10 mM KCl. Sensitivity to Cl⁻ would have resulted in a negative-going potential with increasing Cl⁻. These data may indicate that the selectivity of the H⁺ LIX to changes in K⁺ is greater than reported by the manufacturer, at least under these conditions.

Oscillating differential anion concentration lags growth oscillations

Having identified limitations of the Cl⁻-selective LIX, we then mapped apparent changes in Cl⁻ around growing pollen tubes. Immediately after fabrication, the LIX proved to be toxic to cells. The microelectrodes were calibrated before experiments in 1, 10, and 100 mM KCl solutions, which took less than 10 min. Within minutes after calibration, measurements that were made within 20 μm of the pollen tube tip caused immediate cessation of growth and a slower loss of the clear zone. Electrodes loaded with 10–20 μm columns of the anion LIX were no longer toxic to cells after having been left in culture medium for 30–60 min. These electrodes were not as stable as electrodes made with LIX column lengths of 100–150 μm. However, even after hours of sitting in culture medium, the long column LIX microelectrodes were still

Table 2. On average the t95% values between different Cl⁻ concentrations were not different and were averaged together to obtain the values listed in the chart. The average t95% for changes in Cl⁻ with different concentrations of buffer and in the presence of 1 mM NO₃ range between 24 and 65 msec. These are not significant differences for our method of self-referencing in which the first 30% of the signal is removed from processing. This 30% lasts 0.5 sec and includes the 10 μm move of the probe at a speed of 40 μm/sec −1 (0.25 sec) and equilibration of the probe at its excursion point (0.25 sec). The time response of the LIX to changes in Cl⁻ is sufficiently fast that it will have reached equilibrium with its new chemical environment by the time data begins to be logged.

Dynamic measurement of H⁺ LIX to pH and Cl⁻ changes

As the Cl⁻ LIX can indirectly sense H⁺ gradients, it was necessary to review the sensitivity of the H⁺ LIX (95293) as electrodes made with this LIX were used to measure extracellular the H⁺ gradients around pollen tubes (Feijó et al., 1999; Messerli et al., 1999). The results are shown at the bottom of Table 2. The H⁺ LIX detected only a −22.3 mV difference between pH 5.5 and 6.5 when using the culture medium of Feijó et al. (1999) and Zonia et al. (2002) (in mM): 0.050 MES, 1 KCl, 0.05 CaCl₂, 1.6 H₂BO₃ and 5% sucrose. The time response of the electrode was very long, 1.4 sec. There was an undershoot that sometimes occurred at the beginning of medium exchange that lasted between 0 and 200 msec. We could not determine whether this was a mechanical artifact produced by flow or was due to a rapid chemical change at the surface of the electrode. For comparison, we also characterized the H⁺ LIX to pH changes in Low Calcium Dickinson’s medium that we have used previously (Messerli et al., 1999), consisting of (in mM): 5 MES, 1 KNO₃, 0.13 Cal(NO₃)₂, 0.16 H₂BO₃ and 10% sucrose. The electrode measured a slightly superNerstian response with a −61.7 mV response between pH 5.5 and 6.5 with an average t95% of 155.6 msec. We also checked to see whether the H⁺ LIX could sense changes in Cl⁻. We used a simplified medium consisting of only 5 mM MES, and 5% sucrose set to pH 5.5 with KOH containing 0.1, 1.0, and 10 mM KCl. The LIX measured +0.5 mV between 0.1 and 1 mM KCl and +3.5 mV between 1 and 10 mM KCl. Sensitivity to Cl⁻ would have resulted in a negative-going potential with increasing Cl⁻. These data may indicate that the selectivity of the H⁺ LIX to changes in K⁺ is greater than reported by the manufacturer, at least under these conditions.
toxic to cells. Equilibration of the LIX was unnecessary for electrodes that were made with rinsed LIX. The hydrophobic LIX and aqueous medium were easily separated by centrifugation. A 20-µl volume of LIX was rinsed with five changes of 1 ml of culture medium. LIX that had been equilibrated with culture medium did not have different selectivity for Cl\textsuperscript{−} or the other anions tested compared with fresh LIX, as shown above in Table 1, and was no longer toxic to cells.

Using this Cl\textsuperscript{−}-selective LIX, the anion concentration differences were measured near the grains, shafts, and tips of growing pollen tubes. We report the measurements as the anion concentration-dependent differential voltage collected near the cell and 10 µm further away from the cell surface rather than as the flux of a specific ion because of the lack of selectivity of the probe and our reservations in declaring any single anion as the primary anion that gives rise to the anion concentration differences near the cell surface. A negative differential potential indicates a higher anion concentration near the cell. In 50 µM MES buffer, the differential potential averaged +175 ± 54 µV when the probe was moved between two positions that were 10 and 20 µm from the surface of the grains (n = 10). We noted that the signal varied by at least twofold at different points over the surface of the grain. The differential anion concentration at parts of the grain with underlying vacuole was generally smaller than the differential anion concentration near surfaces with underlying cytoplasm. The differential anion concentration was also found to be positive at a position 50–100 µm up the shaft of the tube away from the grain. Behind the growing tip, the differential anion concentration

![Figure 3. Oscillating differential anion concentration during oscillating growth. Growth rate measurements and anion concentration were acquired for tubes growing in identical media except for 50 µM (a), 0.5 mM (b) and 5.0 mM (c) H\textsuperscript{+} buffer, MES. The difference in anion concentration, between two points 10 µm apart, is reported as the anion concentration-dependent differential voltage. Greater negative voltage indicates a higher concentration of anions near the cell surface than 10 µm further away.](image-url)
gave rise to a potential difference of \(+167 \pm 68 \mu V\) \((n = 3\, \text{tubes})\) at a near distance of 5 \(\mu m\) from the tube surface. During oscillating growth, oscillating differential anion concentrations were detected near the growing tips. Figure 3(a–c) shows representative traces collected from tubes growing in medium containing 50 \(\mu M\) H\(^+\) buffer. The cell in Figure 3(a), 50 \(\mu M\) buffer, had low amplitude growth oscillations on a higher basal rate of growth and a high basal difference in anion concentration between the two points of excursion of about –300 to –400 \(\mu V\). In 50 \(\mu M\) H\(^+\) buffer, the differential anion concentration gave rise to an average peak voltage difference of –908 \(\pm 224\) \(\mu V\) \((n = 5\, \text{tubes})\) and an average voltage difference of –380 \(\pm 119\) \(\mu V\). Assuming that this signal is only due to the Cl\(^-\) anion, the average, peak Cl\(^-\) efflux at the cell surface would be 2853 \(\pm 690\) pmol cm\(^{-2}\) sec\(^{-1}\) while the average efflux would be 951 \(\pm 366\) pmol cm\(^{-2}\) sec\(^{-1}\). The flux is calculated using the subNernstian slope, –41.5 mV, determined for changes in Cl\(^-\) concentration in culture medium. Figure 3(b) shows measurement on a cell with regular oscillations in 0.5 mM H\(^+\) buffer, with a background signal collected 100 \(\mu m\) away from the cell starting at 450 sec. The difference in potential measured at the lowest point of the differential anion concentration ranged between –20 and –50 \(\mu V\). Cells in 0.5 mM H\(^+\) buffer had an average peak differential voltage of –566 \(\pm 111\) \(\mu V\) and an average differential voltage of –126 \(\pm 10\) \(\mu V\) \((n = 5)\). The differential anion concentration at the bases of the oscillations was also in the range of –5 to –50 \(\mu V\). The average differential voltage at the peaks of the oscillations in 5 mM H\(^+\) buffer was –438 \(\pm 105\) \(\mu V\) with an average differential voltage of –94 \(\pm 26\) \(\mu V\) in 5.0 mM MES \((n = 5\, \text{tubes})\).

Figure 4 shows the average cross-correlation analyses for all three conditions. The increase in the oscillating differential anion concentration lagged growth oscillations by 8.2 \(\pm 0.9\) sec \((n = 5\, \text{tubes})\), 9.1 \(\pm 0.9\) sec \((n = 5\, \text{tubes})\) and 6.3 \(\pm 0.6\) sec \((n = 5\, \text{tubes})\) for cells growing in 50 \(\mu M\), 0.5 mM and 5 mM MES buffer, respectively. The average period of oscillation was shorter in 50 \(\mu M\) buffer, 19.8 \(\pm 1.4\) sec, compared to periods of 41.3 \(\pm 4.6\) and 48.4 \(\pm 12.5\) sec in 0.5 and 5 mM buffer, respectively. This corresponds to phase lags of 153 \(\pm 20\), 80 \(\pm 5\), and 54 \(\pm 8\) in the three buffer concentrations. Pollen tubes grew at different rates in the different media as well. Cells in 50 \(\mu M\) buffer grew faster, 0.33 \(\pm 0.02\) \(\mu m\) sec\(^{-1}\), than cells in 0.5 mM MES, 0.27 \(\pm 0.02\) \((P < 0.05;\, \text{one-tailed } t\text{-test})\) and cells in 5.0 mM, 0.24 \(\pm 0.03\) \(\mu m\) sec\(^{-1}\), \((P < 0.05;\, \text{one-tailed } t\text{-test})\). There was no significant difference in peak growth rate among cells in any of the three conditions.

Differential anion concentration measurements were also performed on pollen tubes in the absence of added Cl\(^-\). If Cl\(^-\) is the primary component of the differential anion concentration, then Cl\(^-\) removal would be expected to significantly reduce the measured differential voltage as internal stores of Cl\(^-\) become exhausted. The gluconate salts of Ca\(^{2+}\) and K\(^+\) were used instead of the Cl\(^-\) salts. Under these conditions the probe measured a –0.71 mV difference between Cl\(^-\)-free medium at pH 5.5 and Cl\(^-\)-free medium at pH 6.5 with a time response of 456 msec (Table 2). Measurements on pollen tubes grown in Cl\(^-\)-free medium show oscillating differential

![Figure 4](image-url)
anion concentrations with similar characteristics as cells grown in 1.1 mM Cl\(^{-}\). The average period of the anion oscillations was 23.5 ± 5.7 sec while the peak differential voltage was ~796 ± 145 μV (four tubes). We show these results with reservations because the Cl\(^{-}\) anion exchanger already displays a subNernstian potential in the medium when Cl\(^{-}\) is present in a concentration between 0.1 and 1 mM, and the poor response may be exacerbated at even lower Cl\(^{-}\) concentrations.

**Cl\(^{-}\) concentrations in medium and cells**

Many studies of lily pollen tube growth prior to Zonia et al. (2002) have been carried out in media with no added Cl\(^{-}\) (Brewbaker and Kwack, 1963; Messerli and Robinson, 1997, 1998; Messerli et al., 1999, 2000; Pierson et al., 1995; Rathore et al., 1991; Weisenseel and Jaffe, 1976). The only Cl\(^{-}\) in those media would have come from either contamination from other reagents or from the pollen itself. We used a Dionex ion chromatograph to determine the Cl\(^{-}\) concentration in ‘Cl\(^{-}\)-free’ medium and in pollen grains. Figure 5(a) shows the 100 μM Cl\(^{-}\) standard identified by no. 1. Figure 5(b) shows the ‘Cl\(^{-}\)-free’ medium and Figure 5(c) shows the ‘Cl\(^{-}\)-free’ medium plus 100 μM Cl\(^{-}\), where the Cl\(^{-}\) peak is also identified by no. 1. A sulfate peak is indicated by no. 2 and no. 3 while an unknown anion is identified by no. 1 and no. 2 in Figure 5(b,c), respectively. The Cl\(^{-}\) peak occurred at 1.34 and 1.39 min in the Cl\(^{-}\) standard and

![Figure 5](image-url)

**Figure 5.** Measurement of Cl\(^{-}\) in media and pollen grains. Ion chromatography was used to measure the absolute level of Cl\(^{-}\) in ‘Cl\(^{-}\)-free’ medium and in pollen grains. The 100 μM Cl\(^{-}\) standard (a) shows a clean peak at 1.39 min while the growth medium with no added Cl\(^{-}\) (b) shows only a shoulder off the much larger signal that precedes it. Cl\(^{-}\) (100 μM) was added to the ‘Cl\(^{-}\)-free’ medium (c) to identify the position of the Cl\(^{-}\) peak. The greatest concentration of Cl\(^{-}\) in the medium to which no Cl\(^{-}\) was added is ≤31 μM. Lysed pollen grains were a source of many measured anions (d). Peak no. 1 is the Cl\(^{-}\) peak that was used to identify an average level of 21.8 pg Cl\(^{-}\) per grain. Many of the profiles that arose from the pollen grain lysate were not identified.

Cl\textsuperscript{−} doped, ‘Cl\textsuperscript{−}-free’ medium, but no such peak occurred in the ‘Cl\textsuperscript{−}-free’ medium. Gluconate was used as the anion in the ‘Cl\textsuperscript{−}-free’ medium and may account for the peak beginning at 1 min. For the ‘Cl\textsuperscript{−}-free’ medium, there was a slight shoulder at 1.38 min that may represent the Cl\textsuperscript{−} peak. By using the highest value in the retention time range of 1.34–1.39 min we calculate a final upper limit concentration of 30.7 \( \mu \text{M} \) Cl\textsuperscript{−} in ‘Cl\textsuperscript{−}-free’ medium.

The Cl\textsuperscript{−} content from grains was determined by lysing the grains in distilled water after rinsing the grains five times with an equal volume of distilled water. Figure 5(d) shows the anion content of lysed pollen grains. The peak at no. 1 is the Cl\textsuperscript{−} content which corresponds to 9.8 \( \mu \text{g cm}^{-2} \text{g}^{-1} \) or 21.8 \( \mu \text{g grain}^{-1} \), from 4.5 \( \times 10^{5} \) cells ml\textsuperscript{-1}. With such a small amount of Cl\textsuperscript{−} in each grain, even if 10\textsuperscript{4} grains released all of their Cl\textsuperscript{−} in the 3 ml of culture medium used for each experiment, the final Cl\textsuperscript{−} concentration would only increase by 1.6 \( \mu \text{M} \). Typically, there were fewer than 500 grains per culture dish. We found no difference in Cl\textsuperscript{−} content between ‘Cl\textsuperscript{−}-free’ medium and ‘Cl\textsuperscript{−}-free’ medium with normal amounts of germinated pollen.

The nitrate peak occurs at no. 5 (Figure 5d) and the sulfate peak occurs at no. 11. The other peaks were not identifiable based on the inorganic anions in the standard solutions. As shown for gluconate (Figure 5b,c), organic anions can also be detected by the column and may contribute to the additional peaks.

Discussion

Careful characterization of the Cl\textsuperscript{−}-selective liquid ion-exchanger (24899) based on trioctylpropylammonium chloride in 1,2 Dimethyl-3-nitrobenzene indicates that it has poor selectivity among anions in general. In fact, it is more appropriate to call it a NO\textsubscript{3}\textsuperscript{−}-selective LIX as it has two orders of magnitude greater selectivity for nitrate than Cl\textsuperscript{−}. This ‘Cl\textsuperscript{−}-selective LIX’ has better selectivity for NO\textsubscript{3}\textsuperscript{−} over Cl\textsuperscript{−} than the NO\textsubscript{3}\textsuperscript{−}-selective LIX (72549) sold by Fluka. The Cl\textsuperscript{−}-selective electrode is also a good sulfonate group detector as shown by its ability to sense methylsulfonate nearly as well as Cl\textsuperscript{−}, and also the sulfonate groups on the H\textsuperscript{+} buffer, MES (Table 1), and the Cl\textsuperscript{−} channel blocker, DIDS (Figure 1). The sulfonate group on MES keeps its negative charge while the nitrogen looses its H\textsuperscript{+} at higher pH. Therefore, MES, primarily a zwitterion below its pKa, becomes an anion above its pKa. This indirect pH sensitivity of the Cl\textsuperscript{−}-selective LIX is due to its poor anionic selectivity, which is only about an order of magnitude for Cl\textsuperscript{−} over any of the other anions tested (Table 1). The alternate Cl\textsuperscript{−}-selective LIX (24902) sold by Fluka is directly pH-sensitive, producing nearly a 25-mV response to an order of magnitude change in H\textsuperscript{+} concentration in 1 mM CI\textsuperscript{−} (S.S. Garber, Rosalind Franklin University of Medicine and Science, Chicago, IL, USA, personal communication). The selectivity coefficients in Table 1 were determined through static measurements and are not the best representation of selectivity during self-referencing measurements in which the dynamic response to different anions could alter selectivity coefficients. We investigated the influence of the dynamic response to the anions by comparing the time response of the electrode to changes in the concentration of Cl\textsuperscript{−} and the H\textsuperscript{+} buffer, MES. While the steady-state response of the electrode to Cl\textsuperscript{−} changed significantly with increasing concentrations of MES and NO\textsubscript{3}\textsuperscript{−}, the time response for measuring changes in Cl\textsuperscript{−} did not (Table 2), remaining in the range of 24–65 msec. The temporal response of the electrode showed large differences during changes of anionic MES. With a pH difference of 5.5–6.5, the electrode was exposed to anionic MES concentration differences of 0.91–3.5 mM (5 mM MES), 91–350 \( \mu \text{M} \) (500 \( \mu \text{M} \) MES), and 9.1–35 \( \mu \text{M} \) (50 \( \mu \text{M} \) MES). The response time to MES was shortest, 24.7 msec, in 5 mM MES and longest, 216.7 msec, in the lowest concentration of MES. The Cl\textsuperscript{−} electrode responded to changes in Cl\textsuperscript{−} nearly seven times faster than changes in anionic MES. However, under our measuring conditions, this difference in temporal response will not affect selectivity. The probe moving at 0.3 Hz has 1.66 sec to move from one point of excursion to the other and collect data in the new position. The first 30\% of this time period is ignored as it is the time designated for the probe to move and equilibrate with its new position. Moving at 40 \( \mu \text{m sec}^{-1} \) the probe requires 0.25 sec to reach its new position after which it has another 0.25 sec to equilibrate to the new concentration. This is adequate time for the electrode to reach steady-state with the new Cl\textsuperscript{−} concentration, even at 50 \( \mu \text{M} \) MES, in which it reached 95\% of its steady-state value in 217 msec. The average of the next 1.16 sec of the half-cycle is then used to acquire an activity measurement. One may be concerned that under normal conditions diffusion to the electrode surface could significantly increase the time response. However, diffusion to the center of a microelectrode with a tip size as large as 4 \( \mu \text{m} \) would take only about 0.33 msec for Cl\textsuperscript{−} and 1.0 msec for MES (for three-dimensional diffusion, \( t = L^{2}/6D \) where \( L \) is radius of the tip size and \( D \) is the diffusion coefficient, \( 2.0 \times 10^{-5} \text{ cm}^{2} \text{ sec}^{-1} \) for Cl\textsuperscript{−} and \( 0.66 \times 10^{-5} \text{ cm}^{2} \text{ sec}^{-1} \) for MES (Berg, 1983)). From these data, we conclude that the selectivity coefficients as measured with the separate solutions method are not changed during self-referencing measurements and that the Cl\textsuperscript{−}-selective LIX is only about an order of magnitude more selective for Cl\textsuperscript{−} than most other anions.

It was then necessary to check the Cl\textsuperscript{−} sensitivity of the H\textsuperscript{+} LIX as any sort of Cl\textsuperscript{−} sensitivity may be misinterpreted as H\textsuperscript{+} changes if Cl\textsuperscript{−} fluxes exist. We investigated the H\textsuperscript{+} selectivity of the H\textsuperscript{+} LIX and found that the electrode behaves poorly under the pollen culture medium used for these Cl\textsuperscript{−} flux studies which is identical to the medium used by Feijó et al. (1999) for H\textsuperscript{+} flux studies and Zonia...
et al. (2002) for the Cl⁻ flux studies. It had a subNernstian response to H⁺ of only –22.3 mV between pH 5.5 and 6.5 and a very long time response of 1.4 sec. There must be an interfering ion in this medium that is not in low-calcium Dickinson’s medium that we have used previously (Messerli et al., 1999) as the H⁺ electrode responded in that medium with a –61.7 mV change from pH 5.5 to 6.5 and a time response of 156 msec. Due to the poor response of the H⁺ electrode in the culture medium used in these Cl⁻ flux studies, we used a simplified medium to determine the Cl⁻ selectivity of the H⁺ LIX. Small, but increasingly positive potentials were recorded by the H⁺ LIX for increasing concentrations of KCl, indicating that the H⁺ LIX is not strongly responding to changes in Cl⁻ but may be responding to changes in K⁺. Further investigation of the H⁺ LIX, in these growth media, may help explain discrepancies between previous H⁺ flux measurements of Feijó et al. (1999) and Messerli et al. (1999).

Having characterized the poor selectivity of the electrode, we targeted the measurement of Cl⁻-fluxes from growing lily pollen tubes, but with strong reservations about the interpretation of the results. Apparent Cl⁻ influx was measured near the surface of pollen grains, along the shaft of the tube near the grain and along the shaft immediately behind the growing pollen tube tip. Apparent Cl⁻ efflux was measured at the tip and oscillations in apparent Cl⁻ efflux were detected at the tips of lily tubes that were growing in an oscillating manner. The average peak anion concentration-dependent differential voltage measured by the anion exchanger electrode was 908 µV, which would indicate a Cl⁻ efflux of 2850 pmol cm⁻² sec⁻¹, assuming that the anion gradient is in fact Cl⁻. We find that the changes in the differential anion concentration in lily pollen tubes lag growth by 7.9 ± 1.4 sec on average over the three different H⁺ buffer concentrations. Increasing the H⁺ buffer concentration had a striking effect on tube growth by increasing the average period of oscillation from 20 to 41 to 48 sec over two 10-fold increases in buffer concentration. This changed the phase relationship of the anion and growth oscillations, despite the fact that the temporal lag of apparent anion efflux did not change greatly. Taking this into account, the phase lags were 153°, 80°, and 54° in 50 µM, 0.5 mM and 5.0 mM buffers, respectively. The peak temporal lag in apparent anion efflux is similar to the peak temporal lags found for H⁺, 11 sec, K⁺, 14 sec, and Ca²⁺ 13 sec which correspond to phase lags of 103°, 100°, and 123°, respectively (Messerli et al., 1999). As changes in buffer concentration significantly change the average period of growth oscillations, slight discrepancies between the phase lags reported here and in earlier work could be accounted for by the differences in the medium, specifically, half as much sucrose and Ca²⁺, 100 times less H⁺ buffer, MES, and 10 times more boric acid. Changes in the concentration of Ca²⁺, boric acid, sucrose and MES alter the frequency of oscillation in lily pollen tubes (Holdaway-Clarke et al., 2003; Messerli and Robinson, 2003).

The temporal overlap of the putative Cl⁻ fluxes with other measured fluxes along with the poor selectivity of the Cl⁻ LIX led us to consider that the probe was not measuring Cl⁻. Our doubts about attributing the differential voltages measured by the anion exchanger were further increased by the fact that the peak differential voltages measured at pollen tube tips were not changed by eliminating medium Cl⁻. While this result is subject to other interpretations, the simplest interpretation, requiring the fewest assumptions, is that the differential voltages are not due to Cl⁻ efflux. We considered the possibility that a large component of the apparent anionic concentration difference is due to the Cl⁻ LIX measuring changes in the ionic state of the H⁺ buffer, MES, along with other weak acids, including bicarbonate. If the probe were in fact measuring the differences in MES/MES⁻ due to a H⁺ gradient then we should be able to detect apparent anion influx anywhere we detected a H⁺ efflux and apparent anion efflux anywhere we detected H⁺ influx. This is certainly the case. H⁺ efflux was measured from the grain, a region along the tube just up the shaft from the grain (Messerli et al., 1999), and was measured behind the growing tip in the presence of 50 µM H⁺ buffer by Feijó et al. (1999). We measured anion influx at each of these regions and also Zonia et al. (2002) reported Cl⁻ influx at a region behind the growing tip. In addition, oscillating H⁺ influx occurs at the tip (Feijó et al., 1999; Messerli et al., 1999) and oscillating apparent anion efflux is measured at the tip.

In growth medium containing 50 µM MES, the Cl⁻ LIX responded to a pH change of one unit, 5.5–6.5, by changing its output by 1.1 mV. The average differential voltage measured at the peaks of the oscillations was 908 µV. If we assume that no Cl⁻ flux is present and that the electrode is indirectly sensing changes in pH then we would expect to measure a pH change from 5.5 to 6.3 over a 10-µm distance. Feijó et al. (1999) reported that when grown in 50 µM buffer, pH changes of about 0.5 pH units could be detected over a 10-µm distance near the tube tip. This is approximately the pH difference we would expect from the H⁺ fluxes measured by Messerli et al. (1999) after converting from 5 mM to 50 µM buffering capacity. The 0.8 pH unit difference predicted with the anion exchanger is greater than the 0.5 pH unit difference discussed, indicating the possibility of the release of anions. Certainly we would expect to measure the release of bicarbonate (pKa 6.35) from a respiring cell and the changes in its anionic state due to the large consumption of H⁺ during oscillations. Removal of MES will decrease the magnitude of the anion-dependent differential voltage but will not eliminate the pH sensitivity of the Cl⁻ electrode in culture medium thus eliminating a possible solution to MES interference.
While we were able to detect oscillatory changes in the differential voltage recorded by the Cl\textsuperscript{—}–selective electrode near lily pollen tubes, some of our other findings differed significantly from those of Zonia et al. (2002). We found a quite different phase relationship between the apparent anion fluxes and growth in lily. We found anion fluxes lagged growth by 7.9 sec on average in lily while they found no phase change in tobacco and did not report a temporal relationship in lily. We were unable to confirm the 3 order magnitude dynamic Cl\textsuperscript{—} selectivity of the anion exchanger claimed by Zonia et al. (2002). We provide evidence that most selectivities were ≤1 order of magnitude, similar to those reported by the manufacturer. The dynamic response to Cl\textsuperscript{—} versus MES, under our measuring conditions, did not change the selectivity for MES, a primary interferent. We also found that the Cl\textsuperscript{—} channel inhibitors, NPPB and niflumic acid, did not interfere with Cl\textsuperscript{—} detection while DIDS significantly interfered with Cl\textsuperscript{—} detection. The electrodes responses to Cl\textsuperscript{—} dropped by 63\% on average between 0.1 and 1 mm Cl\textsuperscript{—} in the presence of 100 μM DIDS. This result could be used to explain the approximately 80% drop in Cl\textsuperscript{—} efflux from a tobacco pollen tube in the presence of 80 μM DIDS measured by Zonia et al. (2002). Their tobacco growth medium only contained 0.4 mM Cl\textsuperscript{—}. The addition of such a large amount of DIDS to a lower background concentration of Cl\textsuperscript{—} would essentially blind the electrode to changes in Cl\textsuperscript{—} and mask differences of other anions.

If it is assumed that the anion exchanger does indeed measure Cl\textsuperscript{—} fluxes, there are some disturbing implications. Lily pollen tubes maintain similar average growth rates in 5 mM MES buffer whether they are grown in medium with 1.1 mM Cl\textsuperscript{—}, 0.24 ± 0.03 μm sec\textsuperscript{—1} (this paper) or in medium with only contaminating levels of Cl\textsuperscript{—}, shown here to have an upper limit of 31 μM, 0.24 ± 0.01 μm sec\textsuperscript{—1} (Messerli et al., 2000). The pollen grains themselves are not capable of changing the medium Cl\textsuperscript{—} concentration under normal growth conditions. Thus, a 39-fold reduction in Cl\textsuperscript{—} does not affect the rate of lily pollen tube growth. This supports a previous report indicating that lily pollen germination and tube growth is completely independent of extracellular inorganic anions, including Cl\textsuperscript{—} (Weisenseel and Jaffe, 1976). While contaminating levels of Cl\textsuperscript{—} may be present, it is unclear how intracellular Cl\textsuperscript{—} levels could be maintained in the face of such huge losses with only trace levels of Cl\textsuperscript{—} in the bathing medium. Zonia et al. (2002) reported Cl\textsuperscript{—} influx in a medium containing 1.1 mM Cl\textsuperscript{—}. If Cl\textsuperscript{—} fluxes are an essential component of pollen tube growth how can normal growth occur in the absence of added Cl\textsuperscript{—}? We have shown here that contaminating levels of Cl\textsuperscript{—} are quite low and that pollen grains do not have vast reserves of Cl\textsuperscript{—}, so intracellular Cl\textsuperscript{—} would be rapidly exhausted by the reported fluxes. The small amount of Cl\textsuperscript{—} in lily pollen tubes would be exhausted in 2.3 min assuming average Cl\textsuperscript{—} efflux of 951 pmol cm\textsuperscript{—2} sec\textsuperscript{—1} from a hemispherical tip of 8.5 μm radius with no Cl\textsuperscript{—} uptake.

Additionally, while the opening of Cl\textsuperscript{—} channels was proposed to give rise to the Cl\textsuperscript{—} efflux in tobacco (Zonia et al., 2002), no such channels were found during patch-clamp studies of lily pollen grain, or pollen tube tip protoplasts, by Dutta and Robinson (2004). They characterized a spontaneous K\textsuperscript{+} channel, a stretch-activated K\textsuperscript{+} channel and a stretch-activated Ca\textsuperscript{2+} channel, but no anion channel despite experiments designed specifically to detect anion channels. Therefore, any anion movement across the membrane would have to occur via membrane transporters or exchangers rather than channels.

A third issue for consideration is that the massive efflux of Cl\textsuperscript{—} at the tip would be expected to have significant electrical consequences. The efflux greatly increases the discrepancy between measurements of net ionic current and individual measurements of ion fluxes described in Messerli et al. (1999). An uncompensated Cl\textsuperscript{—} efflux through an ion channel, as proposed by Zonia et al. (2002), of 6000 pmol cm\textsuperscript{—2} sec\textsuperscript{—1} amounts to a net electrical current entry of about 600 μA cm\textsuperscript{—2}. Direct measurements of net current influx oscillations do not exceed 0.5 μA cm\textsuperscript{—2} (Messerli and Robinson, 1998; Weisenseel et al., 1975). If there is large Cl\textsuperscript{—} efflux, there must also be a yet-undetected efflux of a cation or influx of another anion that is of the same magnitude as the reported Cl\textsuperscript{—} efflux. Efflux of a cation would only increase the difficulty of maintaining osmotic pressure making this solution unlikely, while equivalent anion influx seems impossible as the only other anions in the medium are the anionic form of the H\textsuperscript{+} buffer, 0.3 μM borate anion, H\textsubscript{2}BO\textsubscript{3}\textsuperscript{—} and 1.8 μM HCO\textsubscript{3}\textsuperscript{—} at pH 5.5. Likewise, the massive efflux of Cl\textsuperscript{—} should depolarize the membrane potential. We would expect to observe large, oscillating depolarizations of the membrane potential corresponding to the opening of Cl\textsuperscript{—} channels at the tip, but such depolarizations have not been detected.

Thus, we conclude that the self-referencing voltage differences detected by the Fluka 24899 LIX cocktail do not represent Cl\textsuperscript{—} fluxes. This conclusion is based on the poor selectivity of the LIX in the conditions in which it was originally used, and inconsistency with a large body of well-confirmed physiological data. Instead, we conclude that the signals detected by electrodes constructed from this LIX are primarily due to gradients of the anionic form of the buffer that are formed secondarily to the well-known, large pH gradients that accompany pollen tube growth. Our results also reaffirm the necessity to characterize the ion-selective electrodes in the medium in which it is to be used. This is especially relevant to plant biologists, as the bathing media for plant cells are very different from those used for animal cells, but the published characterization of the LIXs are often carried out under conditions appropriate for animal cells.
Experimental procedures

Electrode construction

Ion-selective microelectrodes were made by pulling thin-walled borosilicate glass to 1–2 μm tips, drying at >200°C overnight, coating with N,N-Dimethyltrimethylsilylamine (Sigma-Aldrich, St Louis, MO, USA) at >200°C for 30–60 min and drying for >2 h at >200°C. The silanized microelectrodes were back-filled with 100 mM KCl, 10 mM MES pH 5.5 and were then tip-filled with the 0.10 mM KCl to decrease the large osmotic gradient across the LIX in the absence and presence of different concentrations of chloride ions. Electrodes gave more stable potentials for longer periods of time when the length of the anion-selective column was 100–150 μm. The reference electrode consisted of an Ag/AgCl half-cell connected to the bath via a 100-μm citric acid bridge set to pH 5.5 with KOH during selectivity and tube flux measurements. The H⁺-selective electrode based on the H⁺ ionophore I cocktail B (Fluka, 95293) was constructed in a similar manner with the same backfilling solution but with a 30-μm column of cocktail. During flow conditions, reference electrodes were filled with 3 mM Na acetate or 3 mM KCl. Reference electrodes were downstream of the ion-selective electrode and did not contaminate the measuring solutions.

Separate solutions method

Electrode selectivity was determined using the separate solutions method as described by Umezawa et al. (2000), which involves measuring the potentials from solutions of two different cations or anions of equal activity. The selectivity of the electrode, K, can then be calculated based on the following equation

$$K_{AB} = \frac{a_A^{(1-2z_Az_B)} e^{(V_A-V_B)/(2F/RT)}}{a_B}$$  \hspace{1cm} (1)

where a is activity, z is valence and V is voltage (Umezawa et al., 2000). For our purposes A and B represent the Cl⁻ ion and the interfering ion, respectively. A simplified version of this method employs the use of 100 mM concentrations of each of the anions tested rather than matching ionic activities. As long as the valences of the ions under comparison are the same, the discrepancy between 100 mM concentrations and identical activity of the anions is ≤8% on the linear scale. The separate solutions method was chosen as used by Fluka Chemical Co. (1991) to describe the anionic selectivity of the anion exchanger. They also used the simplified version of this method as described above.

Interference by Cl⁻ channel inhibitors was determined by comparing the response of the electrode to 0.1, 1.0, 10, and 100 mM KCl in the absence and presence of different concentrations of chloride transport inhibitors. Mannitol (5%) was added to 0.1, 1.0, and 10.0 mM KCl to decrease the large osmotic gradient across the LIX thereby preventing it from being dislodged from the tip. Cl⁻ channel inhibitors 4,4’-Disothiocyanostilbene-2,2'-dissulfonic acid disodium (DIDS), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), niflumic acid and tamoxifen were purchased from Sigma-Aldrich. Inhibitors dissolved in DMSO were diluted to the point that the final DMSO concentration was 0.1%. This same concentration of DMSO was placed in the Cl⁻ standards when measuring an inhibitor that was dissolved in DMSO.

Electrode time response

A rapid exchange flow system was used to measure the time response of the electrodes to changes in solution. A syringe pump (Univentor Ltd, Zejtun, Malta) was used to pass different solutions at 0.83 ml min⁻¹ through 0.7 mm i.d. glass tubing (Warner Instruments, Hamden, CT, USA). The measuring electrode remained stationary as the triple-barreled glass was intermittently shifted using the SF-77B Perfusor Fast-Step (Warner Instruments) to expose the electrode to a new solution. Data were acquired by passing the 1x unfiltered signal out of the ion-selective electrode amplifier (BioCurrents Research Center, MBL, Woods Hole, MA, USA) into an EG & G preamplifier (Princeton Applied Research, Oak Ridge, TN, USA) before it was digitized with an Axon Instruments 1322 A/D converter and logged with Axoscope software (Axon Instruments Inc., Union City, CA, USA).

Growth conditions

Lilium longiflorum pollen grains stored at ~20°C were germinated in a modified Dickinson’s medium (in mM: 1.6 H,BO₃, 1.0 KCl, 0.05 CaCl₂, 0.05 MES and 146 sucrose [5% sucrose]) pH 5.5 as described by Feijo et al. (1999) and Zonia et al. (2002). For solutions with greater buffering capacity, 0.5 and 5.0 mM MES were used and set to pH at 5.5 with KOH. Grains were hydrated for 1 h in 1 mL of medium and then spread over the bottom of 35 x 10 mm Falcon tissue culture dishes that had been soaked in a solution containing 0.5 mg mL⁻¹ of high molecular weight polysaccharide, as described by Messerli and Robinson (1998). Flow measurements were acquired a few hours after plating at ambient temperature 22–24°C.

Differential anion concentration and growth measurements

The ion-selective electrodes were used in self-referencing mode (Smith et al., 1999) in order to reduce noise and drift. This method also allows calculation of the ionic flux at the cell surface. Data acquisition during self-referencing consisted of translating the ion-selective electrode with a square wave function at a frequency of 0.3 Hz. Data are acquired at 1000 points sec⁻¹ and binned into 10 blocks for each half cycle. The first 30% of each half cycle, 3 bins, was ignored and the last 70%, 7 bins, was used for measuring the ion activity at the new position. The microelectrode moving at 40 μm sec⁻¹ required 0.25 sec to reach its new position, 10 μm away, and was given 0.25 sec to reach equilibrium at its new position. Under these conditions, electrode drift varied between 0.14 and 3.5 μV sec⁻¹ with a differential voltage, peak to peak noise of ±30–40 μV giving rise to a differential detection limit of ±1.8–2.4 μM in real time. Averaging over blocks of time provides greater sensitivity as the averaged noise converges toward zero.

The probe was stepped between two points, 10 μm apart, near the cell surface. Measurements at the pollen tip were taken with the probe moving either parallel or perpendicular to the growth path. The calculated flux is taken from the total amount of substance moving per unit time between surface 1 with radius a and surface 2 with radius b

$$\frac{2\pi D}{a-b} \cdot dC$$  \hspace{1cm} (2)

where D is the diffusion coefficient and dC is the differential concentration (Crank, 1975) divided by the surface area of the pollen tube tip hemisphere, 2πr². The differential concentration is determined from the differential voltage (dV) by:

$$dC = C_{ave} \cdot 10^{dV/S} - C_{ave}$$  \hspace{1cm} (3)

where C_{ave} is the average background concentration and S is the slope of the calibration curve. Measurements on pollen tubes were made in ‘sample hold’ mode where a DC offset potential was used.
to keep the signal in the dynamic range of the amplifier while applying gain.

**Growth rate measurements**

Analog video was collected of growing pollen tubes magnified with a 32×/0.35 NA objective. Video was digitized at a rate of one frame per second with a home built video to TIF image converter. A video tracker with 0.1 pixel resolution capability was used to measure the change in position of the pollen tube tip between successive images (Messerli et al., 1999) that were on average, 2.5 sec apart. The phase relationship between the growth and the apparent anion efflux oscillations was determined by finding the maximum positive correlation coefficient (Samuels, 1989) of the cross-correlation between the slopes of the two waveforms as the two waveforms were shifted in time with respect to each other. The maximum positive correlation was determined as the apparent anion efflux signal was multiplied by −1 before analysis.

**Total anion concentration determination**

A Dionex IonPac AS4a suppressed conductivity ion chromatograph (Sunnyvale, CA, USA) was used to measure the total anion content of medium and cells. ‘Cl−’ free medium was made by replacing KCl and CaCl2 with the gluconate salts of K+ and Ca2+ and setting the pH with H2SO4. Most samples were simply collected and run through the chromatograph. Only the pollen grain sample itself required pre-processing. For this measurement pollen grains were initially rinsed five times in an equal volume of reverse osmosis 18 MΩ·cm−1 water, to a known volume and sonicated for mixing, before measurement. The sample was allowed to dry over night at 70°C to remove the EtOH and then rehydrated with D.I. water, to a known volume and sonicated for mixing, before measurement. Cells were counted prior to treatment using a hemacytometer and by averaging six different counts of the same sample.

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**References**


