Availability of Chloride Affects the Balance between Potassium Chloride and Potassium Malate in Guard Cells of *Vicia faba* L.

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Received for publication October 26, 1977 and in revised form March 15, 1978

ABSTRACT

Electron probe microanalysis for K and Cl and enzymic determination of malate were performed on epidermal strips of *Vicia faba* L. which had been incubated with 0.1 equivalent of K⁺ per liter in the absence or presence of Cl⁻. In the absence of Cl⁻, iminodiacetate, a presumed impermeant zwitierion, served as anion. With no Cl⁻ in the medium, 91% of the K⁺ imported into the guard cells during stomatal opening was neutralized by malate production; import of Cl⁻ (presumably from the rest of the epidermal tissue) contributed 6%. In the presence of Cl⁻, 50% of the necessary negative charges were provided by malate synthesis, 45% by Cl⁻ import. Stomatal opening was not obviously affected by the chloride concentration in the incubation medium, but malate production declined roughly linearly with the logarithm of [Cl⁻] between 10⁻² and 10⁻¹ equivalent per liter.

Allaway (1) and Outlaw and Lowry (7) have shown that guard cells of *Vicia faba* produce malic acid when the stomata open. In Allaway's experiments, malate neutralized about one-half of the K⁺ imported into the guard cells (1). It is possible that chloride, provided the balance since there is evidence that chloride occurs in guard cells of *V. faba*, compensating between 5% (6) and about 30% (8) of the positive charges of K⁺. The low value of 5% was determined on material that had been obtained from plants watered with a nutrient solution containing Cl⁻ as a trace element only. The high value of 30% was found in epidermal strips that had been exposed to a 10 mM KCl solution while stomata opened. Chloride is also known to participate in the ion shuttle between guard and subsidiary cells in *Zea mays* (11) and *Avena sativa* (2).

The variation in the participation of Cl⁻ as anion in guard cells of *V. faba* may have its cause in the variation in the availability of this ion. This explanation is based on the finding that the amount of malate formed in isolated epidermal strips of *V. faba* during stomatal opening was large when Cl⁻ was absent in the incubation medium, and low when it was present (14). Malate content and stomatal aperture were linearly correlated with each other in the absence of Cl⁻; the correlation broke down in the presence of Cl⁻. Apparently, guard cells of *V. faba* can satisfy their anion requirement by producing malic acid and importing Cl⁻ simultaneously. Regulation of cytoplasmic pH through OH⁻-Cl⁻ exchange and acid metabolism is a likely mechanism by which guard cells balance malate synthesis and chloride uptake in response to an alkalization of the guard cells resulting from an exchange of K⁺ for H⁺ (10).

We tested the effect of the availability of Cl⁻ on the ionic composition of the guard cells of *Vicia faba* by incubating epidermal samples on solutions free of Cl⁻ or containing Cl⁻ and then determining the amounts of K⁺ and Cl⁻ absorbed as well as the amounts of malic acid synthesized. We used established methods; electron probe microanalysis for the determination of K⁺ and Cl⁻ in the guard cells (6), and enzymic oxidation coupled to the reduction of NAD for the determination of malate in the epidermal samples (4, 14).

MATERIALS AND METHODS

Plants. *V. faba* L. plants (cv. Long Pod; seeds from Lagomarsino Seeds Inc., Sacramento, Calif.) were grown for 3 to 4 weeks in a growth chamber in soil consisting of 2 parts Bacto potting soil (Michigan Peat, Houston, Texas) and 1 part Perlite (W. R. Grace & Co.) in pots with a soil depth of about 12 cm. The plants were watered once a week with Hoagland solution (full strength, pH 6.5), and twice a week with deionized H₂O. The day length was 16 hr light intensity 85 W m⁻². The air temperature was 27°C during the light period and 23°C during the dark period. Relative humidity was about 85%, day and night.

Preparation and Incubation of Epidermal Samples. The second, third and fourth fully expanded leaves were removed from the plants in the morning, immediately before the beginning of the light period. The leaves were washed in deionized H₂O. Sections measuring about 5 × 10 mm² were cut from the intercostal areas of the leaves and floated, upper side down, on deionized H₂O in the dark. Then the lower epidermis was peeled in room light (from fluorescent tubes), a process during which most of the epidermal cells are ruptured (3). The epidermal strips were rinsed in deionized H₂O, rubbed with a dissecting needle (bent at an angle of 120°) to remove adhering mesophyll cells, and collected on deionized H₂O. From there, the strips were transferred to the incubation media in small plastic beakers. The beakers were placed under an inverted crystallizing dish (150-mm diameter, 75-mm height). Humidified, CO₂-free air was passed through the inverted dish for 4 hr at 50 1 hr⁻¹ and 21°C. The samples were illuminated by mercury vapor lamps (General Electric H 400 RDX 33-1); the irradiance below the water filter (5 cm deep) was 85 W m⁻². The incubation media were prepared as described before (13). Their composition was: 10 mM MES (pH 5.6); 0.09 to 0.11 eq l⁻¹ K⁺; 0 to 0.1 eq l⁻¹ Cl⁻. Iminodiacetate (J. T. Baker Chemical Co.) was used to balance K⁺ not balanced by Cl⁻. guard cells presumably do not absorb iminodiacetate (12). Impurities in the chemicals used resulted in an estimated maximal contamination of 0.001 meq Cl⁻ l⁻¹. No Cl⁻ could be detected in the double distilled H₂O used for the preparation of the incubating media nor did crushed plastic beakers give off detectable amounts of Cl⁻ (the limit of detection by argentometry was 0.03 meq Cl⁻ l⁻¹ in the original volume; determinations were made on samples whose volumes had been reduced by boiling to one-tenth of their original values).

The area of the epidermal strips was measured with calipers; stomatal aperture was measured by microscopy.

1 Research supported by the U.S. Energy Research and Development Administration under Contract EY-76-C-02-1338.

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**Electron Probe Analysis.** The procedure described by Humble and Raschke (6) was followed. After incubation, epidermal strips were washed by floating them for 3 min on 50 mM Ca(NO₃)₂, followed by 0.2 mM Ca(NO₃)₂ at 0 C. Then they were placed on a drop of deionized H₂O on an aluminum slide, blotted, rapidly frozen in liquid N₂, and freeze-dried at −40 C. Before exposure to the electron beam the epidermal samples were coated with carbon. An Applied Research Laboratories (Sunland, Calif.) model EMX-SM microanalyzer was operated with an acceleration voltage of 15 keV and a current of 22 namp on the slide. This voltage is sufficient to penetrate a frozen-dried epidermal sample but does not lead to rapid decomposition. Stomata were scanned by producing a 40-μm-long line with the flying-spot beam and moving this line for 50 μm across a stoma within 10 sec. Between five and eight epidermal strips were prepared/treatment; ten stomata were scanned in each strip. Background radiation was determined by scanning (ruptured) epidermal cells or by setting the spectrometer off peak. Both methods yielded similar results. Contents of K and Cl were determined from these data for each stoma separately and then used to compute averages. These averages are thus based on measurements on between 50 and 80 stomata each.

The microanalyzer was calibrated with crystals of KCl and K oxalate (6). They were grown on silica slides and shadowed with carbon for 1 min at 30 amp in a Ladd vacuum evaporator (model 4350) from a point source at known angles. The lengths of the shadows cast and the projected areas of a number of crystals were measured with a Zeiss photomicroscope and used to calculate crystal volumes. The crystals were scanned by the electron beam in the same manner stomata were scanned. Stomatal contents of K and Cl fell in the range between 0 and 3 peq. Since the limited resolution of the light microscope does not allow an accurate determination of the dimensions of crystals containing similar amounts of K and Cl larger crystals were included in the measurements. Calibration curves were obtained that permitted a more accurate computation of stomatal contents than the scatter of the data obtained with the smallest crystals indicated (Fig. 1). Atoms of Cl absorb the Kα x-rays of K. The calibration curve for K is therefore not linear if Cl is present in the sample (Fig. 1, and Fig. 4 of ref. 6). Fortunately, the absolute amounts of Cl in guard cells were generally small; correction for Cl was in many cases unnecessary.

**Malate Assay.** Malate was extracted from fresh or freeze-dried epidermal samples in 5 ml of boiling 80% alcoholic ethanol for 20 min. The procedure was repeated three times. The collected extracts were evaporated to dryness in a steam of air at room temperature. The residue was resuspended in 1 to 2 ml of 0.2 M hydrazine buffer (pH 9), and the malate content was determined by fluorometry of the NADH arising from an enzymic oxidation of malate (4). Each malate assay was calibrated individually by the addition of a known amount of malate ("spike"). Details of the procedure have been given previously (14).

**RESULTS**

**Stomatal Aperture and Malate Content in Relation to Chloride Availability.** Stomatal aperture was independent of the Cl⁻ content of the incubation medium (Table I) but the malate content decreased with increasing [Cl⁻] in the medium, from about 100 pmol mm⁻² in the absence of Cl⁻ to about 58 pmol mm⁻² when Cl⁻ was present at 0.1 eq l⁻¹ (Fig. 2).

**Ionic Balance in Guard Cells of Stomata Opened in Absence and Presence of Cl⁻.** Tables II and III summarize the results of simultaneous determinations of the K and Cl contents of guard cells and of malate contents of the epidermal samples. Malate was extracted from the same samples which had been under the electron beam.³ In Table II, ion contents are expressed relative to the unit area of epidermis; Table III lists ion contents/stoma. Computation of stomatal malate content from measurements on epidermal strips is possible because most of the epidermal cells are ruptured during the preparation of the strips and during incubation (3, 14, and our staining experiments with neutral red). Virtually all of the malate found in the strips must have been in the guard cells.

Guard cells exposed to KCl produced only half as much malate as those supplied with K iminodiacetate. This reduction in malate synthesis was compensated by an increased import of Cl⁻. On K iminodiacetate, about 6% of the total positive charges of K⁺ in the guard cells were balanced by Cl⁻; on KCl, the percentage was 35 and in individual pairs of guard cells as high as 50. Chloride content of guard cells increased with stomatal opening even if no Cl⁻ had been added to the incubation medium. Probably, the medium as well as the broken epidermal tissue contained traces of this element.

**DISCUSSION**

Guard cells of epidermal strips incubated with K iminodiacetate imported K⁺ in quantities very similar to those reported before (5, 6). Per pair of guard cells, 0.43 peq were required to increase stomatal aperture by 1 μm (Table III). The increase in anion content (malate + Cl⁻) was of equal magnitude. Participation of no other ion needs be invoked to achieve electroneutrality, although the malate contents of irradiated samples and those that had not been subjected to electron-probe analysis was 0.93 ± 0.14 in strips incubated with K iminodiacetate and 1.05 ± 0.20 in strips incubated with KCl. The levels of significance of the differences in malate content were P > 0.7 and P > 0.8, respectively.

³ Exposure of freeze-dried epidermal strips to the electron beam did not result in significant changes of their malate contents. The ratio between
though guard cells of *V. faba* are also known to produce citrate and aspartate during stomatal opening (7, and own determinations).

Balance between increases in $K^+$ on one hand and the anions, malate, and $Cl^-$, on the other was maintained also in guard cells incubated with KCl. However, the absolute increases/µm of stomatal opening (0.19 peq $K^+ µm^{-1}$) were only half as large as during exposure to K iminodiacetate. Although the standard deviations of the means were large, the probability that the difference in solute requirement is accidental is <0.001. A higher osmotic effectiveness of $K^+$ is to be expected from an association of $K^+$ with $Cl^-$ than from one with malate because each $K^+$ will require the presence of another osmotically active ion in the first case but only one-half of a counterion if the latter is divergent. In addition, the activity coefficient for KCl is higher than that for K malate. Using a Wescor (Logan, Utah) HR-33T dew point microvoltmeter with C-51 sample chambers, we found that at concentrations occurring in guard cells, the activity coefficients of solutions of KCl were 1.12 times higher than those of K malate. If the difference in the number of ions/$K^+$ are combined with the differences in the activity coefficients, a theoretical ratio of 1.5 is obtained for the “osmotic effectiveness” of KCl as compared to that in K malate. From the data listed in Table III a ratio of 2.3:1 is obtained if the calculations are based on the differences between initial and final conditions, and the ratio is 1.7:1 if the total amount of $K^+$ accumulated in each treatment is divided by the respective final aperture. Thus, we can explain part but not all of the observed difference between treatments in the osmotic effectiveness of $K^+$.

Availability of $Cl^-$ reduced malate production by guard cells, as was observed before by Van Kirk and Raschke (14). This reduction was roughly linearly related to the logarithm of the [Cl$^-$] offered (Fig. 2). It was suspected earlier that import of $Cl^-$ compensated for this reduction (14). Tables II and III show that this was indeed the case. Our data confirm that guard cells of *V. faba* produce citrate and aspartate in response to the increased $Cl^-$.

### Table I. Stomatal Aperture in Relation to $Cl^-$ Availability

Epidermal strips were incubated in the light for 4 h on solutions containing between 0.09 and 0.11 eq l$^{-1}$ $K^+$ and the indicated amounts of $Cl^-$ in 0.01 M MES, pH 5.6. The balancing anion was iminodiacetate. The initial stomatal opening was 6.4 µm.

<table>
<thead>
<tr>
<th>$Cl^-$ content (eq l$^{-1}$)</th>
<th>0</th>
<th>10$^{-3}$</th>
<th>10$^{-4}$</th>
<th>10$^{-5}$</th>
<th>10$^{-6}$</th>
<th>10$^{-7}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aperture ($µm$)</td>
<td>11.7 ± 1.7</td>
<td>13.2 ± 2.1</td>
<td>11.2 ± 1.8</td>
<td>12.9 ± 1.9</td>
<td>11.4 ± 2.0</td>
<td>12.2 ± 2.0</td>
</tr>
<tr>
<td>± standard deviation</td>
<td>11.7 ± 1.7</td>
<td>13.2 ± 2.1</td>
<td>11.2 ± 1.8</td>
<td>12.9 ± 1.9</td>
<td>11.4 ± 2.0</td>
<td>12.2 ± 2.0</td>
</tr>
</tbody>
</table>

### Table II. The Balance Between Potassium, Chloride and Malate in Epidermal Strips Incubated in the Absence or Presence of Chloride

Ion content in peq mm$^{-2}$ epidermis ± standard deviation. Exposure to CO$_2$-free air for 4 h, 21°C, irradiance 85 w m$^{-2}$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aperture$^1$ ($µm$)</th>
<th>$K^+$</th>
<th>$Cl^-$</th>
<th>Malate$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>6.4</td>
<td>25 ± 12</td>
<td>1 ± 1</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>K iminodiacetate$^5$</td>
<td>11.7</td>
<td>144 ± 30</td>
<td>8 ± 5</td>
<td>147 ± 16</td>
</tr>
<tr>
<td>KCl$^6$</td>
<td>13.9</td>
<td>98 ± 29</td>
<td>34 ± 18</td>
<td>75 ± 11</td>
</tr>
</tbody>
</table>

1. Measured on the epidermal samples before freeze-drying for the electron microprobe.
2. Average $K$ and $Cl$ contents of pairs of guard cells were multiplied by the average stomatal density of the epidermal strips used (52 mm$^{-2}$).
3. Malate content was determined in the same samples that had been subjected to electron-probe analysis. Results obtained in mol mm$^{-2}$ were converted to eq mm$^{-2}$ by multiplication by two, on the assumption that the pH in guard cells was sufficiently above the $pK_2$ of maleic acid (= 5.1).
4. 0.1 N KOH, 0.01 M MES titrated to pH 5.6 with solid iminodiacetic acid.
5. 0.1 w/v 0.01 M MES, pH 5.6.
6. Downloaded from p1978 American Society of Plant Biologists. All rights reserved.

CHLORIDE AND MALATE IN GUARD CELLS

Table III. The Balance Between Potassium, Chloride, and Malate in Stomata Opened in the Absence and Presence of Chloride

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aperture (±μm)</th>
<th>K</th>
<th>Cl</th>
<th>Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial</strong></td>
<td>6.4</td>
<td>0.47±0.22</td>
<td>0.02±0.04</td>
<td>0.73±0.20</td>
</tr>
<tr>
<td>K iminodiacetate</td>
<td>11.7</td>
<td>2.77±0.58</td>
<td>0.16±0.10</td>
<td>2.83±0.31</td>
</tr>
<tr>
<td>KCl</td>
<td>13.9</td>
<td>1.88±0.56</td>
<td>0.66±0.35</td>
<td>1.43±0.21</td>
</tr>
<tr>
<td><strong>Increases during stomatal opening</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K iminodiacetate</td>
<td>5.3</td>
<td>2.30</td>
<td>0.14</td>
<td>2.10</td>
</tr>
<tr>
<td>KCl</td>
<td>7.5</td>
<td>1.41</td>
<td>0.64</td>
<td>0.70</td>
</tr>
</tbody>
</table>

1All changes in ion contents that occurred during stomatal opening are significant at P < 0.001.
2Malate content was computed from malate content of 1 mm² of epidermis divided by the stomatal density (52 mm⁻²). This procedure was possible because most of the epidermal cells were ruptured after the incubation period (13).

*faba* do not need to import anions for stomatal opening, as was shown before when K⁺ was offered to epidermal strips in combination with presumably nonabsorbable anions (12); but guard cells will import Cl⁻ (and possibly other small anions) if these ions are available. A possible mechanism that balances transfer and metabolism of anions was outlined earlier (14). Without addition of Cl⁻ to the incubation medium, 91% of the increase in K⁺ was balanced by an increase in malate and 6% by Cl⁻. With 0.1 eq Cl⁻ l⁻¹ in the medium, malate neutralized only 50% of the increase in K⁺, and Cl⁻ contributed 45%. These findings compare well with earlier ones obtained on the same species: when *Vicia faba* plants were fertilized with Hoagland solution, which contains Cl⁻ only as a trace element, Cl⁻ compensated only 5% of the K⁺ in guard cells of open stomata (6). When Allaway let stomata open in detached leaves of *V. faba* with their base in 0.1 mm CaCl₂, about half of the K⁺ in epidermal samples was balanced by malate (1); the other half was presumably balanced by Cl⁻. Pallaghy and Fischer found that on the average 30% of the K⁺ was associated with Cl⁻ when epidermal strips of *V. faba* were exposed to 10 mm KCl (8).

The use of chloride by guard cells during stomatal opening is not restricted to *V. faba*. Chloride has been demonstrated histochemically in the guard cells of *Begonia*, *Plantago*, and even of *Ginkgo biloba* and the fern *Ophioglossum engelmanni* (2). In two grasses (*Avena sativa* [2] and *Zea mays* [11]), and in another monocotyledonous plant possessing distinct subsidiary cells, *Commelina communis* (9), chloride participates in the ion shuttle between guard and subsidiary cells.

It will now be necessary to test whether presence of Cl⁻ inhibits starch breakdown and formation of malate or whether absence of Cl⁻ leads to acid formation in response to the alkalization of the cytoplasm of guard cells resulting from an expulsion of H⁺ (10). Evidence already exists that the stomata of *Allium cepa* cannot open in the absence of Cl⁻ because their guard cells lack starch as a source of carbon for the production of malate (13).

Acknowledgments—We thank C. Schmuck for her valuable help in the determination of the crystal volumes which were required for the calibration of the microprobe, and V. Shull, Department of Horticulture, Michigan State University, for the operation of the electron probe microanalyzer.

LITERATURE CITED