Malate-sensitive anion channels enable guard cells to sense changes in the ambient CO$_2$ concentration

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Summary

Malate is a characteristic metabolite in the photosynthesis of C4 and CAM plants. Furthermore, changes in the intracellular concentration of this organic acid provide part of the osmotic motor for guard cells. Since alterations in the malate concentration influence the photosynthetic capacity on one side and stomatal action on the other, it was studied whether the extracellular malate level represents an indicator of changes in the ambient CO$_2$ concentration and a key regulator of ion transport in guard cells.

Here it is demonstrated that alterations in the ambient CO$_2$ level modify the extracellular malate concentration of *Vicia faba* leaves. Elevated external malate caused stomatal closure in a concentration-dependent manner ($K_m^{\text{mal}} = 0.3$ mM). Slight variations in the external malate concentration strongly regulate the voltage-dependent properties of GCAC1, an anion-release channel in the plasma membrane of guard cells. Supersufusion of guard cell protoplasts with malate levels in the physiological range ($K_m^{\text{mal}} = 0.4$ mM) caused the voltage gate to shift towards the resting potential of the cell-activating GCAC1. Single-channel conductance was dependent on the extracellular chloride concentration ($K_m^{\text{Cl}} = 3$ mM). In the absence of extracellular chloride the plasma membrane lacked anion conductance until the addition of malate-induced channel opening. Isophthalate was a powerful agonist in both malate-induced processes, channel regulation and stomatal closure, indicating that modulation of GCAC1 is a key step in stomatal action. It was thus concluded that feedback regulation of volume and turgor with respect to the ambient CO$_2$ concentration via malate-sensitive anion channels may provide a CO$_2$ sensor to guard cells.

Received 11 June 1994; revised 21 July 1994; accepted 12 August 1994.

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steroid-hormones, neurotransmitter/amino acids or nitric oxide). Since highly organized organs like the human brain signal numerous bits of information within a network, enabling neuronal processes such as learning and memory, on the basis of changes in the extracellular glutamate or aspartate level, we cannot exclude the possibility that members of the primary metabolism such as malate are involved in plant signal transduction as well.

In this report we elucidate whether malate, an important metabolite in CO₂-fixation, acid metabolism, control of cytoplasmic pH and the redox state (i) is released in response to CO₂ changes, (ii) affects stomatal movement, and (iii) is recognized via receptor channels in the guard cell plasma membrane.

Results

Does extracellular malate serve as a key metabolite in the signal transduction pathway controlling water-use efficiency?

*Vicia faba* plants were exposed to ambient CO₂ (362 p.p.m., Figure 1a target), elevated CO₂ (1%, filled symbols), or CO₂-free air (open symbols). At the incubation times indicated leaves were excised and the extracellular fluid (apoplast) was analysed with respect to its malate content (Figure 1a). Under the conditions shown in Figure 1(a) average malate concentrations under ambient CO₂ were in the 0.4–0.6 mM range (horizontal bar). The existence of malate gradients along the leaf apoplast, resulting from uneven malate synthesis or release from guard cells, mesophyll and/or epidermal cells could, however, not be excluded. In this context it should be noted, that the absolute values in different experiments showed a large variability while the relative increase in malate content at elevated CO₂ was similar. Upon the switch to high CO₂ the apoplastic malate content reproducibly increased by 50–100% within the first 15 min. A comparable increase in the malate content (e.g. from 1.00 ± 0.60 to 3.10 ± 2.30 mM) was found when the CO₂ concentration was raised to only 672 p.p.m., indicating that the malate pool is saturable. An observation in line with the fact that stomatal closure saturates already at 1000 p.p.m. (Raschke, personal communication). In CO₂-free air the malate level decreased to about 50%. Under both conditions the kinetics of changes in the malate pool were too fast to be resolved by this method (< 5 min; Speer and Kaiser, 1991). Sometimes the rapid change to high CO₂ caused the malate content to oscillate (Figure 1a, open bars between upright filled triangles), well in agreement with fluctuations in stomatal aperture under similar conditions (Raschke, 1965; Kaiser, personal communication). Measuring the diurnal cycle of the apoplastic malate pool of greenhouse plants (light period 5 a.m. to 10 p.m.), we observed a steady increase in malate concentration from, e.g. 0.54 ± 0.29 at noon to 2.39 ± 0.63 mM (n = 6) at night. In the beginning of the light period (8 a.m.) values as low as 0.75 ± 0.21 mM (n = 5) were recorded, suggesting that the malate pool is under light control, too (Raschke et al., 1988; Scheibe, 1987).

Since the apoplastic malate pool seems to reflect the current CO₂ atmosphere, we proved in the following whether the increase in the malate content is able to induce stomatal closure via regulated salt release through plasma membrane ion channels in guard cells. Epidermal strips were excised from the adaxial surface of leaves from predarkened *V. faba* plants. Stomatal opening was induced by light treatment. During the opening phase extracellular chloride was replaced by gluconate or either malate or isophthalate in addition to gluconate while the potassium content remained unchanged (Figure 1b). Changes in aperture were recorded with respect to the amplitudes before stimulus onset (Figure 1b, arrow). In contrast to gluconate (squares) which only prevented a further increase in the pore diameter, malate (filled triangles) caused stomata to close down to the dark level (filled circles). Application of 100 µM isophthalate (open triangles), a powerful malate-agonist of malate-dependent anion transport in vacuoles (Martinoia, personal communication), reduced stomatal opening to the same extent as 10 mM malate. The sensitivity for malate-induced stomatal closure could be expressed by a Michaelis–Menten type behaviour with a half-saturation constant, Kₘ, of 0.3 mM (Figure 1c).

In order to study the CO₂/malate-induced volume decrease in more detail we applied the patch-clamp technique (Hamill et al., 1981) to guard cell protoplasts to monitor the electrical properties of the plasma membrane during effector treatment. GCAC1 (guard cell anion channel 1; Hedrich and Jeromin, 1992), a key element in signal transduction and excitation of this cell type (Hedrich, 1994; Marten et al., 1991, 1992) is modulated by extracellular malate (Hedrich and Marten, 1993). Because of this effect and since malate and isophthalate were anionic under our experimental conditions and the latter was shown to interfere with vascular anion transport, we concentrated on the action of malate and its ‘agonist’ on this plasma membrane channel.

GCAC1 is characterized by a cell-specific voltage-dependence as well as peculiar kinetics, which allowed us to design experimental conditions to record selectively GCAC1 from the guard cell plasma membrane (Hedrich et al., 1990). In order to activate GCAC1 fully in a Ca²⁺- and nucleotide-dependent manner the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was established on protoplasts with 150 mM TEACl or KCl in the pipette and 40 mM CaCl₂ in the
Figure 1.
(a) Time course of changes in extracellular (apoplastic) malate concentration of V. faba leaves in response to CO₂. Ambient CO₂ (horizontal bar), 1% CO₂ (filled symbols), and CO₂-free air (open symbols).
(b) Stomatal closure in response to extracellular malate. Stomatal opening was induced by incubation of epidermal strips from predarkened plants in 50 mM KCl solution under white light (open circles). At the time indicated by the arrow KCl was replaced by 3 mM K₂malate in addition to 3 mM KCl and 41 mM Kgluconate (filled triangles), 50 mM Kgluconate (squares), or 3 mM KCl and 47 mM Kgluconate plus 100 μM isophthalate (open triangles), or in the presence of 50 mM KCl a dark regime (filled circles) was applied. Each data point represents the mean of at least 100 stomatal pore diameters. The solutions were buffered with 10 mM MES pH 5.6 / TRIS.
(c) Concentration dependence of malate-induced stomatal closure. Stomatal apertures were normalized with respect to the light control in KCl after 120 min (arrow b). Each data point represents the mean of three independent experiments 1 h after solution exchange. The solutions contained 3 mM chloride. Potassium concentration was adjusted to 50 mM with Kgluconate. Solutions were buffered with 10 mM MES, pH 5.6 / TRIS.
bath. Lowering the calcium concentration to physiological levels (1 mM), the extracellular membrane surface was superfused with malate-containing media. In the presence of malate the voltage gate of GCAC1 shifted towards the resting potential of the cell (Blatt, 1991; Lohse and Hedrich, 1992; Thiel et al., 1992) in a fully reversible manner while a change in the extracellular calcium or chloride concentration did not alter the voltage dependence of the channel (Hedrich and Marten, 1993; Hedrich et al., 1990). This malate-induced gate shifting was dependent on the malate concentration (Figure 2) and showed a saturation behavior with a half-saturation constant, \( K_{\text{m}} \), of about 0.4 mM (Hedrich and Marten, 1993), well in line with the bioassay (\( K_{\text{m}} \) = 0.3 mM). This striking similarity might indicate that the malate sensitivity has been preserved during protoplast isolation and that malate could represent a potential regulator of stomatal aperture and GCAC1 at physiological malate concentrations (0.3-3.1 mM in this report and 0-3.4 mM in another; Speer and Kaiser, 1991). Within this range GCAC1 is most sensitive towards malate changes which in turn would shift its range of activity by 38 mV (at \( K_{\text{m}}^{\text{mal}} \)) towards the resting potential of the cell. When isophthalate as low as 100 \( \mu \)M was applied to guard cell protoplasts (Figure 3A) it was as effective as 0.5 mM malate. The shape of the current–voltage curve in the presence of malate and isophthalate (Figures 2 and 3, lack or reduction of inward and outward currents positive to the peak current potential) not only results from effector-induced gate shifting but a voltage-dependent block as well. Both effectors, malate and isophthalate, induced stomatal closure in the presence of 3 and 30 mM chloride (Table 1). Thus malate is effective at halide concentrations found to accompany stomatal movement (Bowling, 1987). An increase in extracellular chloride, however, re-
duced the malate− (1 mM) as well as the isophthalate-induced (100 μM) gate shifting on GCAC1 by about 20 mV (Figure 3A and 3B). Thus we deduced that chloride ions shield the 'gate-shifting site'.

When in the presence of isophthalate the anion current was activated by depolarizing 70 msec voltage steps steady-state inward currents (chloride efflux) were only observed in the voltage window between −156 mV and −46 mV (Figure 4) as expected from the current−voltage curve in Figure 3(A). The open probability at further depolarized potentials, however, remained unchanged with respect to effector-free conditions (Figure 4). Since the chloride gradient was unaltered throughout the activation−curve analysis (see legend to Figure 4), at, e.g. 4 mV the high open probability in contrast to the steady-state current amplitude thus indicates that isophthalate caused a voltage-dependent block rather than a decrease in open probability of GCAC1.

From the voltage-dependent activity of single anion channels in outside-out patches (Figure 5a, lower graph) we could deduce that the increase in macroscopic current (Figure 5a, upper graph) in the presence of malate results from channel openings in a voltage range of higher driving force for anion release. Reducing the extracellular CaCl₂ concentration resulted in a decrease in the unit conductance of single anion efflux channels. It should be noted that elevated cytoplasmic Ca²⁺ was essential to activate GCAC1 (Hedrich et al., 1990), but upon activation physiological extracellular calcium concentration as low as 1 mM, maintained steady-state activity throughout an experiment. In excised cell-free patches the absence of extracellular chloride did not allow us to detect GCAC1 conductance (in the whole cell configuration due to unstirred layers and/or membrane invaginations it was not possible to impose chloride-free conditions (see Figure 3A,a): an observation comparable with that on K⁺ release channels in nerve cells (Pardo et al., 1992)). At a concentration of about 3 mM, however, the halide was able to half-saturate the 'regulatory site' with respect to the unitary conductance (Figure 5b). In the presence of extracellular chloride as low as 3 mM (Figure 5a) or in the absence of extracellular chloride (Figure 5c) malate is able to replace the halide in modifying the open channel amplitude of GCAC1. Thus the two external regulatory sites are sensitive to physiological concentrations of malate and chloride. The interaction of malate and isophthalate on these two sites of GCAC1 is in accordance with the different anion-channel blocker sensitivities in relation to inhibition and modification of gating (Marten et al., 1993). Thus changes in concentration of apoplastic chloride relative to malate, which accompany stomatal closure (Raschke, 1979), may interfere with the action of this carbon metabolite on the 'gate-shifting site'.

### Table 1. Effect of malate and isophthalate on stomatal aperture under physiological Cl⁻ concentrations

<table>
<thead>
<tr>
<th>Solute compositions</th>
<th>Stomatal aperture (μm)</th>
<th>Standard deviation (μm)</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (50 mM KCl)</td>
<td>11.5</td>
<td>2.1</td>
<td>7</td>
</tr>
<tr>
<td>Dark control (50 mM KCl)</td>
<td>6.2</td>
<td>1.8</td>
<td>7</td>
</tr>
<tr>
<td>10 mM K₂malate + 30 mM Kgluconate</td>
<td>5.7</td>
<td>2.9</td>
<td>4</td>
</tr>
<tr>
<td>10 mM K₂malate + 3 mM KCl + 27 mM Kgluconate</td>
<td>5.3</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>10 mM K₂malate + 30 mM KCl</td>
<td>7.1</td>
<td>2.2</td>
<td>3</td>
</tr>
<tr>
<td>100 μM isophthalate + 50 mM Kgluconate</td>
<td>6.0</td>
<td>2.2</td>
<td>3</td>
</tr>
<tr>
<td>100 μM isophthalate + 3 mM KCl + 47 mM Kgluconate</td>
<td>6.0</td>
<td>1.3</td>
<td>3</td>
</tr>
<tr>
<td>100 μM isophthalate + 30 mM KCl + 20 mM Kgluconate</td>
<td>6.3</td>
<td>0.7</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 4. Voltage-dependent block of the whole-cell anion current by 100 μM isophthalate. Relative inward currents (circles) and relative open probabilities (squares) were given as a function of the voltage. Inward anion currents were activated by 70 msec voltage steps from a holding potential of −196 mV to depolarized voltages. Relative open probabilities are expressed by tail currents after return to the holding potential normalized with respect to the saturation of the tail currents. The pipette solution was composed of 150 mM TEACl, 2 mM MgCl₂, 10 mM MgATP, 10 mM Na₂GTP, 0.1 mM EGTA and 10 mM HEPES, pH 7.2/TRIS. The bathing medium contained 1 mM Cagluconate₂ (open symbols) and 1 mM Cagluconate₂ plus 100 μM isophthalate (closed symbols), respectively. The extracellular media were buffered with 10 mM MES, pH 5.6/TRIS.
Figure 5.
(a) Whole-cell anion currents in the presence of 84 mM chloride and after solute exchange to 3 mM chloride plus 1 mM malate (upper graph). Corresponding single-channel fluctuations in an outside-out patch at voltages indicated (lower graphs).
(b) Single-channel conductance of GCAC1 as a function of the extracellular chloride concentration. The half-saturation constant (K_{m,CI} = 3 mM) was deduced from lineweaver-burk analysis (inset). Data points represent the mean of five to seven individual experiments.
(c) Malate-induced single-channel activity in the presence of 1 mM malate. Single-channel fluctuations from an outside-out membrane patch at voltages indicated. Note, that single-channel amplitudes can be resolved in the absence of extracellular chloride.

The pipette solution was composed of 150 mM TEA.Cl (a and c) or 150 mM KCl (b), 2 mM MgCl_2, 10 mM MgATP, 10 mM Na_2GTP, 0.1 mM EGTA and 10 mM HEPES, pH 7.2/Tris. The bathing medium contained 40 mM CaCl_2 and 2 mM MgCl_2 (a, right trace); 1.5 mM CaCl_2 and 1 mM TEA.Cl-malate (a, left trace); 40 mM, 20 mM, 5 mM, 3 mM or 1 mM CaCl_2 (b); 1 mM Cagliouonate (c). The extracellular media were buffered with 10 mM MES, pH 5.6/Tris.
We have shown that guard cells possess anion channels which are controlled by extracellular levels of a primary metabolite, a type of regulation reminiscent of glycine- or glutamate-activated channels in nerve cells (Hille, 1992). The apoplastic content of this carbon metabolite provides a measure of the ambient CO₂ concentration and photosynthetic capacity. The effect of malate and its 'agonist' on GCAC1 is well in agreement with the downregulation of volume and turgor of guard cells in their natural environment in response to both external effectors (Figure 1b), indicating the vital role of this channel in stomatal movement. Moreover, the $K_m^{\text{mal}}$ for stomatal closure and for the gate shifting of the channel are almost identical and well within the physiological range of extracellular malate concentrations (Figure 1; Speer and Kaiser, 1991). The voltage-dependent block of GCAC1 by isophthalate and malate at depolarizing potentials may prevent shifts in membrane potential into the non-physiological (positive) voltage range and thus restrict the action of the key metabolite to the physiological voltage window, only. Cross-sensitivity of the malate-site ('gate-shifting site') to elevated chloride may protect guard cells from depleting their residual (closed state) anion content by resetting the voltage gate (Figure 3). Malate comprises the major organic anion osmoticum in guard cells (Raschke, 1979), where during stomatal closure malate-permeable GCAC1 may account for its partial release (Van Kirk and Raschke, 1978). Since isolated guard cells and protoplasts still maintain their responsiveness to changes in CO₂ (Fitzsimons and Weyers, 1986; Gotow et al., 1982; for volume- and day-night cycle-dependent properties of malate-forming enzymes in guard cells see Raschke et al., 1986; Scheibe, 1987; Scheibe et al., 1990; Schnabl, 1981), malate release may feed-forward chloride and malate efflux through GCAC1 (the mechanism is as yet unclear, but it is quite possible that a plasma membrane dicarboxylate carrier or fraction of GCAC1 in the voltage-independent S-type (Schroeder and Hagiwara, 1990) gating mode rather than R-type is involved (Dietrich and Hedrich, 1994; Schroeder and Keller, 1992)). Under our experimental conditions we have not yet observed alterations in the malate-sensitivity of GCAC1, although desensitization and/or long-term modification (e.g. diurnal changes or cultivation under elevated CO₂) could present versatile mechanisms to shift the dynamic range of a receptor channel.

We thus conclude that the two regulatory sites on the extracellular face of GCAC1, when accessed by malate and/or chloride might provide feedback loops which adapt the voltage sensor and anion-transport capacity of GCAC1 to the CO₂ concentration as well as the current anion gradient across the plasma membrane of guard cells to optimize water-use efficiency.

**Discussion**

**Experimental procedures**

**Plants**

Broad bean (Vicia faba L.; Kröbel, Göttingen, Germany) plants were grown in the greenhouse. The photoperiod was 14 h and the photon flux density 300 μmol m⁻² sec⁻¹ (HQL-TS 250 W/D; Osram, München, Germany). Temperatures in the light and dark were 20°C and 14°C, respectively. The humidity was 60–70%.

**Measurement of the apoplastic malate concentration**

Plants were incubated for up to 2 h under ambient CO₂, 1% CO₂, and CO₂-free air in a growth chamber. Leaves were excised and apoplastic solutions collected at times indicated to analyze malate levels as described before (Speer and Kaiser, 1991). In contrast to Speer and Kaiser (1991) we used 250 mM MES/KOH, pH 5.5, to infiltrate the leaves. Malate changes were normalized with respect to the level before stimulus onset (see Figure 1, target). In Figure 1 each symbol represents an independent experiment and data points the mean of 6–12 leaves (1-2 leaves per plant). The standard deviation of the mean (SSD) was about 30%.

**Stomatal-opening assay**

The lower epidermis of almost fully expanded leaflets from 2- to 3-week-old plants, predarkened for 14–16 h, was peeled off. To induce stomatal opening (Table 1 and Figure 1b) the epidermal strips were incubated in 50 mM KCl for 120 min in the light. Stomatal apertures were determined after 210 min in the presence of various effectors in the light as indicated in Table 1 and Figure 1(b). All solutions were buffered with 10 mM MES/TRIS to pH 5.6.

**Protoplast isolation and patch-clamp recordings**

Guard cell protoplasts were enzymatically isolated from 2- to 3-week-old leaves of V. faba (Hedrich et al., 1990). Patch pipettes were sealed on to the plasma membrane to study ion fluxes in the whole-cell configuration and in outside-out patches (Hamill et al., 1981). Current measurements were made with either an EPC-7 or EPC-9 patch-clamp amplifier (List electronic, Darmstadt, and HEKA Lambrecht, Germany) and low-pass filtered with an eight-pole Bessel filter. Data were digitized (VR10, Instrutech Corp., Elmont, NY, USA), stored on hard disc or video tape and analysed using patch-clamp software of Instrutech Corp. on a Mega Atari ST4. Patch pipettes were prepared from Kimax-51 34500 glass (Kimble products, Vineland, NY, USA) to obtain high-resistance seals. After guard cell protoplasts were applied to the recording chamber and stuck to its glass bottom the chamber was continuously perfused with the extracellular solution (0.13 μl min⁻¹). Upon changes in the saline composition of the medium the perfusion rate was increased to 1.3–3.8 ml min⁻¹ until a new steady state had been established. Repetitive voltage ramps of 1 sec duration from −196 to +84 mV and 70 msec voltage steps enabled us to record the activation state (current amplitude) and voltage-dependence of GCAC1 simultaneously during the course of an experiment. The membrane potentials in Figures 2–4 were corrected for the liquid junction potential determined after Neher (1992).
Patch-clamp solutions

The pipette solution (cytoplasm) was composed of 150 mM TEACl, 2 mM MgCl₂, 10 mM MgATP and Na₂GTP, 0.1 mM EGTA, 10 mM HEPES, pH 7.2/TRIS. The standard bathing medium contained 40 mM CaCl₂, 2 mM MgCl₂, 10 mM MES, pH 5.6/TRIS. Modifications of the extracellular medium are mentioned in the figure legends. Sorbitol was used to adjust solutions to a final osmolality of 400 mosmol kg⁻¹, as verified by a water-vapour osmometer (5100 C, Wescor, Logan, U, USA). The reference electrode was filled with 3 M KCl and a 3 M KCl/2% agarose-plug.

Acknowledgements

We gratefully acknowledge helpful discussions with W.M. Kaiser (Würzburg), E. Martinoia (Zürich), and the participants of the 1993 DFG workshop ‘Cellular Transport Physiology’ and the SEB-Congress at Wye College. This work was funded by DFG grants to R.H. and H.W.H.

References


