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Malate-induced feedback regulation of plasma membrane anion channels could provide a CO_2 sensor to guard cells

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Plants have developed strategies to circumvent limitations in water supply through the adjustment of stomatal aperture in relation to the photosynthetic capacity (water-use efficiency). The CO_2 sensor of guard cells, reporting on the metabolic status of the photosynthetic tissue, is, however, as yet unknown. We elucidated whether extracellular malate has the capability to serve as a signal metabolite in regulating the membrane properties of guard cells. Patch-clamp studies showed that slight variations in the external malate concentration induced major alterations in the voltage-dependent activity of the guard cell anion channel (GCAC1). Superfusion of guard cell protoplasts with malate solutions in the physiological range caused the voltage-gate to shift towards hyperpolarized potentials (K_m^{mal} = 0.4 mM elicits a 38 mV shift). The selectivity sequence of the anion channel NO_3^- (4.2) $\geq$ I^-(3.9) > Br^-(1.9) > Cl^- (1) >> mal (0.1) indicates that malate is able to permeate GCAC1. The binding site for shifting the gate is, however, located on the extracellular face of the channel since cytoplasmic malate proved ineffective. Single-channel analysis indicates that extracellular malate affects the voltage-dependent mean open time rather than the unitary conductance of GCAC1. In contrast to malate the rise in the extracellular Cl^- concentration increases the unit conductance of the anion efflux channel. We suggest that stomata sense changes in the intercellular CO_2 concentration and thus the photosynthetic activity of the mesophyll via feedback regulation of anion efflux from guard cells through malate-sensitive GCAC1.

Key words: anion channel/CO_2 sensor/guard cells/malate/patch-clamp

Introduction

Plants exchange carbon dioxide and water with their environment through turgor-operated valves, the stomata. Turgor and volume changes on the other hand result from changes in the potassium salt content of the guard cells. Light, CO_2 and plant hormones have been shown to affect the size of the diffusion pore between the two guard cells (Raschke, 1979). Voltage-dependent ion channels in the plasma membrane of these cells provide a versatile mechanism to control the large and rapid changes of the salt content required for stomatal movement (Hedrich and Schroeder, 1989; Schroeder and Hedrich, 1989; Blatt, 1991).

Patch-clamp studies have demonstrated that guard cell anion channels [GCAC1; see Hedrich and Jeromin (1992) and Marten *et al.* (1992) for nomenclature] possess target sites for extra- and intracellular signals (Hedrich *et al.*, 1990; Marten *et al.*, 1991; Lohse and Hedrich, 1992). These channel modulators can be subdivided into (i) activators, such as calcium and nucleotides, which increase the open probability of GCAC1 and thus the amplitude of the whole-cell anion current; (ii) gating modifiers, such as the growth hormones, auxins (and malate in the present study), which direct the voltage gate and thus the working range of the anion channel towards the resting potential of the cell (Lohse and Hedrich, 1992).

Because of its peculiar characteristics, inactivation following prolonged voltage (depolarization)-activation and deactivation upon hyperpolarization, GCAC1 was supposed to present the permeation pathway for anions during volume decrease as well as to underly transient changes in membrane potential during excitation (Thiel *et al.*, 1992) and transduction of external signals (Bates and Goldsmith, 1983). Activation-inactivation cycles of GCAC1 are thus capable to account for oscillations in stomatal aperture upon changes in the environment.

Since sudden changes in light intensity or ambient CO_2 concentration serve as triggers of oscillations in photosynthetic CO_2 fixation as well, we have asked whether or not guard cells respond to the current photosynthetic status through metabolite (malate)-regulated anion channels. We have further elucidated how stomatal closure and anion release preceed when the extracellular anion concentration and thus the anion gradient across the plasma membrane decays.

Results and discussion

Malate is a gating modifier of GCAC1

GCAC1 is characterized by cell-specific properties such as steep voltage-dependence, as well as peculiar kinetics with fast activation and deactivation, slow inactivation and extremely slow recovery from inactivation (Hedrich *et al.*, 1990). These properties allowed the design of experimental conditions to selectively record GCAC1 from the guard cell plasma membrane (Hedrich and Schroeder, 1989; Schroeder and Hedrich, 1989; Blatt, 1991; Tyerman, 1992).

The whole-cell configuration of the patch-clamp technique was established on protoplasts (Hedrich *et al.*, 1990) with millimolar concentrations of 150 TEACl or 82 TEA_2 malate in the pipette. In the first step (preconditioning) cells were exposed to 40 mM $CaCl_2$ to fully activate GCAC1 in a Ca^{2+}- and nucleotide-dependent manner (Hedrich *et al.*, 1990). In the second step calcium usually was reduced to physiological levels (1 mM; for the role of Ca^{2+} in GCAC1 activation see Hedrich *et al.*, 1990), before the effectors under investigation were applied by bath perfusion. Repetitive voltage ramps of 1 s duration

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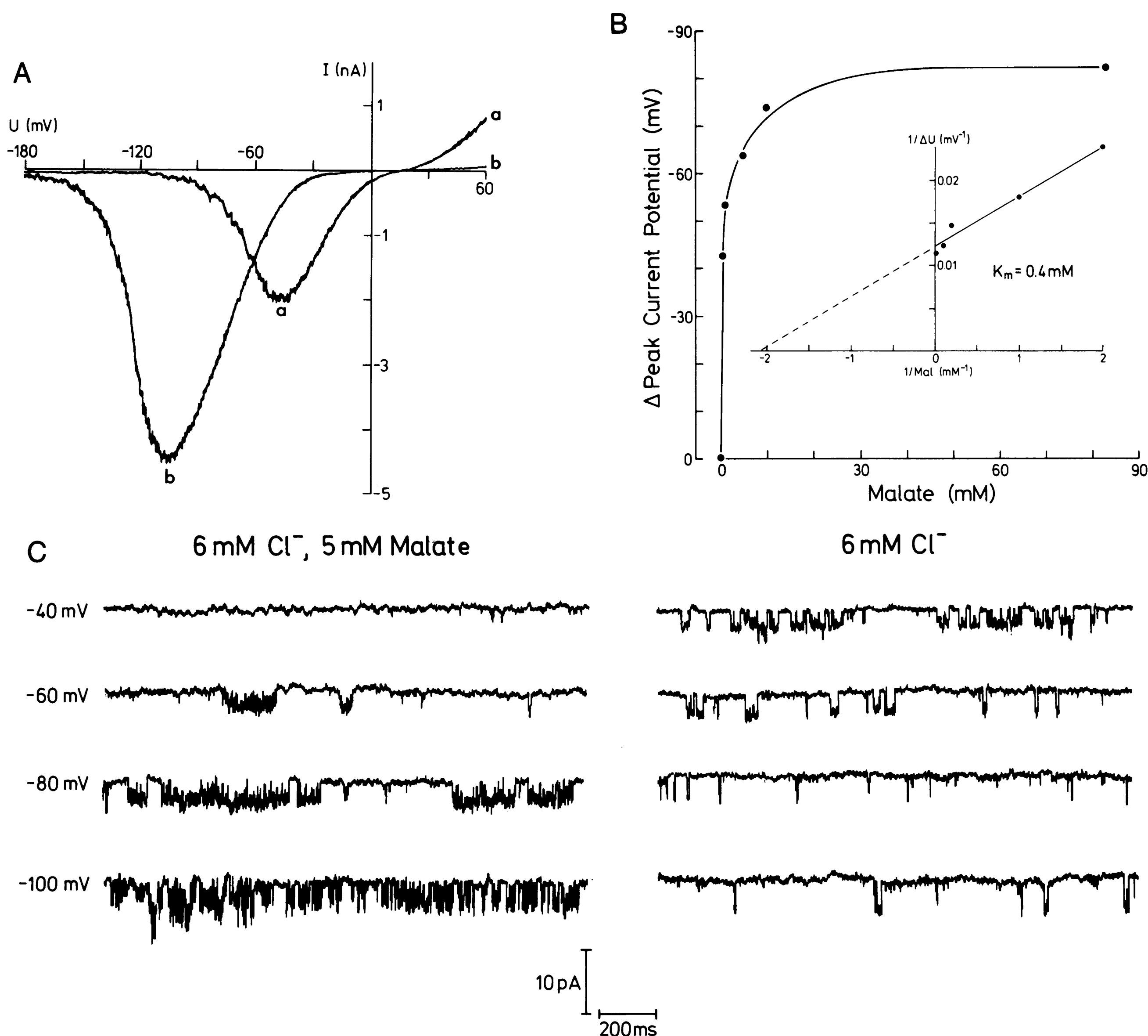

Fig. 1. Modulation of the plasma membrane anion channel of guard cells by malate. Malate-induced modulation of peak amplitude of inward current and shift in activation potential. (**A**) Current–voltage relation of whole-cell anion fluxes before (a) and during (b) bath perfusion with 82 mM malate resulting from 1 s voltage ramps from −200 to +60 mV. (**B**) Concentration-dependence of malate-induced shift in peak current potential of the anion channel. (**C**) Single-channel activity before (right panel) and during malate stimulation (left panel). Channel fluctuations, from an outside-out membrane patch were recorded with the membrane potential clamped to −40, −60, −80 and −100 mV.

from −200 to +60 mV enabled us to simultaneously record the activation state and voltage-dependence during the course of an experiment (Figure 1, see also Marten *et al.*, 1991).

Upon perfusion with malate solutions the peak current amplitude of GCAC1 increased, while the voltage gate shifted towards hyperpolarized potentials (Figure 1A). Dose–response analysis revealed a Michaelis–Menten type behaviour. The half-saturation constant, K_m, for this dicarboxylate of 0.4 mM (Figure 1B) is well within the range of extracellular malate concentrations (0.2–5.7; Speer and Kaiser, 1991; and H.Winter, personal communication; K-J.Dietz, personal communication).

K_m concentrations of D/L-malate shift the peak current potential by ~38 mV more negative, whereas at levels above 1 mM malate a maximal shift of almost 80 mV was observed (Figure 1B). L-malate was equally effective as the D/L-dicarboxylate. Complete recovery was always achieved on returning to malate-free solutions. The increase in current amplitude in the presence of the metabolite at the extracellular side is a consequence of channel openings in a voltage window of higher driving force for anion release (Figure 1A and C).

When the membrane potential of guard cells was clamped around −90 mV, almost no inward current was elicited. Upon the perfusion with malate solutions, GCAC1 was transiently activated. Following activation and inactivation the anion currents reached a steady state amplitude in the 100 pA range (Figure 2).

From single-channel analysis (Figure 1C) we deduced that malate shifted the voltage-dependent open probability towards hyperpolarized potentials rather than affecting the unitary conductance (g_{sc} = 29 pS).

Comparison of malate action on whole-cell currents and single anion channels of outside-out patches (Figure 1) indicates that cytoplasmic factors are not required for modulation of GCAC1 by the metabolite.

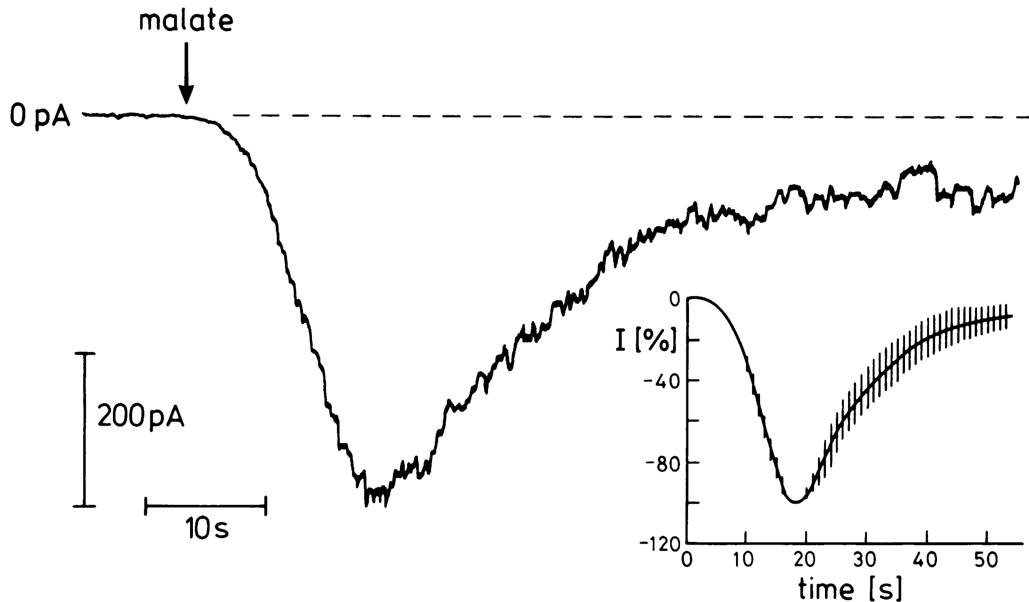

Fig. 2. Transient activation of GCAC1 following malate application. Time-course of malate-induced anion currents in guard cells. Whole-cells were voltage-clamped to −90 mV throughout the experiment. Whole-cell current of a single guard cell and malate-induced (5 mM) anion currents of three different cells normalized with respect to the peak current (inset; error bars represent standard deviation, SSD). Note that during malate treatment rise and decay times of GCAC1 result from the slow exchange rate of the bath perfusion system superimposed by channel inactivation (see Hedrich *et al.*, 1990).

Location of the effector site

In order to locate the site of malate action we compared the gate-shifting efficiency of the metabolite with the relative permeability of GCAC1 to malate. From the permeability ratio analysis we learned that halides followed Eisenman series I (Figure 3, see also Eisenman and Horn, 1983),

NO_3^- ≥	I^-	> Br^-	> Cl^-	>> Mal
4.2	3.9	1.9	1	0.1

indicating anion binding sites of low field strength within the pore (Hille, 1992). Nitrate was about three times more and malate 10 times less permeable to the anion channel than chloride. The fact that external malate in submillimolar concentrations was able to modulate the channel whereas even 82 mM cytoplasmic malate proved ineffective, indicates that channel modulation does not require effector permeation through the channel. An external binding site seems thus to be linked to the voltage gate.

Since malate action was not significantly altered upon a rise in pH from 5.6−7.2, malate^{2-} seems to present the active form of the metabolite (pK_1^{mal1-} = 3.4, pK_2^{mal2-} = 5.3). Besides changes in pH which could result from CO_2 in solution ($CO_2 + H_2O <-> HCO_3^- + H^+ <-> H_2CO_3$) we could also exclude the possibility that modulation of the voltage sensor was caused by changes in the net surface charge (e.g. Ca^{2+} binding to mal^{2-}), field (ionic) strength or redox state of the cell (not shown).

Unit conductance adapts to external chloride

When the chloride concentration in the bath solution was increased the peak amplitude of the inward current (Cl^- efflux) revealed an inverse dependence on the Cl^- gradient across the plasma membrane (Figure 4A and B), which is reminescent to the dependence of the K^+ channel subtype, RCK4, on external potassium (Pardo *et al.*, 1992). Thus a rise in extracellular chloride caused anion efflux through GCAC1 to increase. Figure 4 shows that the increase in

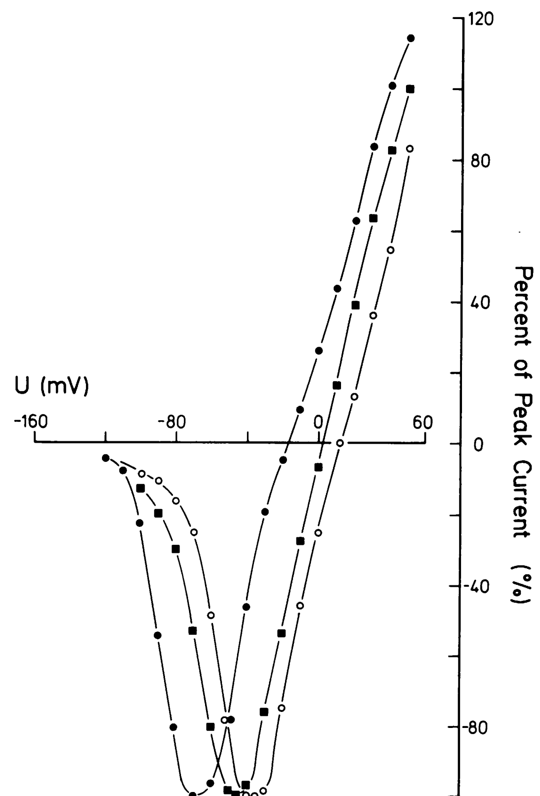

Fig. 3. Relative permeability of GCAC1. Current−voltage curves for GCAC1 in the whole-cell configuration under biionic conditions. TEAI (●), $CaBr_2$ (■) and $CaCl_2$ (○) were applied at halide concentration of 80, 80 and 84 mM, respectively, by bath perfusion in the presence of 150 mM TEACl in the pipette. Currents were normalized with respect to the peak current. When 82 mM TEA_2 malate was used in the pipette it replaced Cl^- as intracellular anion. The shift in reversal potential reflects the reduced permeability of GCAC1 to malate of P_{mal}/P_{Cl} of <0.1. When malate was applied to the extracellular side (150 mM TEACl in the pipette), voltages up to +60 mV did not elicit outward currents. This observation is in line with the malate permeability deduced from the modified Goldman−Hodgkin−Katz equation (Fatt and Ginsborg, 1958).

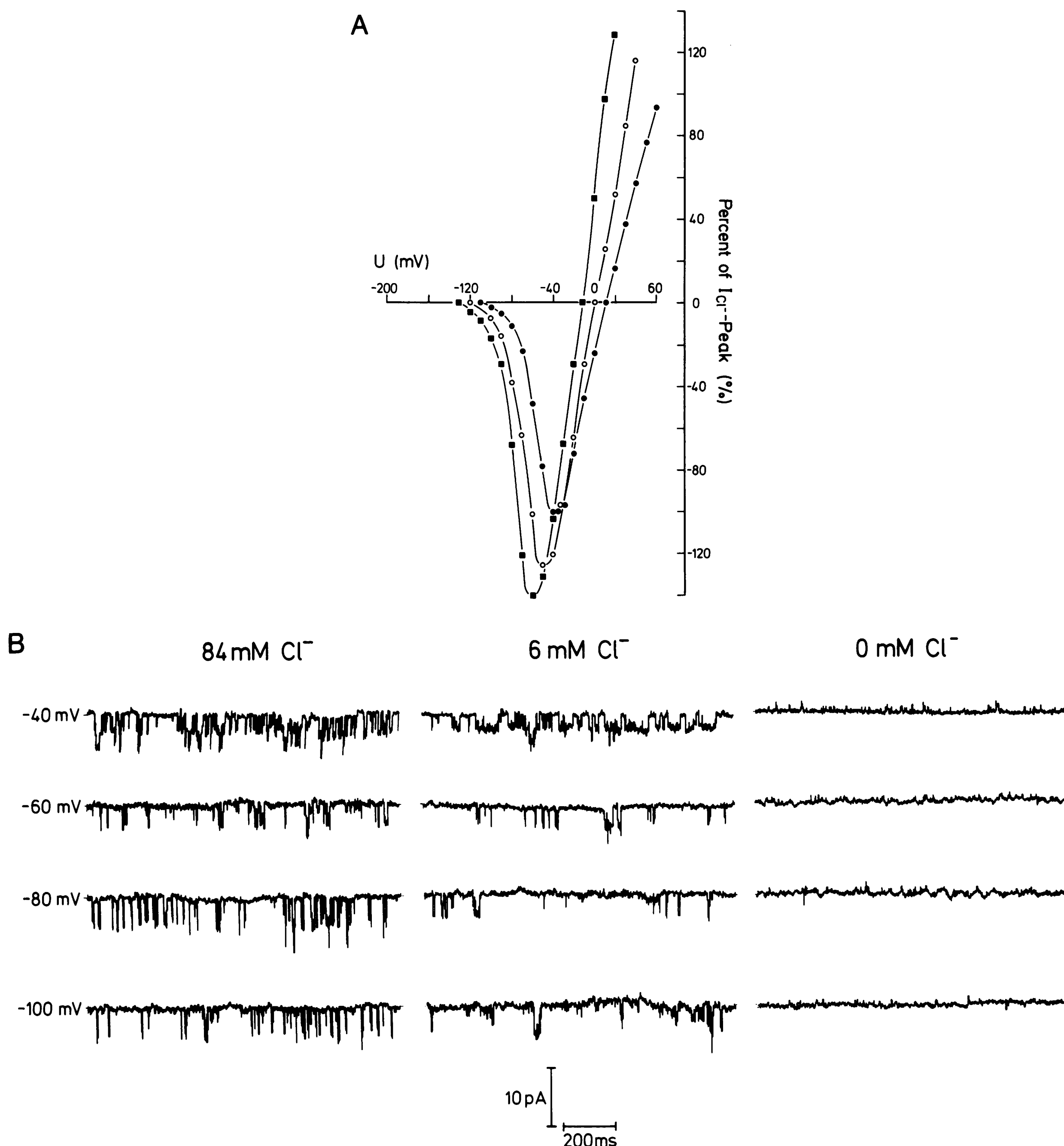

Fig. 4. Decrease in extracellular chloride concentration suppresses anion release through GCAC1. (A) Whole-cell current–voltage curves for GCAC1 in the presence of 84 (●), 152 (○) and 249.5 mM chloride (■). In order to maintain the osmolarity and Ca^{2+} concentration the bath solution was adjusted with TEACl. Current–voltage relations of GCAC1 resulted from 1 s voltage ramps from a holding potential of −200 to +60 mV. Currents were normalized with respect to the peak current of 84 mM external Cl^-. (B) Single-channel amplitude of GCAC1 in the presence of 84, 6 and 0 mM chloride in the bath solution. Note that the outward and inward currents increase upon a rise in external chloride concentration. Thus inward currents increase inverse to the chemical gradient, indicating a regulatory anion-binding site on the extracellular surface of GCAC1.

whole-cell current is the result of an anion-induced increase in single-channel conductance. Whereas the presence of 84 and 6 mM chloride in the bath resulted in a mean channel amplitude at −40 mV of ~2.9 and 2.3 pA (34 and 29 pS) the absence of the anions did not allow to resolve the unit conductance (Figure 4B, right hand trace).

We thus postulate two sites on the extracellular surface of GCAC1: one when accessed by malate modulates the voltage sensor and another adapting its conductance to the current anion concentration. It is tempting to speculate that the inverse relation of GCAC1 to the chemical gradient of the anion allows anion efflux and thus stomatal closure to preceed even at increasing external anion levels.

How do guard cells sense the intercellular CO_2 concentration?

Upon an increase in intercellular CO_2, reflecting the inability of the photosynthetic apparatus to take advantage of excess CO_2, stomata close (Raschke, 1979). In C3 and C4 plants low CO_2 levels in the light or even in the dark cause stomatal opening. CAM plants, on the other hand, which accumulate CO_2 in the form of malate are

characterized by an inverse day–night cycle in stomatal aperture (Lüttge, 1988). These photosynthesis subtypes have been distinguished by the pattern of their assimilation products, where the malate content, transport and its cellular and subcellular compartmentation report on the metabolic level of each cell type (Lüttge, 1988).

External malate released from the photosynthetic tissue, guard cells or neighbouring cells would shift GCAC1 towards the resting potential of the guard cell. The increase in concentration of this metabolite in turn induces the release of anions through GCAC1, a fraction of which is malate (Van Kirk and Raschke, 1978). 'Endogenous malate' will thus activate GCAC1 to feed forward anion release.

Thus CO_2 sensitivity of guard cells may result from malate action on GCAC1. Therefore malate-sensitive anion channels may provide a mechanism to adapt stomatal aperture to the mode of CO_2 fixation and current photosynthetic capacity. Upon changes in the environmental conditions cross-talk between guard cells and mesophyll cells through the extracellular malate level should allow to control water-use efficiency.

Materials and methods

Protoplast isolation and patch–clamp recording

Guard cell protoplasts were enzymatically isolated from 2–3 week-old leaves of the broad bean *Vicia faba* (Hedrich *et al.*, 1990). Patch pipettes were sealed against 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 an EPC-7 or EPC-9 patch–clamp amplifier (List Electronic, Darmstadt and HEKA Lambrecht, FRG), 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.

Solutions

Solutions in Figures 1, 2, 3 and 4 were composed of 1 or 40 mM $CaCl_2$ or 1 mM $Ca(gluconate)_2$, ± 2 mM $MgCl_2$, 10 mM MES–TRIS pH 5.6 in the bathing medium and 150 mM TEACl (or KCl in Figures 1C, 2 and 4B), 2 mM $MgCl_2$, 10 mM MgATP and Na_2GTP, 0.1 mM EGTA, 10 mM HEPES–TRIS pH 7.2 in the pipette (cytoplasm).

Bath solution

After guard cell protoplasts were applied to the recording chamber and stick to its glass bottom the chamber was continuously perfused with the extracellular solution (≥ 0.13 ml/min). Upon changes in the solute composition of the medium the perfusion rate was increased to 1.3–3.8 ml/min until a new steady state has been established.

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Note added in proof

Since submitting this paper we have been able to demonstrate that (i) darkness and increase CO_2 concentrations increase the extracellular malate content and (ii) that elevated malate levels cause stomatal closure.