Anion Metabolism as Correlated with the Volume Changes of Guard Cell Protoplasts

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Isolated and purified guard cell protoplasts of starch-containing (*Vicia faba*) and starch-lacking (*Allium cepa*) stomata were used to study the metabolism of malate and Cl^- anions as correlated with the K^+-induced swelling of protoplasts. The correlation between swelling and malate synthesis in starch-containing and the inhibition of this correlation in starch-lacking guard cell protoplasts were demonstrated. A model system was proposed involving the role of malate and its place of synthesis in these two contrasting stomatal types.

Introduction

Stomatal opening is the result of solute accumulation in the guard cells. The uptake of K^+ by guard cells accounts for a major part of the solute build up leading to stomatal opening. Some of the K^+ uptake is balanced by external Cl^- anions. However, the malic acid in the guard cells plays a key role in stomatal functioning by compensating mainly the K^+ ions and by releasing the protons during the K^+ import (Allaway [1], Raschke and Humble [2]).

This proton-cation exchange which is associated with the synthesis and the subsequent accumulation of malic acid by dark CO_2-fixation via phosphoenolpyruvate carboxylase (Jacobson and Ordin [3], Jacobson [4], Ting and Dugger [5, 6], Hiatt [7], Hiatt and Hendricks [8], Cram [9], and Osmond [10]) is not only restricted to stomatal movements. The transport mechanism is also reported to be involved in K^+/H^+ exchange with the concomitant synthesis of malic acid during the elongation growth stimulated by indoleacetic acid (Haschke and Lüttge [11, 12]), in the ion uptake by roots (Torri and Laties [13]) and in the storage tissue (Osmond and Laties [14]).

The effect of environmental factors on malate accumulation in starch-containing and starch-lacking stomata of *Vicia faba* and *Allium cepa* during stomatal opening has been described (Raschke and Schnabl [15], Schnabl and Raschke [16]): In *Vicia* about 91% of the K^+ influx into the guard cells during stomatal opening was balanced by malate; import of Cl^- contributed 6%. In the presence of Cl^- anions in the external medium, 50% of the positive K^+-charge was provided by malate anions, and 45% by Cl^-. The stomatal opening was not influenced by the distribution of the anions (Raschke and Schnabl [15]).

In *Allium,* KCl was the major osmoticum during the stomatal opening; in its absence guard cells did not open. The malate appeared not to participate in stomatal ion transfer (Schnabl and Raschke [16], Schnabl and Ziegler [17]). – As the stomatal movements are correlated with the volume changes of guard cell protoplasts (Schnabl *et al.* [18]) the analysis of the anion metabolism in these protoplasts separated from the mesophyll and epidermal cells seemed to be reasonable. In this paper data are presented on the K^+-induced swelling of guard cell protoplasts in relation to the malate synthesis and the Cl^--influence on the formation of the organic anions. A sensitive method of malate determination by fluorometry was used which enables us to detect small differences of the anion concentrations in the guard cell protoplasts during the swelling procedure.

Materials and Methods

Plants

Small bulbs of *Allium cepa* L. (Stuttgarter Riesen) obtained in the previous year from seeds in the greenhouse at 23 ± 1 °C and seeds of *Vicia faba* L. (cv. weißkernige Hangdown) were germinated and grown in peat moss in a growth chamber at 22 ± 1 °C (day) and 17 ± 1 °C (night) with a 12 h photoperiod (15000 lx). *Allium* plants were watered twice weekly, once with KCl (5 mM; 100 ml per 1000 cm^3 soil). *Vicia* plants were watered three times weekly.

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Preparations of guard cell protoplasts

The isolation of guard cell protoplasts has been described earlier (Schnabl *et al.* [18]). The guard cell protoplasts were purified by resuspending them in 5 ml of 0.6 M sucrose containing 1 mM $CaCl_2$. On top of the suspension 1 ml of 0.6 M mannitol, 1 mM $CaCl_2$, 5 mM MES-KOH (pH 6.0) was layered. After 5 min at $600 \times g$, the protoplasts banded at the interphase between the uppermost two layers, were collected carefully and stored in ice. The diameters of 30 guard cell protoplasts per experiment were measured microscopically.

Malate assay

Guard cell protoplasts were incubated in K^+-iminodiacetate (KIDA, 10 mM in 0.6 M mannitol, 1 mM $CaCl_2$), a non permeable zwitterion (Raschke and Humble [2]), in KCl (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$) and for the control experiment only in mannitol (0.6 M, 1 mM $CaCl_2$) at light conditions (15000 lx) with CO_2 free air at room temperature. For accomplishing the time courses of the volume changes and malate contents of guard cell protoplasts (after 5, 10, 15, 20 min) the samples were taken simultaneously from the same assay.

For malate determination aliquots were added to boiling 80% alkaline ethanol for 20 min and centrifuged. The supernatant was evaporated to dryness in a stream of air at room temperature. The residue was resuspended in 1 ml 0.2 M hydrazine buffer (pH 9.0), and the malate content was determined by fluorometry of NADH arising from an enzymatic oxidation of malate (Goldberg and Passonneau [19]). Details of the procedure have been given previously (Van Kirk and Raschke [20]).

The protein content was measured with xylene-brilliant cyanin G after Bramhall *et al.* [21].

Results

The starch-containing system

The time courses of experiments presented in Fig. 1a show that the swelling of *Vicia* guard cell protoplasts can be induced by addition of KCl or KIDA (10 mM) in the external medium. This effect is also observed in the presence of K^+-solutions lower than 10 mM, up to 1 mM.

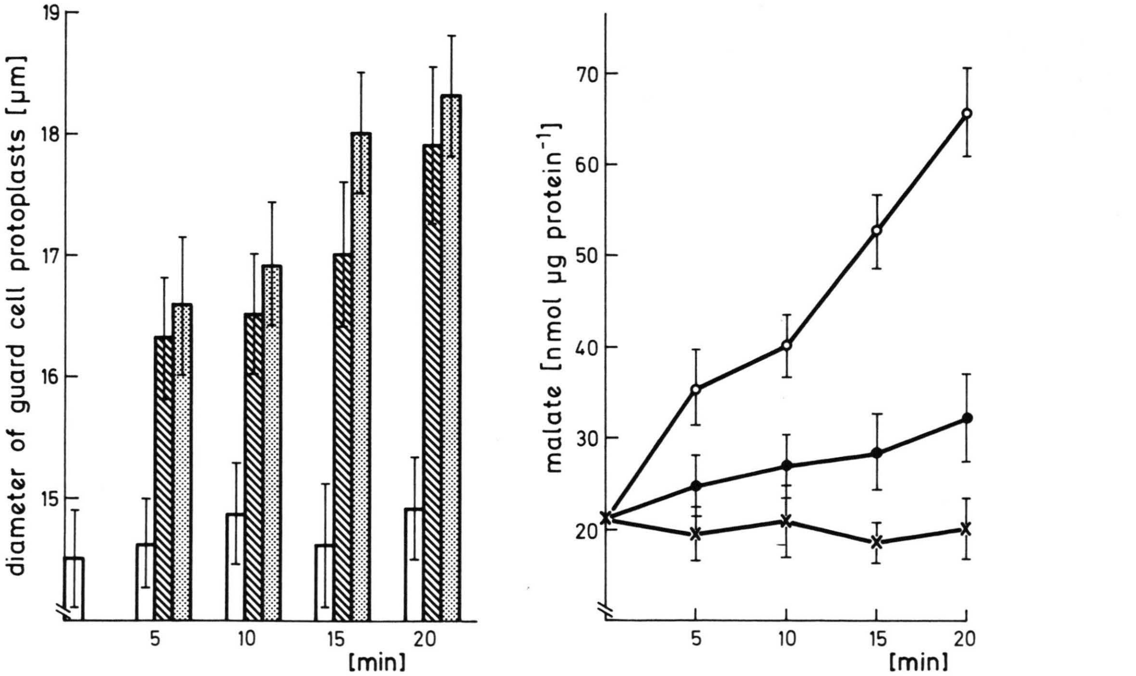

Fig. 1. Swelling of guard cell protoplasts (*Vicia faba*) as correlated with the malate content. 1a: Time course of the increasing diameters of guard cell protoplasts. ☐ Control (0.6 M mannitol, 1 mM $CaCl_2$); ▨ KIDA (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$); ▒ KCl (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$). 1b: Time course of malate content determined in the swelling guard cell protoplasts. ×—× Control (0.6 M mannitol, 1 mM $CaCl_2$); ○—○ KIDA (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$); ●—● KCl (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$).

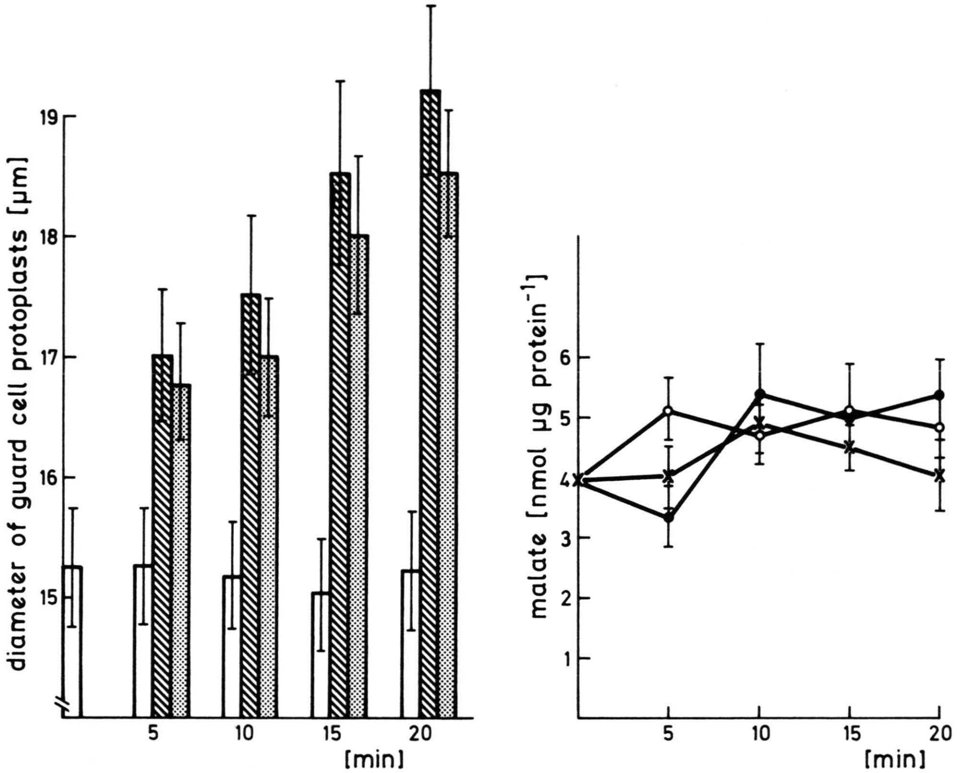

Fig. 2. Swelling of guard cell protoplasts (*Allium cepa*) (plants were watered once with KCl 5 mM; 100 ml per 1000 cm^3 soil) not correlated with the malate content. 2a: Time course of the increasing diameters of guard cell protoplasts. □ Control (0.6 M mannitol, 1 mM $CaCl_2$); ▨ KIDA (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$); ▒ KCl (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$). 2b: Time course of malate content determined in the swelling guard cell protoplasts. ×—× Control (0.6 M mannitol, 1 mM $CaCl_2$); ○—○ KIDA (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$); ●—● KCl (10 mM in 0.6 M mannitol, 1 mM $CaCl_2$).

The guard cell protoplasts of *Vicia faba* doubled their volume of 1600 µm^3 in the mannitol medium (= control value) to about 3200 µm^3 within 20 min after adding K^+ ions. The results are calculated on the basis of the microscopically measured diameters of 30 protoplasts per measurement with standard errors. No significant differences are indicated in the volumes of protoplasts obtained with KIDA or with KCl.

The close correlation between the swelling of protoplasts and the malate synthesis is demonstrated by Fig. 1b showing that K^+ not only enhances the swelling of the protoplasts but also concomitantly increases malate accumulation. The malate concentrations of the protoplasts incubated in KIDA for 20 min are strongly increased from 20 nmol µg protein^{-1} to 65 nmol µg protein^{-1}. However, this stimulation of malate synthesis in protoplasts by KIDA is not obtained by KCl: the presence of Cl^- induces only less than a doubling of the malate formation (from 20 to 35 nmol µg protein^{-1}).

The starch-lacking system

In the same way, the swelling of *Allium* guard cell protoplasts (the plants were watered once with KCl) is induced by KIDA and KCl without any change of the controls (Fig. 2a).

However, the correlation between the malate accumulation and the volume changes of protoplasts is inhibited in protoplasts of *Allium* (Fig. 2b). The malate content is levelled out to about 4–5 nmol µg protein^{-1} in *Allium* protoplasts incubated in mannitol, KIDA or KCl-solution.

In contrast, the malate content in these protoplasts is reduced to $^1/_4$ to $^1/_5$ of that measured in *Vicia* protoplasts under control conditions (*Allium* guard cell protoplasts: about 4 to 5 nmol malate µg protein^{-1}; *Vicia* guard cell protoplasts: about 20 nmol malate µg protein^{-1}).

Discussion

The swelling of guard cell protoplasts in the starch-containing and the starch-lacking system is both induced by K$^+$ ions. However, the *Allium* plants have to be watered with an adequate Cl$^-$-supply.

In a previous report using intact starch-containing *Vicia* stomata (Raschke and Schnabl [15]) the kind of anions important for K$^+$ balancing in the incubation medium (KIDA or KCl) seems to play no decisive role for stomatal opening. This is also confirmed by the experiments with *Vicia* protoplasts swelling to the same extent both in KIDA and in KCl.

After the exposure of *Vicia* protoplasts in KIDA solution K$^+$ ions imported into the protoplasts appear to be compensated completely by malate (65 nmol µg protein^{-1}) being provided presumably via glycolysis from starch to phosphoenolpyruvate and by phosphoenolpyruvate carboxylase to malate (Schnabl [22], Outlaw *et al.* [23]). After adding KCl, the malate synthesis in the guard cell protoplasts is reduced to about 50% (32 nmol µg protein^{-1}), thus the remainder of the positive charge seems to be taken by Cl$^-$ anions. The data are in agreement with those obtained with intact stomata by electron microanalysis (Raschke and Schnabl [15]). The correlation between the increase of the volumes of the guard cell protoplasts and the malate content both after KIDA and KCl-treatment supports the idea of the requirement of starch within the guard cells to produce malate anions for compensating K$^+$ (Raschke and Schnabl [15]; Schnabl [22]).

There is evidence that the guard cells, isolated and deprived of their source of malate from the mesophyll and epidermal tissue are able to produce sufficient amounts of malate by themselves from the reservoir of starch accumulated in the chloroplasts. This is confirmed also by Travis and Mansfield [24]. Therefore, the stomata are suggested to have the ability to synthesize and to remove malate as part of a self-contained mechanism.

The synthesis of malic acid plays an important role in contributing to the balance of excess cation over anion uptake (Allaway [1] and Raschke and Humble [2], Osmond [10]). The movement of ions produces changes in the acid-base-balance. However, the pH in the cytoplasm has to be maintained at a constant value. By a balance between the carboxylation and the decarboxylation of malic acid this control can be achieved. Thus, the malic acid synthesis is based on a general mechanism of homeostatic pH control proposed by Davies [25], Raven and Smith [26] and Smith Raven [27].

The basis of the multiple role of malate in this mechanism is suggested to be effective in all plant systems providing (1) protons for H$^+$/K$^+$ exchange (2) anions for balancing of the positive charges taken

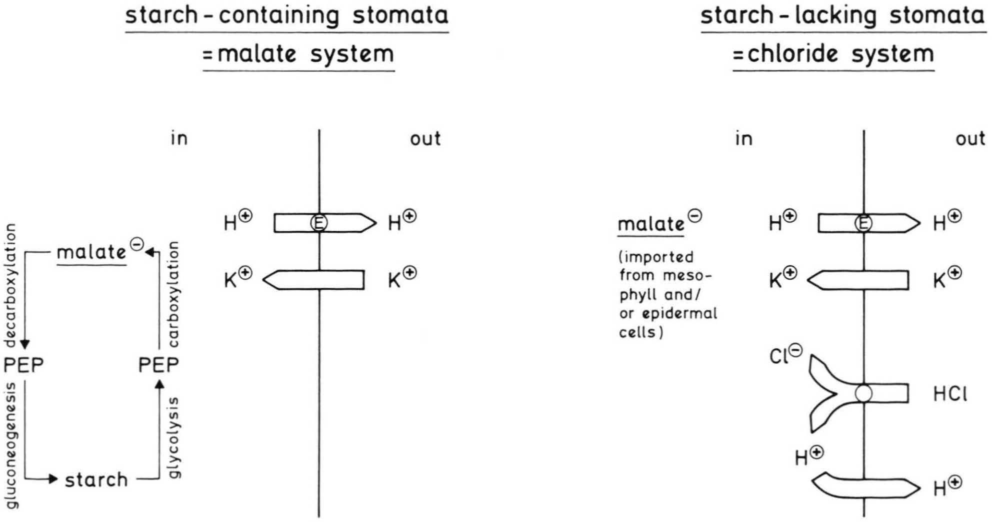

Fig. 3. A model system involving the role of malate and the place of its synthesis in the two contrasting stomatal types.

up and (3) a stabilization of the intracellular pH during the H^+ extrusion. The involvement of a H^+ extruding mechanism coupled with an enhanced malate synthesis on the basis of H^+/K^+ exchange is described in auxin-induced elongation growth of coleoptile segments of *Avena* (Haschke and Lüttge [11, 12]), in roots (Torri and Laties [13], Hiatt [7]) and in storage tissues (Osmond and Laties [14]). There is also evidence that the phytotoxin fusicoccin produces similar effects (Marrè *et al.* [28]). Fusicoccin and indoleacetic acid seem to act on the system which catalyzes the H^+ extrusion coupled to the uptake of K^+, thje consequential rise of pH activates the phosphoenolpyruvate carboxylase, thus leading to an increase of malate.

This mechanism of compensating the intracellular pH plays also a key role during the stomatal movement. The synthesis and the degradation of malic acid in the starch-containing system of guard cells as a response to K^+ induced H^+ extrusion is demonstrated in a scheme (Fig. 3) showing a reversible malate-starch-balance as correlated with the volume changes of guard cell protoplasts. The degradation of malate via decarboxylation and the subsequent gluconeogenesis and the reversible formation of malate via glycolysis and carboxylation has been described (Schnabl [22] and [29]). The mechanism proposed in starch-containing system involves the malate as an anion generated from starch and transformed to starch within the guard cells during stomatal movement.

In contrast, in the starch-lacking system the role of malic acid and its place of synthesis seem to be quite different and should be summarized as follows:

Without any Cl^- watering of the *Allium* plants the swelling of their guard cell protoplasts by K^+ ions is inhibited (unpublished results). The absence of starch from guard cells of *Allium* deprived them of the ability to produce malate, thus the supply of external given KCl being necessary for stomatal opening (Schnabl and Ziegler [17] and Schnabl and Raschke [16]). For providing the guard cells with sufficient Cl^- anions the *Allium* plants were treated once with KCl. As a result of the Cl^- treatment, in the starch-lacking system the swelling of guard cell protoplasts was stimulated by K^+ ions. It has been described by Schnabl [30] that the Cl^- supply should not exceed a definite limit otherwise the stomatal sensitivity is inhibited.

In contrast to the starch-containing system, there was no correlation between the swelling of *Allium* guard cell protoplasts treated with Cl^- and malate synthesis supporting the hypothesis of Schnabl and Ziegler [17] and Schnabl and Raschke [16] that malate does not participate in stomatal ion transfer. Assuming it is not involved in stomatal movement the role of malate which is accumulated in the guard cells of *Allium* to a small degree ($^1/_4$ to $^1/_5$ of the amount found in *Vicia*, Figs. 1b, 2b) is an open question. It is proposed by Schnabl [30] that the presence of the organic anion should be effective as an initial proton-primer delivering H^+ as a substrate for the proton-pump, thus maintaining H^+/K^+ exchange (Fig. 3).

As mentioned above, the guard cells lost their ability for generating malate by themselves due to the absence of starch. However, evidence exists for the presence of minimal amounts of malate in the guard cell protoplasts of *Allium* (4 to 5 nmol µg protein^{-1}) confirming also the results done with intact stomata (Schnabl and Raschke [16]). As a consequence, it is suggested that the malate is imported into the guard cells either from the epidermal cells which are able to fix CO_2 (Schnabl [31]) or from the underlying photosynthesizing mesophyll cells.

Summarizing, malate is hypothesized not to be participated in stomatal ion transfer due to its import from the surrounding cells metabolically active. Therefore, in Fig. 3 malate is involved in stomatal mechanism of the starch-lacking system as a proton-primer necessary for providing H^+ and for stimulating the proton-pump.

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