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STIMULATION AND INHIBITION BY BICARBONATE OF STOMATAL OPENING IN EPIDERMAL STRIPS OF *COMMELINA BENGHALENSIS*

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SUMMARY

The effect of 0 to 100 μM bicarbonate on stomatal opening in epidermal strips of *Commelina benghalensis* was examined in the presence or absence of fusicoccin in light or darkness. Low concentrations of bicarbonate (up to 10 μM in the absence and 25 μM in presence of fusicoccin) stimulated stomatal opening while higher concentrations inhibited. The enhancement of opening by low concentrations of bicarbonate and phosphoenol pyruvate (PEP), and prevention of bicarbonate stimulation by malate or oxaloacetate suggested PEP carboxylase as a CO_2 sensor in the guard cells. However, the inhibition of PEP carboxylase did not completely suppress the opening caused by fusicoccin. The action of fusicoccin therefore appears to involve a site other than CO_2 fixation, presumably through the stimulation of proton excretion.

INTRODUCTION

Stomatal movements are very sensitive to ambient CO_2 concentration (Meidner and Mansfield, 1968; Raschke, 1975; Allaway and Milthorpe, 1976) but the mechanism of CO_2 response by guard cells is not yet understood clearly. Epidermal tissues fix $^{14}CO_2$ predominantly into malate and aspartate through the β-carboxylation of phosphoenol pyruvate (PEP) (Pearson and Milthorpe, 1974; Willmer and Dittrich, 1974) and contain high amounts of PEP carboxylase (Willmer, Pallas and Black, 1973; Das and Raghavendra, 1974; Milthorpe, Pearson and Thrower, 1974). The kinetics of the stomatal response to CO_2 suggested the involvement of PEP carboxylase (Raschke, 1972). However, it has been difficult to reconcile the observations on malate formation with the closing response to CO_2. A positive correlation is found between the malate levels in guard cells and stomatal opening (Allaway, 1973; Van Kirk and Raschke, 1978). Although the malate levels in guard cells increase as stomata open (Allaway, 1973; Travis and Mansfield, 1977), an increase in ambient CO_2 level from 0 to 350 μl l^{-1} causes stomatal closure, over which range malate formation from PEP carboxylation should markedly increase (cf. Travis and Mansfield, 1979).

Here we report our observations on stomatal opening at varying levels of bicarbonate in the presence or absence of fusicoccin. We found that low concentrations of bicarbonate (HCO_3^-/CO_2) could, in fact, stimulate opening while higher concentrations inhibited. The characteristics of bicarbonate-stimulated opening suggested the involvement of PEP carboxylase as a CO_2/HCO_3^- sensor.

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MATERIALS AND METHODS

The preparation of epidermal strips from leaves of *Commelina benghalensis* and the measurement of stomatal aperture have been described before (Das *et al.*, 1976; Raghavendra, Rao and Das, 1976). All the reagents were freshly prepared every day with boiled and cooled distilled water. The basal medium contained 50 mM sodium phosphate buffer (pH 7·0) with 50 mM KCl and 0·5 mM $CaSO_4$. Requisite amounts of $NaHCO_3$ were added to the media prior to each experiment so as to provide the described final concentrations. The experiments were performed in 8 cm diameter Petri dishes. After placing the epidermal strips in the medium, CO_2-free air was passed over them before the Petri dishes were closed.

The light source consisted of a bank of incandescent bulbs providing a light intensity of 10 klx and the temperature during experimentation in light or darkness was 28 ± 2 °C. Stomatal aperture was measured with a precalibrated occular micrometer and the values presented are the averages of three readings of 30 stomata each in randomly selected microscopic fields.

The 'bottoms' of Petri dishes were always kept covered with their corresponding 'tops' so as to minimize the contact with the atmospheric air. In most of the experiments the strips were examined quickly at the end of 3-h incubation period. However, for periodical examination of stomatal movement, replicate Petri dishes were maintained and the epidermal strips from one of them were sampled every 30 min, thus avoiding the opening of the Petri dish during incubation. In some of the experiments the strips were transferred from CO_2/HCO_3^--free medium to a HCO_3^--containing medium and vice versa. Care was taken to flush the Petri dish with CO_2-free air before closing it.

RESULTS

In the absence of fusicoccin, bicarbonate up to 6 μM stimulated stomatal opening (Fig. 1), while in the presence of fusicoccin the opening was stimulated by levels of bicarbonate up to 25 μM (Fig. 2). Higher concentrations of bicarbonate restricted stomatal opening. The enhancement by bicarbonate was noticed in both light and darkness although the opening was greater in light than in darkness (Table 1). PEP, but not ribulose-bisphosphate (RuBP), enhanced further

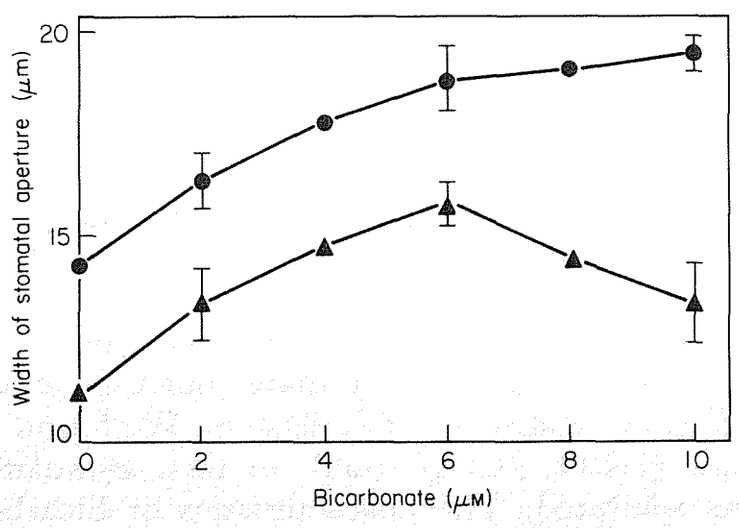

Fig. 1. The response of stomatal opening to low concentrations of bicarbonate (2 to 10 μm) in the presence (●) or absence (▲) of fusicoccin. The values represent the width of stomatal aperture after incubation in light for 3 h. The initial opening was 3·5 μm.

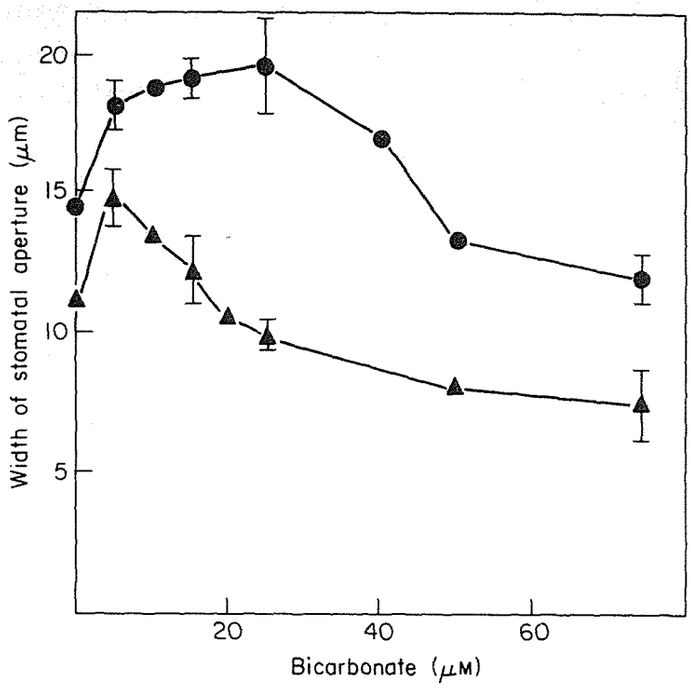

Fig. 2. The degree of stomatal opening at varying concentrations of bicarbonate (5 to 75 μm) in presence (●) or absence (▲) of fusicoccin. The values represent the width of stomatal aperture after incubation in light for 3 h. The width of stomatal aperture before incubation was 4·7 μm.

Table 1. *Stomatal responses to bicarbonate in the presence or absence of fusicoccin in light or darkness*

Treatment	Light	Darkness
Control (zero fusicoccin)		
zero HCO_3^-	12·6 ± 2·8	8·8 ± 1·5
6 μM HCO_3^-	14·2 ± 1·9	10·1 ± 1·3
25 μM HCO_3^-	11·0 ± 2·1	8·0 ± 1·3
Fusicoccin (+10 μM)		
zero HCO_3^-	15·3 ± 1·4	13·6 ± 1·0
6 μM HCO_3^-	18·7 ± 2·6	15·4 ± 1·7
25 μM HCO_3^-	19·2 ± 2·9	15·8 ± 1·2

The values represent the stomatal apertures (± s.e.) after an incubation of 3 h. The initial stomatal opening was 5·5 μm.

Table 2. *Bicarbonate stimulation of stomatal opening in relation to fusicoccin, PEP and RuBP*

Treatment	Bicarbonate (μM)		
	0	6	25
Control	11·8 ± 2·5	14·5 ± 2·8	9·5 ± 1·2
+1 mM PEP	13·5 ± 1·1	17·2 ± 2·6	10·4 ± 1·2
+1 mM RuBP	11·6 ± 3·2	13·6 ± 1·8	9·8 ± 1·6
+10 μM fusicoccin	14·6 ± 1·8	19·3 ± 2·5	20·0 ± 4·1
+10 μM fusicoccin + 1 mM PEP	17·5 ± 2·8	22·9 ± 2·1	23·8 ± 4·2
+10 μM fusicoccin + 1 mM RuBP	14·2 ± 1·9	19·8 ± 1·7	20·6 ± 31·5

The data represent the stomatal apertures (μm ± s.e.) after an incubation of 3 h in light. The initial opening was 3·5 μm.

Table 3. *Sensitivity of bicarbonate-stimulated stomatal opening to organic acids*

Treatment	HCO_3 (μM) (zero fusiccocin)			Fusicoccin (10 μM) + HCO_3 (μM)		
	0	6	25	0	6	25
Control	10·9 ± 1·7	14·2 ± 2·1	9·6 ± 1·8	15·5 ± 3·2	19·2 ± 3·0	20·3 ± 2·8
+ Oxaloacetate	1·6 ± 0·4	1·5 ± 0·7	2·2 ± 0·1	8·6 ± 0·9	10·3 ± 1·2	11·7 ± 1·7
+ Malate	2·2 ± 0·5	0·9 ± 0·3	1·8 ± 0·4	9·4 ± 0·9	11·6 ± 1·5	10·5 ± 2·2
+ Succinate	12·2 ± 1·2	15·8 ± 2·1	9·8 ± 0·6	16·6 ± 1·8	19·5 ± 2·5	20·2 ± 3·4
+ Oxoglutarate	12·6 ± 1·6	15·5 ± 1·2	10·2 ± 1·1	17·0 ± 2·1	20·1 ± 2·8	20·5 ± 1·9

The values represent the stomatal apertures (± s.e.) after an incubation in light for 3 h. The initial opening was 4·2 μm. All the organic acids tested were sodium salts, and the concentration in the incubation medium was 5 mM.

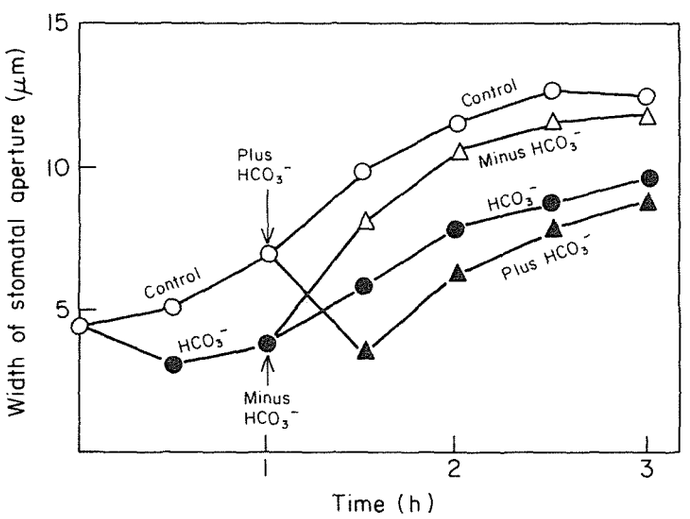

Fig. 3. The course of stomatal opening in the presence (●) or absence (○) of 100 μM bicarbonate. After 1 h (indicated by arrow) the strips were transferred from bicarbonate containing medium to that without bicarbonate (△) or vice versa (▲).

bicarbonate-stimulated stomatal opening either in presence or absence of fusicoccin (Table 2). The stimulation by bicarbonate of stomatal opening was suppressed by malate or oxalacetate, inhibitors of PEP carboxylase (cf. Raghavendra and Das, 1977), but succinate or oxoglutarate had no affect (Table 3).

Stomatal opening was partially suppressed by 100 μM bicarbonate (Fig. 3), and when bicarbonate was removed from the medium, opening returned to the control level. When bicarbonate was added to the medium the stomata closed immediately and thereafter exhibited a slow recovery.

DISCUSSION

The effect of externally added 100 μM HCO_3^- simulated the influence of CO_2 on stomata in intact leaves, because its addition either in light or darkness caused rapid closure, and normal opening was restored upon its removal (Fig. 3). The pattern of these changes was similar to those observed with intact leaves and gaseous CO_2. This experiment confirms that epidermal strips can be as effective as the intact system for studies of stomatal responses to carbon dioxide, as indicated earlier by Willmer and Mansfield (1969).

Since our experiments were conducted at pH 7·0, the optimal level of bicarbonate

to cause stomatal opening (6 μM) corresponds to about 1·5 μM CO_2, which is equivalent to 50 p.p.m. CO_2. Raschke (1977) reported that stomata open over the range 0 to 200 μl l^{-1} and then close as the concentration is further increased, which is similar to our observations.

The two carboxylating enzymes known to occur in higher plants are RuBP and PEP carboxylase. Three points from the data of our experiments suggest the involvement of PEP carboxylase in the CO_2 response of stomata.

(i) The range of optimal concentration of HCO_3^-/CO_2 for stimulating stomatal opening, 5 to 10 μM in the absence and 10 to 20 μM in the presence of fusicoccin (Fig. 2) falls near the K_m for HCO_3^- of PEP carboxylase.

(ii) PEP, but not RuBP, further stimulates the bicarbonate promoted stomatal opening (Table 2).

(iii) Malate and oxaloacetate, inhibitors of PEP carboxylase, prevent bicarbonate stimulation of stomatal opening (Table 3). Two other organic acids of the tricarboxylic acid cycle, succinate and oxoglutarate, are ineffective.

The stimulation by bicarbonate at concentrations lower than 25 μM in the presence of fusicoccin is similar to the observations of Travis and Mansfield (1979). The inhibition by higher bicarbonate concentrations (Fig. 2) indicates that fusicoccin extends the optimal limit of bicarbonate. However, the failure of PEP-carboxylase inhibitors to suppress stomatal opening (Table 3) reveals that fusicoccin acts at another site apart from that of CO_2 fixation. For example, fusicoccin stimulates proton excretion (Marré, 1977; Nelles, 1978), and during stomatal opening H^+ are released to be exchanged for K^+ (Raschke and Humble, 1973).

Bicarbonate ions could also alter the membrane permeability, thereby affecting stomatal movements. Glinka and Reinhold (1962) found that an exposure to CO_2 altered the permeability of cell membranes, but the concentrations they used were several fold higher than in the present work. The formation of malate, on the one hand to facilitate K^+ uptake and, on the other, the leakage of K^+ at elevated CO_2 levels through an increased permeability, could perhaps explain the dual role of CO_2 causing both opening or closure (Travis and Mansfield, 1979). Bicarbonate concentrations above 50 μM may exert a feed back inhibition of PEP carboxylase because of the malate accumulation (Kluge, 1976).

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