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The Effects of Light and CO_2 on the Changes of Electrical Potential Difference in Isolated Epidermis and Intact Leaves of *Commeina communis* L

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Abstract - The effects of light and CO_2 on the electrophysiological characteristics of guard cells in the intact leaf and isolated epidermis have been investigated. Fast hyperpolarization of guard cell apoplastic PD in the intact leaf was recorded reaching up to around 7 mV and 20 mV in response to light and CO_2. Whenever the experiments were attempted with isolated epidermis, there was no response to light and CO_2. In order to determine the influence of the mesophyll cells, the apoplastic PD of guard cells in isolated epidermis was measured in the presence of the mesophyll supernatant or the control medium. The apoplastic PD in isolated epidermis was hyperpolarized to −7 mV, changing from −22 mV to −29 mV at 40 min. But, when isolated epidermis was incubated with the supernatant from mesophyll cells incubated in the light, the apoplastic PD in isolated epidermis was hyperpolarized to −19 mV, changing from −22 mV to −40.5 mV. CO_2 also caused a change of 0.1 to 0.3 pH unit in the intact leaf. However, this change was absent in isolated epidermis. A vibrating probe was used to detect the change in electrical currents at the surface of excised intact leaves and isolated epidermis. The reading of excised intact leaves in the dark was 0.5 μA cm^{-2}, remaining steady until illuminated. Light increased the current on the surface of excised leaves to about 0.8 μA cm^{-2}. However, light had no effect in the current on the surface of isolated epidermis. Apoplastic pH changes across the stomatal complex in response to light and dark were measured both in the intact leaves and isolated epidermis over the same time period using pH micro-electrodes. The guard cell wall of intact leaf was acidified to 2.5 pH unit, falling from pH 7.5 to pH 5.0 in the first 10 min. in the light. At the same time the guard cell wall pH of isolated epidermis fell from pH 7.5 to pH 7.0 at 10 min. The guard cell wall pH of isolated epidermis incubated in the mesophyll supernatant fell from pH 7.6 to pH 6.7 at 10 min. Likewise, It could be imagined that an electrical signal, chemicals and hormones propagated from the mesophyll in response to light and CO_2 could control a fast stomatal response.

Key words : apoplastic potential difference, apoplastic pH, mesophyll cells, stomata

INTRODUCTION

Environmental factors such as light and low CO_2 concentrations trigger events which may result in stomatal opening. Stomatal aperture is controlled by the solute content of guard cells, largely, but not exclusively, by their content of potassium salts, with K^+ balanced either by Cl^- or malate, depending on the species and conditions. A number of physiological data have demonstrated that proton efflux originating in guard cells precedes stomatal opening, suggesting that proton efflux is a necessary precursor of stomatal opening (Raschke and Humble 1973; Gepstein *et al.* 1982; Edwards *et al.* 1988). Therefore, when stomata open, protons are first pumped out of the guard cell, resulting in a potential gradient across the plasmalemma. This

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gradient stimulate stomatal opening of inward K^+ channels which may allow K^+ influx to guard cells resulting in an increase in water potential. This is the theory which is now widely accepted to explain stomatal opening by light. However, how these environmental signals are sensed and how they are transduced into driving the ion fluxes which control stomatal movements are not still fully understood.

There is a possibility of an indirect effect of light on stomata which could be mediated from the mesophyll cells. Gunar *et al.* (1975) found that a fairly rapid polarization of the potential difference (PD) by 10~15 mV was observed upon the switching on of the light in the intact leaves of *Tradescantia albiflora*. They suggested that since epidermal and subsidiary cells do not have chlorophyll, changes in their PD induced by light could be associated with electrical excitation propagated from the mesophyll cells. Cheesman *et al.* (1982) also found that membrane potentials in isolated strips were considerably lower than those in intact sections and were insensitive to light. In addition, there are a number of reports suggesting that photoinduced ion fluxes in green plant tissue are probably associated with pumps regulated by photosynthesis (Jeschke 1970; Luttge 1973; Higinbotham 1973), photosynthetic electron transport (Hartman 1975; Lawson *et al.* 2002) and cyclic-phosphorylation (Spanswick 1973). The above results evoke the question that membrane hyperpolarization of guard cells in respond to light might be related to mesophyll cells. Moreover, a slow wave of depolarization has been reported following localized wounding by heating or burning, in many species, including *Tradescantia* (Pfirsch 1966), *Lupinus* (Paszewski and Zawadzki 1976), *Gossypium, Cucurbita, Xantium* (Van Sambeek and Pickard 1976), *Vicia, Mimosa* (Roblin 1985; Roblin and Bonnemaim 1985), and *Lycopersicon* (Van Sambeek and Pickard 1976; Roblin 1985; Wildon *et al.* 1989). Roblin (1985) stated that "the slow wave appears general in herbaceous plants". Similar slow waves of apoplastic depolarization may follow other types of wounding, including pricking of the midrib in leaves or petioles of *Bidens pilosus* (Frachisse *et al.* 1985) and crushing or squeezing of leaf or petiole in various species (Pickard 1973; Van Sambeek and Pickard 1976; Wildon *et al.* 1989). Research over many years has established that a signal is transmitting from the wounded region with an apparent velocity of about 1~6 mm s^{-1} and that the signal, when received by metabolically active tissue, causes rapid depolarization of the extracellular potential as measured with surface contact electrodes or with noble-metal wires inserted into apoplast (Pickard 1973; Van Sambeek and Pickard 1976; Roblin and Bonnemaim 1985; Frachisse and Desbiez 1989). The transmitted signal is termed "Ricca's factor" after its proposer (Ricca 1916). Pickard (1973) suggested that Ricca's factor may represent a hormone or a group of hormones of widespread distribution in higher plants which coordinate aspects of the plant response to breakage, abrasion and perhaps water deficit. Malone and Stankovic (1991) suggested that arrival of the wave alters leaf water potential and thereby induces stomatal activity.

However, no attempts to investigate how the mesophyll can contribute to guard cell apoplastic potential difference, pH and electrical currents have been made. Therefore, this study was carried out to investigate the influence of the mesophyll on guard cell apoplastic potential difference, pH and electrical currents in response to light or CO_2.

MATERIALS AND METHOD

1. Plant materials

Commelina communis was potted in John Innes No. 2 compost supplemented with phostrogen and watered every morning. They were grown in a glass house (minimum temperature of 20°C during the day and 15°C at night) under a light regime of 18 hours day and 6 hours night (natural daylight supplemented by high pressure sodium lighting (150 μmol m^{-2} s^{-1}).

2. Isolation of mesophyll cells

Mesophyll cells were isolated according to the method based on Paul and Bassham (1977). Plants were selected for leaf materials in the early stage of maturation (3~4 weeks old). The abaxial epidermis of the leaf was removed and the remainder of the leaf cut into small sections with a razor blade. The samples were vacuum-infiltrated with 20 mL digestion medium for 1 min. (500 mm Hg). The vacuum was released slowly to prevent damage to cell untrastructure. The digestion medium contained the following, pectolyase (0.4%, Seishin Pharm., Ltds, Tokyo, Japan), sorbitol (350 mM), BSA (0.1%), succinic acid (20 mM, pH 5.7), KNO_3 (1 mM), KH_2PO_4 (0.5 mM), $MgSO_4$ (0.5 mM), and EDTA (1

mM). Incubation in the digestion medium was carried out on a rotary shaker (80 rotations/min.) for 90 min. The cells were filtered through a 100 μm nylon screen, collected by centrifugation at 100 g (1 min.) and washed three times with suspension medium. The cell pellet was finally suspended in suspension medium to a chlorophyll concentration of 380～420 μg mL^{-1} and used for experiments. All isolated mesophyll cells possessed an intact tonoplast and peripherally arranged chloroplasts. The light intensity used for incubation of mesophyll cells was 550 μmole m^{-2} s^{-1}. Suspension medium contained, 50 mM HEPES, 10 mM NaCl, 70 mM KCl, 2 mM EDTA, 2 mM $MgSO_4$ and the pH was adjusted to 7.3 with KOH.

To obtain the mesophyll supernatant, batches of isolated mesophyll cells were incubated with suspension medium either in the light or dark for a hour which was aerated with normal air. Then the cells were removed by centrifugation at 100 g for 5 min. The resulting supernatant was completely free of cells and showed no absorption bands characteristic of chlorophyll.

3. Apoplastic PD measurements

The measurements were conducted on the abaxial surface of the leaves of *Commelina*. Four or five week-old fully expanded leaves were excised early in the morning and floated on distilled water in the dark for 1 hour to ensure that the stomata were tightly closed. After 1 h, the leaf was put, abaxial side uppermost, on a slide which was placed in a chamber with two holes provided for air stream. The slide was inclined at 30° to facilitate micro-electrode implantations. The cut end of the leaf was dipped in KCl (10 mM). The chamber was installed on the stage of a stereomicroscope and the leaf surface was viewed at a magnification of ×100 with the microscope lamp giving 100 μmol m^{-2} s^{-1}. Light intensity used in the measurements was 550 μmol m^{-2} s^{-1}, provided by a Lux 150 4-port Projector. When a steady reading for the pH had been obtained, CO_2 was given to the leaf. A stream of CO_2 obtained from a cylinder (pure CO_2) passed into the box chamber via air flow meter.

Micro-electrode for PD measurements were pulled from glass capillaries (2.0 mm outside, 1.6 mm inside diameter with internal filament, Clark Electromedical Instruments, Pangbourne, Berks, U.K.) filled with 3 M KCl and connected by way of a Ag/AgCl half cell to a voltage follower and chart recorder. The circuit was completed by a Ag/AgCl reference electrode dipping into the bathing solution. Micro-electrodes were inserted into the cells using a micromanipulator (Research Instruments TVC 300). In some experiments, apoplastic PD of guard cells of detached epidermis in response to light was measured. Small pieces of strip, about 1.0 cm^2, was held in 5.0 cm^3 of the medium (10 mM KCl) by a fixed plastic ring in a Petri-dish so that the strips was just submerged. The PD and pH was measured under an Olympus CK2 inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at a magnification of × 200.

4. The measurements of electrical currents on the surface of intact leaf and isolated epidermis

Electrical currents at leaf surface were measured using a vibrating probe (Model NJ 806, The Vibrating Probe Co., Davis, California, U.S.A.). It was mounted on an XYZ micromanipulator (Line Tool Co., New York, U.S.A.) on a vibrating free bench. The probe was calibrated using a constant current source of 15 μA cm^{-2} in 10 mM MES-KOH (pH 6.15, 100 mM KCl, resistivity 540 Ωcm^{-2} using a vibrating amplitude of 30 cm apart (Fig. 1). Thus;

$$\text{current density} = \frac{2.83\ V}{3 \times 10^{-3} \times 540}\ A\ cm^{-2}$$

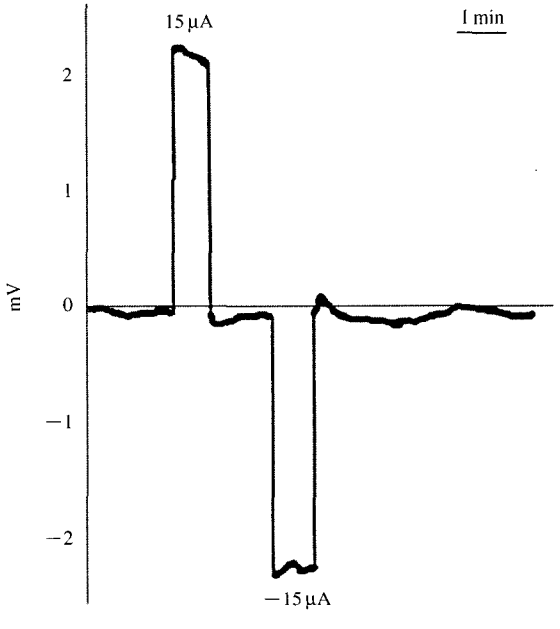

Fig. 1. The electrical current calibration curve.

Leaves of *Commelina communis* grown in a glass house were used for the measurements. Before measurements of currents, leaves were kept in the dark for 1 hour. After 1 h., both isolated epidermis and whole leaves were rolled and then fixed to the base of an empty plastic Petri-dish with 'Silflex' elastomer, the abaxial side of the leaf being upward, and then the dish was filled with 10 mM MES-KOH buffer (pH 6.15) with 100 mM KCl. The dish was then mounted under a stereomicroscope and the leaf surface viewed at 25 x magnification. The vibrating probe was lowered into the solution until it was 30~40 μm above the sample surface. The currents were recorded on a chart recorder. When a steady reading for the currents had been obtained, the leaf surface was illuminated (550 μmol m^{-2} s^{-1}) by a lux 150 4-port projector. Cells were subjected to successive short light or dark cycles.

5. Micro-electrode determination of apoplastic pH

Direct measurement of apoplastic pH was carried out using pH sensitive micro-electrodes. pH sensitive micro-electrodes were made by the same method used by Bowling (1989). Micro-electrode resistance was typically 0.4~0.5 gigaohms. The calibration of the pH sensitive micro-electrode was carried out with standard pH buffer solutions (pH 4, 5, 6, 7 and 9.2). The slope of the calibration curve was always greater than 50 mV per pH unit (Fig. 2). Reference electrodes were made by pulling micropipepttes as before and filling with 3 M KCl. The pH and reference micro-electrodes were connected via Ag/AgCl half cells to a high impedance electrometer (437 Electrometer, Pitman Instruments, Weybridge, England). The samples of intact leaf for pH measurements were prepared using the same method described for PD experiments. The pH was measured by lowering both micro-electrodes on to the same cell as close together as possible without touching (5~10 μm apart) using Zeiss micromanipulator (C-J Jena). When a steady reading for the pH had been obtained, a stream of CO_2 or light was given to the leaf disc. The source of light and CO_2 was the same as for the PD measurements.

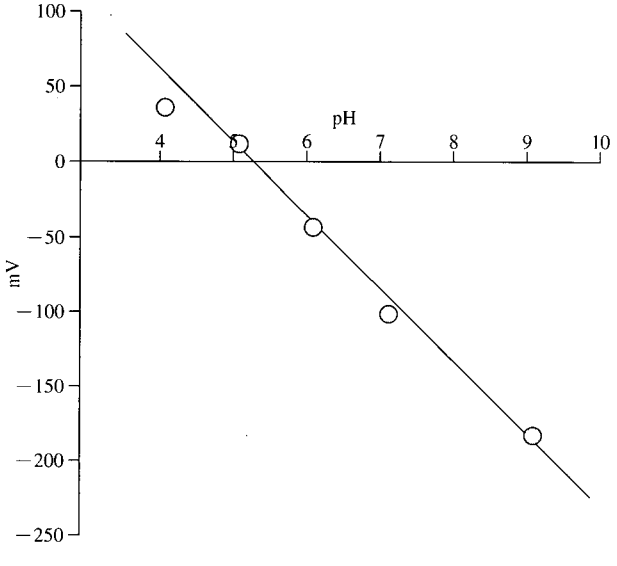

Fig. 2. Calibration of a pH micro-electrode used to measure apoplastic pH. Line fitted by eye, slope 50 mV/pH unit.

RESULTS AND DISCUSSION

The effects of light and CO_2 on apoplastic PD of guard cells in a closed stomata in the intact leaf and isolated epidermis are shown in Fig. 3. Fast hyperpolarization of guard cell apoplastic PD was recorded reaching up to around 7 mV in response to light. The initial responses were fastest and the saturation point of hyperpolarization was recorded within 30 sec. It then slowly depolarized to near the start point while the light was on. In darkness, a stable base reading of PD was recorded and sometimes, there was a very slow depolarization. When a steady reading for the PD had been obtained, a stream of CO_2 was passed into a chamber which the leaf disc was put inside. At the onset of CO_2 the PD showed a rapid hyperpolarization around 20 mV. Whenever the experiments were attempted with detached epidermis, there was no response to light or

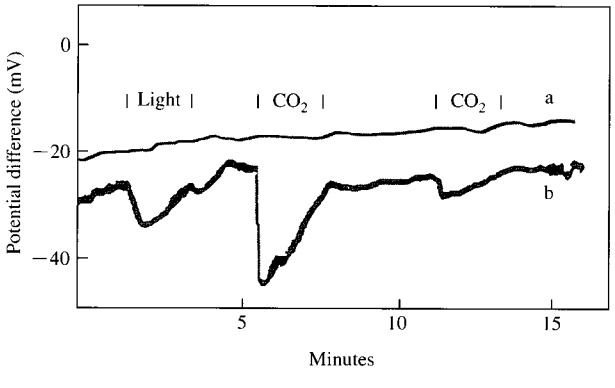

Fig. 3. The effect of white light and CO_2 on the changes of apoplastic PD of the guard cell in a closed stoma in the intact leaf and isolated epidermis of *Commelina communis*.

CO_2.

As was already reported by Lee and Bowling (1992), freshly isolated mesophyll cells incubated with isolated epidermis caused the stomata to open almost as wide as those in the intact leaf. It was thought that the mesophyll producing a compound which diffused out of cells into the medium. To study the mechanism of the action of the unknown compound, particularly at the membrane level, the apoplastic PD of guard cells in isolated epidermis was measured in the presence of the mesophyll supernatant or the control medium.

In the initial stomatal opening response, the apoplastic PD in isolated epidermis started to hyperpolrize and around 40 min. there was a maximum PD change followed by a return to the original level of the PD (Fig. 4). The apoplastic PD in isolated epidermis was hyperpolarized to −7 mV, changing from −22 mV to −29 mV at 40 min. But, when isolated epidermis was incubated with the supernatant from mesophyll cells incubated in the light, the apoplastic PD in isolated epidermis was hyperpolarized to −19 mV, changing from −22 mV to 40.5 mV. This apoplastic hyperpolarization seems to be coincide with the apoplastic acidification under illumination (Edwards *et al.* 1988).

To investigate if the apoplastic hyperpolarization is the result of the proton efflux, the effect of apoplastic pH of guard cells in the intact leaves was investigated (Fig. 5). Apoplastic pH of guard cells in the intact leaf was acidified about 0.25 pH unit at the onset of CO_2 in the intact leaf and then rapidly alkaliesed a little bit more than the first pH. After that it maintained a uniform level of pH near to the start point after 6 min. However, this change was absent in isolated epidermis.

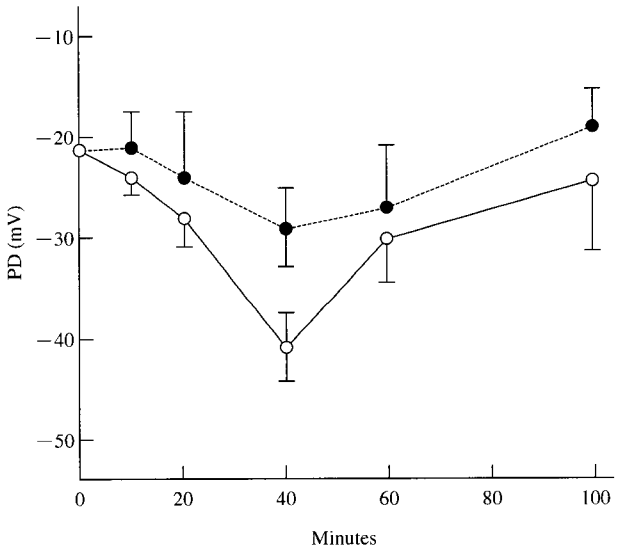

Fig. 4. The effect of the supernatant from the mesophyll cells which were pretreated in light on the change of guard cell apoplastic PD in isolated epidermis of *Commelina communis*. Each pont is the mean of (±s.e.m.) of three replicate experiments. Epidermis was incubated in 50mM HEPES-KOH (pH 7.3) containing 10 mM NaCl, 70 mMKCl, 2 mM EDTA, 2 mM $MgSO_4$ under illumination (200 μmol m^{-2} s^{-1}). Closed circles, isolated epidermis incubated with normal medium; open circles, isolated epidermis incubated with light treated mesophyll supernatant.

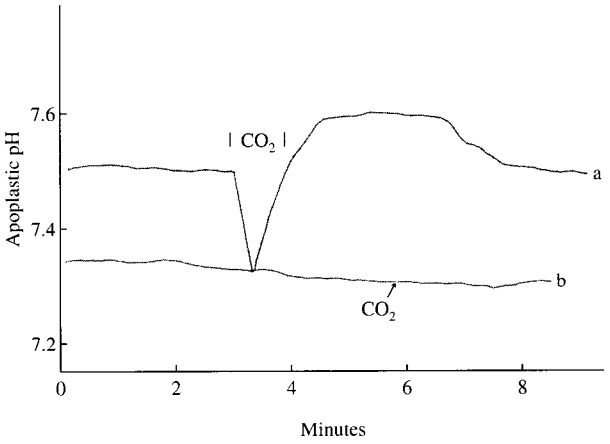

Fig. 5. The effect of CO_2 on the changes of apoplastic pH of the guard cell in a closed stoma in the intact leaf and isolated epidermis of *Commelina communis*.

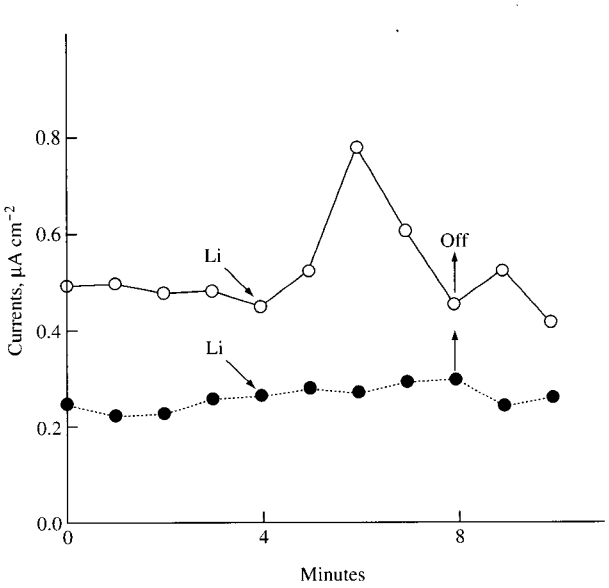

Fig. 6. The effect of the white light on the currents flowing on abaxial side of the intact leaf (open circles) and isolated epidermis (closed circles) in *Commelina communis* L.

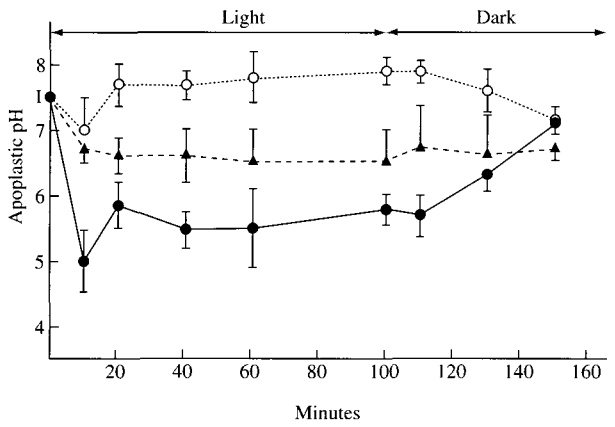

Fig. 7. The effect of light and dark on the changes of apoplastic pH of the guard cell in a closed stoma in the intact leaf and isolated epidermis of *Commelina communis*. The effect of the supernatant from the mesophyll cells which were pretreated in light was also investigated. Each pont is the mean of (±s.e.m.) of three replicate experiments. Epidermis was incubated in 50 mM HEPES-KOH (pH 7.3) containing 10 mM NaCl, 70 mMKCl, 2 mM EDTA, 2 mM $MgSO_4$ under illumination (200 μmol m^{-2} s^{-1}). Open circles, isolated epidermis incubated with normal medium; Closed circles, intact leaf; closed triangles, isolated epidermis incubated with light treated mesophyll supernatant.

A vibrating probe was used to detect the change in electrical currents at the surface of excised intact leaves and isolated epidermis (Fig. 6). When exposed to light, the current on the surface of excised intact leaves was higher than that on the surface of isolated epidermis. Both intact leaf and isolated epidermis were subjected to successive short light-dark cycles. The reading of excised intact leaves in the dark was 0.5 μA cm^{-2}, remaining steady until illuminated. Light increased the current on the surface of excised leaves to about 0.8 μA cm^{-2}. However, light had no effect in the current on the surface of isolated epidermis. Bowling *et al.* (1986) used the vibrating probe to measure currents over the surface of detached leaves and over isolated portions of leaf epidermis of *Commelina*. They found a linear relationship between current and stomatal aperture. They also suggested that the current was brought about by a flow of H^+ from the leaf surface. Therefore, the present results indicate that light stimulate H^+ efflux from the surface of the intact leaf but not for the isolated epidermis.

Apoplastic pH changes across the stomatal complex in response to light and dark were measured both in the intact leaves and isolated epidermis over the same time period using pH micro-electrodes (Fig. 7). There was a big difference in guard cell apoplastic pH in response to light and dark between intact leaf and isolated epidermis. The guard cell wall of intact leaf was acidified to 2.5 pH unit, falling from pH 7.5 to pH 5.0 in the first 10 min. in the light. After 40 min. in the light, cell wall pH in the intact leaf had reached a uniform level approximately between pH 5.4 and pH 6.3. On transfer to the dark after 100 min. in the light there was an immediate rise in apoplastic pH and the level observed at the beginning of the experiment was regained after 50 min. At the same time the guard cell wall pH of isolated epidermis fell from pH 7.5 to pH 7.0 at 10 min. and after that it maintained a uniform level of around pH 7.7 ~ 7.8 in the light. When the isolated epidermis was transferred to the dark, guard cell wall was slowly acidified. The difference between intact leaf and isolated epidermis demonstrate that stomatal change of guard cell wall pH when stomata open is dependent on the presence of the mesophyll. In order to find out if the mesophyll is important in the change of apoplastic pH of isolated epidermis, batches of isolated mesophyll cells were incubated in the light for an hour and then the cells were removed by centrifugation. The light treated mesophyll supernatant was then incubated with isolated epidermis. The apoplastic pH of isolated epidermis incubated in the supernatant dropped lower than that of isolated epidermis. The guard cell wall pH of isolated epidermis incubated in the supernatant fell from pH 7.6 to pH 6.7 at 10 min. and then maintained a uniform level between pH 6.7 and pH 6.5.

From the all above results, it could be concluded that mesophyll participation in stomatal control might be more important than we originally thought. It also could be imagined that an electrical signal, or chemicals and hormones propagated from the mesophyll in response to light and CO_2 could produce a fast stomatal response.

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