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Changes in Stomatal Behavior and Guard Cell Cytosolic Free Calcium in Response to Oxidative Stress[1]

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We have investigated the cellular basis for the effects of oxidative stress on stomatal behavior using stomatal bioassay and ratio photometric techniques. Two oxidative treatments were employed in this study: (a) methyl viologen, which generates superoxide radicals, and (b) H_2O_2. Both methyl viologen and H_2O_2 inhibited stomatal opening and promoted stomatal closure. At concentrations $\leq 10^{-5}$ M, the effects of methyl viologen and H_2O_2 on stomatal behavior were reversible and were abolished by 2 mM EGTA or 10 μM verapamil. In addition, at 10^{-5} M, i.e. the maximum concentration at which the effects of the treatments were prevented by EGTA or verapamil, methyl viologen and H_2O_2 caused an increase in guard cell cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), which was abolished in the presence of EGTA. Therefore, at low concentrations of methyl viologen and H_2O_2, removal of extracellular Ca^{2+} prevented both the oxidative stress-induced changes in stomatal aperture and the associated increases in $[Ca^{2+}]_i$. This suggests that in this concentration range the effects of the treatments are Ca^{2+}-dependent and are mediated by changes in $[Ca^{2+}]_i$. In contrast, at concentrations of methyl viologen and H_2O_2 $>10^{-5}$ M, EGTA and verapamil had no effect. However, in this concentration range the effects of the treatments were irreversible and correlated with a marked reduction in membrane integrity and guard cell viability. This suggests that at high concentrations the effects of methyl viologen and H_2O_2 may be due to changes in membrane integrity. The implications of oxidative stress-induced increases in $[Ca^{2+}]_i$ and the possible disruption of guard-cell Ca^{2+} homeostasis are discussed in relation to the processes of Ca^{2+}-based signal transduction in stomatal guard cells and the control of stomatal aperture.

Many environmental stresses result in the enhanced production of active oxygen species in plants, including $O_2^{\cdot -}$ radicals and H_2O_2 (for reviews, see Bowler et al., 1992; Foyer et al., 1994; Inzé and Van Montagu, 1995). Oxidative stress resulting from the production of active oxygen species has been shown to occur in response to high and low temperatures, high light intensities, UV irradiation, drought, exposure to pollutant gases (e.g. O_3 and sulfur dioxide), herbicides (e.g. Paraquat), transition metal toxicity, and senescence (for reviews, see Bowler et al., 1992; Foyer et al., 1994; Inzé and Van Montagu, 1995). By themselves $O_2^{\cdot -}$ radicals and H_2O_2 are relatively unreactive. Indeed, several metabolic processes use activated oxygen species in a beneficial manner (for review, see Inzé and Van Montagu, 1995). For example, the H_2O_2 produced from the oxidative burst generated during hypersensitive plant-pathogen interactions functions as a local trigger of programmed cell death and causes rapid cross-linking of cell-wall proteins (Bradley et al., 1992; Levine et al., 1994). Furthermore, H_2O_2 also appears to act as a signal molecule that induces the transcription of defense-related genes (Chen et al., 1993, 1995; Levine et al., 1994). However, $O_2^{\cdot -}$ radicals and H_2O_2 can react to produce additional activated oxygen species that are damaging to essential cellular components. The formation of $OH^{\cdot}$ radicals, which are catalyzed by transition metals through the Haber-Weiss reaction, accounts for much of the toxicity of $O_2^{\cdot -}$ radicals and H_2O_2. $OH^{\cdot}$ radicals are capable of causing indiscriminate lipid peroxidation, protein denaturation, and damage to DNA (for reviews, see Cadenas, 1989; Halliwell and Gutteridge, 1989; Inzé and Van Montagu, 1995).

Stomata regulate the uptake of CO_2 for photosynthesis and the loss of water vapor during transpiration (for reviews, see Mansfield et al., 1990; MacRobbie, 1992; Assmann, 1993). Consequently, any factor that alters their function will affect both photosynthesis and water relations and, as a consequence, will be expected to influence plant productivity. Preliminary studies suggest that oxidative stress resulting from exposure to methyl viologen (which generates $O_2^{\cdot -}$ radicals [Foyer et al., 1994]) or H_2O_2 has a marked effect on stomatal aperture (Price, 1990). Similarly, the pollutant gas O_3, which generates a number of highly reactive activated oxygen species in solution (such as $OH^{\cdot}$ radicals, singlet oxygen, $O_2^{\cdot -}$, and H_2O_2 [Heath, 1994; Wellburn, 1994]), also affects stomatal responses together with plant growth (for reviews, see Heath, 1994; Wellburn, 1994). Marked reductions in stomatal conductance have been observed in a number of species in response to O_3 (see Pearson and Mansfield, 1993; Hassan et al., 1994; Le Thiec et al., 1994a). In addition, O_3 may partially inhibit the normal closing response of stomata to drought (Pearson and Mansfield, 1993). Little is known about the cellular mechanisms underlying the effects of oxidative stress on stomatal behavior. However, these are likely to include changes in the permeability of the guard-cell plasma membrane, which result from ozonolysis and

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+}; FDA, fluorescein diacetate.

lipid peroxidation, and disruption of ion transport processes (for reviews, see Heath, 1994; Wellburn, 1994).

Ca^{2+} has been shown to play a key role in the regulation of guard-cell turgor and stomatal aperture (Mansfield et al., 1990; MacRobbie, 1992; Assmann, 1993; Ward et al., 1995; Webb et al., 1996b). Increases in external Ca^{2+} (De Silva et al., 1985; McAinsh et al., 1995) and $[Ca^{2+}]_i$ (Gilroy et al., 1990) are known to promote stomatal closure, and Ca^{2+} has been shown to act as a second messenger in the response of stomata to both opening and closing stimuli (Gilroy et al., 1990, 1991; McAinsh et al., 1990, 1992, 1995; Schroeder and Hagiwara, 1991; Irving et al., 1992; Allan et al., 1994; Webb et al., 1996a). Therefore, in the absence of other compensatory mechanisms, it can be predicted that factors that interfere with cellular Ca^{2+} homeostasis will have a marked effect on stomatal behavior. Recent studies using recombinant aequorin techniques have demonstrated that H_2O_2 stimulates a transient increase in whole-plant $[Ca^{2+}]_i$ in tobacco seedlings (Price et al., 1994). In addition, O_3 has been shown to influence Ca^{2+} transport in mixed membrane preparations isolated from pinto bean (Castillo and Heath, 1990). "Inside-out" membrane vesicles isolated from plants exposed to O_3 exhibit greater $^{45}Ca^{2+}$ efflux (corresponding to Ca^{2+} influx in intact cells) than those isolated from unexposed plants. There is also indirect evidence to suggest a role of Ca^{2+} in the response of stomata to both H_2O_2 and methyl viologen (Price, 1990). Furthermore, exposure of Norway spruce to O_3 has been found to induce a marked redistribution of Ca^{2+} oxalate crystals from the apoplast into the vacuoles of both epidermal cells and cells of the stomatal complex (Fink, 1991). This implies a substantial movement of Ca^{2+} through the cytoplasm of these cells. The total Ca^{2+} content of stomatal guard cells, determined by x-ray microanalysis, has also been shown to be higher in Norway spruce grown in an O_3-enriched atmosphere than those grown under ambient conditions (Le Thiec et al., 1994b). This is associated with a decrease in stomatal aperture. These data suggest that oxidative stress has the potential to affect cellular Ca^{2+} homeostasis, possibly through the stimulation of an influx of Ca^{2+} into the cytosol.

We have used H_2O_2 (which generates $OH^{\cdot}$ radicals directly [Bowler et al., 1992]) and methyl viologen (which generates $OH^{\cdot}$ radicals as a secondary activated oxygen species after the formation of $O_2^{\cdot -}$ radicals [Babbs et al., 1989]) to investigate changes in stomatal behavior in response to oxidative stress at the cellular level. Epidermal-bioassay and fluorescence ratio photometric techniques were used to determine if the effects of oxidative stress on stomata result from changes in the guard cell Ca^{2+} homeostasis, with a concomitant disruption of Ca^{2+}-based signal transduction. In addition, alterations in membrane integrity and guard-cell viability in response to the two oxidative treatments were assessed through "washout" experiments and the FDA viability test.

MATERIALS AND METHODS

Plant Material

Commelina communis was grown from seed (McAinsh et al., 1991). Immediately prior to each experiment, the epidermis was peeled carefully from the abaxial surface of the youngest, fully expanded leaves of 4-week-old plants and floated on CO_2-free, 10 mM Mes/KOH, pH 6.15, at 25°C (McAinsh et al., 1991).

Epidermal Strip Bioassay

Freshly prepared epidermis was cut into 5-mm lengths. To study inhibition of stomatal opening, epidermal strips were incubated for 3 h under conditions promoting stomatal opening (McAinsh et al., 1991) in CO_2-free, 50 mM KCl, 10 mM Mes/KOH, pH 6.15 (KCl-Mes), in the presence and absence of methyl viologen or H_2O_2 (10^{-9} to 10^{-3} M) with and without 2 mM EGTA or 10 μM verapamil. In washout experiments, strips were subsequently transferred to fresh CO_2-free KCl-Mes and incubated for 2 h under opening conditions in the absence of test chemicals. To study promotion of stomatal closure, epidermal strips were incubated under conditions promoting stomatal opening for 3 h and then transferred to CO_2-free KCl-Mes in the presence and absence of methyl viologen or H_2O_2 with and without EGTA or verapamil, after which they were incubated for another 2 h under opening conditions. In all cases, the strips were subsequently examined under the microscope to determine the aperture of the stomatal pores.

Microinjection of Stomatal Guard Cells

Stomatal guard cells were microinjected according to McAinsh et al. (1990, 1992, 1995). Freshly prepared epidermis (>2 cm in length) in which stomata were open to <1 μm were mounted cuticle-side down in a perfusion chamber. Guard cells were impaled with filamented glass microelectrodes (<0.25 μm tip diameter) containing 10 mM fura-2 pentapotassium salt (Calbiochem-Novabiochem, Nottingham, UK) in their tips. Fura-2 was microinjected into the cytosol of cells iontophoretically. Injected cells were subsequently maintained under conditions promoting stomatal opening for 45 min (McAinsh et al., 1992, 1995). Fluorescence measurements were made only on stomata that opened to the same aperture as those on the rest of the epidermal strip (6–10 μm) and in which both the injected and noninjected cells of a single stoma exhibited the same increase in turgor. Epidermal strips in which injected guard cells met all of the criteria for estimating viability (Gilroy et al., 1991; McAinsh et al., 1992, 1995) were perfused with CO_2-free KCl-Mes in the presence or absence of 10^{-5} M methyl viologen or H_2O_2 with and without 2 mM EGTA at 25°C, and the fluorescence was monitored. Methyl viologen and H_2O_2 had little effect on the fluorescence of fura-2 in vitro. Epidermal strips in which the injected guard cells failed to exhibit a change in the Ca^{2+}-dependent ratio were subsequently perfused with KCl-Mes containing 0.1 mM $CaCl_2$ to determine whether they were capable of maintaining Ca^{2+} homeostasis through a regulated increase in $[Ca^{2+}]_i$ and were, therefore, physiologically viable (McAinsh et al., 1995; Webb et al., 1996a).

Fluorescence Ratio Photometry

The Ca^{2+}-dependent and Ca^{2+}-independent fura-2 fluorescence values were determined according to McAinsh et al. (1995). The perfusion chamber was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Excitation wavelengths (Ca^{2+}-dependent fluorescence, 340 and 380 nm; Ca^{2+}-independent fluorescence, 360 nm; 10-nm half-bandwidth interference filters [Cairn Research, Kent, UK]) were selected using a spinning filter changer (Cairn Research). Fluorescence emissions (510 nm; 20-nm half-bandwidth interference filter [Nikon]) were quantified using a Cairn Research spectrophotometer system. The autofluorescence of each guard cell was determined at each excitation wavelength prior to microinjection. Autofluorescence subtraction was calculated online. Methyl viologen and H_2O_2 had no effect on guard-cell autofluorescence. Typically, the spinning filter changer was run at 64 revolutions per second, which allows fluorescence measurements to be obtained at a rate of 64 readings per second. To increase the signal-to-noise ratio, values were calculated as the mean of 64 individual readings, giving a data point every 1 s. Ratios (340:380 nm) were calculated every 1 s online and were subsequently converted into measurements of whole-cell $[Ca^{2+}]_i$ using a predetermined calibration curve. Similar results were obtained for both in vitro and in vivo calibration of the Ca^{2+}-dependent ratio in guard cells (Gilroy et al., 1991; McAinsh et al., 1992). Consequently, an in vitro calibration was routinely used.

FDA Viability Test

Membrane integrity and guard-cell viability were assessed using FDA, a nonfluorescent, nonpolar molecule that enters cells freely across the plasma membrane, where it is hydrolyzed by the action of membrane-bound esterases releasing the highly fluorescent, polar fluorescein molecule (Larkin, 1976; Dixon, 1985). Fluorescein is much less permeant than FDA and accumulates in cells that possess an intact plasma membrane. Viable, intact cells exhibit a discrete distribution of fluorescence often associated with the cell membranes and organelles. However, accelerated leakage of the fluorescent product can occur after the hydrolysis of FDA if the plasma membrane of the cell has been damaged (Larkin, 1976). Epidermal strips were incubated for 3 h under conditions promoting stomatal opening (McAinsh et al., 1991) in CO_2-free KCl-Mes in the presence and absence of methyl viologen or H_2O_2. Subsequently, the epidermis was mounted in a 0.01% (w/v) solution of FDA in KCl-Mes and examined microscopically using an epifluorescence microscope. It is important that methyl viologen and H_2O_2 have no effect on the fluorescence of FDA in solution. Fluorescence (450- to 490-nm excitation filter; 515-nm emission filter [Leitz, Milton Keynes, UK]) and bright-field photographs of strips were taken approximately 5 min after mounting. To prevent bleaching of the fluorescein, the bright-field photographs were always taken first, using very low illumination. The intensity of fluorescein production was quantified using a JVC (Sussex, UK) color video camera and an ARGUS-50 image analysis system (Hamamatsu Photonics, Middlesex, UK) to generate two-dimensional response surfaces of FDA-stained stomatal complexes.

RESULTS

Changes in Stomatal Behavior in Response to Oxidative Stress

Methyl viologen and H_2O_2 had a marked effect on stomatal behavior. Both treatments inhibited stomatal opening (Fig. 1) and promoted stomatal closure (Fig. 2), but these effects were dose-dependent. Little effect was observed at concentrations of methyl viologen and H_2O_2 $<10^{-7}$ M, whereas at concentrations $\geq 10^{-7}$ M, both treatments caused increasing inhibition of stomatal opening and promotion of stomatal closure. However, at concentrations $>10^{-5}$ M, the effect of methyl viologen on stomatal aperture was significantly ($P < 0.05$) less than that of H_2O_2. The maximum inhibition of stomatal opening was observed at 10^{-3} M methyl viologen and H_2O_2, giving stomatal apertures of 7.8 ± 0.1 and 4.2 ± 0.1 μm, respectively, which were 45% (see Fig. 3A) and 27% (see Fig. 3B) of control values. Similarly, the maximum promotion of clo-

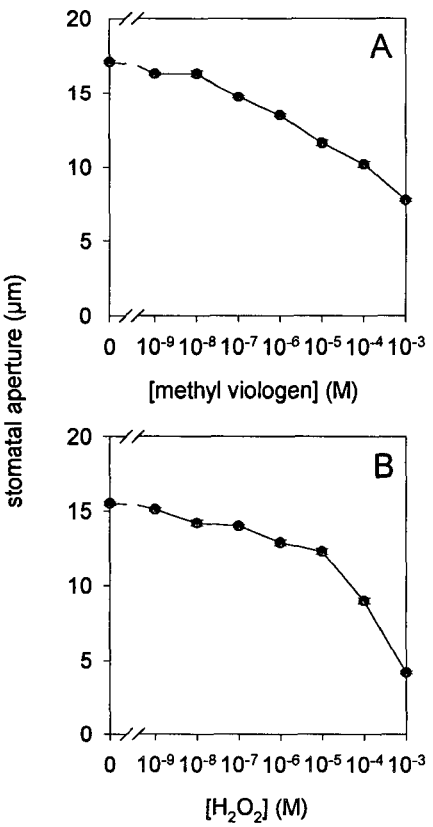

Figure 1. Inhibition of stomatal opening by methyl viologen (A) and H_2O_2 (B). Freshly prepared epidermis was incubated in CO_2-free KCl-Mes containing different concentrations (10^{-9} to 10^{-3} M) of methyl viologen or H_2O_2 under conditions promoting stomatal opening. Stomatal apertures were determined after 3 h. Values are the means of 180 measurements ± SE values.

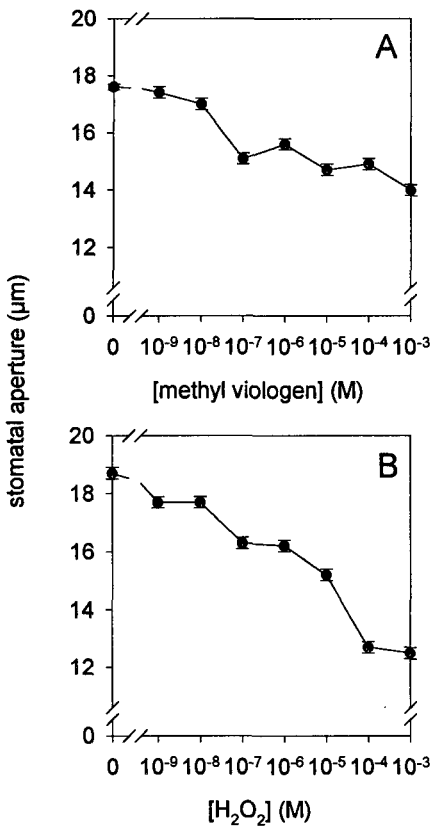

Figure 2. Promotion of stomatal closure by methyl viologen (A) and H_2O_2 (B). Freshly prepared epidermis was incubated in CO_2-free KCl-Mes under conditions promoting stomatal opening for 3 h and then transferred to fresh CO_2-free KCl-Mes containing different concentrations (10^{-9} to 10^{-3} M) of methyl viologen or H_2O_2. Stomatal apertures were determined after another 2-h incubation. Values are the means of 180 measurements ± SE values.

sure was observed at 10^{-3} M methyl viologen or H_2O_2, giving stomatal apertures of 10.0 ± 0.2 and 12.5 ± 0.2 μm, respectively, which were 79% (see Fig. 4A) and 66% (see Fig. 4B) of the control values.

Ca^{2+}- and Oxidative Stress-Induced Changes in Stomatal Behavior

The role of Ca^{2+} in the response of stomata to oxidative stress was investigated using EGTA (reducing the concentration of external Ca^{2+} to vanishingly low levels) and verapamil, a Ca^{2+} channel blocker that has been shown to be active in stomatal guard cells (MacRobbie, 1989; McAinsh et al., 1991). EGTA had a marked effect on the inhibition of stomatal opening (Fig. 3) and the promotion of stomatal closure (Fig. 4) caused by both methyl viologen and H_2O_2. The effect was concentration-dependent. At concentrations of methyl viologen and H_2O_2 $\leq 10^{-5}$ M, EGTA completely abolished the effects of the two treatments on stomatal aperture. However, at concentrations of methyl viologen and H_2O_2 $>10^{-5}$ M, EGTA had little or no effect on the inhibition of stomatal opening or promotion of stomatal closure.

The effect of verapamil was more varied. This may be due to the incomplete characterization in plants of the pharmacology of the phenylalkylamine-derived series of compounds to which this Ca^{2+} channel blocker belongs (for review, see Hetherington et al., 1992). At concentrations of methyl viologen and H_2O_2 $\leq 10^{-5}$ M, verapamil completely abolished the methyl viologen-induced inhibition of stomatal opening (Fig. 3A) and the H_2O_2-induced promotion of stomatal closure (Fig. 4B), whereas at concentrations of methyl viologen and H_2O_2 $>10^{-5}$ M, verapamil had little effect. In contrast, verapamil had no effect on either the promotion of closure caused by methyl viologen (Fig. 4A) or the inhibition of stomatal opening observed in response to H_2O_2 (Fig. 3B). A similar degree of variability has been observed in the effects of verapamil on the response of stomata to ABA (De Silva et al., 1985; McAinsh et al., 1991).

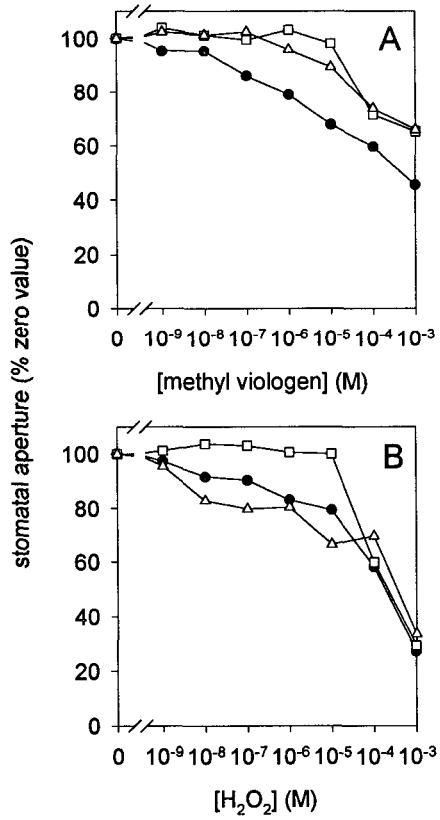

Figure 3. The effect of 2 mM EGTA (□) and 10 μM verapamil (△) on the inhibition of stomatal opening by methyl viologen (A) and H_2O_2 (B). The effect of methyl viologen and H_2O_2 alone on stomatal aperture, calculated from values in Figure 1, A and B, respectively, is included for comparison (●). Freshly prepared epidermis was incubated in CO_2-free KCl-Mes containing methyl viologen or H_2O_2 (10^{-9} to 10^{-3} M), in the presence of either EGTA or verapamil, under conditions promoting stomatal opening. Stomatal apertures were determined after 3 h. Values are expressed as percentages of the aperture in the absence of methyl viologen or H_2O_2 calculated from the means of 180 measurements.

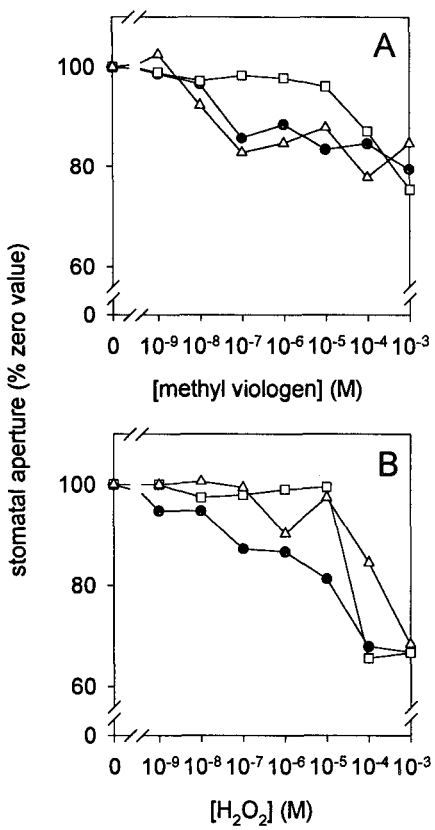

Figure 4. The effect of 2 mM EGTA (□) and 10 μM verapamil (△) on the promotion of stomatal closure by methyl viologen (A) and H_2O_2 (B). The effect of methyl viologen and H_2O_2 alone on stomatal aperture, calculated from values in Figure 2, A and B, respectively, is included for comparison (●). Freshly prepared epidermis was incubated in CO_2-free KCl-Mes under conditions promoting stomatal opening for 3 h and then transferred to fresh CO_2-free KCl-Mes containing methyl viologen or H_2O_2 (10^{-9} to 10^{-3} M), in the presence of either EGTA or verapamil. Stomatal apertures were determined after another 2-h incubation. Values are expressed as percentages of the aperture in the absence of methyl viologen or H_2O_2 calculated from the means of 180 measurements.

The Effects of Oxidative Stress on Membrane Integrity and Cell Viability

The effects of methyl viologen and H_2O_2 on membrane integrity and guard-cell viability were assessed (a) in washout experiments (Fig. 5) to determine whether changes in stomatal aperture were reversible or a consequence of permanent cell damage (McAinsh et al., 1991), and (b) through the production of fluorescein from FDA by the action of membrane-bound esterases (Figs. 6 and 7). Fluorescein accumulates in viable, intact cells (Larkin, 1976; Dixon, 1985). At concentrations of methyl viologen and H_2O_2 $\leq 10^{-5}$ M, the effects of both treatments on stomatal aperture were completely reversible (Fig. 5). With the exception of stomata treated with 10^{-5} M methyl viologen, at which only approximately 50% recovery was observed in the recovery period, there was no significant ($P < 0.05$) difference between the apertures of stomata treated with methyl viologen or H_2O_2 for 3 h followed by a 2 h washout and those incubated under the same conditions for 5 h in the absence of the two treatments. The final stomatal aperture was approximately 19 μm in all cases. This indicates that there is no reduction in membrane integrity as a result of treatment with low concentrations of methyl viologen or H_2O_2, and that the plasma membrane remains capable of regulating the transport of ions required for both stomatal opening and closure. In contrast, at a concentration of 10^{-3} M the effects of methyl viologen and H_2O_2 on stomatal aperture were irreversible, indicating a loss of membrane integrity; stomatal apertures remained at approximately 7 and 5 μm, respectively.

Epidermis that had not been exposed to either treatment exhibited a discrete distribution of fluorescence. This was particularly intense in the guard cells, and was associated with the cell membranes and organelles (Figs. 6A, 6F, 7A, and 7F). There was no effect of methyl viologen and H_2O_2 on the fluorescence of FDA in solution (data not shown). Methyl viologen and H_2O_2 caused a marked change in the distribution and intensity of the fluorescence in epidermis. The effects of both treatments were concentration-

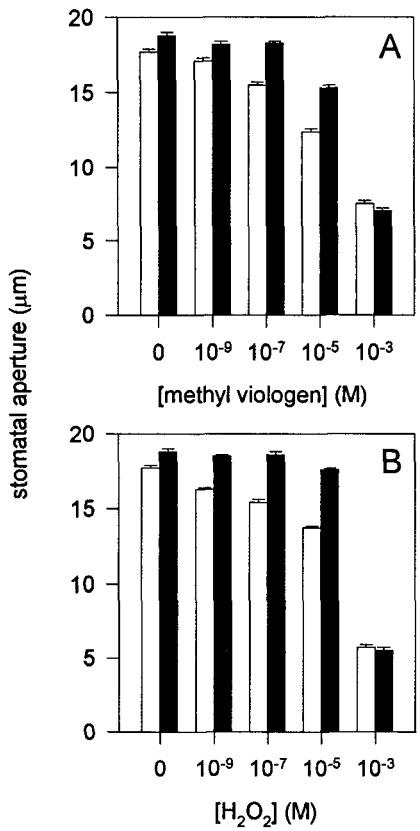

Figure 5. Reversible inhibition of stomatal opening by methyl viologen (A) and H_2O_2 (B). Freshly prepared epidermis was incubated in CO_2-free KCl-Mes containing methyl viologen or H_2O_2 (10^{-9}, 10^{-7}, 10^{-5}, and 10^{-3} M) under conditions promoting stomatal opening for 3 h. Strips were then transferred to fresh, CO_2-free KCl-Mes and incubated for another 2 h under opening conditions. Stomatal apertures were determined at the end of the initial 3-h incubation (□) and at the end of the subsequent 2-h washout period (■). Values are the means of 180 measurements ± SE values.

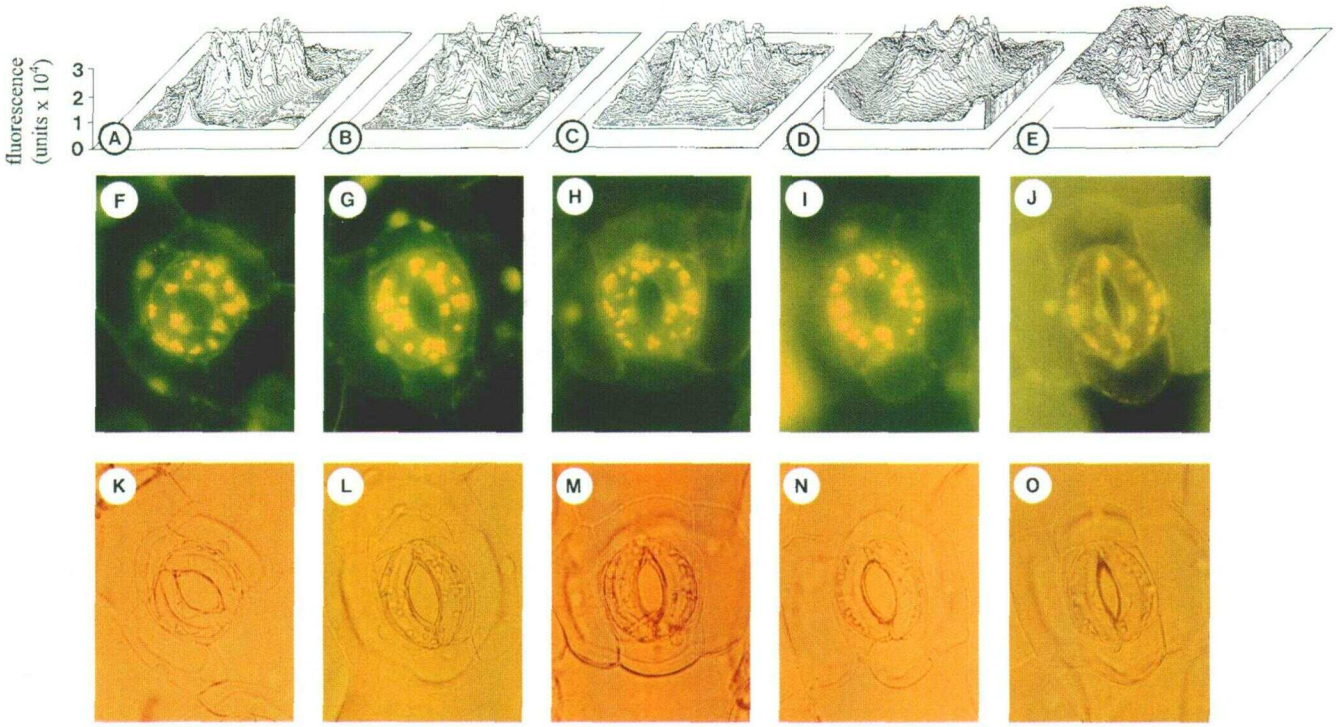

Figure 6. The effect of methyl viologen on membrane integrity and guard-cell viability. Freshly prepared epidermis was incubated in CO_2-free KCl-Mes containing 0 (A, F, and K), 10^{-9} (B, G, and L), 10^{-7} (C, H, and M), 10^{-5} (D, I, and N), and 10^{-3} M (E, J, and O) methyl viologen under conditions promoting stomatal opening for 3 h. Membrane integrity and cell viability were assessed subsequently through the pattern of FDA staining (A–E, two-dimensional response surfaces; F–J, fluorescence micrographs). K–O are light micrographs. Magnification ×400.

dependent. There was little change in the distribution of fluorescence in epidermis treated with 10^{-9} M (Fig. 6, B and G) or 10^{-7} M (Figs. 6, C and H) methyl viologen, concentrations that cause no alterations in stomatal aperture (Figs. 1 and 2). However, in epidermis treated with 10^{-5} M methyl viologen (Figs. 6, D and I), which causes slight changes in stomatal aperture that are completely abolished by EGTA (Figs. 3 and 4), there was a reduction in both the intensity and the discreteness of the fluorescence. In addition, there was diffuse fluorescence present in the epidermal cells. These differences were more apparent in epidermis treated with 10^{-3} M methyl viologen (Fig. 6, E and J), a concentration that causes changes in stomatal aperture that are unaffected by EGTA (Fig. 3 and 4).

Similar changes in the distribution and intensity of the fluorescence were observed in epidermis treated with H_2O_2 (Fig. 7, A–J). However, diffuse fluorescence was also observed in the subsidiary cells of epidermis treated with H_2O_2 (Fig. 7, B–E, and G–J). The appearance of fluorescence in the subsidiary and epidermal cells may be due to the accelerated leakage of fluorescein from the guard cells, where it is produced, into the surrounding cells following a reduction in the integrity of the plasma membrane and intracellular membranes of all three cell types (Larkin, 1976). There was no effect of EGTA on the distribution and intensity of the fluorescence observed in epidermis treated with either methyl viologen or H_2O_2 (data not shown). With the exception of epidermis treated with 10^{-3} M H_2O_2 (Fig. 7O), there was no visible change in the morphology of guard cells exposed to either methyl viologen or H_2O_2 (Figs. 6, K–O, and 7, K–O).

Oxidative Stress-Induced Changes in $[Ca^{2+}]_i$

Fluorescence ratio photometry was used to investigate the effects of methyl viologen and H_2O_2 on $[Ca^{2+}]_i$. Both treatments were applied at a concentration of 10^{-5} M, i.e. the maximum concentration at which the effects appeared to be Ca^{2+}-dependent (Figs. 3 and 4). At this concentration, methyl viologen and H_2O_2 had no effect on guard-cell autofluorescence and the fluorescence of fura-2 in vitro (data not shown). Resting $[Ca^{2+}]_i$ ranged from 90 to 250 nM (n = 46 cells). This is similar to that reported previously in guard cells (Gilroy et al., 1990, 1991; McAinsh et al., 1990, 1992, 1995; Schroeder and Hagiwara, 1990; Irving et al., 1992; Allan et al., 1994; Webb et al., 1996a). Methyl viologen (Fig. 8) and H_2O_2 (Fig. 9) both stimulated rapid increases in guard cell $[Ca^{2+}]_i$. The magnitude of the increases in $[Ca^{2+}]_i$ varied from 100 to 750 nM above the resting concentration in the case of methyl viologen and 100 to 500 nM above the resting concentration in the case of H_2O_2. These increases can be grouped into "classes" on an arbitrary basis, depending on the magnitude and pattern of the increases (McAinsh et al., 1992; Webb et al., 1996a). As can be seen in Figure 8, methyl viologen stimulated a small (less than 250 nM above the resting concentration), sus-

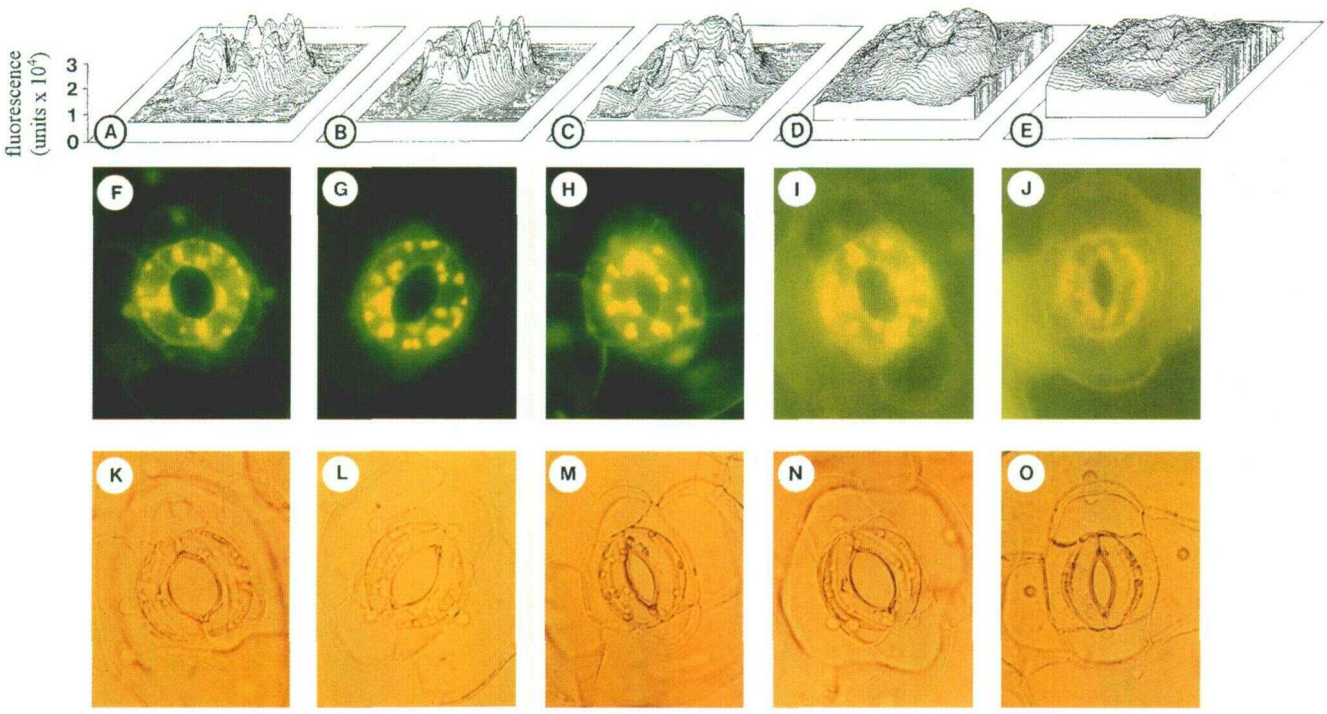

Figure 7. The effect of H_2O_2 on membrane integrity and guard-cell viability. Freshly prepared epidermis was incubated in CO_2-free KCl-Mes containing 0 (A, F, and K), 10^{-9} (B, G, and L), 10^{-7} (C, H, and M), 10^{-5} (D, I, and N), and 10^{-3} M (E, J, and O) H_2O_2 under conditions promoting stomatal opening for 3 h. Membrane integrity and cell viability were assessed subsequently through the pattern of FDA staining (A–E, two-dimensional response surfaces; F–J, fluorescence micrographs). K–O are light micrographs. Magnification ×400.

tained increase (n = 13 cells) (Fig. 8A); a medium (between 250 and 500 nM above the resting concentration), sustained increase (n = 3 cells) (Fig. 8B); or a large (greater than 500 nM above the resting concentration), sustained increase (n = 3 cells) (Fig. 8C) in $[Ca^{2+}]_i$. In contrast, Figure 9 shows that H_2O_2 stimulated a small (less than 250 nM above the resting concentration), sustained increase (n = 6 cells) (Fig. 9A); a medium (between 250 and 500 nM above the resting concentration), transitory increase (n = 3 cells) (Fig. 9B); or a small (less than 250 nM above the resting concentration), transitory increase (n = 5 cells) (Fig. 9C) in $[Ca^{2+}]_i$. EGTA prevented both the methyl viologen (n = 6 cells) and H_2O_2 (n = 7 cells) -stimulated increases in $[Ca^{2+}]_i$ (Fig. 10). Eleven of the 13 guard cells that had been exposed to EGTA exhibited a regulated increase of $[Ca^{2+}]_i$ in response to 0.1 mM $CaCl_2$ in the absence of EGTA (Fig. 10, A and B). However, in 2 cells H_2O_2 had an adverse effect on the Ca^{2+}-dependent ratio (see below) so that it no longer reflected changes in $[Ca^{2+}]_i$ (Fig. 10C). These data indicate that 85% of the cells were physiologically viable and were capable of maintaining Ca^{2+} homeostasis (McAinsh et al., 1995; Webb et al., 1996a). Therefore, EGTA abolished both the oxidative stress-induced increase in $[Ca^{2+}]_i$ (Fig. 10) and the oxidative stress-induced changes in stomatal aperture (Figs. 3 and 4).

In 8 of the 14 cells treated with 10^{-5} M H_2O_2 in the absence of EGTA, the increases in guard-cell $[Ca^{2+}]_i$ were transitory. In these cells the Ca^{2+}-dependent ratio dropped rapidly below the value at which it reflected $[Ca^{2+}]_i$ accurately, approximately 2 min after treatment with H_2O_2 (Fig. 9, B and C). During this lag period, the ability of the Ca^{2+}-dependent ratio to report $[Ca^{2+}]_i$ remained unaffected by changes in the amount of fura-2 present in the cells (see below). Similarly, 5 of the 7 cells treated with 10^{-5} M H_2O_2 in the presence of EGTA also exhibited a small decrease in the Ca^{2+}-dependent ratio (Fig. 10B). In the remaining 2 cells the Ca^{2+}-dependent ratio dropped rapidly below the value at which it reflected $[Ca^{2+}]_i$ accurately, which made it impossible to demonstrate an increase in $[Ca^{2+}]_i$ in response to 0.1 mM $CaCl_2$ (Fig. 10C). In all cases, the decrease in the Ca^{2+}-dependent ratio after treatment with H_2O_2 was associated with a concomitant decrease in the Ca^{2+}-independent fura-2 fluorescence (Figs. 9C and 10C). The Ca^{2+}-independent fluorescence of fura-2 can be used to monitor the amount of fura-2 present in cells (McAinsh et al., 1995; Webb et al., 1996a). Therefore, the decrease in the Ca^{2+}-independent fura-2 fluorescence triggered by H_2O_2 is indicative of a loss of fura-2 from cells after exposure to the treatment.

DISCUSSION

Methyl Viologen and H_2O_2 as Oxidative Stressors in Plants

There are two advantages of using the oxidative treatments employed in this study. First, methyl viologen,

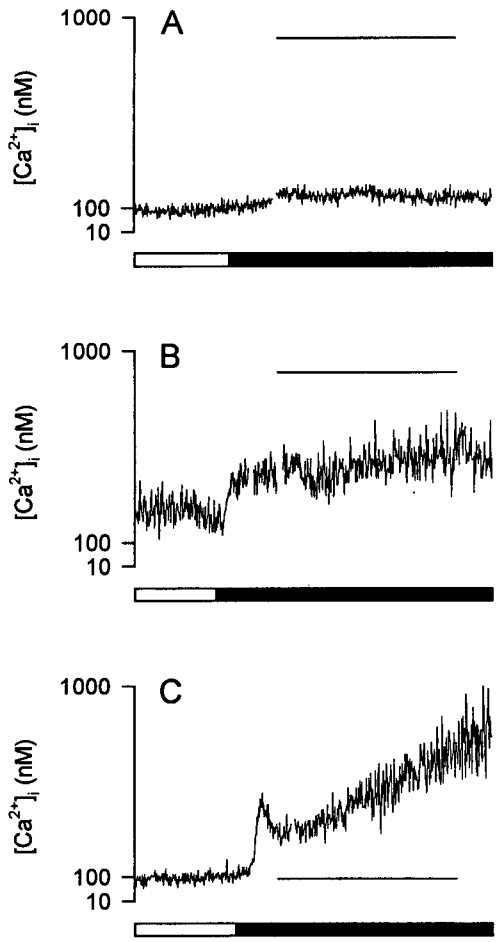

Figure 8. Changes in guard-cell $[Ca^{2+}]_i$ in response to 10^{-5} M methyl viologen. Guard cells of closed stomata were microinjected with fura-2 into the cytosol and the stomata opened to 6 to 10 μm. Resting $[Ca^{2+}]_i$ was determined in CO_2-free KCl-Mes (□). Guard cells were subsequently perfused with CO_2-free KCl-Mes containing 10^{-5} M methyl viologen (■). Changes in $[Ca^{2+}]_i$ were grouped into three "classes": a small (less than 250 nM above the resting concentration), sustained increase (n = 13 cells) (A); a medium (between 250 and 500 nM above the resting concentration), sustained increase (n = 3 cells) (B); and a large (greater than 500 nM above the resting concentration), sustained increase (n = 3 cells) (C). Bars = 5 min.

which generates $O_2^{\cdot -}$ radicals (Foyer et al., 1994), and H_2O_2 provide attractive model systems for studying the effects of oxidative stress in plants. For example, $O_2^{\cdot -}$ radicals and H_2O_2, both of which form $OH^{\cdot}$ radicals (Babbs et al., 1989; Bowler et al., 1992), are two of the major activated oxygen species formed by O_3 in solution (for reviews, see Heath, 1994; Wellburn, 1994). H_2O_2 can also be applied externally to mimic the chilling-induced oxidative stress response in maize (Prasad et al., 1994). In addition, activated oxygen species, and in particular H_2O_2, have been implicated as being important signaling molecules during plant-pathogen interactions (for review, see Inzé and Van Montagu, 1995). Second, methyl viologen and H_2O_2 are important environmental oxidative stressors. Methyl viologen (Paraquat) has been widely used as an herbicide, and tropospheric H_2O_2, formed from hydroperoxyl radicals ($HO_2^{\cdot}$) (for review, see Gunz and Hoffmann, 1990), is becoming an increasing problem. Concentrations of H_2O_2 up to 247 μM in cloud water have been reported, and it has been predicted that by the year 2030 the levels of tropospheric H_2O_2 could have increased by 20 to 40% in remote areas and by approximately 100% in urban areas (Gunz and Hoffmann, 1990).

Methyl Viologen Has Less Effect on Stomata Than H_2O_2

The two oxidative treatments used in this study have a marked effect on stomatal aperture (Figs. 1 and 2), membrane integrity, and guard-cell viability (Figs. 5–7). However, the effects of methyl viologen are consistently smaller

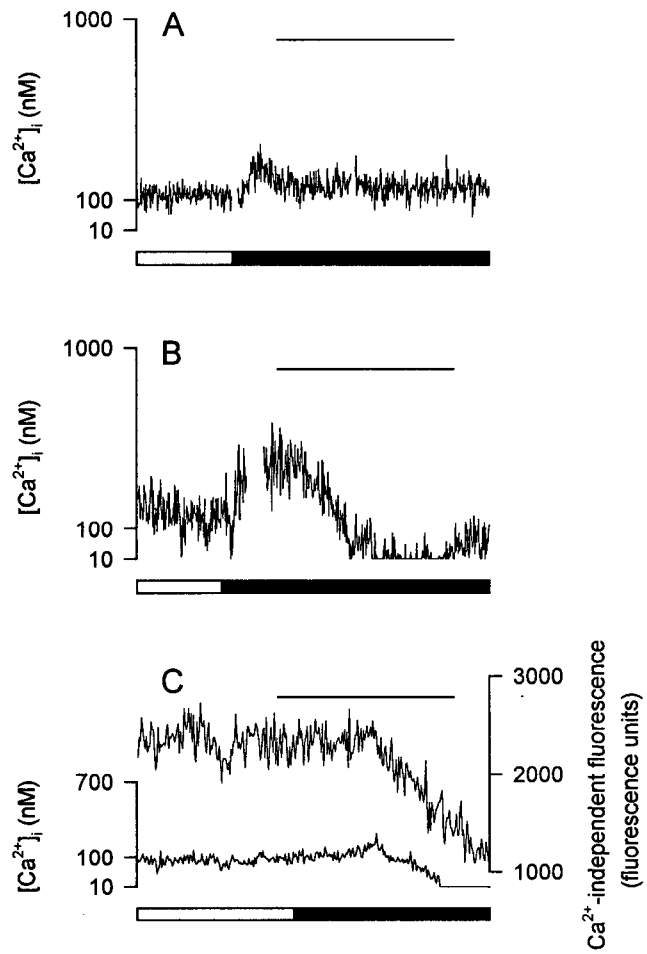

Figure 9. Changes in guard-cell $[Ca^{2+}]_i$ in response to 10^{-5} M H_2O_2. Guard cells of closed stomata were microinjected with fura-2 into the cytosol and the stomata opened to 6 to 10 μm. Resting $[Ca^{2+}]_i$ was determined in CO_2-free KCl-Mes (□). Guard cells were subsequently perfused with CO_2-free KCl-Mes containing 10^{-5} M H_2O_2 (■). Changes in $[Ca^{2+}]_i$ were grouped into three "classes": a small (less than 250 nM above the resting concentration), sustained increase (n = 6 cells) (A); a medium (between 250 and 500 nM above the resting concentration), transitory increase (n = 3 cells) (B); and a small (less than 250 nM above the resting concentration), transitory increase (n = 5 cells) (C). The amount of fura-2 present in cells was monitored using the Ca^{2+}-independent fluorescence of fura-2 (C). Bars = 5 min.

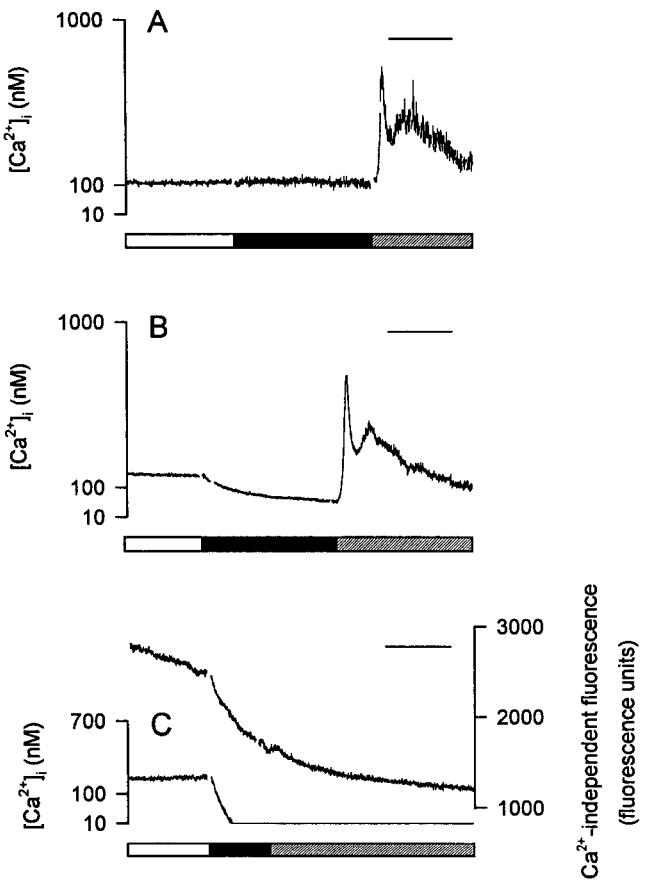

Figure 10. The effect of 2 mM EGTA on changes in guard-cell $[Ca^{2+}]_i$ in response to 10^{-5} M methyl viologen (A) or H_2O_2 (B and C). Guard cells of closed stomata were microinjected with fura-2 into the cytosol and the stomata opened to 6 to 10 μm. Resting $[Ca^{2+}]_i$ was determined in CO_2-free KCl-Mes in the presence of 2 mM EGTA (□). Guard cells were then perfused with CO_2-free KCl-Mes, containing either 10^{-5} M methyl viologen (n = 6 cells) or H_2O_2 (B, n = 5 cells; C, n = 2 cells), in the continued presence of EGTA (■). Subsequently, cells were perfused with CO_2-free KCl-Mes containing 0.1 mM $CaCl_2$ (▨) to determine whether the guard cell Ca^{2+} homeostasis had been affected adversely by the prolonged exposure to EGTA. The amount of fura-2 present in the cells was monitored using the Ca^{2+}-independent fluorescence of fura-2 (C). Bars = 5 min.

than those of H_2O_2. Three factors may contribute to this difference: (a) the ability of the $O_2^{\cdot-}$ radicals generated by methyl viologen to penetrate cell membranes is usually much lower than that of H_2O_2 (Halliwell and Gutteridge, 1989); (b) methyl viologen and H_2O_2 are both capable of generating $OH^{\cdot}$ radicals (for reviews, see Cadenas, 1989; Halliwell and Gutteridge, 1989; Bowler et al., 1992). However, methyl viologen generates $OH^{\cdot}$ radicals as a secondary activated oxygen species after the formation of $O_2^{\cdot-}$ radicals (Babbs et al., 1989), whereas H_2O_2 can generate $OH^{\cdot}$ radicals directly (Bowler et al., 1992). Therefore, the effects of $OH^{\cdot}$ radicals generated from methyl viologen via the production of $O_2^{\cdot-}$ radicals are likely to be less immediately apparent than those due to $OH^{\cdot}$ radicals generated directly from H_2O_2 (Cadenas, 1989; Halliwell and Gutteridge, 1989; Bowler et al., 1992); and (c) there may be differences in the activity of the enzymatic antioxidant defense mechanisms, the most important of which are superoxide dismutase for $O_2^{\cdot-}$ radicals and catalase for H_2O_2 (for review, see Bowler et al., 1992), which are responsible for converting these potentially dangerous activated oxygen species to O_2.

Methyl Viologen and H_2O_2 Disrupt Guard Cell Ca^{2+} Homeostasis

Our results suggest that at low concentrations ($\leq 10^{-5}$ M) the effects of methyl viologen and H_2O_2 on stomatal behavior are Ca^{2+}-dependent (Figs. 3 and 4) and are due to oxidative stress-induced increases in $[Ca^{2+}]_i$ (Figs. 8 and 9). EGTA prevented the oxidative stress-induced changes in stomatal aperture (Figs. 3 and 4) and completely abolished the oxidative stress-induced increase in $[Ca^{2+}]_i$ (Fig. 10). This strongly suggests that at concentrations of methyl viologen and H_2O_2 $\leq 10^{-5}$ M, an increase in $[Ca^{2+}]_i$ is required to evoke the stomatal responses to both treatments.

The range of increases in $[Ca^{2+}]_i$ observed in response to 10^{-5} M methyl viologen (Fig. 8) and H_2O_2 (Fig. 9) is consistent with that reported in guard cells in response to a number of different physiological stimuli (Gilroy et al., 1990, 1991; McAinsh et al., 1990, 1992, 1995; Schroeder and Hagiwara, 1990; Irving et al., 1992; Allan et al., 1994; Webb et al., 1996a). Increases in $[Ca^{2+}]_i$ also have been demonstrated in animal cells exposed to $O_2^{\cdot-}$ radicals (Baladi et al., 1994; Murata et al., 1994) and H_2O_2 (Toraason et al., 1994; Dreher and Junod, 1995; Munns and Leach, 1995), although these effects have also been attributed to the formation of $OH^{\cdot}$ radicals (Dreher and Junod, 1995).

Changes in stomatal behavior and guard-cell $[Ca^{2+}]_i$ induced by low concentrations ($\leq 10^{-5}$ M) of methyl viologen and H_2O_2 appear to be at least partially mediated by external Ca^{2+} (Figs. 3, 4, and 10), possibly through the entry of Ca^{2+} into guard cells. Washout experiments (Fig. 5) and application of the FDA viability test (Figs. 6 and 7) demonstrate that in this concentration range there is no effect by either treatment on membrane integrity and guard cell viability. Methyl viologen and H_2O_2 induced changes in stomatal aperture that were reversible, indicating that the plasma membrane remains integral and is capable of regulating the transport of ions required during stomatal opening and closure. Furthermore, there was little effect by either treatment on the intensity or distribution of fluorescein fluorescence. Therefore, it is unlikely that leakage of Ca^{2+} into the cytoplasm following the oxidative stress-induced loss of membrane integrity contributes to this process. In addition, although methyl viologen is known to disrupt photosynthetic electron transport (for review, see Bowler et al., 1992), and photosynthetic activity has been linked to the level of $[Ca^{2+}]_i$ (Miller and Sanders, 1987; Johnson et al., 1995), it is unlikely that this will influence the observed increases in $[Ca^{2+}]_i$. When calculated on a per cell basis, the activities of PSI and PSII are low in guard cells compared with mesophyll cells (Gautier et al., 1991; for review, see Willmer and Fricker, 1996), although they appear similar if calculated on a per milli-

gram of chlorophyll basis (Shimazaki and Zeiger, 1985). Instead, Ca^{2+} may enter cells through disruption of Ca^{2+} transport across the plasma membrane (Heath, 1994; Wellburn, 1994) due to changes in the activity of plasma membrane Ca^{2+} channels and Ca^{2+} efflux mechanisms (for reviews, see Hetherington et al., 1992; Johannes et al., 1992; Schroeder, 1992; Evans, 1994; Ward et al., 1995; Webb et al., 1996b).

At high concentrations ($>10^{-5}$ M) the effects of methyl viologen and H_2O_2 are either independent of external Ca^{2+} or are caused by a loss of membrane integrity that causes complete disruption of intracellular Ca^{2+} homeostasis. The changes in membrane integrity and guard-cell viability observed in washout experiments (Fig. 5) and in studies using FDA (Figs. 6 and 7) support the latter explanation. At this concentration the effects of methyl viologen and H_2O_2 on stomatal aperture were irreversible. In addition, both treatments caused a reduction in the intensity and discreteness of the fluorescein fluorescence in the guard cells that coincided with the appearance of fluorescence in the epidermal cells. This may indicate accelerated leakage of fluorescein from the guard cells into the surrounding cells after a reduction in the integrity of the plasma membrane and intracellular membranes (Larkin, 1976). Furthermore, the rapid loss of fura-2, which occurred in 57% of cells in response to 10^{-5} M H_2O_2 (Fig. 9C) and in 29% of cells treated with 10^{-5} M H_2O_2 in the presence of EGTA (Fig. 10C), also implies a reduction in membrane integrity. Together, these data suggest strongly that at high concentrations ($>10^{-5}$ M) the deleterious effects of methyl viologen and H_2O_2 on stomatal behavior are directly related to loss of membrane integrity. This has the potential to cause the disruption or total breakdown of intracellular Ca^{2+} homeostasis through an unregulated increase in guard cell $[Ca^{2+}]_i$ to critical levels. Ca^{2+} overload of this type has been observed in animal cells after exposure to oxidative treatments (for reviews, see Orrenius et al., 1992; Harman and Maxwell, 1995).

Significance of Oxidative Stress-Induced Increases in Guard Cell $[Ca^{2+}]_i$

Increases in guard cell $[Ca^{2+}]_i$ may occur through either disruption of Ca^{2+} transport across the plasma membrane or release of $[Ca^{2+}]_i$ from internal stores. Oxidative stresses have been shown indirectly to have the potential to influence Ca^{2+} transport in plants (Castillo and Heath, 1990; Price, 1990; Fink, 1991; Le Thiec et al., 1994b). However, there is little direct evidence regarding their effect on the regulation of Ca^{2+} homeostasis (Price et al., 1994).

In animals many oxidative treatments have been shown to interfere with the mechanisms of Ca^{2+} transport across the plasma membrane through the inhibition of Ca^{2+} efflux and the stimulation of Ca^{2+} influx (for reviews, see Orrenius et al., 1992; Harman and Maxwell, 1995). If the mechanisms responsible for the regulation of intracellular Ca^{2+} homeostasis in plants are affected in a manner similar to those in animals, this may explain the increases in guard-cell $[Ca^{2+}]_i$ and the changes in stomatal reactions observed in response to low concentrations ($\leq 10^{-5}$ M) of methyl viologen and H_2O_2. However, at high concentrations ($>10^{-5}$ M) an additional explanation is required for the extreme effects of the two treatments on stomatal reactions. This may reflect oxidative stress-induced loss of membrane integrity.

The changes in intracellular Ca^{2+} homeostasis observed in response to oxidative stress have the potential to profoundly influence the processes of Ca^{2+}-based signal transduction in guard cells. These are responsible for the regulation of guard-cell turgor and the control of stomatal aperture (for reviews, see Mansfield et al., 1990; MacRobbie, 1992; Assmann, 1993; Ward et al., 1995; Webb et al., 1996b). This may explain the present observations. At low concentrations of methyl viologen and H_2O_2 ($\leq 10^{-5}$ M) changes in stomatal behavior, which can be inhibited by EGTA and/or verapamil (Figs. 3 and 4) and are reversible (Fig. 5), may be due to alterations in the Ca^{2+} homeostasis of guard cells, which causes the activation of Ca^{2+}-dependent enzymes involved in signal transduction (for review, see Poovaiah and Reddy, 1993) and modulates the activity of the ion channels involved in the regulation of guard-cell turgor (for reviews, see Mansfield et al., 1990; MacRobbie, 1992; Assmann, 1993; Ward et al., 1995; Webb et al., 1996b). In this concentration range the oxidative treatments also have the potential to stimulate Ca^{2+}-mediated changes in the organization of the cytoskeleton, particularly the microtubule component (Kim et al., 1995), of guard cells (for review, see Cyr, 1994). However, the reductions in membrane integrity and guard cell viability observed at high concentrations ($>10^{-5}$ M) of methyl viologen (Fig. 6J) and H_2O_2 (Figs. 7J, 9 and 10) may be the result of either (a) direct effects of the oxidative treatments through ozonolysis and indiscriminate lipid peroxidation (for reviews, see Heath, 1994; Wellburn, 1994), or (b) indirect effects through the activation of Ca^{2+}-dependent enzymes following an increase in $[Ca^{2+}]_i$, e.g. Ca^{2+}-dependent phospholipases and proteases. These enzymes have been implicated in the accelerated phospholipid turnover during anoxia (Chien et al., 1979) and the oxidative damage of cytoskeletal proteins (Mirabelli et al., 1989) that result in membrane damage in animals.

CONCLUSIONS

Our data show that methyl viologen and H_2O_2 affect stomatal behavior in a concentration-dependent manner. At low concentrations, the effects of both treatments are Ca^{2+}-dependent and appear to be the result of increases in guard-cell $[Ca^{2+}]_i$ and changes in guard-cell Ca^{2+} homeostasis. This has the potential to affect the regulation of guard-cell turgor and stomatal aperture and the control of gas exchange through alterations in the processes of Ca^{2+}-based signal transduction. However, at high concentrations the effects of methyl viologen and H_2O_2 appear to be independent of external Ca^{2+} and are associated with a reduction in membrane integrity and guard-cell viability. Whether this represents a typical response of plants to oxidative stress remains to be determined. We are currently addressing this question with particular reference to modified stomatal responses due to O_3 exposure.

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