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A POSSIBLE ROLE FOR CALCIUM IN OXIDATIVE PLANT STRESS

ADAM H. PRICE

Institute of Environmental and Biological Sciences, Lancaster University, Lancaster, LA1 4YQ, U.K.

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Disruption of calcium homoeostasis may be crucially important in damaging animal cells under oxidising conditions. In hepatocytes, oxidative stress causes an increase in cytosolic calcium, a process which is thought to be largely responsible for subsequent cell death. The oxidation of glutathione precedes a flux of calcium into the cytoplasm from internal and external sources. The possibility that similar events occur in plant cells has been tested using strips of epidermis taken from leaves of *Commelina communis*. The closure of stomata in response to many chemical or physical stimuli is thought to involve a transient rise in cytosolic calcium. Paraquat (10^{-4} M) and hydrogen peroxide (10^{-3} M) caused a marked reduction in stomatal aperture when included in the incubation medium of illuminated epidermal strips. The calcium channel blocker verapamil (10^{-5} M) protected stomata from hydrogen peroxide-induced closure while the ion chelator EGTA (2×10^{-3} M) protected stomata in both oxidising treatments. These results are discussed in so far as they support the hypothesis that a rise in cytosolic calcium may play a role in the pathology of oxidative stress in plant cells through a mechanism analogous to the liver cell system.

KEY WORDS: Calcium, oxidative stress, stomatal aperture, verapamil, EGTA, plant.

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INTRODUCTION

Accumulated evidence over recent years has shown that perturbation of calcium homoeostasis is an important event in the cytotoxicity of oxidising stress to animal cells.[1-3] A clear involvement of glutathione has been shown[4] and the subject has been recently reviewed.[5] In summary, the following sequence of events has been suggested; oxidative stress, oxidation of glutathione, oxidation of critical protein thiol groups, inhibition of calcium transport out of the cytosol, sustained increased cytosolic calcium concentration, loss of cell viability.

If oxidative stress in plant cells results in an impairment of calcium flux analogous to the liver cell system, then the stomatal guard cell may prove a valuable tool for initial investigation. Using strips of leaf epidermis it has been shown that verapamil (a calcium channel blocker[6]) and EGTA (ethylene glycol-O,O′-bis (2-aminoethyl)-N,N,N′, N-tetracetic acid) (a metal ion chelator with a relatively high affinity for calcium) reduce the response of stomatal guard cells to the hormone abscisic acid (ABA)[7] and to carbon dioxide,[8] both of which promote stomatal closure. The possibility that calcium acts as a secondary messenger in plant cells has recently been suggested following the observation of an increase of cytosolic calcium concentration in stomatal guard cells upon treatment with ABA at concentrations which subsequently cause stomatal closure.[9] These and other authors[7,8,10,11] conclude that increased calcium concentrations triggers the intracellular machinery responsible for the loss of turgor pressure which causes stomatal closure.

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If oxidative stress in plant cells causes increased cytoplasmic calcium concentrations and if elevated cytoplasmic calcium results in reduction of the stomatal aperture, then oxidising compounds should promote stomatal closure and, moreover, that closure may be modulated by the presence of calcium chelators or channel blockers. The results of a brief experiment to test this using oxidising agents (hydrogen peroxide and paraquat) in conjunction with verapamil and EGTA are presented here.

MATERIALS AND METHODS

Seedlings of *Commelina communis* L. were grown from seed.[10] Epidermis was peeled carefully from the abaxial surface of the youngest fully expanded leaf,[12] floated on 10^{-2} M MES (2-[N-morpholino]ethane sulphonic acid), pH6.2, and cut into 5 mm lengths. To promote stomatal opening, pieces of epidermis were incubated for 3 h at 25°C under a photon flux density of 200 umol $m^{-2}s^{-1}$ in 10^{-2} M MES, 5×10^{-2} M KCl, pH 6.2, aerated with CO_2-free air.[10] Treatment consisted of the inclusion of 10^{-3} M hydrogen peroxide, 10^{-4} M paraquat (methyl viologen), 10^{-5} M verapamil and 2×10^{-3} M EGTA, in the combinations indicated. The apertures of 40 stomata per treatment were measured under the microscope and the experiment repeated three times.

MES, EGTA, and KCl were obtained from Fluka Chemicals Ltd., Glossop. Paraquat, hydrogen peroxide and verapamil were obtained from Sigma Chemical Company, Poole.

Statistics For each of the four combinations of oxidising agent with a calcium antagonist, twoway analysis of variance was conducted to reveal oxidising agent/antagonist interactions.

RESULTS

After 3 hrs incubation the stomatal aperture of control strips was 14.2 $\pm$ 2.5 μm ($\pm$ standard deviation) (see Figure 1). Treatment with 10^{-5} M verapamil or 2×10^{-3} M EGTA resulted in apertures of 13.7 $\pm$ 1.8 μm and 14.9 $\pm$ 3.1 μm respectively. In neither case was this significantly different from the control (using a t-test). Both 10^{-3} M H_2O_2 and 10^{-4} M paraquat caused marked and significant reductions in stomatal aperture (2.7 $\pm$ 2.3 μm and 8.5 $\pm$ 4.01 μm respectively). When verapamil was present with the oxidising stress, the aperture was 4.1 $\pm$ 2.7 μm for H_2O_2 and 8.9 $\pm$ 3.8 μm for paraquat. When EGTA was present, the stomatal aperture was 4.1 $\pm$ 3.3μm for H_2O_2 and 12.5 $\pm$ 3.8 μm for paraquat.

These results were statistically analysed using twoway analysis of variance for each calcium antagonist/oxidising stress combination which reveals any interaction between calcium antagonists and oxidising compound. There was a highly significant interaction ($P > 0.001$) between EGTA and paraquat and between hydrogen peroxide and verapamil. That is, the effect of paraquat and EGTA in combination, for example, cannot be explained by the effects of the two compounds separately. EGTA reduces the response of stomata to paraquat treatment. Likewise, verapamil reduces the response of stomata to H_2O_2 treatment. Interaction between EGTA and H_2O_2 was not significant at $P = 0.05$ (presumably due to the slight opening of stomata by EGTA alone) whilst verapamil and paraquat did not interact.

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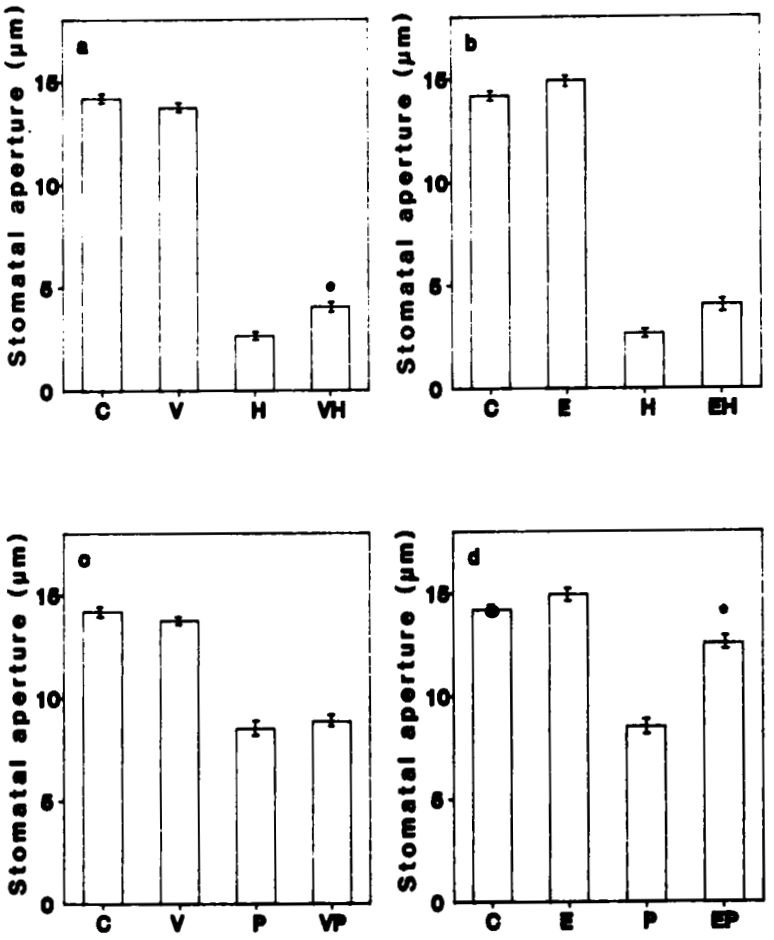

FIGURE 1 The effect of calcium antagonists and oxidative stress agents, alone and in combination, on stomatal aperture in epidermal strips; a: 10^{-5} M verapamil (V), 10^{-3} M hydrogem peroxide (H) and both (VH); b: 2×10^{-3} M EGTA (E), 10^{-3} M hydrogen peroxide (H) and both (EH); c: 10^{-5} M verapamil (V), 10^{-4} M paraquat (P) and both (VP); d: 2×10^{-3} EGTA (E), 10^{-4} M paraquat (P) and both (EP). In each case C represents no treatment control. Bars are standard errors ($n = 120$).

*Highly significant interaction ($P < 0.001$) between verapamil and hydrogen peroxide, and between EGTA and paraquat. Significant interaction was not present between verapamil and paraquat, nor between EGTA and hydrogen peroxide.

DISCUSSION

This experiment demonstrated that stomatal closure resulted from the treatment of epidermal strips with paraquat and hydrogen peroxide and that, in some cases, inclusion of verapamil or EGTA reduced the closure.

Paraquat acts by generating the superoxide ion in illuminated chloroplasts[13] which are present in stomatal guard cells but not other epidermal cells. Superoxide dis-

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mutase in the chloroplast will convert superoxide to hydrogen peroxide[14] which is able to cross the chloroplast membrane.[15] Both superoxide and hydrogen peroxide cause oxidation of glutathione, either directly or through coupling with ascorbate antioxidant function via the enzyme dehydroascorbate reductase.[15]

The evidence presented here suggests that verapamil and EGTA protect stomatal guard cells from closure induced by oxidative stress. While EGTA chelates many di- and trivalent ions it also chelates calcium and it has been shown to influence calcium-dependent stomatal closure.[8,10,11] It is possible that both paraquat and hydrogen peroxide treatment cause an influx of calcium ions into the stomatal guard cell. As with hepocytes, this may be preceeded by oxidation of glutathione. The increased calcium would then cause stomatal closure in the oxidised strips in a manner analogous to ABA treatment (although the cause and mechanism of calcium influx is different in the two cases).

The failure of verapamil and EGTA to prevent stomatal closure entirely in this experiment is expected since oxidative stress in liver cells mobilises calcium from internal[2] as well as external[16] sources and since verapamil and EGTA do not entirely prevent stomatal closure induced by ABA[7] or carbon dioxide[8] respectively, probably for similar reasons.

The pathology of calcium in animal cells subjected to oxidative stress is thought to involve calcium activation of a number of degratative enzymes.[5] For example, DNA fragmentation is thought to occur through the action of a calcium-activated endonuclease[17] while membrane damage may result from activation of a calcium-dependent phospholipase.[18] Similar calcium-activated degradation processes may occur in plant cells under oxidative challenge.

In conclusion, it is possible that disruption of calcium homoeostasis plays a crucial role in the pathology of oxidative stress in plants, including many environmental conditions such as drought[19,20] and air pollution.[21-24] Clearly, a more thorough investigation is warranted. The epidermal strip may provide a valuable system in this endeavour, particularly since direct measurements of cytosolic calcium concentration in guard cells using fluorescent dyes has recently been achieved.[9]

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