Partial inhibition of ABA-induced stomatal closure by calcium-channel blockers

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SUMMARY

ABA-induced increases in $[Ca^{2+}]_{cyt}$ (cytosolic free Ca^{2+}) may result from Ca^{2+} influx from the apoplast and/or release from intracellular stores. In this paper, Ca^{2+}-channel blockers have been used to investigate this question in the detached epidermis of *Commelina communis*. Examples from the benzothiazepine, dihydropyridine and phenylalkylamine series all inhibited ABA-induced stomatal closure: (±) verapamil > nifedipine > diltiazem. Inhibition was partial, the magnitude of the effect being dependent on both the concentration of ABA and that of the channel blocker. The maximum inhibition observed in the presence of 100 nM ABA was approximately 66% at high (100 nM) concentrations of (±) verapamil or nifedipine. In the near absence of extracellular Ca^{2+} (2 mM EGTA) ABA-induced stomatal closure was reduced by approximately 22% and the inhibition by Ca^{2+}-channel blockers abolished. Inhibition by (±) verapamil was totally reversible and exhibited signs of stereospecificity, the S(−) enantiomer being a more potent inhibitor of ABA-induced stomatal closure than the R(+) enantiomer. Bay K 8644 (a fluorinated analogue of nifedipine) exhibited biphasic action on 500 μM Ca^{2+}-induced stomatal closure, i.e. agonistic at low concentrations (10 nM), antagonistic at high concentrations (> 10 nM to 100 μM), but did not affect ABA-induced stomatal closure. These results suggest that Ca^{2+} release from intracellular stores may be important in the ABA-induced increase in $[Ca^{2+}]_{cyt}$ associated with stomatal closure. They do not, however, exclude a contribution of Ca^{2+} influx from the apoplast.

1. INTRODUCTION

The ability of abscisic acid (ABA) to stimulate stomatal closure and inhibit stomatal opening has been recognized for many years (Mittelheuser & van Steveninck 1969). This involves alterations in guard cell turgor driven by anion and cation effluxes (MacRobbie 1988, 1989). It has been suggested that ABA stimulates an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) which then acts to trigger the intracellular machinery responsible for stomatal closure (see Mansfield *et al.* (1990) for a review of the evidence upon which this hypothesis is based). We have shown an increase in $[Ca^{2+}]_{cyt}$ in the guard cells of *Commelina communis* in response to ABA, which precedes stomatal closure (McAinsh *et al.* 1990*a*). Such an increase in $[Ca^{2+}]_{cyt}$ may be the result of Ca^{2+} influx from the apoplast and/or release from internal stores. From flux studies, MacRobbie (1989, 1990) has concluded that an exclusively apoplastic origin is unlikely, whereas Smith & Willmer (1988), examining the effects of ABA on guard cell protoplasts, exclude any contribution of extracellular Ca^{2+}.

In this paper we report the results of studies designed to further investigate the origin of the ABA-induced increase in $[Ca^{2+}]_{cyt}$. Previous studies have shown that EGTA, verapamil and nifedipine reduce the ability of stomata to respond to ABA (De Silva *et al.* 1985). In the present study we have chosen to make use of various classes of organic compounds known collectively as Ca^{2+}-channel blockers (see Tester (1990) for a review of the activities of Ca^{2+}-active compounds in plants). Examples from three major groups of Ca^{2+}-channel blockers, diltiazem (a benzothiazepine), nifedipine (a dihydropyridine) and verapamil (a phenylalkylamine), are discussed. It has previously been established that these compounds bind to isolated plant membranes (Hetherington & Trewavas 1984; Andrejauskas *et al.* 1985, 1986; Dolle & Nultsch 1988*a*, *b*) and will also inhibit the entry of $^{45}Ca^{2+}$ into carrot protoplasts (Graziana *et al.* 1988). In addition, a verapamil-binding protein has been partly purified from higher plants (Harvey *et al.* 1989) which when reconstituted into planar lipid bilayers exhibits Ca^{2+}-channel activity (Tester & Harvey 1989). The Ca^{2+} agonist BAY K 8644, which is a fluorinated analogue of nifedipine (Nayler 1988), is also examined.

2. MATERIALS AND METHODS

(*a*) *Plant Material*

Seedlings of *Commelina communis* L. were grown from seed in John Innes No. 2 potting compost in a heated glasshouse

(minimum temperature 20 °C, 16 h day length at a minimum photon flux density of 100 μmol m^{-2} s^{-1}, 400–700 nm), and then transferred to a controlled environment chamber (temperature 25 ± 1 °C, 16 h day length at a photon flux density of 100 μmol m^{-2} s^{-1}) several days before use. Plants were kept free from water stress at all stages of development. Epidermis was peeled carefully from the abaxial surface of the youngest fully expanded leaf (Weyers & Travis 1981), floated on 10 mM MES, pH 6.15, and cut into 5 mm lengths.

(*b*) *Epidermal strip bioassay*

Pieces of epidermis were incubated under conditions promoting stomatal opening (at 25 ± 1 °C under a photon flux density of 150 μmol m^{-2} s^{-1} in 5 cm Petri dishes containing 10 ml of 10 mM MES, 50 mM KCl, pH 6.15, aerated with CO_2-free air) for 3 h (De Silva *et al.* 1985). The test chemicals were either then added to the incubation buffer as 10 μl aliquots of concentrated stock solutions, giving final concentrations ranging between 100 μM to 1 nM, or the epidermal strips transferred to 10 ml of fresh MES–KCl buffer, pH 6.15, containing the test chemicals at the required concentrations. The strips were incubated for a further 1 h and then mounted in the appropriate medium and examined microscopically to determine the aperture of the stomatal pores.

(*c*) *Perfusion system*

The effects of the light-sensitive dihydropyridines on stomatal aperture were examined by using a purpose-built perfusion system. This allowed pieces of epidermis to be incubated in a constant volume (10 ml) of continuously flowing (1 ml min^{-1}) media aerated with CO_2-free air. Pieces of epidermis were incubated under conditions promoting stomatal opening for 3 h (De Silva *et al.* 1985) and then transferred to the perfusion system. Epidermis was incubated for 1 h at 25 ± 1 °C under a photon flux density of 150 μmol m^{-2} s^{-1} in media containing 10 mM MES, 50 mM KCl, in which the test chemicals were dissolved. All solutions were maintained in the dark before entering the perfusion chamber. After incubation the epidermal strips were examined microscopically to determine the aperture of the stomatal pores.

A similar procedure was used to investigate the reversible effects of (±) verapamil on stomatal aperture. Pieces of epidermis were incubated under conditions promoting stomatal opening for 3 h (De Silva *et al.* 1985) and then incubated for a further 1 h in the presence of the test chemical. Subsequently, the epidermal strips were transferred to the perfusion system and washed for 1 h with media in the absence of the test chemical. The aperture of the stomatal pores was determined at each successive stage.

(*d*) *Chemicals*

MES and EGTA were obtained from Fluka (U.K.). (±) *cis–trans* ABA was obtained from Calbiochem (U.K.). Diltiazem, nifedipine and (±) verapamil were obtained from Sigma (U.K.). s(−) and R(+) verapamil were obtained from Research Biochemical Inc. (U.S.A.). BAY K 8644 was a gift from Bayer U.K. Ltd. ABA, BAY K 8644 and nifedipine were initially made up as concentrated stock solutions in ethanol. The final concentration of ethanol was never greater than 0.001 % (by volume), a level shown to have no effect on stomatal aperture.

3. RESULTS

(*a*) *Partial inhibition of ABA-induced stomatal closure by Ca^{2+}-channel blockers*

Ca^{2+}-channel blockers caused partial inhibition of ABA-induced stomatal closure. Diltiazem caused the least and (±) verapamil the greatest inhibition. The magnitude of the effect was dependent upon both the concentration of ABA and that of the channel blocker. Treatment with 10 μM channel blocker caused a reduction in the dose response to ABA (figure 1). This effect decreased with increasing ABA concentration, being significant ($p < 0.05$) for nifedipine and (±) verapamil in the range of 10 nM to 10 μM ABA and for diltiazem at 10 nM ABA. None of the channel blockers had a significant effect ($p < 0.05$) at either 1 nM or 100 μM ABA. For a fixed concentration of ABA (100 nM) inhibition of stomatal closure by Ca^{2+}-channel blockers was highly dose-dependent (figure 2). At channel blocker concentrations of 100 nM or greater, inhibition increased with concentration, reaching a maximum (*ca.* 33 % for diltiazem and 66 % for nifedipine and (±) verapamil) at 100 μM. EGTA (2 mM) significantly reduced ($p < 0.05$) 100 nM ABA-induced stomatal closure, by approximately 22 %, whilst abolishing totally the inhibition caused by 10 μM channel blocker (table 1). There was no significant effect ($p < 0.05$) of any of the channel blockers alone on stomatal aperture.

(*b*) *Ca^{2+}-channel agonist BAY K 8644*

There was no effect of 1 μM BAY K 8644 on ABA-induced stomatal closure (figure 3). However, Ca^{2+}-induced closure exhibited a marked response to BAY K 8644, the nature of which was highly dose-dependent. Treatment with 500 μM $CaCl_2$ reduced stomatal

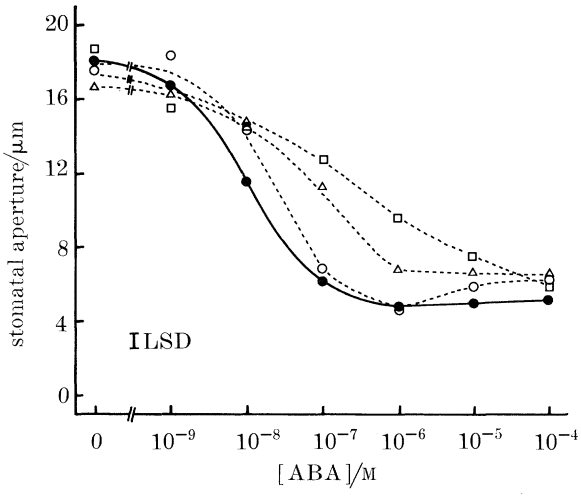

Figure 1. The effects of Ca^{2+}-channel blockers on the ABA-induced stomatal closure: ABA alone (filled circles), ABA + 10 μM diltiazem (open circles), ABA + 10 μM nifedipine (triangles) and ABA + 10 μM (±) verapamil (squares). Values are expressed as the means of measurements made on 90 randomly selected stomata. The least significant difference, LSD, ($p < 0.05$) is indicated.

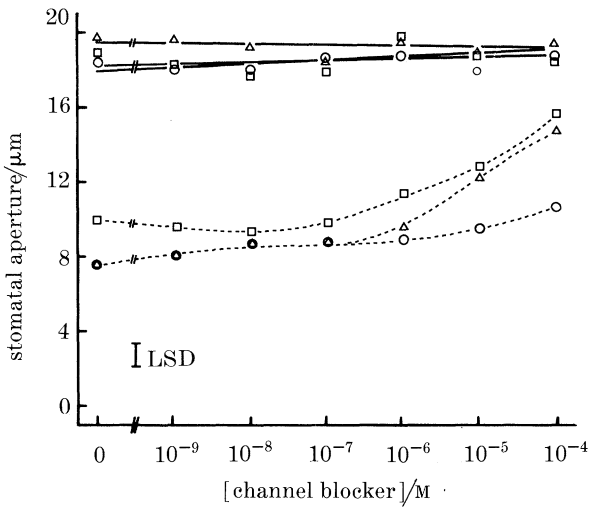

Figure 2. The effects of diltiazem (circles), nifedipine (triangles) and (±) verapamil (squares) on stomatal aperture: alone (solid lines) and in the presence of 100 nM ABA (broken lines). Values are expressed as the means of measurements made on 90 randomly selected stomata. The least significant difference, LSD, ($p < 0.05$) is indicated.

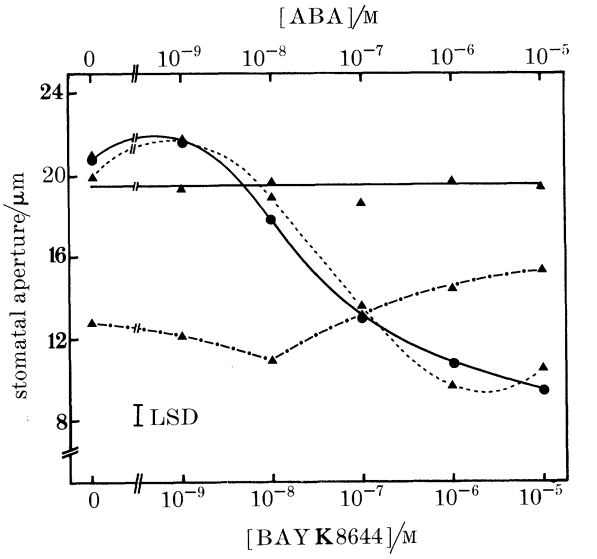

Figure 3. The effects of BAY K 8644 on ABA and Ca^{2+}-induced stomatal closure: BAY K 8644 alone (triangles, solid line), BAY K 8644 + 500 μM Ca^{2+} (triangles, broken-and-dotted line), ABA alone (circles) and ABA + 1 μM BAY K 8644 (triangles, broken line). Values are expressed as the means of measurements made on 90 randomly selected stomata. The least significant difference, LSD, ($p < 0.05$) is indicated.

aperture by approximately 6.5 μm (*ca.* 32%). At high concentrations (100 nM or greater) BAY K 8644 showed Ca^{2+}-antagonist properties, producing a partial inhibition of Ca^{2+}-induced stomatal closure (inhibition increasing with concentration), whereas at 10 nM it showed significant ($p < 0.05$) Ca^{2+}-agonist properties, appearing to promote closure. Below 10 nM the effects of BAY K 8644 were negligible. There was no significant effect ($p < 0.05$) of BAY K 8644 alone on stomatal aperture.

Table 1. *The effects of EGTA (2 mM) and/or Ca^{2+}-channel blockers (10 μM) on the stomatal closure in response to treatment with 100 nM ABA*

(Values are expressed as the means of 90 randomly selected stomata ± s.e.m.)

treatment	stomatal aperature/μm
none (control)	17.0 ± 0.2
EGTA	16.1 ± 0.2
ABA	5.6 ± 0.2
ABA + EGTA	8.1 ± 0.2
ABA + EGTA + (±) verapamil	8.4 ± 0.2
ABA + EGTA + nifedipine	8.2 ± 0.2
ABA + EGTA + diltiazem	8.0 ± 0.2

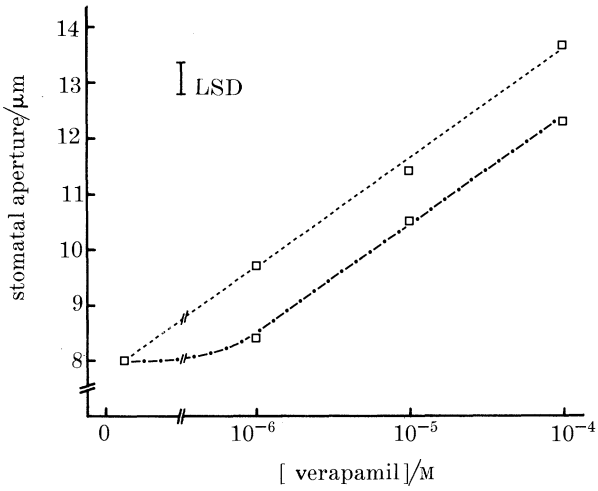

Figure 4. The effects of stereoisomers of verapamil on ABA-induced stomatal closure: S(−) verapamil (broken line) and R(+) verapamil (broken-and-dotted line). Values are expressed as the means of measurements made on 90 randomly selected stomata. The least significant difference, LSD, ($p < 0.05$) is indicated.

(c) *Differential inhibition of ABA-induced stomatal closure by the S(−) and R(+) enantiomers of verapamil*

Both S(−) and R(+) verapamil produced a significant reduction ($p < 0.05$) in 100 nM ABA-induced stomatal closure (figure 4). Inhibition increased linearly with verapamil concentration for both the S(−) and R(+) enantiomers. At all concentrations of verapamil tested, the S(−) enantiomer was a more potent inhibitor of ABA activity, achieving approximately 1.3 μM (*ca.* 11%) greater inhibition of ABA-induced stomatal closure, than the R(+) enantiomer. There was no significant effect ($p < 0.05$) of either the S(−) or R(+) enantiomer of verapamil alone on stomatal aperature (data now shown).

(d) *Reversible inhibition of ABA-induced stomatal closure by (±) verapamil*

Treatment with 10 μM (±) verapamil for 1 h caused a reduction of *ca.* 37% in 100 nM ABA-induced stomatal closure (table 2). This effect was totally

Table 2. *Reversible inhibition of ABA-induced stomatal closure by (±) verapamil*

(Epidermal strips were incubated in the presence of 100 nM ABA and/or 10 μM (±) verapamil for 1 h. Values are expressed as the means of 90 randomly selected stomata ± s.e.m.)

treatment	stomatal aperture/μm
none (control)	18.4 ± 0.2
verapamil	18.0 ± 0.2
ABA	6.5 ± 0.2
ABA + verapamil	10.8 ± 0.2
ABA washout*	6.7 ± 0.3

* Strips pre-treated with 100 nM ABA + 10 μM (±) verapamil for 1 h before washing for 1 h with 100 μM ABA only.

reversed by washing with 100 nM ABA for 1 h in the absence of (±) verapamil. There was no effect of (±) verapamil alone on stomatal aperture.

4. DISCUSSION

Our results show that ABA-induced stomatal closure is reduced in the presence of EGTA and inhibited by Ca^{2+}-channel blockers. This is consistent with earlier observations of De Silva *et al.* (1985). Inhibition by Ca^{2+}-channel blockers is partial, dose-dependent, abolished totally in the near-absence of extracellular Ca^{2+} (2 mM EGTA) and, in certain instances, totally reversible. The abolition of inhibition at vanishingly low concentrations of extracellular Ca^{2+} suggests that in guard cells the effect of these chemicals is to block Ca^{2+}-channels. Tester & MacRobbie (1990) have also noted reversible inhibition of the 'Ca^{2+}-dependent' inward current during the action potential in *Chara* by 1,4-dihydropyridines. Therefore, an easily accessible site of action, notably plasma membrane Ca^{2+}-channels, is indicated. ABA induces an elevation of $[Ca^{2+}]_{cyt}$ which precedes stomatal closure (McAinsh *et al.* 1990*a*), and it has been suggested that this increase triggers the intracellular machinery responsible for stomatal closure (see Mansfield *et al.* (1990) for review). Inhibition of ABA-induced stomatal closure by EGTA and chemicals that are known to block Ca^{2+}-channel activity in animals (Nayler 1988), which bind to isolated plant membranes (Andrejauskas *et al.* 1985, 1986) and which appear only to effect Ca^{2+}-channels in plants may, on first consideration, seem to suggest an apoplastic origin for the ABA-induced increase in $[Ca^{2+}]_{cyt}$. However, the different sensitivities of Ca^{2+}-channels in animal and plant systems (Tester & MacRobbie 1990) necessitate care when assigning direct cause-and-effect relations to these chemicals. Notably, for both EGTA and all of the Ca^{2+}-channel blockers tested inhibition was only partial, the maximum achieved being approximately 66% at high (100 μM) concentrations of either (±) verapamil or nifedipine. These results, therefore, suggest that influx of Ca^{2+} from the apoplast into guard cells may not constitute the only source of the ABA-induced increase in $[Ca^{2+}]_{cyt}$.

Smith & Willmer (1988), examining the effects of ABA and Ca^{2+} in guard cell protoplasts of *C. communis*, concluded that there was no involvement of extracellular Ca^{2+} in the ABA response. They did not rule out the involvement of Ca^{2+} released from intracellular stores. MacRobbie (1989), measuring $^{45}Ca^{2+}$ fluxes in isolated stomatal guard cells of *C. communis*, showed that the ABA response did not involve a sustained increase in Ca^{2+} influx at the plasma membrane. However, studies of the effects of Cd^{2+}, Ba^{2+} and tetraethylammonium chloride (TEA) on $^{86}Rb^{+}$ efflux (MacRobbie 1990) were consistent with an ABA-induced release of Ca^{2+} from intracellular stores, possibly mediated by a second messenger. Consequently, the observation that both EGTA (reducing extracellular Ca^{2+} to vanishingly low concentrations) and Ca^{2+}-channel blockers (particularly at concentrations believed to be of the most physiological relevance) only cause partial inhibition of ABA-induced stomatal closure may be indicative of the importance of the release of Ca^{2+} from intracellular stores.

This conclusion is consistent with the model of signal transduction in stomatal guard cells (McAinsh *et al.* 1990*b*) in which it has been suggested that one mechanism by which ABA could induce an increase in $[Ca^{2+}]_{cyt}$ is to initiate the release of Ca^{2+} from intracellular stores, possibly through stimulation of phosphoinositide metabolism. Inositol 1,4,5–trisphosphate ($InsP_3$) is known to stimulate the release of Ca^{2+} from isolated vacuoles of higher plants (Schumaker & Sze 1987; Ranjeva *et al.* 1988; Alexandre *et al.* 1990) and *Neurospora* (Cornelius *et al.* 1989) in a manner analogous to the $InsP_3$-mediated movement of Ca^{2+} across endomembranes in animal cells (Altin & Bygrave 1988; Berridge & Irvine 1989). Recently, Gilroy *et al.* (1990*b*) have reported an increase in $[Ca^{2+}]_{cyt}$ following the photolytic release of $InsP_3$ from inactive caged $InsP_3$ introduced into the guard cells of *C. communis*. Similarly, Blatt *et al.* (1990) have shown a reduction in the inward-rectifying K^{+} current in the guard cells of *Vicia faba* after the photolytic release of $InsP_3$ from inactive caged $InsP_3$. This provides a link between the putative $InsP_3$-mediated release of intracellular Ca^{2+} stimulated by ABA, the ABA-induced increase in $[Ca^{2+}]_{cyt}$ (McAinsh *et al.* 1990*a*) and the closure of plasma membrane K^{+}-channels during ABA-induced stomatal closure.

Apoplastic Ca^{2+} may still make an important contribution to the ABA-induced increase in $[Ca^{2+}]_{cyt}$. It has been shown that the plasma membrane anion channels fundamental to the proposed action of ABA in guard cells require a 'predepolarization' of the plasma membrane for their activation. One way in which this may be achieved is through the opening of plasma membrane Ca^{2+}-channels, allowing Ca^{2+} influx from the apoplast (Schroeder & Hagiwara 1990). In addition, there may be a Ca^{2+}-mediated Ca^{2+} influx. Ca^{2+} released from intracellular stores could activate plasma membrane Ca^{2+}-channels through phosphorylation by a Ca^{2+}-dependent protein kinase. There is evidence that phosphorylation controls Ca^{2+}-channel activity in plants (Shimmen & Tazawa 1977; Zhere-

lova *et al.* 1985; Shiina & Tazawa 1986; Shiina *et al.* 1988; Hodick & Sievers 1988) and Ca^{2+}-dependent protein kinases are known to be widely distributed in higher plant tissues (Ranjeva & Boudet 1987; Hetherington *et al.* 1990; Budde & Randall 1990). Stimulation of Ca^{2+} influx from the apoplast may be vital to the elevations in $[Ca^{2+}]_{cyt}$ reported in response to ABA (McAinsh *et al.* 1990*a*).

It must be noted, however, that the interpretation of data obtained from Ca^{2+}-channel blocker studies may be complicated by several factors. For example, incomplete blocking of plasma membrane Ca^{2+}-channels, owing to the presence of Ca^{2+}-channels in the guard cell plasma membrane which are either insensitive to or have a different sensitivity to blockers of Ca^{2+}-channels in animals, could result in partial inhibition of ABA-induced stomatal closure. In addition, although the present studies suggest the importance of Ca^{2+} released from intracellular stores they do not preclude the possibility: (i) that Ca^{2+} forms part of, but may not be the key regulator in, the signal transduction pathway; or (ii) that there may be both a Ca^{2+}-dependent and a Ca^{2+}-independent signal transduction pathway through which ABA initiates stomatal closure. In both of these alternatives, blocking Ca^{2+} influx at the plasma membrane would cause a partial inhibition of the signal transduction pathway without abolishing the response to ABA. A recent preliminary report (Gilroy *et al.* 1990*a*) may provide some support for the latter. However, no additional mechanism of signal transduction has, as yet, been identified in higher plant systems.

The reduction in ABA-induced stomatal closure observed in the presence of EGTA (*ca.* 22%) is less than that reported previously (De Silva *et al.* 1985). This may arise through the use of different experimental protocols; earlier workers examining inhibition of opening over 3 h compared with the present 1 h inhibition of closure studies. A similar degree of inhibition (up to 30% in 2 mM EGTA) has also been detected in separate closure studies (A. Webb, personal communication). However, it must be noted that no effect of EGTA on ABA-induced contraction of guard cell protoplasts has been detected (Smith & Willmer 1988).

The extent to which Ca^{2+}-channel blockers inhibited ABA-induced stomatal closure varied; (±) verapamil > nifedipine > diltiazem. The substantial inhibition observed with (±) verapamil might be expected if Ca^{2+} influx from the apoplast is involved in the ABA response. Verapamil is known to inhibit $^{45}Ca^{2+}$ fluxes in plants (Zherelova *et al.* 1985; Tsutsui *et al.* 1987; Graziana *et al.* 1988; Zherelova 1989), and the binding of verapamil to plant membranes (Andrejauskas *et al.* 1985, 1986; Graziana *et al.* 1988; Dolle & Nultsch 1988*a*) and to membrane proteins (Harvey *et al.* 1989) that exhibit Ca^{2+}-channel activity (Tester & Harvey 1989) has been clearly demonstrated. Similarly, the lesser effects of diltiazem are consistent with the results of earlier studies on *Nitellopsis* (Shiina & Tazawa 1987) and microsomes and carrot protoplasts (Graziana *et al.* 1988) in which there was no effect on $^{45}Ca^{2+}$ entry and in which binding to microsomes was only detected at low ionic concentrations. The inhibition of Ca^{2+}-mediated processes by nifedipine is more controversial in plant systems. Graziana *et al.* (1988) have been unable to identify 1,4-dihydropyridine receptors in microsomes or carrot protoplasts and have found no effect on $^{45}Ca^{2+}$ entry. Hetherington & Trewavas (1984), however, have reported low levels of [^{3}H]-nitrendipine binding sites in pea membranes, and in *Chara* (MacRobbie & Banfield 1988) and *Nitellopsis* (Shiina & Tazawa 1987; Zherelova 1989) inhibition of $^{45}Ca^{2+}$ fluxes by 1,4-dihydropyridines has been shown. It has therefore been suggested that the channels in carrot protoplasts are different, possibly as a result of the enzyme treatment (Tester 1990). This may also be true in stomatal guard cells, although further clarification of this question is required.

The present studies provide evidence to suggest a degree of stereospecificity in the inhibition of ABA-induced stomatal closure by Ca^{2+}-channel blockers. This conclusion has also been drawn for a range of Ca^{2+}-active compounds in microsomes and in carrot protoplasts (Graziana *et al.* 1988).

In animals, BAY K 8644, a fluorinated analogue of nifedipine, exhibits a biphasic action on Ca^{2+}-channel activity, i.e. agonistic at low concentrations, antagonistic at high concentrations (Schramm *et al.* 1983; Nayler 1988). The present results show a similar mode of action in stomatal guard cells. Tester & MacRobbie (1990) have also observed an inhibitory effect of BAY K 8644 on the 'Ca^{2+}-dependent' inward current during the action potential in *Chara* at high concentrations (1 μM to 100 μM), and MacRobbie & Banfield (1988) have shown inhibition of $^{45}Ca^{2+}$ influx into depolarized cells of *Chara* by 10 μM BAY K 8644. These observations have been explained in animal systems by postulating that either the dihydropyridine receptor contains two binding sites, one associated with activation and another with inhibition (Kokubun *et al.* 1986), or that subtypes of dihydropyridine receptors exist which mediate activation and inhibition (Hosey & Lazdunski 1988). Tester & MacRobbie (1990) have suggested that an analogous situation exists in plants. If correct, this idea may help to explain some of the anomalies surrounding the activity of dihydropyridines in plant systems (i.e. through the detection of different populations of receptors or different sites within the same receptor). In contrast to Ca^{2+}-induced stomatal closure there was no effect of BAY K 8644 on ABA-induced closure, irrespective of concentration. This provides corroborative evidence for the involvement of Ca^{2+} of intracellular origin in ABA-induced increases in $[Ca^{2+}]_{cyt}$.

In conclusion, the results of this study, along with other evidence discussed here, suggest an important role for Ca^{2+} released from intracellular stores in guard cell stimulus response coupling. However, the contribution of Ca^{2+} influx from the apoplast to increases in $[Ca^{2+}]_{cyt}$ is not ruled out. Digital ratio imaging techniques may be used to determine the relative contributions of these two sources of Ca^{2+} (work currently in progress). This paper also raises interesting questions as to the nature of Ca^{2+}-channel blocker receptors in stomatal guard cells, particularly dihydro-

pyridine receptors, the answers to which lie in the further characterization of the mode of action of Ca^{2+}-channel blockers in higher plant systems.

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