A CRITICAL ASSESSMENT OF THE ROLE OF ABSCISIC ACID AND CALCIUM IONS IN REGULATING STOMATAL MOVEMENTS IN *COMMELINA COMMUNIS* L.

BY

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ABSTRACT

The inhibition of stomatal opening in Commelina communis L. by abscisic acid (ABA) appeared to be dependent on the availability of calcium ions. Synergism between calcium ions and ABA in suppressing stomatal opening was observed, and a factorial experiment revealed a highly significant interaction between calcium and abscisic acid. The effect of calcium appeared to be on the latter stages of the opening process, and might have been the result of an inhibition of potassium accumulation in the guard cells. The ABA-induced stomatal closure was barely detectable when the apoplastic calcium was chelated by EGTA.

The divalent cation ionophore, A 23187, reduced stomatal opening presumably because it mimicked the effect of ABA in increasing the permeability of the plasma membrane of the guard cells to calcium. Agents such as La^{3+}, nifedipine and verapamil, which in animal tissues are known to block calcium channels, and thus the free passage of calcium, reduced the ability of stomata to respond to ABA.

It is proposed that increased calcium levels in the cytosol of the guard cells could activate the calcium-binding modulator protein, calmodulin, which in turn activates one or more of the enzymes which are responsible for reducing K^{+} accumulation in the guard cells: compounds which are known to antagonize calmodulin reduced the effects of abscisic acid in the same quantitative way as calcium channel blockers.

It is suggested that the action of ABA on guard cells requires a free passage of calcium ions into the cytosol. The calcium ions may then act as 'second messengers' interacting with calmodulin to produce the overt cellular response to ABA.

A study on the differential behaviour in opening between adaxial and abaxial epidermes revealed that different endogenous levels of ABA

and/or calcium could be responsible for the observed disparity, and that EGTA and La^{3+} could reduce the normal differences in stomatal opening between the two epidermes.

From the results of a preliminary study, it is concluded that electron probe X-ray microanalysis is not a suitable method for the measurement of intracellular calcium ion concentrations.

The results are discussed in relation to the concept of hormones in plants, which has recently been attacked by a number of plant physiologists on the basis that there is no convincing evidence that plants have hormones which work in a comparable way to those in animals.

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CHAPTER ONE

GENERAL INTRODUCTION

Stomata are pores formed by a pair of highly specialized cells, guard cells, which are found on the surface of aerial parts of most higher plants. They are the portals through which plants communicate with the aerial environment. They provide higher plants with a primary means of adjusting to their continuously changing environment (Cowan, 1982). In his recent review, Zeiger (1983) pointed out the functional analogy between stomata and cell membranes: stomatal movements result in a preservation of biological integrity (at organismal level) while allowing physical exchange between the plant body and the surrounding air. Stomata ensure gaseous exchange necessary for photosynthesis and respiration while retaining an adequate water content essential for cellular activities (Mansfield, Wellburn & Moreira, 1978; Raschke, 1975).

Zeiger (1983) further described the central role of stomata as the maintenance of plant homeostasis. Homeostasis is a fundamental biological property of living organisms, which they need in order to regulate their internal milieu while interacting with their environment (Curtis, 1979). The optimization theory explained by Cowan and others (Cowan, 1982; Cowan & Farquhar, 1977; Farquhar & Sharkey, 1982) which proposes that stomata maximize the rate of carbon gain while minimizing water loss, describes a particular case of homeostasis. Guard cells integrate internal and external stimuli and modulate stomatal apertures to balance the opposing priorities of gaining atmospheric CO_2 and conserving water. The ability of guard cells to modulate stomatal aperture accordingly, and the factors (or stimuli) which bring about changes in stomatal aperture are of immediate importance with respect to the survival of land plants (Mansfield, 1985; Mansfield & Davies, 1985).

One of the physical problems encountered by land plants is the

maintenance of an aqueous environment within the leaves exposed to the desiccating influence of the atmosphere (Davies & Mansfield, 1982). When, as commonly occurs, the amount of water lost by transpiration exceeds the amount of water taken up by roots, leaf water deficits develop, and these may exert a considerable influence over plant growth and development. If, however, plants can maintain a minimum leaf water content, severe effects of water deficits may be avoided. The most effective combination of responses that will ensure that a favourable leaf water status is maintained in a plant exposed to dry air and/or growing in drying soil is a limitation of leaf water loss coupled with the maintenance of an adequate rate of water uptake by roots. The control of the expenditure of water in this manner, however, would inevitably lead to a reduction in CO_2 gain, and in consequence crop yield would fall.

Stomata play a major role in controlling the rate of transpiration. There seem to be two major ways in which stomata respond to dry conditions to reduce water loss and to maintain the turgor of the plant. The first consists of direct responses of stomata to water deficit in the atmosphere (generally called the 'humidity response') and the second occurs when parts of the plant experience a water deficit, and endogenous chemical changes occur to induce partial or total closure of stomata (Appleby & Davies, 1983; Fanjul & Jones, 1982; Mansfield, 1983, 1985). Fanjul & Jones showed that stomatal conductance of well-watered apple trees decreased within one minute of an increase in vapour pressure saturation deficit (VPD) of the atmosphere. Nevertheless, stomata of all species are not sensitive to changes in humidity, and also the degree of responsiveness to humidity can change within a species pretreated in different environments (Appleby & Davies, 1983; Bunce, 1981; Osonubi &

Davies, 1980).

The second method of controlling stomatal movements in response to a condition(s) which would lead to plant water deficits is hormonal regulation. There is considerable controversy over the use of the term 'hormone' in relation to plants (Trewavas, 1981; Trewavas & Cleland, 1983; Weyers, 1984), and this will be dealt with in a later part of this thesis. For the time being the term 'hormone' will be used, because the alternatives such as 'growth substance' or 'growth regulator', are inappropriate in the context of their regulatory role over stomata, that is, when the process under control is not a growth phenomenon (Mansfield, 1985).

Role of abscisic acid in stomatal movements

The involvement of plant hormones in the control of stomatal movements has long been recognized (Wright, 1969; Wright & Hiron, 1969). These workers found that when wheat leaves were allowed to wilt they accumulated the 'inhibitor-β' complex, which was first recognized on paper chromatograms and which had been reported as having growth inhibitory properties (Bennett-Clark & Kefford, 1953). The main component of this complex was found to be abscisic acid (ABA) (Milborrow, 1967). Since then ABA has been found to increase in a wide range of plant species during any treatment that will lead to a decrease in leaf water potential (Allaway & Mansfield, 1970; Beardsell & Cohen, 1975; Blackman & Davies, 1985; Hiron & Wright, 1973; Loveys & Kriedemann, 1973; Mansfield & Davies, 1981; Milborrow & Robinson, 1973; Ogunkanmi, 1974; Radin & Ackerson, 1982; Walton, 1980). Further to the discoveries of the increase in ABA levels in response to leaf water stress, it was found that application of exogenous ABA could reduce transpiration or bring about stomatal closure in many plants. Little & Eidt (1968) showed that ABA could

effectively reduce transpiration rate by about 50% in Picea glauca and Abies balsmea, when cuttings were immersed in an aqueous solution of ABA. Mittelheuser & Van Steveninck (1969) presented evidence that an application of ABA to the transpiration stream of excised leaves of wheat and barley, resulted in partial closure of stomata. A similar effect was observed in excised leaves of tobacco, by Jones & Mansfield (1970), who also observed stomatal closure in Xanthium strumarium L. as a result of foliar application of an aqueous solution of ABA to the whole plant.

Such stomatal closure induced by application of low concentrations of abscisic acid occurs very rapidly (Mittelheuser & Van Steveninck, 1971), and can occur within minutes of the application (Cummins et al., 1971; Kriedemann et al., 1972). It was estimated by these workers that the closure occurred when the endogenous level of ABA had increased two-fold. It was found that the closing effect of ABA was not a result simply of an increase in intracellular CO_2 concentrations (Jones & Mansfield, 1970) since the effect could not be reversed by flushing the leaves with CO_2-free air. A direct effect of ABA on the function of stomata was, therefore, implied.

Furthermore, Mittelheuser & Van Steveninck (1971) reported that ABA decreased CO_2 assimilation rates after an increase in stomatal resistance. They found that stomatal responses occurred earlier than the effects on CO_2 incorporation, and ergo, they suggested that ABA-induced increase in stomatal resistance was the cause of the decrease in rates of photosynthesis in treated leaves. The work of Jones & Mansfield (1972), showed that a single foliar application of ABA or its methyl and phenyl esters to the young barley plants, caused a reduction by about 50%, in the amount of water transpired over a nine day experimental period. This finding is in agreement with that

of Little & Eidt (1968). Jones & Mansfield (1972), further observed that in spite of the reduction in transpiration (and possibly the rate of photosynthesis), there was no substantial change in the rate of dry matter accumulation, which one would have expected if ABA directly affected CO_2 assimilation. A direct involvement of ABA in determining stomatal movements was recognized with the discovery of a mutant of tomato (flacca) which was unable to maintain its turgor under normal atmospheric conditions; this was found to be a result of an inability to close its stomata and, consequently, the excessive transpiration which occurred (Imber & Tal, 1970; Tal, 1966). These workers found that if flacca seedlings were sprayed with an aqueous solution of ABA once a day, then the plants regained turgor within several days and showed no phenotypic difference from the normal genotype. Analyses of endogenous hormone levels showed higher than normal amounts of indol-3-yl-acetic acid and reduced levels of ABA in the mutant (Tal & Imber, 1970; Tal et al., 1970). Tal et al. (1970), found that flacca plants contained only 10-20% as much ABA as normal plants, even though the flacca plants were often wilted, a condition that normally stimulates ABA synthesis. These reports were further supported by the subsequent findings by Tal & Nevo (1973), Bradford (1983) and Bradford et al. (1983), and it was suggested that the primary cause of the 'wilty' nature in the mutant was due to an interruption in the biosynthesis of ABA; there was, therefore, a lack of ABA which was accompanied by several other changes such as rise in IAA content.

Although there is substantial evidence that ABA inhibits stomatal opening and that in water-stressed leaves there are often considerable increases in endogenous ABA levels, there remain certain difficulties in understanding its mode of action. Especially there have been some

doubts about the timing, with respect to stomatal movements, of both the initial accumulation and the eventual decline of ABA levels recorded in water-stressed leaves. It has been shown that, as water-stress developed the stomata of some plants closed prior to any detectable accumulation of ABA (Beardsell & Cohen, 1975). These results indicated that the stomatal apertures decreased before the bulk-tissue level of ABA had risen appreciably, and, moreover, the recovery of full opening lagged considerably behind the disappearance of ABA. It was also observed by some other workers, that a rise in ABA level was often evident after 20 minutes of stress (Pierce & Raschke, 1980) although stomatal closure was initiated much earlier, even as early as five minutes after the stress was imposed (Gratziani & Livne, 1971; Henson, 1981; Ludlow et al., 1980). It was on grounds of this sort that Trewavas (1981) raised the question whether ABA can, in fact, be the primary agent responsible for stomatal closure in water-stressed plants. However, there are some feasible but not proven answers to the questions of timing of stomatal responses of water-stressed plants. It is now known that ABA is present in the mesophyll chloroplasts of well-watered plants (Railton et al., 1974) and this may be released in the early stages of water-stress (Loveys, 1977; Mansfield et al., 1978). If ABA is released from the mesophyll chloroplasts, and redistributed to sites of action, such as the guard cells, in response to a stress signal, then there need not be any change in the bulk ABA level of the leaf during the initial stages of stomatal closure. Alternatively, there might be a de novo synthesis of ABA in the guard cells themselves when water-stress is developed (Weiler et al., 1982), although there are some reports to the contrary, namely that ABA is preferentially synthesized in the cytoplasm rather than in the chloroplasts (Hartung et al., 1981). The work by Weiler

et al. (1982) was carried out using a sensitive immuno-assay capable of detecting picogramme quantities of ABA with highly purified guard cell protoplasts. Their data, however, have left some doubts in extrapolating these findings to in situ conditions (Mansfield & Davies, 1983). In particular, the temperature at which they carried out the experiment (i.e. $0^{o}C$) and the fact that the initiation of ABA production was said to occur at zero turgor, a situation which is unlikely to be found under natural conditions in guard cells.

It has been calculated that only about 10^{-14}g of ABA per stomatal complex is needed to induce stomatal closure even in a species whose stomata are not the most sensitive to the hormone (Jewer et al., 1981; Weyers & Hillman, 1979). Under these circumstances, one must doubt whether the sensitivity of the immunoassay techniques is sufficient for them to be employed for the detection of any immediate increase in ABA levels with the onset of water-stress, or of the persistence of physiologically active amounts of ABA in the guard cells after the restoration of turgor (Mansfield, 1985; Mansfield & Davies, 1983).

Hartung et al. (1982) found that ABA biosynthesis in protoplasts could not be stimulated by a hyperosmotic treatment. This is in contrast to the findings of Weiler et al. (1982). There is further evidence to suggest that guard cells are not the sites of ABA production. Loveys (1977) could not detect any increase in the ABA content of the detached epidermis of Vicia faba after exposure to an osmotic treatment sufficient to induce ABA production in the intact leaf. However, he found a four-fold increase in the level of ABA in epidermis which had been removed from previously water-stressed leaves. Dörffling et al. (1980) also could not detect any stress-induced ABA biosynthesis in isolated epidermis. This appeared to confirm the earlier finding of Loveys (1977) that ABA is transported to the guard cells from else-

where (probably the mesophyll chloroplasts) in response to a stress signal. However, it is important not to disregard evidence which supports at least some biosynthesis of ABA in the guard cells themselves.

It can now be accepted that much of the ABA in leaves is synthesized in the cytoplasm of mesophyll cells (Loveys, 1977). Heilman et al. (1980) found that ABA is synthesized in the cytosol of spinach mesophyll cells and then trapped in the chloroplasts as a result of the relatively high pH gradient. The pH of the chloroplast stroma is higher than that of the cytoplasm, particularly when illuminated. ABA is a weak acid (pka 4.5), and the dissociated ABA molecule is able to penetrate the chloroplast membrane, where it becomes trapped due to the alkalinity within the chloroplast. Milborrow (1984) calculated that, if the pH of chloroplast stroma is 7.5 and that of cytoplasm is 6.5, the distribution ratio of ABA between chloroplast and cytoplasm of turgid leaves would be 10:1. In contrast, mesophyll chloroplasts of wilted leaves contained a much smaller proportion of total ABA (15%) than in those of turgid leaves (95%) (Loveys, 1977). This could be due to the stress-induced ABA release from the chloroplasts (Hartung et al., 1981) providing a mechanism for its rapid appearance in the cytoplasm, without the need for synthesis of new ABA (Mansfield, 1986).

Milborrow (1979) suggested that a reduction in intrachloroplastic ABA would initiate a feedback mechanism responsible for the biosynthesis of ABA. However, Schultz & Dörffling (1981) were unable to confirm this from their experiments with spinach and Commelina communis plants. They did, however, present evidence for changes in both the chloroplast envelope and thylakoid membrane of farnesol-treated spinach plants, that had previously been reported in Sorghum (Fenton et al., 1976; Mansfield et al., 1978). Such changes did not induce an increase in

ABA biosynthesis.

Although it is now well established that ABA inhibits stomatal opening and that is accumulated in water-stressed leaves bringing about stomatal closure, the mechanism of its action on stomata or any other system has not yet been elucidated. Nonetheless, a careful scrutiny of the recent discoveries would suggest that its mode of action may be on membrane function, especially on the membrane permeability to, and active transport of, potassium ions and even perhaps on hydraulic conductivity (Van Steveninck & Van Steveninck, 1983). There have been controversial views on the effects of ABA on K^+ transport, as to whether the action of ABA is to inhibit proton transport and, consequently, a reduction in K^+ uptake (Raschke, 1977, 1979) or alternatively as to whether it is due to a stimulation of K^+ efflux (MacRobbie, 1981; Weyers & Hillman, 1980). Evidence is now accumulating in favour of the latter. This will be presented in detail in Chapter 3 of this thesis.

Role of calcium as a regulator in living systems

Many naturally occurring and synthetic chemicals have been shown to induce stomatal closure. One of the natural agents known to be highly effective is calcium, but its physiological role as a regulator of guard cell turgor has received little consideration. Iljin (1957) and Fujino (1967) found that calcium ions could interfere with stomatal opening and Willmer & Mansfield (1969) reported that 1 mol m^{-3} $CaCl_2$ almost totally inhibited opening in epidermal strips of Commelina communis.

It is well known that calcium plays an important role in plant growth and development. The function of calcium as a 'second messenger' in animal cells has been acknowledged for many years (Rasmussen, 1970). However, it is only recently that most botanists have come to appreciate

the concept that activities of plant cells are also regulated by calcium ions (Hepler & Wayne, 1985).

The concept of the 'second messenger' has been successful in explaining a diverse array of physiological events involving transduction of extracellular signals into an intracellular language which can be understood by the biochemical and biophysical machinery of the cell. The first such intracellular second messenger to be discovered was cyclic 3',5'-adenosinemonophosphate (cAMP) (for review see Robison et al., 1971).

Cyclic AMP was, for a long time, thought to be the second messenger for all hormonal responses in animal cells, and calcium was considered as the second messenger in a limited number of physiological events such as muscle contraction, secretion and egg activation. With the discovery of calmodulin, a calcium-binding modulator protein (a detailed description of calmodulin is given in Chapter 4 of this thesis) by W.Y. Cheung and S. Kakiuchi (for a review see Cheung, 1980a), it became apparent that Ca^{2+} fulfils all the criteria for being a second messenger. With these extensive experimentation and elegant discoveries, it became evident that the participation of Ca^{2+} was more widespread than had been thought originally, and a universal Ca^{2+} messenger system emerged with cAMP as an additional system built, in part, upon its interaction with Ca^{2+} (Rasmussen, 1981). Ca^{2+} couples excitation to contraction in nearly all contractile systems, to secretion of neurotransmitters and hormones, and to many other metabolic processes in a large variety of cells (see Dieter, 1984 and Marmè, 1984).

The general mechanism by which Ca^{2+} modulates a response is through a localized change in its concentration. In most cases, the cytoplasmic Ca^{2+} concentration is elevated in response to an excitatory or a stimulatory signal. Then, Ca^{2+} binds either directly to a protein

response element or it binds to the modulator protein calmodulin (CaM) (Hepler & Wayne, 1985). In the latter case the Ca^{2+}-CaM complex binds to the response element. In either example, a conformational change is induced in the response element that causes activation, and allows for the subsequent reactions that comprise the response (Hepler & Wayne, 1985; Marmè, 1982). The magnitude of the response is proportional to the concentration of the Ca^{2+}-CaM-response element complex and can be regulated by the Ca^{2+} concentration or by changing the affinity of the receptor protein, or the response element, to Ca^{2+} (Hepler & Wayne, 1985). Rasmussen (1983) referred to these as 'amplitude' and 'sensitivity' modulation, respectively.

Amplitude modulation by the Ca^{2+}-messenger system involves an interaction of an agonist with the plasma membrane and the opening of the channels that allow an increase in the rate of calcium influx (Hepler, 1985; Saunders & Hepler, 1983; Von Willert & Kluge, 1973). The most common source of Ca^{2+} for this process is the external space. Nevertheless, internal compartments such as the endoplasmic reticulum (ER) and vacuole (Slocum & Roux, 1983; Wagner & Rossbacher, 1980; Wick & Hepler, 1978) and mitochondria (Roux *et al.*, 1981) may also act as Ca^{2+} sources.

Sensitivity modulation involves the binding of an agonist which results in an increase in the activity of phospholipase-*c*, an enzyme that catalyses the hydrolysis of phosphatidylinositol 4,5 bis phosphate to diacylglycerol and inositol triphosphate (Rasmussen, 1983; Rasmussen & Barrett, 1984). Diacylglycerol binds directly to a Ca^{2+}-dependent protein kinase (*c* kinase), and increases its affinity for Ca^{2+}, thus activating the enzyme at resting levels of Ca^{2+}. However, during this process, it is possible for Ca^{2+} concentration to be changed, and hence sensitivity modulation is not completely separate

from amplitude modulation (Rasmussen, 1983; Rasmussen & Barrett, 1984).

Apart from the activation of an enzyme as described above, a covalent modification of response elements may be involved in sensitivity modulation, e.g. phosphorylation of response elements by protein kinase: i.e. phosphorylation of phosphorylase b kinase increases its affinity for Ca^{2+}, by about 10-fold the half maximal calcium concentration otherwise required. This mode of action is called positive sensitivity modulation. Sometimes phosphorylation of response-elements may decrease the affinity towards Ca^{2+}, thus increasing the half maximal Ca^{2+} concentration. Such a situation is known as a negative sensitivity modulation, e.g. phosphorylation of myosin light chain kinase (Hepler & Wayne, 1985).

The next requirement of Ca^{2+} to function effectively as a second messenger, is the transition of the cell back again to its initial state, which requires a lowering of intracellular Ca^{2+} concentration (Marmè, 1983). Endoplasmic reticulum and vacuole (Slocum & Roux, 1983; Wagner & Rossbacher, 1980; Wick & Hepler, 1978) and mitochondria (Roux et al., 1981) could act as intracellular Ca^{2+} sequestrators. Alternatively, Ca^{2+} could be removed from the cytoplasm by active extrusion through the plasma membrane (Maklon & Sim, 1981). A variety of calcium pumps has been characterized from different membrane fractions (Schumaker & Sze, 1984) including plasma membrane (Dieter & Marmè, 1981; Gross & Marmè, 1978; Stosic et al., 1983), rough endoplasmic reticulum (Buckhout, 1983, 1984), tonoplast (Gross, 1982), microsomes (Vaughan et al., 1984) and mitochondria (Dieter & Marmè, 1980a; Hertel et al., 1980, 1981; Hodges & Hanson, 1965; Roux et al., 1981). The degree of effectiveness of the Ca^{2+} pumps, in terms of affinity for Ca^{2+}, varies from one organelle to another, and also differs between plants and animals (Hepler & Wayne, 1985). The

organelle which has the highest affinity would be responsible for setting the lowest limit of intracellular Ca^{2+} concentration (Hepler & Wayne, 1985).

Most of the early investigations on Ca^{2+} as a second messenger system have been carried out using animal systems. Since the discovery of cAMP as a second messenger in such systems (see review by Robison et al., 1971) plant physiologists have tried to establish a similar role for cAMP in higher plants, expecting to obtain some insight into the molecular mechanism of the transduction of extracellular signals such as light, hormones or gravity (Marmè, 1982). There is now convincing evidence that cAMP also exists in higher plants (Van Onckelen et al., 1982), but there is no evidence for the existence in plants of a cAMP-dependent protein kinase, the only physiologically important receptor of cAMP (Brown & Newton, 1981). Furthermore, cAMP has never been shown to be required for any physiological response. Therefore, Ca^{2+} alone may contribute to the coupling of stimulus to response in plants (Hepler & Wayne, 1985).

There are many physiological processes which have been reported to be under the regulatory or mediatory control of Ca^{2+}: for example, plant growth hormone action: auxins (Cleland & Rayle, 1977; Cohen & Nadler, 1976; De La Fuente, 1984; Elliott et al., 1983; Lee et al., 1984; Leopold, 1977; Leopold et al., 1973), cytokinins (Elliott, 1983; Elliott et al., 1983; Lau & Yang, 1975; Saunders & Hepler, 1981) and gibberellic acid (Elliott et al., 1983; Jones & Carbonell, 1984; Jones & Jacobsen, 1983; Moll & Jones, 1981); cytoplasmic streaming in Nitella (Hayma et al., 1979) and in Chara (Williamson, 1975), pollen tip growth (Reiss & Herth, 1979), polarized growth (Robinson & Jaffe, 1975), secretion of cell wall material (Griffing & Ray, 1979), chloroplast movement in Mougeotia (Wagner et al., 1984),

mitosis and cytokinesis (Hepler et al., 1981), activity of NAD kinase (Anderson & Cormier, 1978) and Ca^{2+}-transport ATPase (Dieter & Marmè, 1981), transmembrane Ca^{2+} fluxes (Dieter & Marmè, 1980a, 1980b) and phytochrome action (Haupt & Weisenseel, 1976).

The above list is far from complete. An ever increasing number of calcium-mediated processes in plants is being discovered. The impact of these findings is so great that many scientists are now trying to re-evaluate the old discoveries in terms of an involvement of Ca^{2+}. In seeking proof that Ca^{2+} is involved, it is helpful to keep in mind the three rules proposed by Jaffe (1980). They are: 1. the response should be preceded or accompanied by an increase in intracellular calcium concentration ($[Ca^{2+}]$); 2. blockage of the natural $[Ca^{2+}]$ increase should inhibit the response, and 3. experimental generation of an increase in the intracellular $[Ca^{2+}]$ should stimulate the response.

Although, as Hepler & Wayne (1985) have suggested, a revolution is occurring in our thinking about the physiology and development of plants as we begin to realize the importance of calcium ions in mediating many different processes, the progress of the investigations on the effects of Ca^{2+} on stomatal physiology has been virtually at a standstill for a long time. Iljin (1957) reported that Ca^{2+} had no effect in inducing stomatal opening. Ten years later, Fujino (1967) found that Ca^{2+} at low concentrations, reduced stomatal aperture remarkably and, that this was associated with a reduction in net K^+ accumulation. He showed that $CaCl_2$ was effective in preventing stomatal opening as well as in inducing stomatal closure, and therefore, he dismissed the idea that $CaCl_2$ could alter the plasma membrane structure and thereby reduce its permeability to K^+. He argued that if that were so, $CaCl_2$ could not possibly induce open stomata to

close, since it would help guard cells to retain K^+ accumulated during opening. He found (on relatively crude evidence) that ATPase activity was increased with the addition of $CaCl_2$, and he therefore concluded that increased ATPase activity was responsible for excretion of K^+, and as a result guard cells lost their turgor which led to stomatal closure.

Fujino's investigations further revealed that the addition of NaF, or ethylenediamenetetraacetic (EDTA), to the incubation medium prevented the closing response of stomata in the dark. Furthermore, NaF had no remarkable effect on stomatal opening whereas EDTA had a significant stimulation. NaF is known to inactivate enzymes which employ calcium or magnesium as an activator, by coupling with those metals. As he found that $MgCl_2$ had no effect on stomatal movement, he inferred that the effect of NaF was due to the inhibition of ATPase activity and of potassium excretion through coupling with Ca^{2+} ions. EDTA is an unspecific metal ion chelator, but in Fujino's experiments, it is likely that its action was related to calcium ion chelation. This is because no other metal ions which could be chelated by EDTA are known to influence stomatal movements (magnesium ions may be present in the guard cells and are chelatable, but had no apparent effect in Fujino's experiments). However, it is worth noting that other investigators have found that $MgCl_2$ could influence stomatal movements (Willmer & Mansfield, 1969). Willmer & Mansfield found that neither Ca^{2+} nor Mg^{2+} had any apparent effect on Vicia faba stomata (any small effects would have been masked by great variability in opening within treatments) and that Mg^{2+} did not stimulate stomatal closure in Commelina communis during the first two hours of incubation, but in the next hour it decreased the apertures significantly. In contrast, they found that one millimolar (mol m^{-3})

$CaCl_2$ in the incubation medium prevented stomatal opening altogether in C.communis.

Pallaghy (1970) reported that stomatal opening in Vicia faba was also much reduced by the presence of Ca^{2+} in the medium. He presented evidence for the involvement of Ca^{2+} in the control of specificity over the uptake of monovalent cations. Donovan et al. (1985) also observed Ca^{2+}-induced reduction in stomatal apertures in combined treatments of +/- CO_2 and light/dark, the most dramatic effect being in the dark, $-CO_2$ treatment.

A very recent report by Schwartz (1985) indicates that Ca^{2+} (0.1-1.0 mol m^{-3}) can reduce stomatal apertures in light and accelerate dark-induced stomatal closure. Ethylene-glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), a calcium ion chelator, (2 mol m^{-3}) was able to overcome the inhibitory effects of Ca^{2+}.

Therefore, there can be little doubt that calcium ions inhibit stomatal opening, but the precise mechanism of their action remains unknown. MacRobbie (1986), using 'isolated' guard cells, showed that Ca^{2+} ions may be involved in regulating ion fluxes across the guard cell plasma membrane. Results of her preliminary investigation suggest that the presence of Ca^{2+} reduces membrane permeability to Rb^+ (analogous to K^+) influx, but not efflux. However, some of these results are yet to be confirmed and therefore it is advisable to keep an open mind concerning the ion fluxes controlled by Ca^{2+}.

Project aims

It is now clear that there are certain features shared in common by both ABA and calcium ions in their mode of action at the cellular level (for example, both appear to affect cell membrane permeability; they both stimulate stomatal closure accompanied by reduced K^+ accumulation and this process can be blocked by metabolic inhibitors), and

the aim of this study was to investigate these similarities in some detail. Specifically, the mode of action of ABA on stomatal movements of Commelina communis was examined in relation to processes involving calcium ions.

A re-examination of the effects of calcium ions on stomatal movements, is reported in Chapter Two. As Ca^{2+} ions have been shown to be involved in the action of other plant hormones (viz. auxins, cytokinins and gibberellins), and ABA is known to interact with those in a number of physiological processes, the experiments described in Chapter Three were designed to investigate any interactive effects between ABA and Ca^{2+}. Following these experiments, a more detailed study was carried out to elucidate the role of calcium ion channels and calmodulin in stomatal movement and Chapter Four deals with these aspects.

A well known characteristic of many amphistomatous leaves is the disparity in opening of adaxial and abaxial stomata. Previous reports (e.g. Pemadasa, 1982) have suggested that endogenous auxin levels might be responsible for this. Since later research has indicated that there is an interplay between ABA and IAA (Snaith & Mansfield, 1982a) it is very likely that ABA has some role in this phenomenon. Chapter Five of this thesis is, therefore, devoted to a re-assessment of the factors responsible for the disparity between adaxial and abaxial stomatal opening, considering in particular the roles of ABA and Ca^{2+}.

In assigning a second messenger role to Ca^{2+} in the stimulus-response coupling processes in plant cells, it is very important to determine changes in the intracellular $[Ca^{2+}]$. If calcium mediates such stimulus-response coupling, then, an increase in its cytoplasmic free concentrations should occur. Evidence confirming this view is

scanty to date, mainly due to the unavailability of precise techniques capable of detecting any micromolar quantities of Ca^{2+}. Nevertheless, the importance of knowing the internal $[Ca^{2+}]$, and whether it changes following a stimulus, is absolutely central to understanding the role of Ca^{2+} (Hepler & Wayne, 1985). Therefore, an attempt has been made to locate and estimate intracellular $[Ca^{2+}]$ using scanning X-ray microanalysis.

In his article Ebashi, S. (1983) wrote:

> "Our knowledge about Ca^{2+} is still increasing; the more we learn about Ca^{2+}, the more impressed we are by a wide variety of the mode of action of Ca^{2+}. It is almost like life itself."

Briefly, the aim of this study was to keep pace with this rapidly expanding area of research, and to throw some light onto the yet unresolved molecular mechanism of the action of the plant hormone, abscisic acid.

CHAPTER TWO

THE ROLE OF CALCIUM IN STOMATAL MOVEMENTS

INTRODUCTION

Importance of potassium in guard cell functioning

The importance of ion movements in producing the turgor changes in guard cells, responsible for the opening and closing movements of stomata, has now been clearly recognized. It was nearly two decades ago that the central role for potassium fluxes into and out of guard cells during stomatal movements was convincingly established (Fisher, 1968a, 1968b; Fisher & Hsiao, 1968; Fujino, 1967; Humble & Hsiao, 1969). This was not, however, a new discovery, since the presence of large amounts of potassium in tulip guard cells had been detected long before in 1905 by Macallum. Also, about four decades later, Imamura (1943) demonstrated that K^+ levels in the guard cells could be correlated with stomatal aperture. Yet, it was Fujino (1967) who first suggested explicitly that K^+ uptake could lower the osmotic potential of guard cells and could lead to stomatal opening. About the same time Willmer & Mansfield (1969) found that monovalent cations (K^+ and Na^+) stimulated stomatal opening in *Vicia faba* and *Commelina communis*.

Now it is well documented that increases in stomatal aperture are paralleled by potassium influx to the guard cells during opening, e.g. induction by illumination on leaves or epidermal peels (Fisher, 1971); induction by fusicoccin (Squire & Mansfield, 1974; Turner, 1973) and rhythmic and nocturnal opening in *Mimosa pudica* and *Crassula argentia*, respectively (Dayanandan & Kaufman, 1975). However, there are conflicting reports on K^+ accumulation during acid-induced stomatal opening. Jinno & Kuraishi (1982) reported that HCl-induced stomatal opening was not accompanied by an increase in guard cell potassium content in *C.communis* but, Loucks & Ownby (1978) found a concomitant uptake of K^+ with low pH-induced (HCl) stomatal opening in *Crassula argentia*. Apart from this discrepancy, it appears, therefore, that

irrespective of the mechanism through which the various factors may influence stomatal opening, accumulation of potassium seems to be a common feature.

On the other hand, treatments which cause stomatal closure (or inhibit opening) result in reduced amounts of K^+ in the guard cells, e.g. treatments with ABA (Horton & Moran, 1972; Mansfield & Jones, 1971; Squire & Mansfield, 1972b) and herbicidal auxins and metabolic inhibitors (Pemadasa, 1979b; Pemadasa & Jeyaseelan, 1976a, 1976b; Pemadasa & Koralege, 1977).

Potassium is normally the most abundant inorganic element in leaves and, therefore, it is almost universally used to assist in decreasing and increasing guard cell osmotic potentials regardless of the evolutionary level of the plant (Willmer, 1983). For example K^+ accumulation upon stomatal opening has been observed in leaves of Pteridophytes (Equisetum, Ophioglossum engelmanni and Polypodium aureum), Gymnosperms (Ginkgo biloba and Pinus sylvestris) and about 50 Angiosperms including both monocots and dicots (Dayanandan & Kaufman, 1975; Willmer & Pallas, 1973). Also K^+ has been observed to accumulate in guard cells of CAM plants during night opening (Dayanandan & Kaufman, 1975). This suggests that besides light, lowered CO_2 levels can also stimulate K^+ accumulation by guard cells, since the dark opening of stomata in CAM plants is considered to be due to lowered CO_2 levels within the leaves (Willmer, 1983).

The high specificity for potassium ions in the normal functioning of guard cells has been highlighted by the work of Humble & Hsiao (1970), Mansfield, Travis & Jarvis (1981), Pemadasa (1982, 1983b) and Thomas (1970). Nevertheless, under saline conditions at least in one halophyte, Cakile maritima, Na^+, preferentially to K^+, was found to accumulate in guard cells during stomatal opening (Eshel et al., 1974).

Under experimental conditions, monovalent cations other than K^+ have been found to affect stomatal opening. According to Imamura (1943), the order of effectiveness in promoting opening, $K^+ > Na^+ > Li^+$, at lower concentrations, is reversed at higher concentrations. The order that Iljin (1957) observed was $Li^+ > Na^+ > Cs^+ > K^+ > Rb^+$. Willmer & Mansfield (1969) found that among many other alkali cations which stimulated opening, Na^+ and Li^+ were the most effective, while Humble & Hsiao (1969) observed the reverse. These variations might be the reflections of the differences of both the experimental methods employed and the species specificity of stomatal responses to different cations.

Role of calcium in regulating K^+ accumulation

Although there are many reports on the effects of monovalent cations on stomatal movement, very limited information is available on that of divalent cations, and moreover, some contradictory results have been published on this subject. Pallaghy (1969) found that the stimulation, by Na^+ and K^+, of light-dependent stomatal opening in Vicia faba epidermal strips was dependent on the presence or absence of Ca^{2+} and the concentration of the monovalent cation. Calcium inhibited stomatal opening induced by low concentrations (1-20 mol m^{-3}) of Na^+ while, it had little or no effect when the Na^+ concentration was increased (40-50 mol m^{-3}). However, a completely opposite situation was observed when Na^+ was replaced by K^+; that is, at low concentrations of K^+, Ca^{2+} had little or no effect whereas, at higher concentrations, it inhibited opening. Fisher (1972) reported that 0.1-1.0 mol m^{-3} calcium reduced opening in light plus CO_2-free air and the effect was present at least up to 50 mol m^{-3} KCl, in contradiction to his own previous findings that Ca^{2+} had no effect on K^+-stimulated opening in Vicia faba (Fisher, 1968). In considering the early reports

(e.g. Fujino, 1967; Iljin, 1957; Willmer & Mansfield, 1969) together with the recent ones (MacRobbie, 1986; Schwartz, 1985), it appears therefore, that the evidence supporting the antagonistic effects of Ca^{2+} at preventing stomatal opening, outweighs any evidence against it.

It is now becoming apparent that the effect of Ca^{2+} on guard cells is involved with K^+ fluxes (MacRobbie, 1986; Schwartz, 1985). Employing a labelled Rb^+ ion flux-measuring technique on 'isolated' guard cells, MacRobbie (1986) found that $^{86}Rb^+$ influx was reduced significantly in the presence of calcium compared with the presence of magnesium. She further reported that dark-induced inhibition of $^{86}Rb^+$ influx in the presence of calcium was abolished by $LaCl_3$, an agent which has successfully been used in blocking calcium entry (via specific channels) in animal tissues. Based on these crude data, it was speculated that membrane permeability to K^+ might have been increased under Ca^{2+}-free conditions and reduced in the presence of Ca^{2+}. However, these results are not in complete agreement with those of Schwartz (1985). He specifically linked the role of calcium to regulation of K^+ efflux from the guard cells. He showed that 2 mol m^{-3} EGTA prevented closure in the dark, and accelerated opening under illumination. Furthermore, he found no significant EGTA-induced opening in the dark and, therefore, concluded that EGTA did not increase ion uptake but, it rather prevented ion efflux. He also found that for the maintenance of open stomata in the presence of 2 mol m^{-3} EGTA, only 10 mol m^{-3} KCl was required (external supply), whereas, in the presence of 1 mol m^{-3} $CaCl_2$ the required concentration of KCl was as high as 150 mol m^{-3}. This suggests that Ca^{2+}-induced K^+ leakage (and thus the stomatal closure) could be diminished by lowering the concentration gradient across the plasma membrane, and that EGTA could effectively reduce plasma membrane leakiness to K^+, by chelating the apoplastic Ca^{2+} ions. These findings,

therefore, appear to contradict MacRobbie's findings.

As it has been shown above, there are a number of controversial reports on the action of Ca^{2+} on stomata. Therefore, it seemed essential to reassess and establish the part played by Ca^{2+} and EGTA in regulating the ion fluxes of guard cells, and the experiments reported in this Chapter examined this in some detail.

MATERIALS AND METHODS

Plant material

Plants of Commelina communis were grown from seed in John Innes No.2 potting compost in a heated greenhouse (minimum temperature 20 ± 1°C with supplementary light using 250W metal halide discharge lamps (Thorn Lighting Ltd., London), to give a photoperiod of 16 hours and a minimum photon flux density of 100 μmol m^{-2} s^{-1} between 400-700 nm, measured using a LICOR model LI-185 quantum photometer (Lambda Instruments Corporation, Nebraska, U.S.A.).

Ten days after sowing, the seedlings were transplanted in individual pots and at the age of 4-5 weeks, the plants were transferred to a controlled environment room in which the minimum temperature was 25 ± 1°C and the photoperiod was 16 hours with a minimum photon flux density of 150 μmol m^{-2} s^{-1} (400-700 nm) supplied by either metal halide discharge lamps or 'daylight' fluorescent tubes. Plants were placed on a self-watering gravel tray. Relative humidity was not controlled, but readings taken at regular intervals showed that it did not change appreciably. The minimum relative humidity was around 68%. Plants were kept in these controlled conditions for several days before use and both at this stage, and during early growth in the greenhouse, they were free of water deficit stress.

Epidermal peels from the abaxial surface were taken from the youngest fully expanded leaf of each plant using the method of Weyers &

Travis (1981). The leaf was detached from the plant and cut into 5 mm wide strips using a Perspex template avoiding the mid-rib. The leaf strips were floated on distilled water, adaxial surface downwards, to avoid dehydration. Using a razor blade, near one end of the strip, a shallow incision was made on the upper epidermis leaving a tab of leaf tissue attached to the strip by the lower epidermis. The strip was then turned over using forceps and the tab of leaf tissue was pulled back to reveal the mesophyll. Then the strip was held down with forceps and the tab was gently pulled upwards at right angles to the leaf strip, to detach the epidermis. Alteration of the peeling angle can affect both the extent of mesophyll cell contamination and epidermal cell death (Weyers & Travis, 1981). While the epidermis was still attached to the leaf strip, it was placed cutical side uppermost, on distilled water and the epidermal tissue was then separated from the rest of the leaf strip by pulling gently. Any epidermal peel found to be contaminated with mesophyll was rejected for further experimental use. The remaining strips were then cut into 10 mm pieces using dissecting scissors.

Incubation of epidermis

The epidermal pieces were floated cuticle side up on 10 cm^3 of incubation medium in 5 cm diameter 'deep form' plastic Petri dishes (three pieces in each). These Petri dishes were then placed on a Perspex plate which was mounted on a temperature-controlled water bath (Plate 2.1), the dishes were allowed to rest on the Perspex plate partially submerged in the water bath which was maintained at 25 ± 1^{o}C and illuminated from below by a bank of Phillips 'daylight' fluorescent tubes. The photon flux density at the level of the Petri dishes was 175 ± 10 µmol m^{-2} s^{-1} (400-700 nm). Air from either a compressed gas cylinder or pumped from the laboratory, was supplied to each Petri

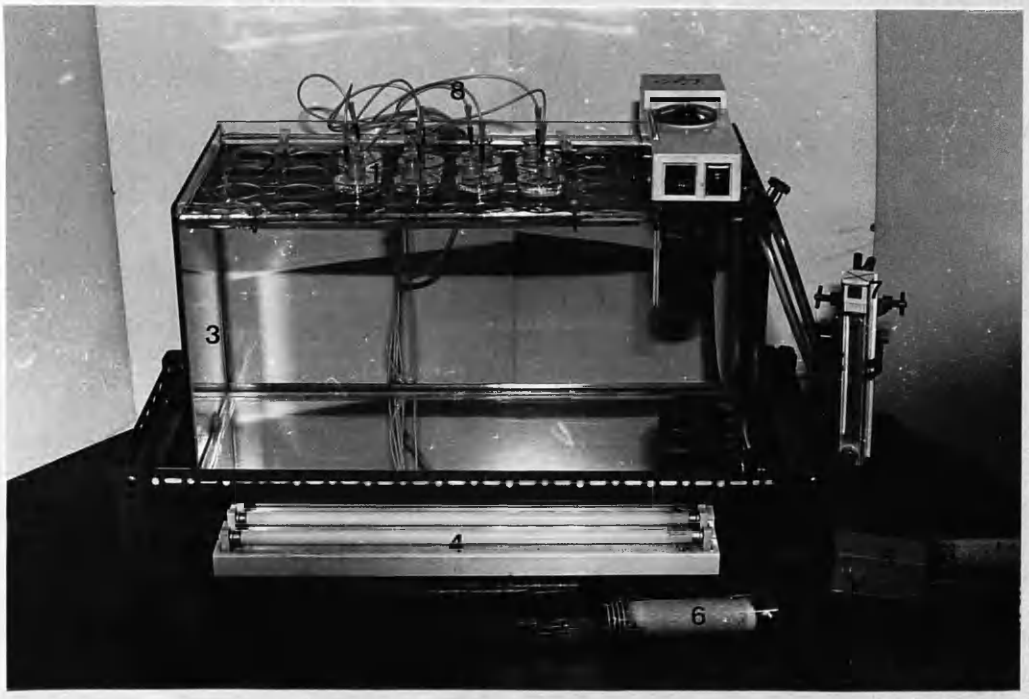


Plate 2.1

View of the experimental set-up showing:

1. Petri dishes
2. Perspex holder for Petri dishes
3. Temperature controlled water bath
4. Light source
5. Air pump
6. Tube filled with soda lime
7. Air flow meter
8. Tubings which supply air to the Petri dishes through hypodermic needles.

dish at 100 cm^3 min^{-1} via hypodermic needles dipping into the medium. In treatments involving CO_2-free conditions a tube filled with soda lime (self-indicating, 10-16 mesh) was positioned between the air source and hypodermic needles. Soda lime was replaced frequently to ensure an air supply totally free from CO_2.

The incubation medium was buffered by 10 mol m^{-3} 2[N-morpholino] ethanesulphonic acid (MES) adjusted to pH 6.15 with KOH which contributed 5 mol m^{-3} K^+ (Travis & Mansfield, 1979b). Additional compounds required by each experiment were dissolved in this medium.

The chemicals used in the experiments described in this Chapter were obtained from BDH Chemicals Ltd., Poole, Dorset (calcium chloride hexahydrate 98% purity and potassium hydroxide 85% purity), Hopkin & Williams, Chadwell Heath, Essex (potassium chloride 99.8% purity), Italchemia S.A., Milan, Italy (fusicoccin) and from Sigma Chemical Company, Poole, Dorset (EGTA 97-98% purity and MES).

In most instances, the epidermal peels were incubated for a period of 3 ± 0.25 hours. After incubation, they were mounted in an appropriate medium on a microscope slide and examined under a Watson Hilux 70 microscope fitted with a projection eyepiece. Pore widths of ten randomly selected stomata were measured on each 5 x 10 mm strip of epidermis. Most experiments were conducted on at least three occasions. All experiments were completed during the first half of the photoperiod. This standard experimental protocol was widely adopted in this investigation and any variation to it (for example, the time course of stomatal opening, experiments involving a pre-incubation etc.) will be described accordingly, where employed.

The results illustrated in Fig. 2.5 were obtained in an experiment which included pre-incubation. The epidermal pieces were pre-incubated for 3 hours in 50 mol m^{-3} KCl in light in the absence of CO_2 and then

transferred into 50 or 100 mol m^{-3} KCl containing 0, 0.1, 0.25, 0.5 and 1.0 $CaCl_2$ and incubated for 3 hours. The mean stomatal aperture at the beginning of the experiment was 15.35 ± 0.18 μm.

Histochemical staining of potassium

Potassium was stained by the histochemical procedure described by Willmer & Mansfield (1970): After incubation, epidermal peels were rinsed in ice-cold distilled water and immersed in ice-cold solution of freshly prepared sodium cobaltinitrite for 30 minutes. They were then washed in ice-cold distilled water until no excess stain flowed from the material, and were next placed in 5% yellow ammonium sulphide for 2 minutes. Finally, the specimens were washed briefly, and then observed under a microscope. The amount of black deposits of cobalt sulphide is believed to correspond to the amount of K^+ present in the cells. Sodium cobaltinitrite was made up as follows: 20g cobaltous nitrate and 35g sodium nitrite were dissolved in 75 cm^3 dilute acetic acid (10 cm^3 glacial acetic acid diluted to 75 cm^3 with distilled water); air was bubbled through the mixture until all the nitrogen peroxide fumes had been evolved. The filtrate was diluted to 100 cm^3 with distilled water prior to use.

Cell viability of the epidermis

After incubation, the epidermal pieces were floated on a solution of 0.01% neutral red in tap water for a few minutes. They were then washed in distilled water and observed under a microscope. Living cells, particularly guard cells, are able to accumulate neutral red and become purple-red. Dead cells do not accumulate the stain.

RESULTS AND DISCUSSION

Preliminary trials indicated that a considerable inhibition of abaxial stomatal opening was caused by 0.25-1.0 mol m^{-3} $CaCl_2$ in the presence of 50 mol m^{-3} KCl. Subsequently, a more detailed study was made to investigate the effects of external KCl concentration on Ca^{2+}-inhibited stomatal opening in the presence of light. The effect of CO_2 was also investigated under the same treatments.

The method of floating epidermal strips on buffered solution would effectively impose the original strength of the medium in the apoplastic space of the epidermis, because the two solutions (i.e. the external medium and free space) are in equilibrium and the volume of the medium is much larger than that of the apoplast. Moreover, there are no plasmodesmatal connections between mature guard cells and their subsidiary cells (Carr, 1976) and it is, therefore, appropriate to consider that any change in aperture in response to an agent present in the apoplast would be due to a direct effect of that agent on the guard cells.

The mean stomatal apertures measured after incubation for 3 hours, in a range of KCl concentrations (0-150 mol m^{-3}) in the presence or absence of 0.5 mol m^{-3} $CaCl_2$ and of 350 µl l^{-1} CO_2 are shown in Figure 2.1. An increase in KCl concentration stimulated stomatal opening under all treatments. The highest apertures were observed in the absence of both Ca^{2+} and CO_2 over the whole range of KCl concentrations examined, and the lowest being when both Ca^{2+} and CO_2 were present simultaneously. The response to $CaCl_2$ was calculated by subtracting the treatment means for Ca^{2+} from the corresponding ones for zero calcium and this is shown in Fig. 2.2. The response to CO_2 was calculated in a similar way from the treatment means for 350 µl l^{-1} CO_2 and zero CO_2 (Fig. 2.3). As can be seen in Fig. 2.3, the effect of

Figure 2.1

The influence of KCl on the responses of stomata to $CaCl_2$ and CO_2. Epidermal pieces were incubated in light in 10 mol m^{-3} MES buffer, pH 6.15 (KOH) at 25 ± 1°C for 3 hours. The concentration of $CaCl_2$ and CO_2 were 0.5 mol m^{-3} and 350 µl l^{-1} respectively. Each point represents the mean of 120 measurements of individual stomata and the vertical bar represents the least significant difference (L.S.D.) at P = 0.05.

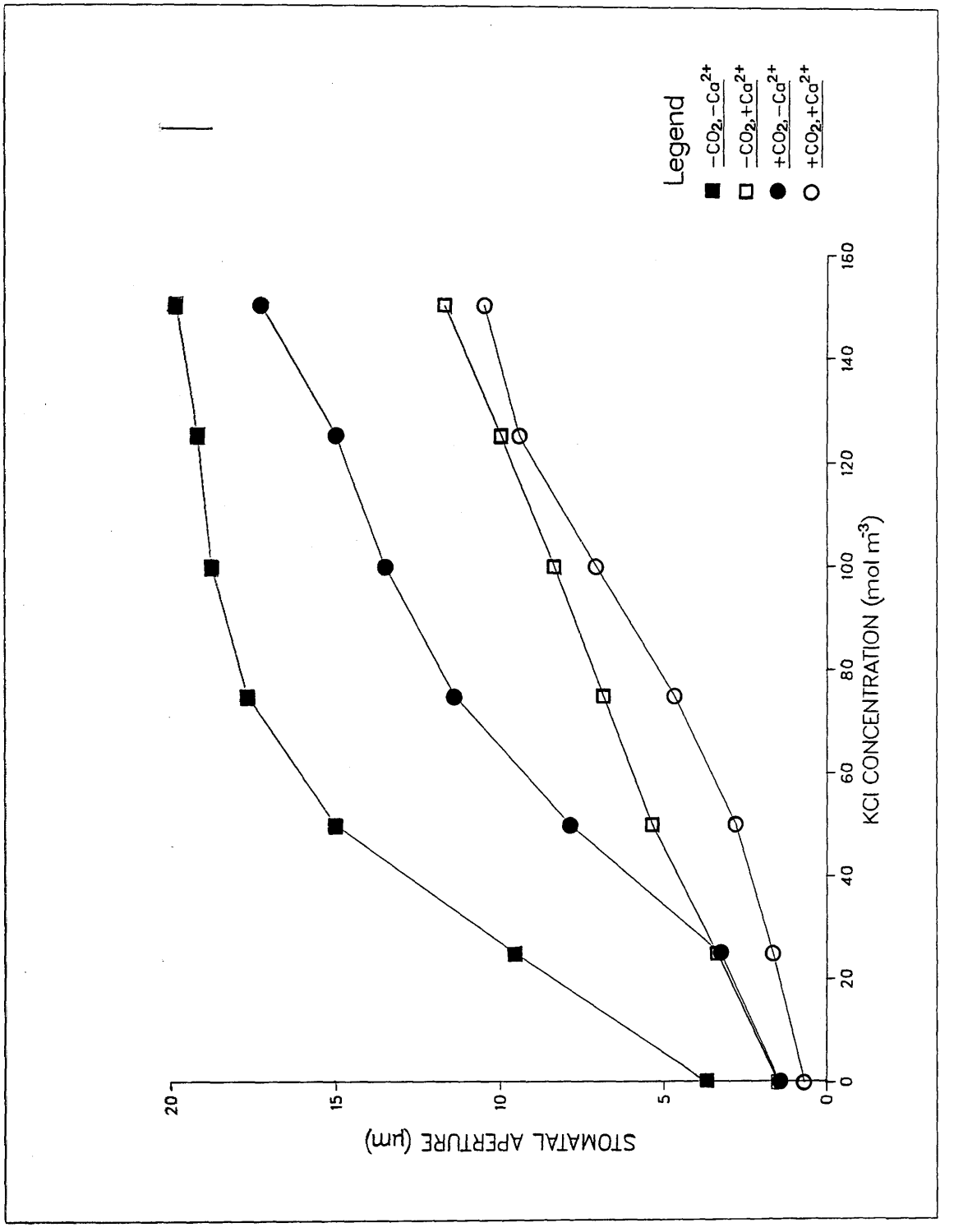

Legend
$-CO_2,-Ca^{2+}$
$-CO_2,+Ca^{2+}$
$+CO_2,-Ca^{2+}$
$+CO_2,+Ca^{2+}$
STOMATAL APERTURE (μm)
KCl CONCENTRATION (mol m^{-3})
0
5
10
15
20
0
20
40
60
80
100
120
140
160

Figure 2.1

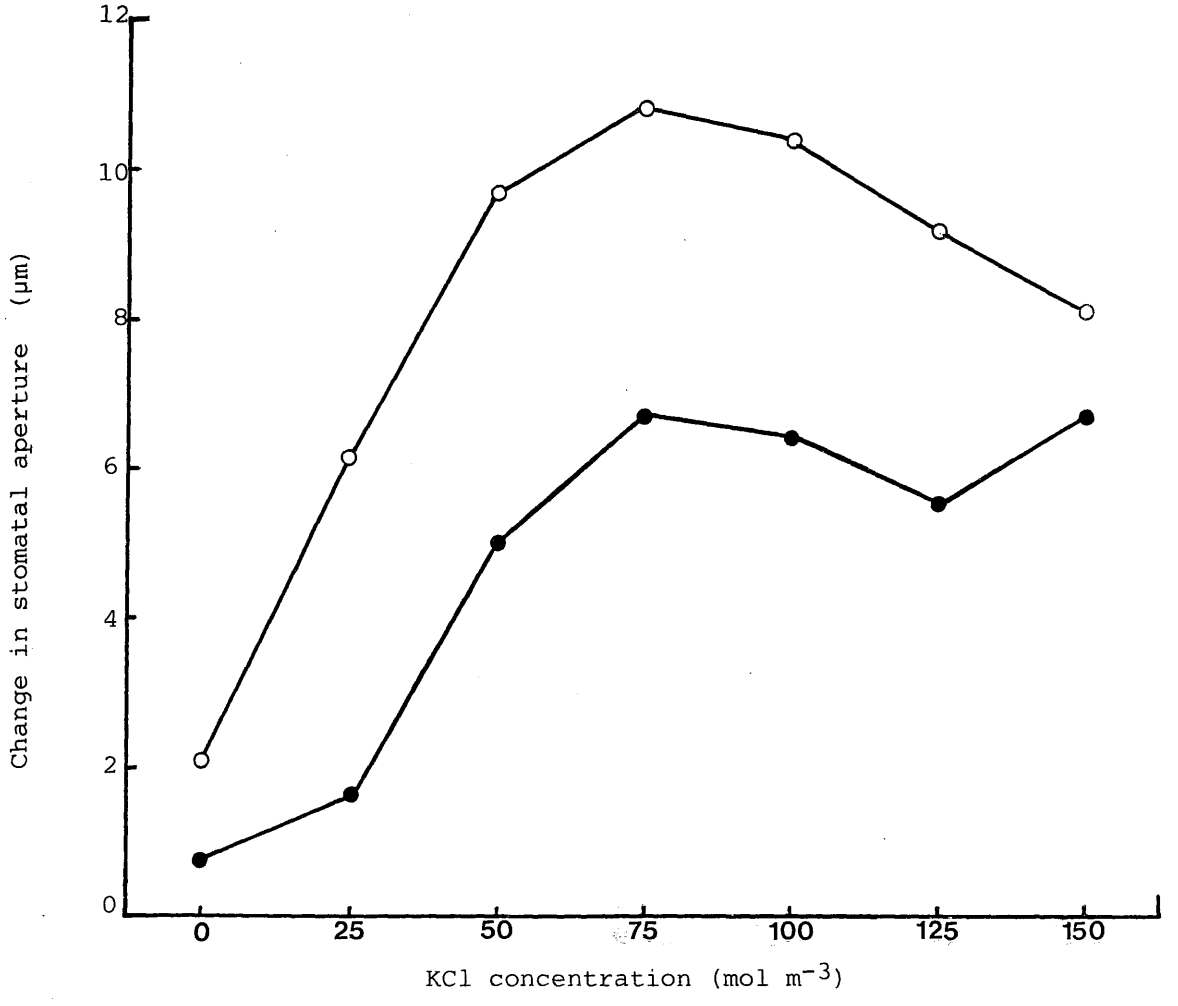


Figure 2.2

Effect of KCl concentration on the absolute stomatal responses to $CaCl_2$ (0.5 mol m^{-3}) in the presence (●) and absence (o) of 350 µl l^{-1} CO_2. Each point represents the difference in aperture between a particular $CaCl_2$ treatment and the relevant control (calculated from the data shown in Fig. 2.1).

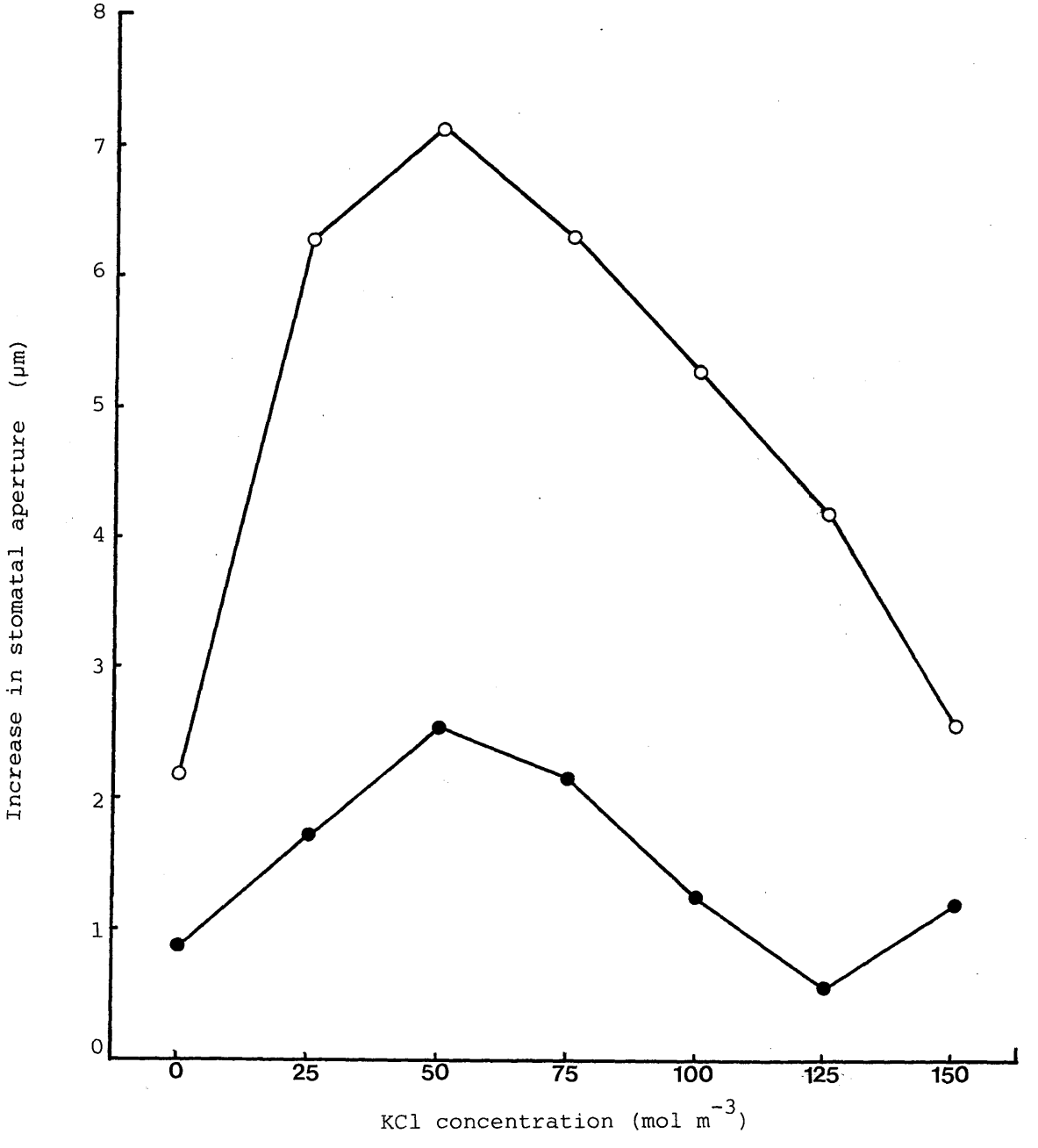


Figure 2.3

Effect of KCl concentration on the absolute stomatal responses to CO_2 (350 µl l^{-1}) in the presence (●) and absence (o) of 0.5 mol m^{-3} $CaCl_2$. Each point represents the difference in aperture between a particular CO_2 treatment and the relevant control (calculated from the data shown in Fig. 2.1).

CO_2 was statistically significant over the whole range of KCl concentrations, but the magnitude of the response was highest at 50 mol m^{-3} KCl irrespective of the presence or absence of $CaCl_2$. This is in close agreement with the results of Travis & Mansfield (1979b) who observed the maximum response for light and CO_2 at 50 mol m^{-3} KCl. As can be seen in Fig. 2.2, the maximum response for $CaCl_2$, however, was found at a higher KCl regime (75 mol m^{-3}). Nevertheless, there was a substantial response to Ca^{2+} in 50 mol m^{-3} KCl. Furthermore, when the same experiment was repeated on another occasion the maximum was observed at 50 mol m^{-3} K^+. Therefore, it seemed appropriate to choose 50 mol m^{-3} as the optimal K^+ concentration for subsequent studies and, hereinafter when reference is made to the 'control medium' this consists of 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer, unless otherwise stated (a more detailed discussion related to the 'choice of incubation medium' is given by Travis & Mansfield, 1979b).

A dose-response study for $CaCl_2$ was performed next, and the results are shown in Fig. 2.4. There was a significant reduction in stomatal opening when a low concentration (0.1 mol m^{-3}) of $CaCl_2$ was incorporated into the control medium. As the $CaCl_2$ concentration increased to 1.0 mol m^{-3} there was a considerable reduction in aperture, but only a small further response between 1.0 and 2.0 mol m^{-3}. A similar pattern of response was observed when 100 mol m^{-3} KCl was used in the incubation medium. When the Ca^{2+} concentration was below 0.5 mol m^{-3}, the two curves were significantly different from each other, but this was not observable at higher concentrations. It is worth noting that there was a significant difference in aperture between 50 and 100 mol m^{-3} KCl treatments containing 0.5 mol m^{-3} $CaCl_2$, in the previous experiment (cf. apertures at 50 and 100 mol m^{-3} KCl on $-CO_2$ + Ca^{2+} curve in Fig. 2.1). Such variations might be a reflection of

Figure 2.4

Effect of $CaCl_2$ concentration on stomatal opening. Epidermal pieces were incubated for 3 hours at 25 ± 1°C in light, in the absence of CO_2. Means of 90 measurements of individual stomata. Vertical bar represents the L.S.D. at P = 0.05.

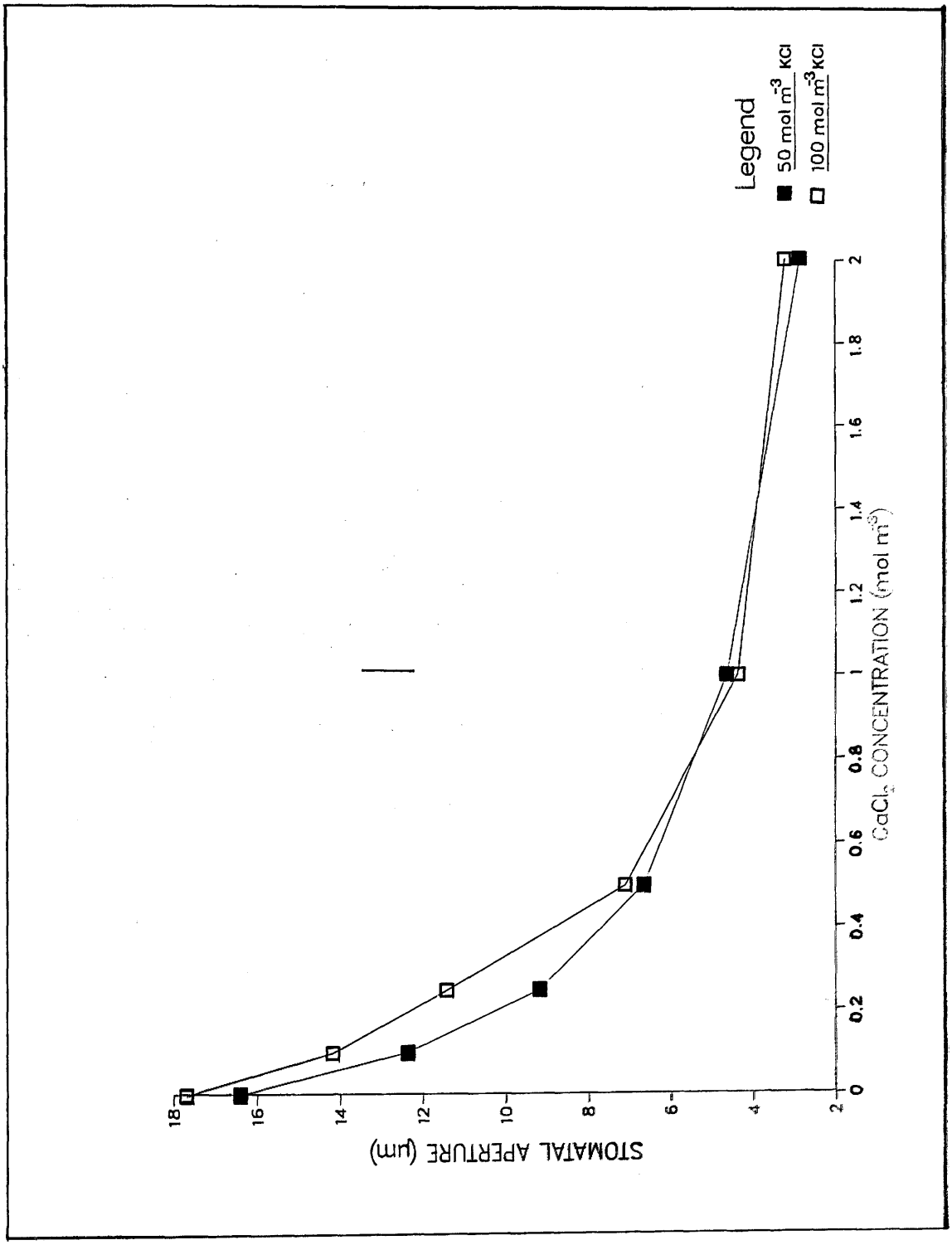

Legend
50 mol m-3 KCl
100 mol m-3 KCl
STOMATAL APERTURE (μm)
CaCl2 CONCENTRATION (mol m-3)
2
4
6
8
10
12
14
16
18
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2

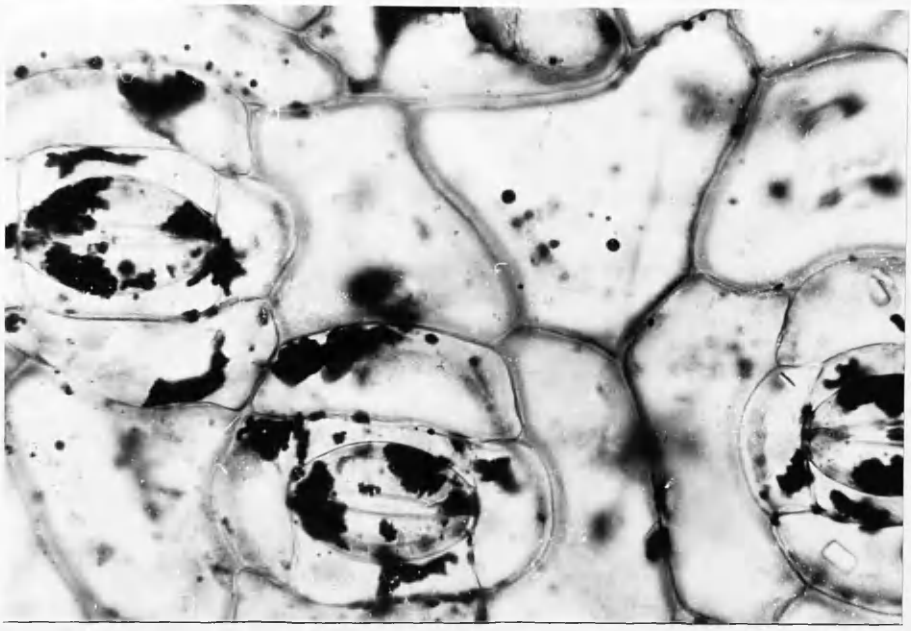
Fig. 2.4

differences in the plants used in different experiments. The pre-treatment of plants can have a major effect on the behaviour of stomata in subsequent experiments (Mansfield & Davies, 1985). Great care was taken in this research to avoid major variations in the conditions affecting the plants prior to isolating the epidermis, but it was impossible to achieve absolute uniformity, and this accounts for differences in stomatal opening under similar treatments in different experiments. Such small variations found between experiments are not likely to affect the major conclusions reached in this research. It is advisable, however, not to draw further conclusions by comparing the data from experiments performed on different occasions.

The reduction in opening caused by 0.25 mol m^{-3} Ca^{2+} in the control medium was about 50 per cent and that by 1.0 mol m^{-3} Ca^{2+} was about 75 per cent. The degree of closure observed here is somewhat lower than those which have been reported previously (e.g. Fujino, 1967; Willmer & Mansfield, 1969). However, such a discrepancy is not surprising, since there are considerable differences in methodology between the earlier studies and those reported here.

Kirkby & Pilbeam (1984) reported that the free calcium concentration in the xylem sap could exceed 1 mol m^{-3}, and solutes in the transpiration stream are thought to move readily to the vicinity of the guard cells (Maier-Maercker, 1983). Therefore, it seems possible that the range of calcium concentration over which the stomatal response occurred (Fig. 2.4) could be present in the apoplast surrounding the guard cells in the intact leaf. The abundant crystals of calcium oxalate in the epidermal cells of C.communis also suggest that calcium is readily supplied to the epidermis from the xylem.

The data in Fig. 2.4 confirm the early reports on the inhibitory effects of Ca^{2+} on stomatal opening. It was interesting to know



A- Control

B- 0.5 mol m^{-3} $CaCl_2$

Plate 2.2

Photomicrographs of epidermal tissues incubated in the presence (B) and absence (A) of $CaCl_2$ followed by histochemical staining for potassium ions (X. **435**).

Figure 2.5

Effect of $CaCl_2$ concentration on open stomata. Epidermal peels were pre-incubated for 3 hours at 25 ± 1°C in 50 mol m^{-3} KCl under CO_2-free air, and then transferred into test solutions and further incubated for 3 h. Means of 90 individual measurements. The vertical bar represents the L.S.D. at P = 0.05. The mean stomatal aperture at the beginning of the incubation was 15.35 ± 0.18 μm.

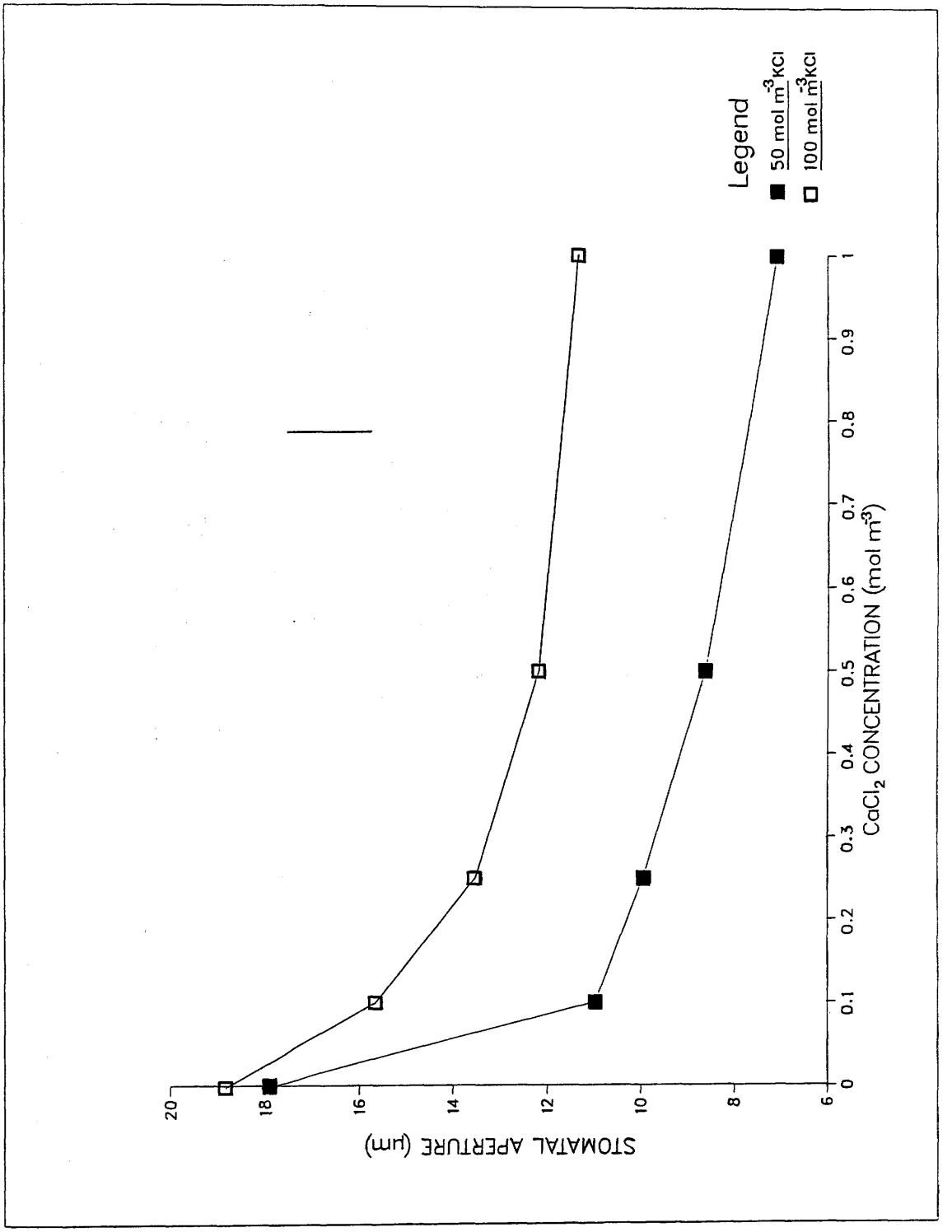

Legend
50 mol m-3 KCl
100 mol m-3 KCl
STOMATAL APERTURE (μm)
CaCl2 CONCENTRATION (mol m-3)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
6
8
10
12
14
16
18
20

Figure 2.5

whether these effects were associated with different levels of ion accumulation in the guard cells. Accordingly, a histochemical test for potassium was carried out on epidermal pieces after the incubation, and it was found that external supply of Ca^{2+} greatly reduced the K^{+} content of the guard cells (Plate 2.2).

It is known that K^{+} fluxes can be controlled by Ca^{2+} both in animal (Atwater et al., 1979) and plant tissues (Bengtsson, 1982; Bisson, 1984; Raschke, 1979; Schwartz, 1985). Schwartz (1985) attributed the involvement of Ca^{2+} in the control of K^{+} accumulation in guard cells, to an enhancement in the efflux process. Figure 2.5 shows that abaxial epidermal pieces were not able to maintain their initial wide-open apertures when transferred to a medium containing Ca^{2+}. The apertures decreased as the concentration of Ca^{2+} in the medium was increased. These data support Schwartz's (1985) conclusions. Furthermore, when 100 mol m^{-3} KCl was present in the medium the responsiveness to Ca^{2+} of open stomata was greatly reduced, compared with that in 50 mol m^{-3}. This was presumably due to the decrease in the gradient of K^{+} concentration between cytosol and apoplast, and, consequently, to a reduction in the rate of efflux.

The time course of stomatal opening on isolated epidermis in the presence and absence of 0.25 mol m^{-3} $CaCl_2$, was then investigated. Measurements were made at 20-minute intervals and mean apertures of 60 individual stomata are shown in Fig. 2.6. There was only a small effect of 0.25 mol m^{-3} calcium on the initial stages of opening, but after about 40 minutes the inhibition due to calcium became increasingly apparent. MacRobbie (1981) pointed out that the accumulation of potassium salts is inadequate to account for osmotic changes in the guard cells of C.communis during the early stages of opening when apertures are below 10 μm. The curves in Fig. 2.6 show clearly that

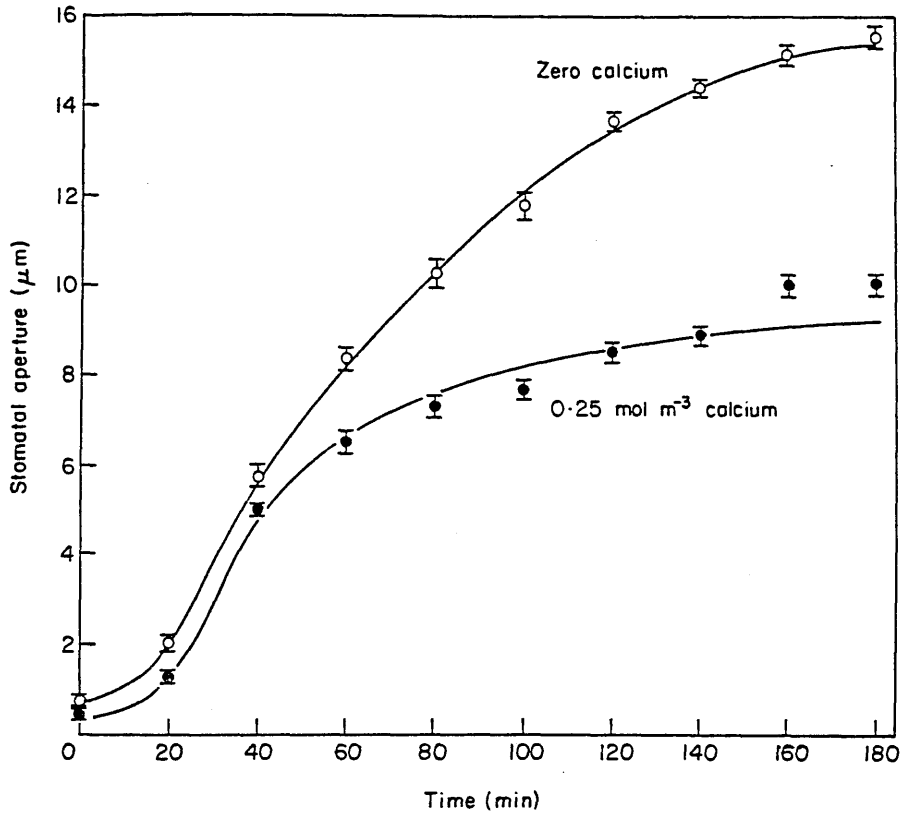


Figure 2.6

Time course of stomatal opening on isolated epidermis in the presence and absence of 0.25 mol m^{-3} $CaCl_2$. Epidermal pieces were incubated in light and in the absence of CO_2. Means of 60 measurements with 95% confidence limits.

the initial phase was little affected by calcium, but there was a marked suppression in the second phase. In the light of MacRobbie's analysis one possible interpretation of these biphasic curves is that Ca^{2+} exerts its effects on the net flux of K^{+} into guard cells and not on the formation of non-ionic solutes which may be involved in the turgor changes of guard cells (i.e. if calcium affects potassium accumulation which does not account for the initial opening, then calcium would not alter the dynamics of that phase). An alternative interpretation would be that the similarity in the initial phase of these curves is indicative of the time required for calcium to equilibrate across the plasma membrane before exerting its effects. At the moment, there is no evidence to distinguish between these two possibilities.

It has already been suggested that a considerable amount of calcium is likely to be present in the apoplastic region of guard cells, and this may have some control over stomatal opening in both isolated epidermis and intact leaf. It was interesting to examine the effects of removing all the apoplastic calcium on stomatal opening in isolated epidermis. The specific calcium ion chelator, EGTA can be employed to remove calcium ions from the apoplast. It has successfully been used in modifying Ca^{2+}-mediated processes in a variety of plant tissues, e.g. arresting the normal development of Micrasterias (Lehtonen, 1984), initiating/accelerating cytoplasmic streaming in the cells of dark grown Vallisneria leaf (Yamaguchi & Nagai, 1981), prolonging metaphase in dividing stamen hair cells of Tradescantia (Hepler, 1985) and inhibiting gravitropic curvature in Avena coleoptiles without inhibiting growth (Daye et al., 1984). The concentrations of EGTA used in those investigations ranged from 0.1-20 mol m^{-3}. Since the effective concentration of EGTA for epidermal strips was not

Figure 2.7

Dose-response curves for EGTA in the presence (●) and absence (O) of 0.1 mol m^{-3} $CaCl_2$. In light, zero CO_2, 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer, pH 6.15 (KOH) at 25 ± 1°C. Means of 90 measurements of individual stomata, with standard errors.

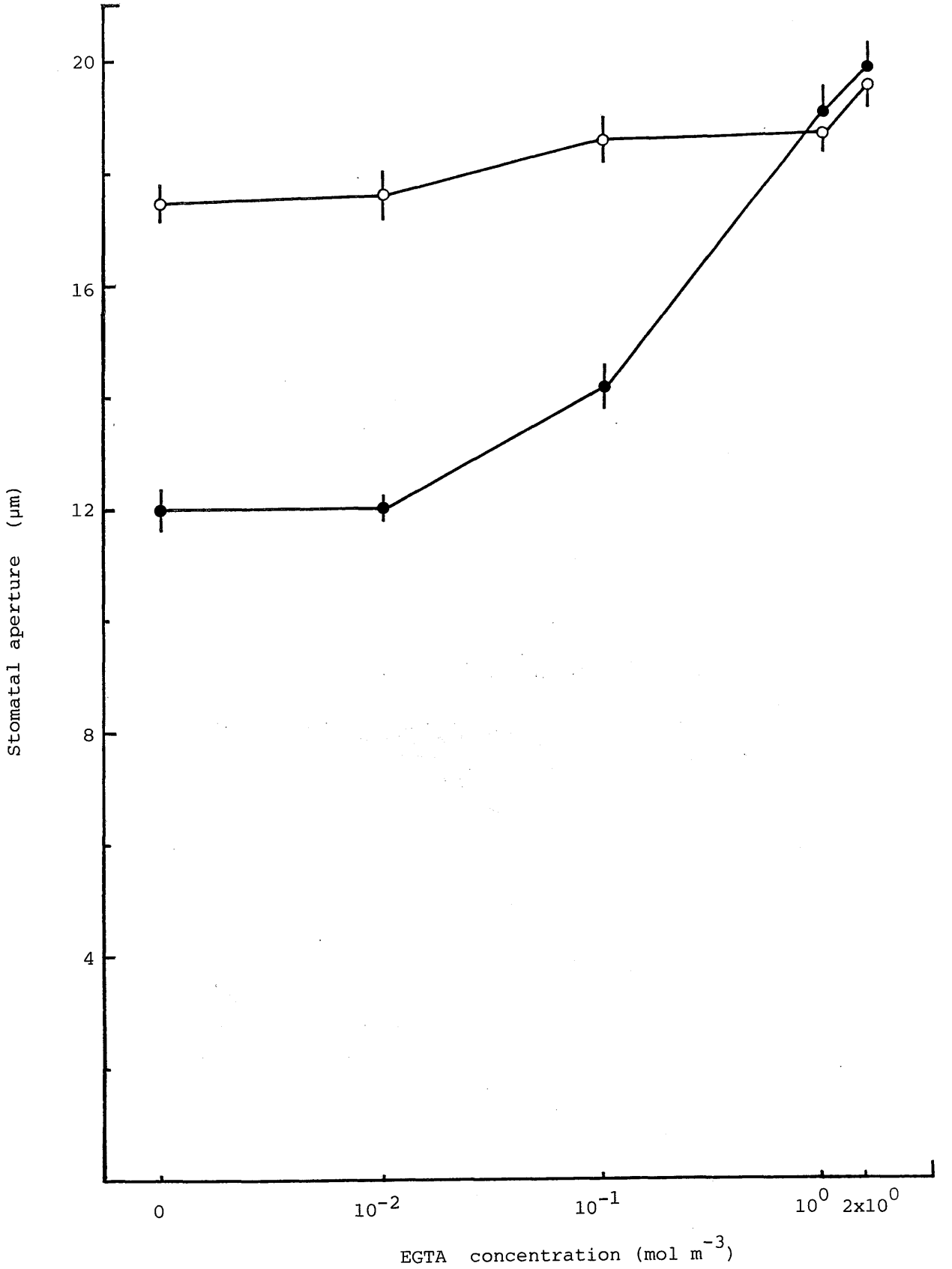

20
16
12
8
4
Stomatal aperture (µm)
0
10^{-2}
10^{-1}
10^{0}
$2x10^{0}$
EGTA concentration (mol m^{-3})

Figure 2.7

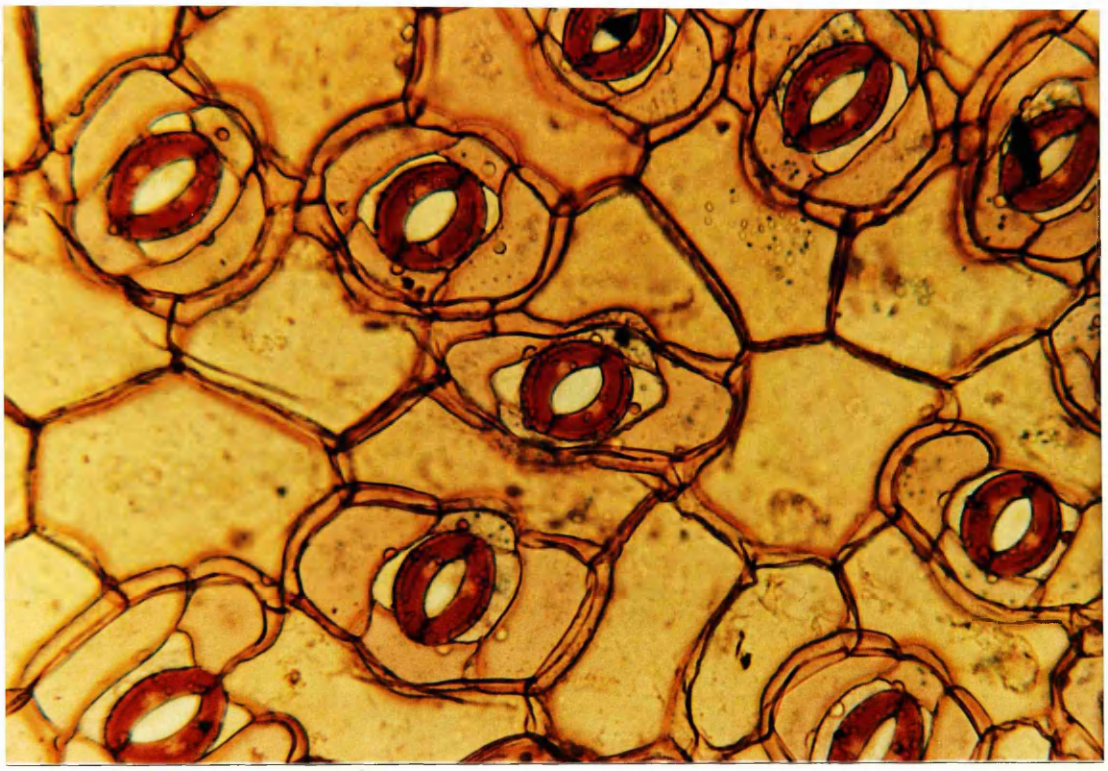


Plate 2.3

Photomicrograph of an abaxial epidermal tissue stained with neutral red after the treatment with 2 mol m^{-3} EGTA (X.310).

known, a dose-response study was carried out in the presence and absence of 0.1 mol m^{-3} Ca^{2+}. The results in Fig. 2.7 show clearly that chelation of calcium from the apoplast of Commelina epidermis widened the aperture both in the presence and absence of exogenous Ca^{2+}, when the EGTA concentration was above 0.1 mol m^{-3}. However, 0.1 mol m^{-3} EGTA did not reverse completely the effect of exogenous calcium, and therefore, it was decided to use 1-2 mol m^{-3} EGTA in subsequent experiments with epidermal strips. In his recent paper Schwartz (1985) reported the successful use of 2 mol m^{-3} EGTA in reversing Ca^{2+}-induced stomatal closure. There might, however, be some doubts about the viability of cells in high concentrations of EGTA, and hence cell viability was examined after treatments with EGTA. The guard cells continued to accumulate the vital stain neutral red (Plate 2.3). Furthermore, it was found from the X-ray scanning technique (see Chapter 6), that in EGTA-treated epidermal tissues, large stomatal apertures were associated with increased accumulations of potassium in the guard cells (Plates 2.4 and 2.5).

Another important point worth mentioning here is that the different regimes of calcium and EGTA employed in this investigation might be expected to modify the deformation properties of the cell wall to some extent. However, it has already been shown that the effects of these two agents are closely associated with potassium accumulation in guard cells, and thus it seems that intracellular events are mainly responsible for the observed effects of those agents.

Fusicoccin is a toxin produced by the fungus Fusicoccum amygdali Del. and it is known to influence a number of important physiological processes, the most fundamental of which is thought to be a stimulation of H^+/K^+ exchange (Marrè, 1979). Fusicoccin induces wide opening of stomata (Graniti & Turner, 1970). It has also been found that

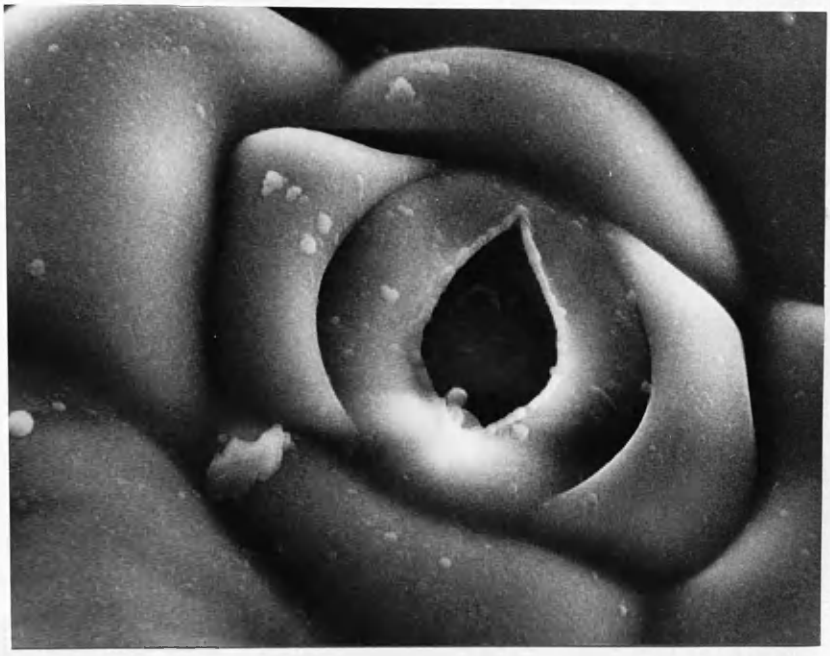


Plate 2.4a

Scanning electron micrograph of an abaxial stomatal complex incubated for 3 h in 50 mol m^{-3} KCl under CO_2-free air (X.1200).

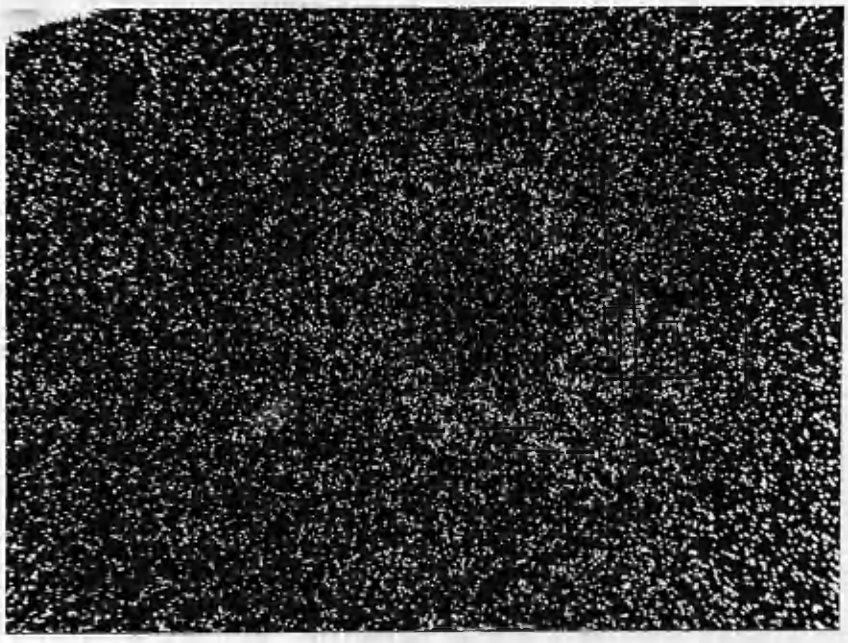


Plate 2.4b

Potassium X-ray emission micrograph of above stomatal complex taken after 8 scans (X.1200).

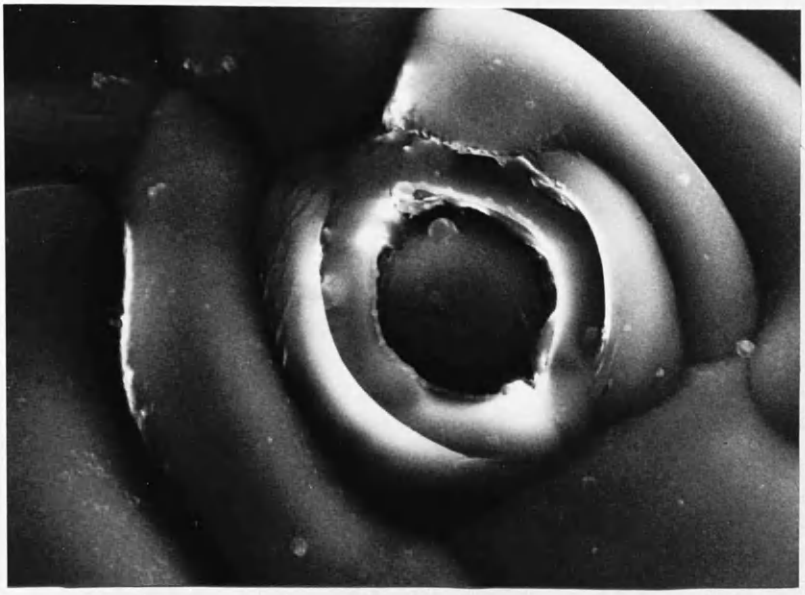


Plate 2.5a

Scanning electron micrograph of an abaxial stomatal complex treated with 2 mol m^{-3} EGTA in 50 mol m^{-3} KCl for 3 h under CO_2-free air (X.1200).

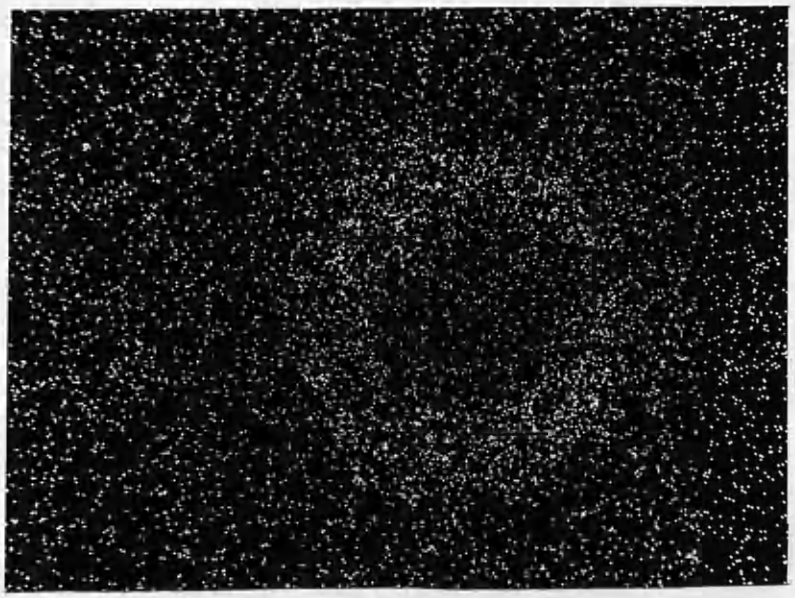


Plate 2.5b

Potassium X-ray emission micrograph of the above stomatal complex taken after 2 scans (X.1200).

Figure 2.8

Dose-response curves for fusicoccin in the presence (●) and absence (O) of 0.5 mol m^{-3} $CaCl_2$. In light, zero CO_2, 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer, pH 6.15 (KOH) at 25 ± 1°C. Means of 90 measurements of individual stomata, with standard errors.

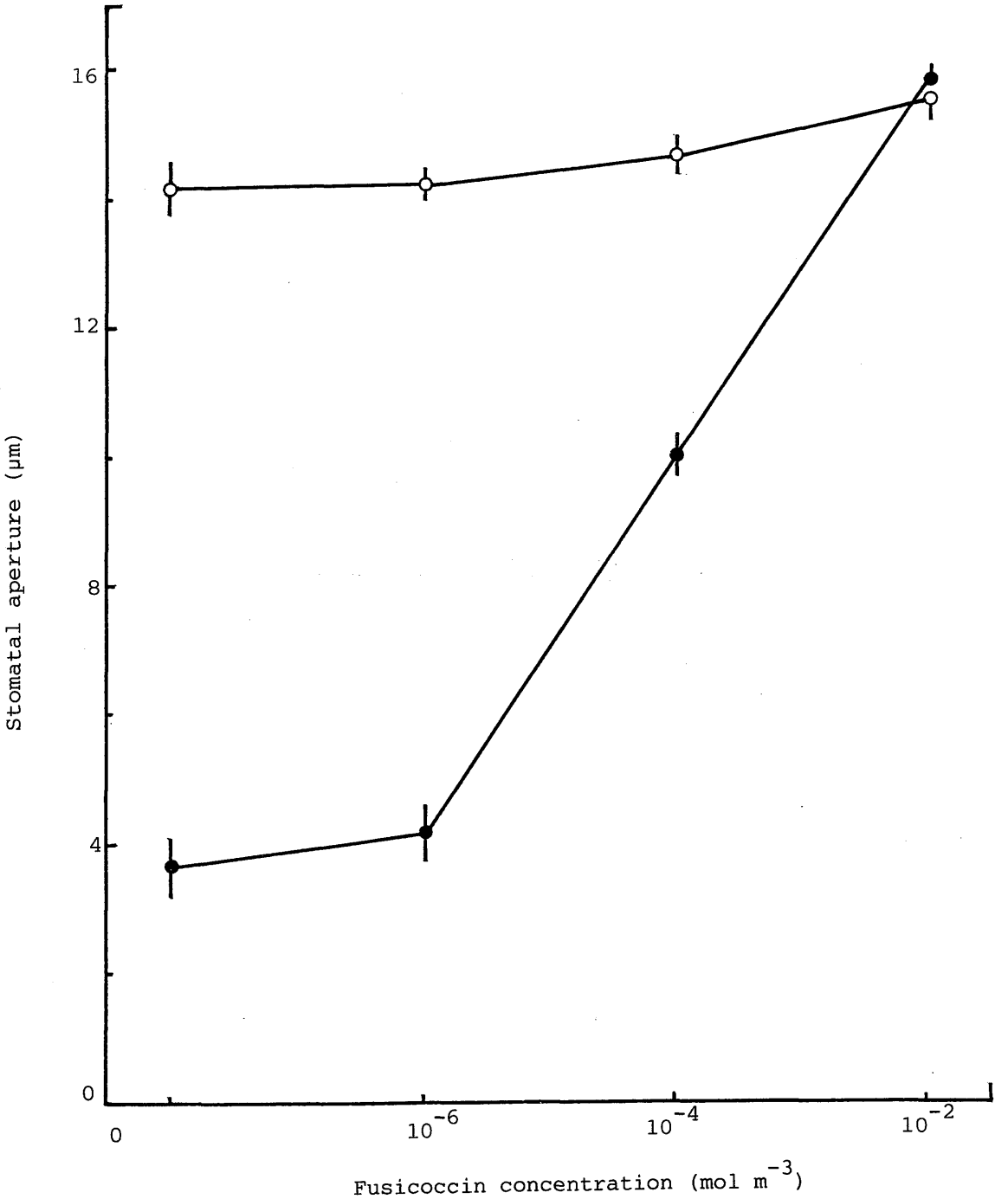

16
12
8
4
0
Stomatal aperture (µm)
0
10^{-6}
10^{-4}
10^{-2}
Fusicoccin concentration (mol m^{-3})

Figure 2.8

Figure 2.9

Effect of calcium ions on stomatal opening in the presence of fusicoccin and/or CO_2. Epidermal pieces were incubated for 3 h, in light, in the absence of CO_2, in 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer, pH 6.15 (KOH) at 25 ± 1°C. Means of 90 measurements of individual stomata. All points except for 'zero calcium plus fusicoccin', were significantly different from one another at P = 0.05.

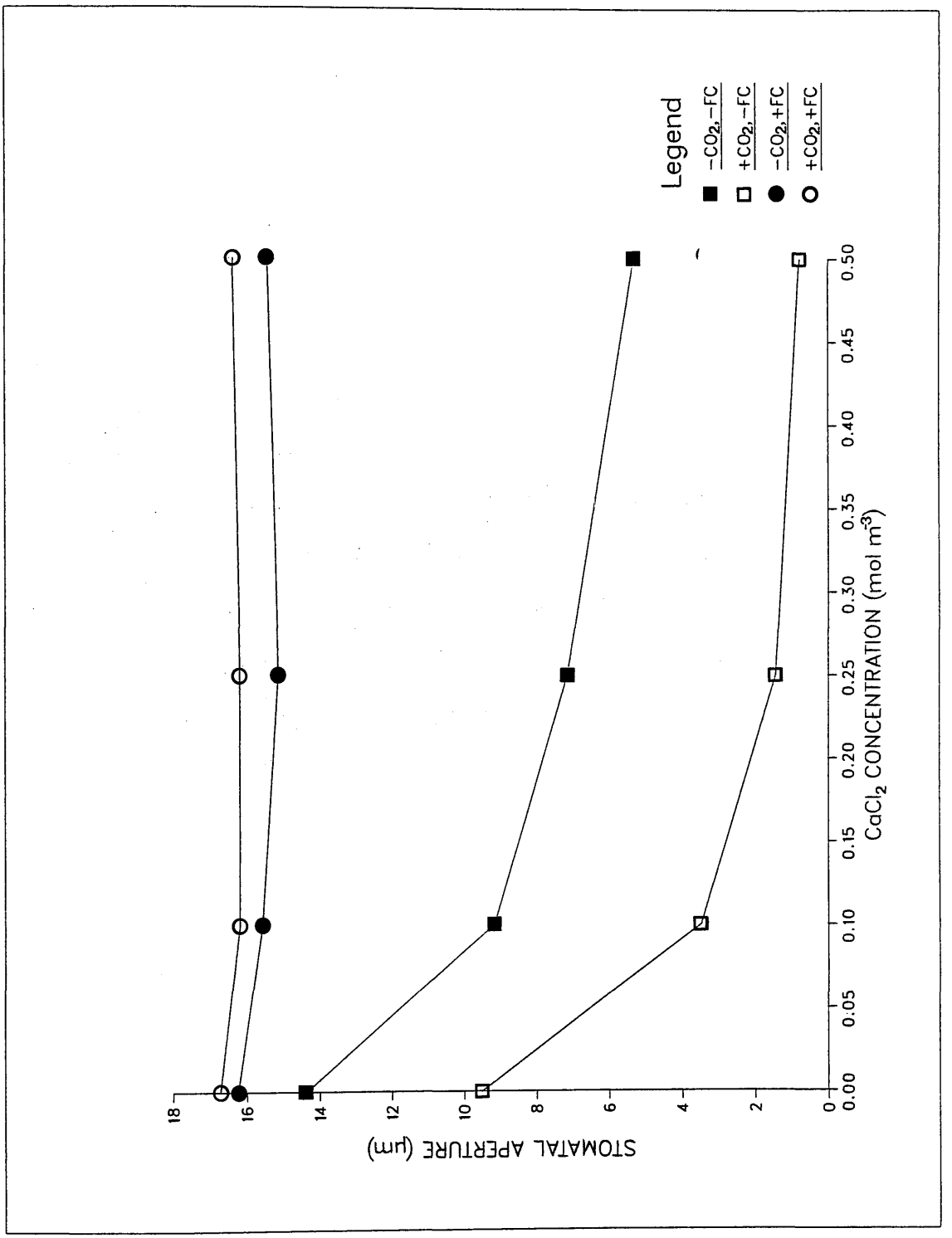

Legend
−CO2,−FC
+CO2,−FC
−CO2,+FC
+CO2,+FC
STOMATAL APERTURE (μm)
0
2
4
6
8
10
12
14
16
18
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
CaCl2 CONCENTRATION (mol m-3)

Figure 2.9

it overrides effects of factors which are known to close stomata, such as darkness, ABA and CO_2 (Squire & Mansfield, 1972, 1974; Travis & Mansfield, 1979a), and it causes a stimulation of the influx of Rb^+ (analogous to K^+) and a decrease in its efflux from 'isolated' guard cells (Clint & MacRobbie, 1984). This striking capacity of fusicoccin in inducing stomatal opening/preventing closure, invited an investigation of its role in relation to calcium-induced stomatal closure.

Preliminary studies to examine dose-response relationship revealed that at 10^{-2} mol m^{-3} fusicoccin could overcome the inhibitory effect of 0.5 mol m^{-3} Ca^{2+} (Fig. 2.8). The effect of fusicoccin was further examined employing a range of calcium concentrations and 350 µl l^{-1} CO_2 in the treatments. Data in Fig. 2.9 show that in the presence of fusicoccin, the previously observed inhibition of opening by calcium was barely detectable. Furthermore, these results confirm the previous findings of Snaith (1984) and Travis & Mansfield (1979a), that fusicoccin in combination with CO_2 enhances stomatal opening rather than simply overriding the inhibitory effects of CO_2. When fusicoccin was supplied, stomata opened more widely in the presence of CO_2 than they did in its absence. This effect was significant ($P < 0.05$) in all treatments except in zero calcium. This increase in stomatal aperture in the presence of CO_2 and fusicoccin could be due to enhanced production of malate in the guard cells (Travis & Mansfield, 1979a).

Another interesting feature of the curves in Fig. 2.9 is that, in the absence of fusicoccin, the responses of stomata to the presence and absence of CO_2 were almost parallel to each other throughout the range of Ca^{2+} tested. This is also true when one compares the two curves in Fig. 2.2 within the range of 25-125 mol m^{-3} KCl. This suggests that the inhibitory action of CO_2 is independent of that of

Ca^{2+}.

The results presented in this Chapter have clarified some earlier unelaborated reports regarding the effects of calcium on stomata. It can now be concluded that in Commelina communis calcium ions inhibit opening/induce closure by reducing net accumulation of potassium ions in the guard cells. It seems very likely that the effect of Ca^{2+} is on the efflux of K^{+}, but not on the influx. The molecular mechanism by which Ca^{2+} modulates ion fluxes in guard cells has not yet been identified, but based on the reports of its regulatory role in other systems (both in animals and plants), it can be speculated that calmodulin mediated processes might be involved in this phenomenon. This possibility will be explored in a later Chapter.

CHAPTER THREE

THE ROLE OF ABSCISIC ACID AND ITS INTERACTION WITH CALCIUM IONS IN PREVENTING STOMATAL OPENING

INTRODUCTION

In the preceding Chapter, the importance of potassium ions in stomatal movements has already been highlighted. Massive fluxes of K^+ to and from guard cells appear to be mainly responsible for the turgor changes between the guard cells and their immediate neighbours (usually specialized subsidiary cells) during stomatal opening and closing. However, potassium is not the only solute which contributes to the turgor changes: other solutes such as malate, citrate, hexose and sucrose may also act as guard cell osmotica (MacRobbie & Lettau, 1980b; Outlaw & Lowry, 1977; Outlaw & Manchester, 1979). Nevertheless, the correlation between K^+ content and the degree of opening is usually high (MacRobbie, 1977; Raschke, 1979). Penny & Bowling (1974) found that the K^+ concentration in epidermal cells of C.communis decreased from 448 mol m^{-3} when stomata were closed, to 73 mol m^{-3} when they were open. Somewhat comparable results have been obtained later by MacRobbie & Letau (1980b), using a different potassium-sensitive microelectrode. They estimated that the K^+ concentration in epidermal cells of the same species, decreased from between 250-450 mol m^{-3} when the stomata were closed to 100 mol m^{-3} when they were open. On the other hand, during opening, guard cells of C.communis were found to accumulate compatible amounts of potassium. Such large fluxes of potassium have been reported by a number of other workers using different plant materials and different techniques (for a review see Outlaw, 1983). It is apparent that the different cells in the stomatal complex operate in opposite phase with one another, and when the guard cells accumulate K^+ ions, the adjacent cells lose them, and vice versa. Such an opposite pattern of behaviour between guard cells and their neighbouring cells clearly implies coordinated, and to some degree opposing, functions of the plasma membranes of these cells

and, Mansfield & Davies (1983) considered that the lack of plasmodesmatal connections between the mature guard cells and their neighbours might be necessary in this situation (Carr, 1976; Sanchez, 1977; Thomson & de Journett, 1970).

The amounts of potassium accumulated in guard cells during opening are very large by normal physiological standards (Mansfield & Davies, 1983), changing 4-6 fold on average. MacRobbie (1982) noted that this could sometimes be above 10 fold (that is, an increase in concentration from 80 mol m^{-3} when stomata were closed to over 800 mol m^{-3} when they were open). Such a high ion uptake is not normally seen in other plant cells. Another peculiarity of guard cells is their ability to regulate this salt accumulation in response to a variety of signals from both the environment and from within the plant itself (MacRobbie, 1982). However, no fundamental difference between the basic mechanisms of ion transport between guard cells and other cells is evident.

The transport of protons is believed to be the primary active process behind ion transport in the guard cells and this occurs from the cytoplasm through the plasma membrane into the aqueous layer around the guard cell (Penny & Bowling, 1975; Raschke & Humble, 1973). The consequent generation of gradients of electrical potential and of pH across the membrane provides a driving force for the uptake of K^+. The electroneutrality within the guard cells following a massive influx of K^+ may be maintained either by accompanied intake of Cl^- or by internal generation of malate (Allaway, 1981; Penny et al., 1976; Van Kirk & Raschke, 1978). Willmer & Rutter (1977) suggested that the well known loss of starch from the chloroplasts of guard cells which normally accompanies stomatal opening might be due to its conversion to malate. However, the relative proportions of Cl^- and malate^{2-} found in the guard cells of open stomata vary widely with species and experimental conditions.

For example, in guard cells of some species (e.g. Allium cepa) there is no starch and there is no malate production, and thus K^+ intake to the guard cells appears entirely to be compensated by Cl^- (Schnabl & Ziegler, 1977). On the other hand in other species such as Commelina communis and Vicia faba, under experimental conditions, if the concentration of Cl^- in the incubation medium is lowered, then the malate level increases (Raschke & Schnabl, 1978; Van Kirk & Raschke, 1978).

The intimate correlation between the degree of stomatal opening and abscisic acid content (both endogenous and exogenous), has already been described in the introductory chapter of this thesis. Although the molecular basis of the action of ABA in guard cells as well as in other cell types is still unresolved, it is believed that it affects membrane function, particularly in relation to potassium permeability and hydraulic conductivity (Cram & Pitman, 1972; Van Steveninck & Van Steveninck, 1983). The effect of ABA on guard cells is found to be a reduction in the net accumulation of K^+. Reduction in potassium accumulation could be a result of a restricted influx or enhanced efflux or both. Influx may be hindered either by interrupting the supply of energy for active ion transport or by preventing H^+ extrusion. Discussing possible mechanisms for ABA action, Raschke (1977, 1979) suggested an effect of ABA on the proton pump at the plasma membrane, or, alternatively, a direct effect of it on malate synthesis. He also suggested that the low cytoplasmic pH or high cytoplasmic malate concentration thereby produced, might lead to a leakage of K^+, Cl^- and malate from the guard cells. This means that the effect of ABA on K^+ uptake would be a secondary one. There is, however, substantial evidence that, this is not so: a direct influence of ABA on the transport of K^+ into the guard cells has been reported by several workers.

Snaith and Mansfield (1982b), Weyers and Hillman (1979) and

Wilson et al. (1978) showed that the ability of Commelina stomata to respond to ABA is highly dependent on the concentration of K^+ surrounding the guard cells. Furthermore, Jarvis & Mansfield (1980) found that the response to ABA was much reduced when Na^+ was supplied to the epidermis as an alternative to K^+. If the proton pump is checked by ABA, the replacement of K^+ by Na^+ is unlikely to overcome the inhibitory effects of ABA, since the H^+ efflux is coupled with influx not only of K^+, but also of other monovalent cations including Na^+ (Marrè, 1979). On the other hand, if ABA were to inhibit the proton pump, the external K^+ would not be expected to modify the responsiveness of guard cells to ABA. Additional evidence has been accumulated from experiments using potassium ionophores. Macrocyclic 'crown' polyethers (e.g. benzo-18-crown-6) which form complexes with potassium ions, and which are thought to be fairly cation-specific, have been found to act as inhibitors of stomatal opening (Georgiou et al., 1982, 1983; Richardson et al., 1979). Like ABA, benzo-18-crown-6, induces stomata to close but, when it was applied in the presence of ABA, the response of stomata to the latter was reduced (Pemadasa, 1983a). Georgiou et al. (1982) postulated that the ionophores might partition into the guard cell plasma membrane, creating channels allowing leakage of the specific ion for which the compound has an affinity and thereby resulting in an enhanced ion efflux down a concentration gradient. If this were so, increasing the K^+ concentration surrounding the guard cells should progressively moderate the ionophore induced closure, because the decreasing concentration gradient would reduce the K^+ leakage from the guard cells. The results of Pemadasa (1983a) of the ionophore effects on adaxial and abaxial stomatal opening, support this view and a situation of that sort is implicated from the work of Wilson et al. (1978), who found a reduced effect of ABA on guard cells supplied with high concentrations

of KCl. MacRobbie (1981a), using radioactive tracers with guard cells of C.communis, obtained evidence that the reduction in the concentration of potassium salts in the guard cells was the result of stimulation of efflux, and was not due to inhibition of influx. She found very high rates of efflux of rubidium and bromide during the first 20 minutes of treatment with ABA. Weyers & Hillman (1980) also found enhanced leakage of rubidium from epidermal strips after addition of ABA. Lea & Collins (1979) and Wassal et al. (1985) using artificially produced lipid bilayer membranes, demonstrated that ABA could increase ion conductance in a manner consistent with the formation of channels. It thus seems likely that the effect of ABA on guard cells is to enhance efflux of potassium, and relatively simple mechanisms are known by which it might operate.

Paleg (1965) proposed that the mechanism of plant hormone action first involves a specific interaction with a perceiving molecule and subsequently a series of biochemical events leading to a physiological response. In this context of sub-cellular action, as far as ABA is concerned, the mechanism of action has not yet been fully characterized. Hartung (1983) found that guard cells of Valerianella locusta did not take up labelled ABA at pH 8, and yet it induced stomatal closure as effectively as when ABA was taken up readily at lower pH values (e.g. 5). He concluded that ABA need not enter the cytosol of the guard cells in order to induce their loss of turgor, and suggested that the primary site of action must either be at the outer surface of plasmalemma or at a location easily accessible from outside. Hornberg & Weiler (1984) reported the presence of proteins which they thought were located in the plasma membrane of the guard cells, and which appeared to bind ABA with a high affinity. This was the first demonstration of proteins which might be involved in the recognition of ABA and its

apparent site of action in plant cells, but has so far not been confirmed by other workers.

It was confirmed in the preceding Chapter that Ca^{2+} ions inhibit stomatal opening by reducing the net accumulation of K^{+} in the guard cells. The action of calcium on guard cells is somewhat comparable to that of ABA in several respects. For example, the inhibition of opening and induction of closure, both of which are associated with a lowered potassium content in the guard cells; the dependence of the external K^{+} concentration for the manifestation of their effects on isolated epidermis; and the reversal by fusicoccin of their inhibitory effects. There is, also, some evidence to suggest that calcium ions are involved in the molecular events underlying the action of auxins, cytokinins and gibberellins (Dieter, 1984; Elliott et al., 1983; Kelly, 1984). In addition, it has previously been shown that auxins appear to interact with ABA in the regulation of stomatal movements. Therefore, an interplay between abscisic acid and calcium ions was strongly insinuative, and the experiments reported in this Chapter explore this possibility. The effects of EGTA on the action of ABA on stomata were also investigated.

MATERIALS AND METHODS

The plant material and its preparation for experimentation were similar to that described in the previous Chapter. Abaxial epidermal pieces were incubated for 3 ± 0.25 h in light in the absence of CO_2. The basic incubation medium (control) contained 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer (pH 6.15) in which the required concentrations and combinations of each additional compound were dissolved. ABA (cis-trans, 95% purity) was obtained from Sigma Chemical Co., Poole, Dorset.

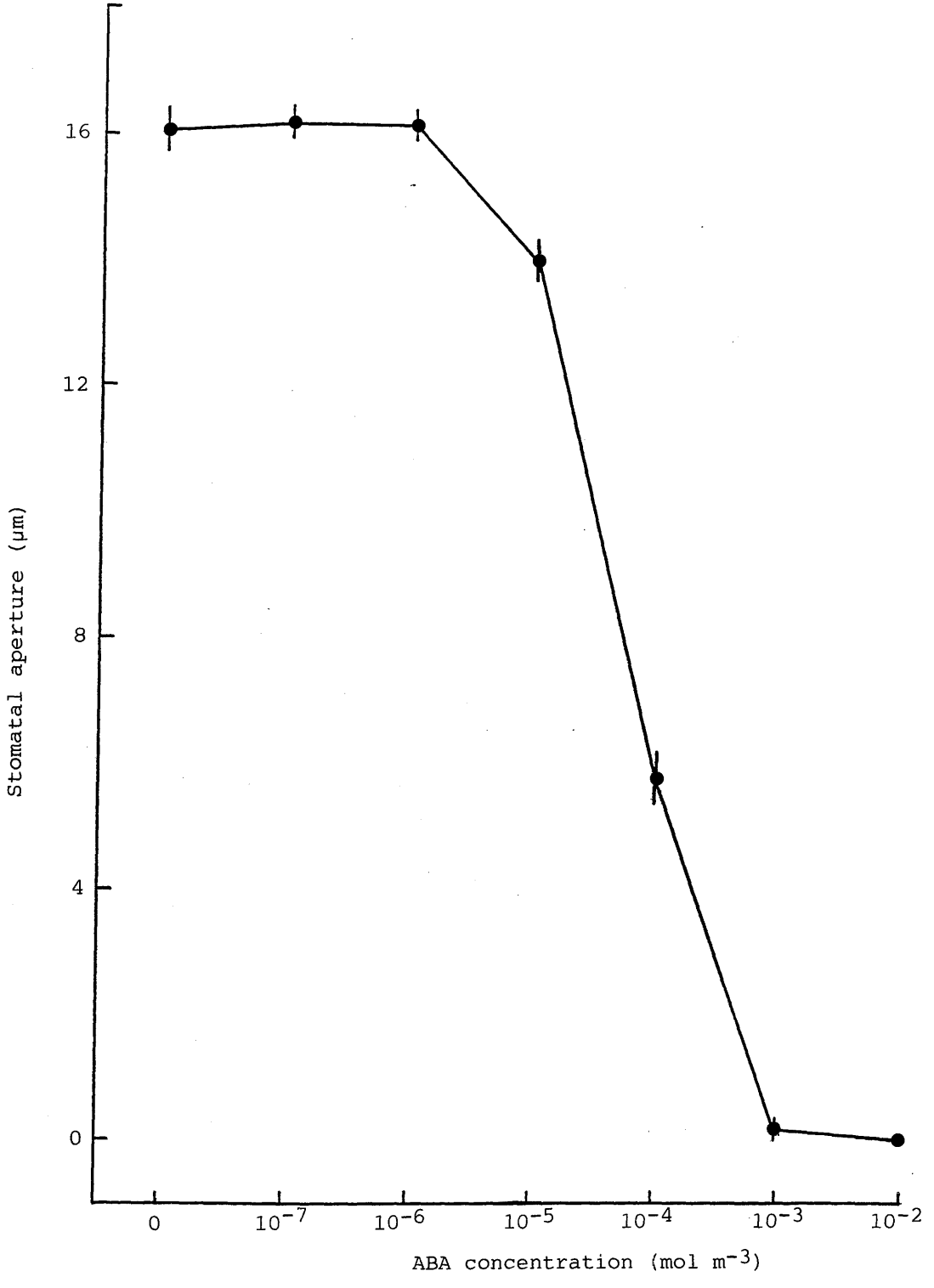


Figure 3.1

Effect of ABA concentration on stomatal opening. Abaxial epidermal peels were incubated for 3 h, in light, in the absence of CO_2, in 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer pH 6.15 (KOH) containing a range of ABA concentrations. Means of 90 individual measurements with standard errors.

Figure 3.2

Inhibition of stomatal opening after incubation of isolated epidermis for 3 h in various combinations of ABA and $CaCl_2$. Histogram treatment

A, control

B, 10^{-6} mol m^{-3} ABA

C, 10^{-5} mol m^{-3} ABA

D, 0.1 mol m^{-3} $CaCl_2$

E, 0.25 mol m^{-3} $CaCl_2$

F, 10^{-6} mol m^{-3} ABA + 0.1 mol m^{-3} $CaCl_2$

G, 10^{-6} mol m^{-3} ABA + 0.25 mol m^{-3} $CaCl_2$

H, 10^{-5} mol m^{-3} ABA + 0.1 mol m^{-3} $CaCl_2$

I, 10^{-5} mol m^{-3} ABA + 0.25 mol m^{-3} $CaCl_2$.

Means of 90 measurements of individual stomata with 95% confidence limits.

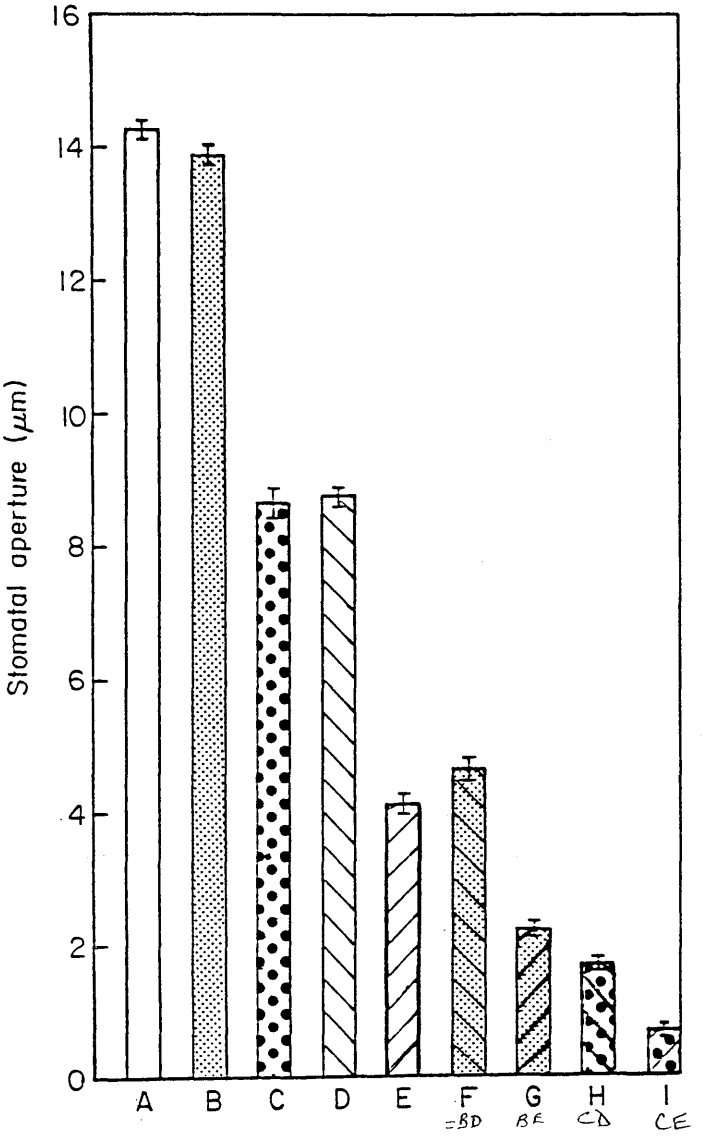


Fig.3·2

RESULTS AND DISCUSSION

The response of abaxial stomata to a wide range of ABA is shown in Figure 3.1. There was no significant effect on stomatal opening, of ABA at 10^{-7} or 10^{-6} mol m^{-3}. Increasing ABA concentrations beyond 10^{-6} mol m^{-3} caused stomatal closure and the stomata were almost completely closed at 10^{-3} mol m^{-3}. These results are in broad agreement with those reported previously on Commelina communis epidermal strips, by Snaith & Mansfield (1982b) and Wilson (1981). To look for possible interactions between calcium and abscisic acid, epidermal pieces were incubated in the presence of low concentrations of calcium (0.1 and 0.25 mol m^{-3}) and ABA (10^{-6} and 10^{-5} mol m^{-3}), and the results are shown in Fig. 3.2. In this experiment there was a very small reduction in aperture at 10^{-6} mol m^{-3} ABA; experience has shown that there is a slight variation in the sensitivity of stomata to ABA in different experiments (compare histogram B of Fig. 3.2 with Fig. 3.1 in which no effect of 10^{-6} mol m^{-3} ABA was observed. A possible explanation for such variations is given in the previous Chapter). The little effect observed at 10^{-6} mol m^{-3} ABA was amplified when it was applied in the presence of 0.1 mol m^{-3} calcium and it became significant. This clearly shows a synergistic response of stomata to ABA and calcium, and the examination of data for other concentrations, with the exception of 10^{-5} mol m^{-3} ABA + 0.25 mol m^{-3} Ca^{2+}, supports this conclusion. That exception rather shows an antagonism between the two agents. This is attributed to some of the stomata being closed and thus unable to respond further. A similar situation was observed in the next experiment in which the interaction was examined further in a 3 x 5 factorial design. The analysis of variance revealed a highly significant ($P < 0.001$) interaction between ABA and calcium, and the appearance of the isometric projection of the data (Fig. 3.3) suggests

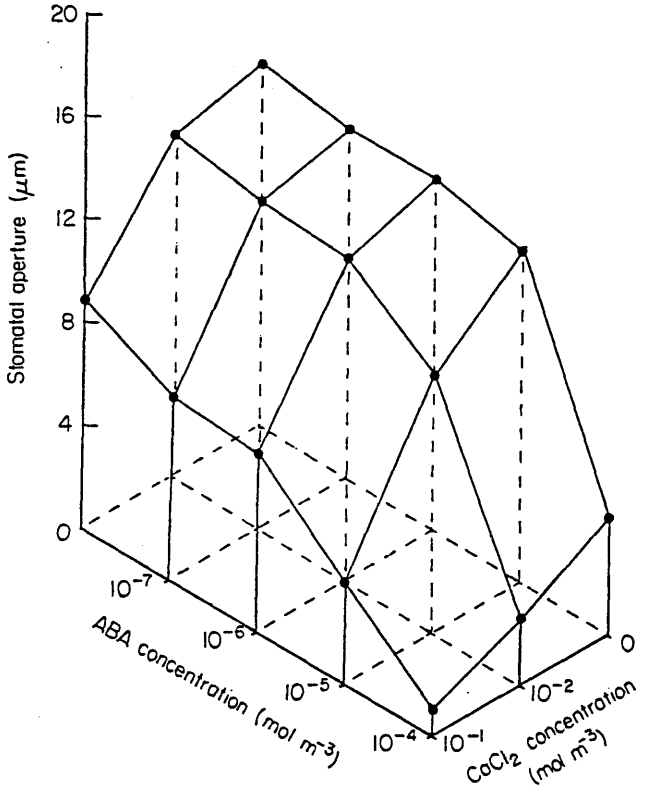


Figure 3.3

Isometric projection of the results of a 3 x 5 factorial experiment showing effects of ABA and calcium in preventing opening of stomata on isolated epidermis. Means of 90 measurements of individual stomata.

that this occurs mainly in the range of 10^{-6} to 10^{-4} mol m^{-3} ABA.

The strong suppression of opening caused by ABA and calcium when present simultaneously, was closely associated with a reduction in guard cell K^+ content. Therefore, the observed greater-than-additive effects may be explained in terms of their effects on ion accumulation. Since the net accumulation is determined by the degree of both influx and efflux, an inhibition of influx by one agent occurring simultaneously with a stimulation of efflux by the other could be manifested as a synergistic suppression of guard cell turgor, because the relationship between turgor and the influx/efflux process may not be linear (MacRobbie & Lettau, 1980b). The synergism observed between ABA and the synthetic auxin, naphth-1-ylactic acid, has tentatively been explained in this way (Snaith & Mansfield, 1984).

Alternatively, if the action of one agent depended in some way on the presence of the other, that could lead to synergism. For example, if the attachment of ABA to its recently discovered binding protein (Hornberg & Weiler, 1984), resulted in an increase in membrane permeability to calcium, and the subsequent increase in intracellular calcium affected the guard cell K^+ channels reported by Schroeder, Hedrich & Fernandez (1984), then a synergistic effect of ABA and calcium would be expected. This seems to be a feasible explanation for this synergism and, therefore, it suggests that the action of ABA is dependent on the availability of calcium ions in the free space. Perhaps, one might then argue that ABA would not exert its effects on the guard cells with no added calcium in the incubation medium. However, as it has already been pointed out, there is likely to be free calcium in the apoplast in sufficient amounts and that may account for the observed effects of ABA when calcium was not provided in the external medium.

It was, therefore, interesting to investigate the effects on ABA

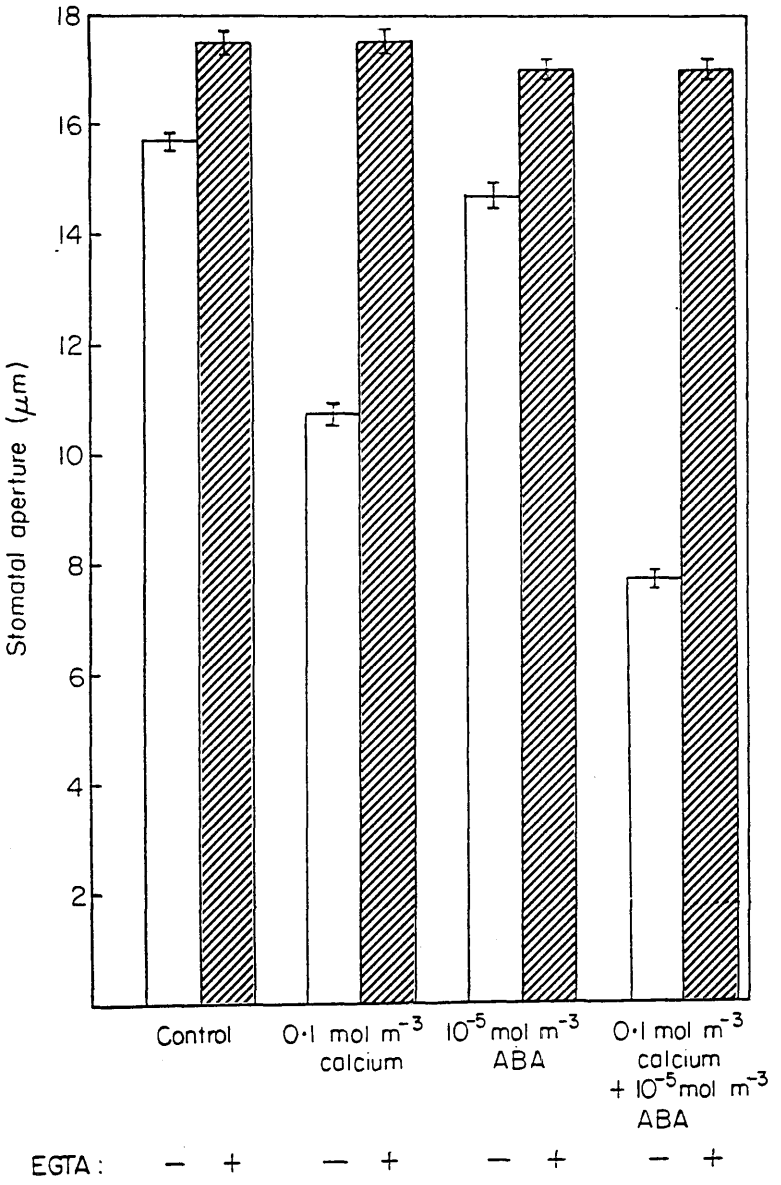


Figure 3.4

Effect of 2 mol m^{-3} EGTA on the stomatal responses to ABA and/or $CaCl_2$. Epidermal pieces were incubated for 3 h in the absence of CO_2, in light, at 25 ± 1°C. The treatments were: control, 0.1 mol m^{-3} $CaCl_2$, 10^{-5} mol m^{-3} ABA and 0.1 mol m^{-3} $CaCl_2$ + 10^{-5} mol m^{-3} ABA. Each treatment was given with or without 2 mol m^{-3} EGTA. Means of 90 measurements of individual stomata with 95% confidence limits.

action of the removal of apoplastic calcium using the chelating agent EGTA. Data in Figure 3.4 show that when 2 mol m^{-3} EGTA was present in the incubation medium, the individual and synergistic suppression of stomatal opening by ABA and Ca^{2+} was abolished, and the opening in each case was greater than that in the control without EGTA. The stimulation of opening in the controls by EGTA was probably due to the removal of endogenous apoplastic calcium which might have prevented opening to some degree.

In view of the results presented in this Chapter, it appears that free calcium in the apoplast is essential to enable guard cells to respond to ABA. Although the molecular basis of the synergism is not known, ABA seems to increase cytosolic Ca^{2+} concentration. The subsequent action of calcium ions in the guard cells is as yet unknown, but it can be speculated that calmodulin may be involved, and this possibility will be examined in the following Chapter.

CHAPTER FOUR

THE INVOLVEMENT OF CALCIUM CHANNELS AND CALMODULIN IN THE ACTION OF ABA ON STOMATA

INTRODUCTION

A key requirement in understanding the role of calcium as a cellular control mediator is the ability to manipulate the activity of the cation in subcellular compartments and to alter specifically, transmembrane Ca^{2+} flux with a minimal disturbance to other properties of the system. An important advance in this area was the discovery of the divalent cation ionophore, A23187 (Reed, 1972; Reed & Lardy, 1972).

The term "ionophore" (= ion bearer) is used in a biophysical context to mean a compound which facilitates the transport of an ion through a natural or artificial lipid membrane from one aqueous medium to another. This definition was given by B.C. Pressman (see Pressman et al., 1967) and dates from his discovery that certain antibiotics stimulated the uptake of potassium by respiring mitochondria (Pressman, 1965). Ionophores are compounds of moderate molecular weight (about 200-2000) that form lipid-soluble complexes with polar cations of which K^+, Na^+, Ca^{2+} and Mg^{2+} are the most significant biologically (Pressman, 1976). Ionophores may, in general, be regarded as molecules with backbones of diverse structures that contain strategically spaced oxygen atoms. The backbone is capable of assuming critical conformations that focus these oxygens about a ring or cavity in space into which a complexible cation may fit more or less snugly. The ligand-binding oxygens consist of various functional groups, such as ether, alcohol, carboxyl and amide. The neutral oxygens ligand to cations via ion-dipole interaction analogous to the solvation of ions in high-dielectric solvents. Thus, in a sense, ionophores act as solvators for cations displacing more or less completely, the solvation shell of the 'bulk solvent' (Pressman, 1976; Truter, 1976). The polar groups of ionophore-complexes orient towards the interior, the exterior bristling with various hydrocarbon groupings. The lipid solubility of the

resulting complex may be partially explained by the effective shielding of its polar interior which delocalizes the cation charge, and partially by the compatibility of the complex exterior with low dielectric solvents (Pressman, 1976; Truter, 1976). The cation is transported to the opposite side where it is decomplexed. Physical studies indicate that the complexation-decomplexation kinetics and diffusion rates of ionophores and their complexes across lipid barriers are so favourable that their transport turnover numbers across biological membranes attain values of thousands per second exceeding the turnover numbers of most macromolecular enzymes (Ovchinnikov, 1974; Haynes et al., 1974). This, along with their high cation selectivity, inspired consideration of ionophores as model carriers for use in biological systems (Pressman, 1976).

Ever since the discovery of A23187, a few thousand publications have appeared in which it has been employed to investigate Ca^{2+} metabolism and its involvement in control mechanisms in a wide variety of tissues, cells and subcellular organelles, and it has been found that it mimics, at least qualitatively, the effect of many physiological stimuli, the responses to which are thought to be mediated by a calcium-involved regulatory process (Campbell, 1983; Pressman, 1976; Rasmussen & Goodman, 1977).

A23187 is a carboxylic acid antibiotic that is crystallized from broths of the bacterium Streptomyces chartreusensis as the magnesium plus calcium salt, and can be converted to and crystallized as the free acid (Reed, 1972; Reed & Lardy, 1972). The free acid has a molecular weight of 523 and the elemental analysis of $C_{29}H_{37}N_3O_6$ (Reed, 1972). The structure of A23187 shown in Fig. 4.1 was established by Chaney et al. (1974) using X-ray crystallography. This molecule has a high degree of conformational rigidity and its fitness for being an ionophore

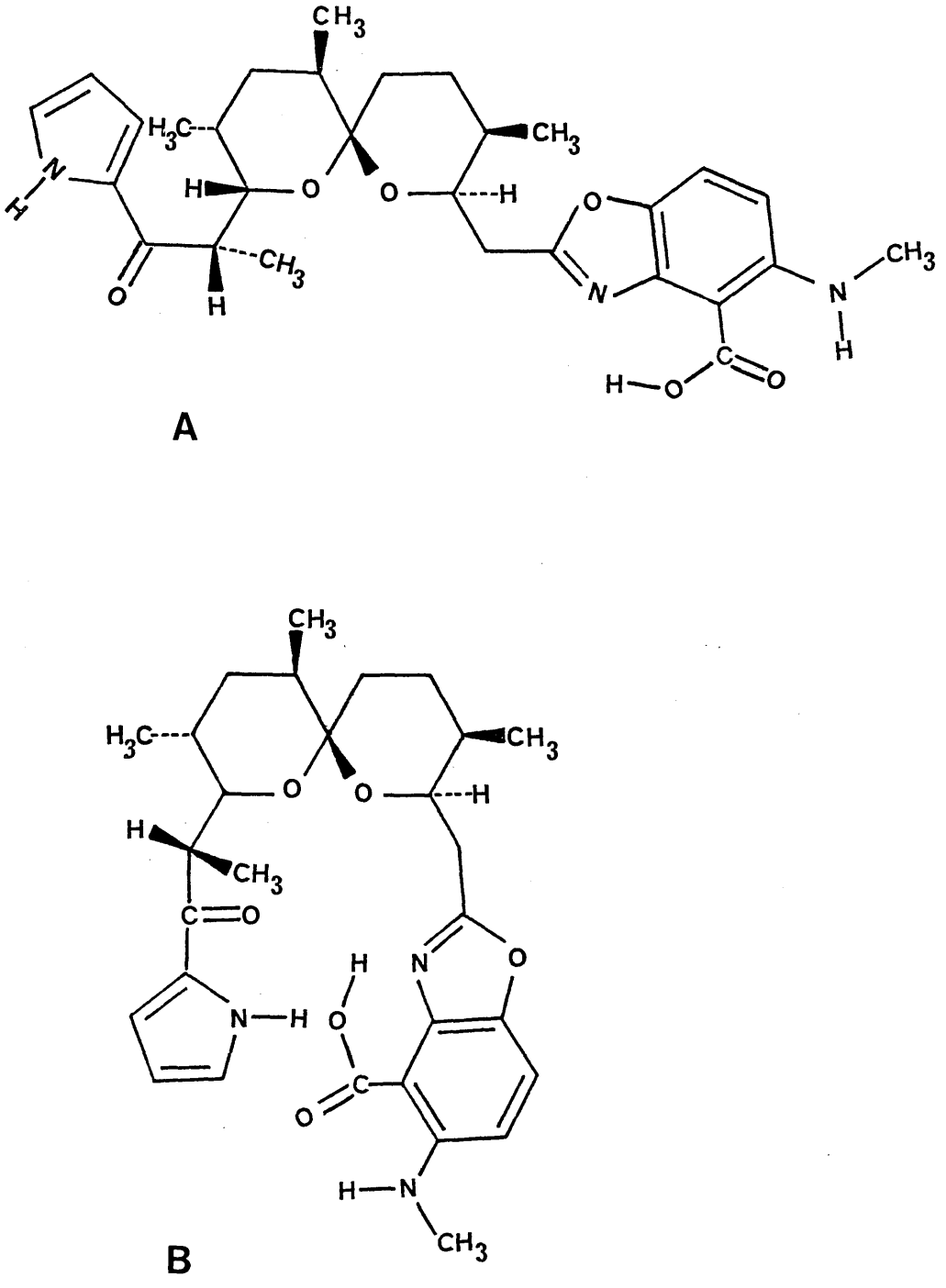


Figure 4.1

The chemical structure of A23187 as the free acid. This was determined by Chaney et al. (1974). A and B show the structure in 'open' and 'closed' conformations, respectively. Reproduced from Pfeiffer et al. (1974).

has been described in detail by Campbell (1983), Chaney et al. (1974) and Pfeiffer et al. (1974).

A23187 has a linear backbone to which oxygen containing heterocyclic rings are attached. The molecule is effectively cyclized by head-to-tail hydrogen bonding between the head carboxyl group and >NH group of the tail-end heterocyclic ring (Chaney et al., 1974; Pfeiffer et al., 1974).

Unlike most other cation ionophores, A23187 is a unique ionophore tool since it is so predominantly selective for divalent over monovalent ions (Reed & Lardy, 1972; Pfeiffer et al., 1974). Pressman attributed this to the probable inclusion of two nitrogen atom ligands in the complexion sphere (Fig. 4.1b). It is not certain, however, which oxygens and nitrogens ligand in complexing with divalent cations, since the structure is based on X-ray crystallography of uncomplexed A23187. Although this ionophore transports Mg^{2+}, the gradients of which across biological membranes seldom participate in biological control, it has been successfully used to study various Ca^{2+}-dependent biological reactions without disturbing pre-existing balances of Na^{+} and K^{+}. Nevertheless, recently, Hinds and Vincenzi (1985) have highly criticized the use of the term 'calcium ionophore' for A23187. They pointed out that it also promotes a passive movement of Mg^{2+}, and suggested that it would be better to use selective Ca^{2+} ionophores such as ETH 1001. However, it is thought that A23187 is more specific for Ca^{2+} relative to Mg^{2+} (Pressman, 1976). The initial work on the biological application of A23187 has centred on the regulatory role of calcium in animal systems and this has been reviewed by Pressman (1976) and Rasmussen & Goodman (1977).

Although A23187 has been employed extensively in animal research during the last decade, it was not until the 1980's that most plant

physiologists became aware of its importance for their investigations on calcium. However, since then, they have found that the ionophore mimicked the action of a number of stimuli, the primary effect of which is thought to be a flux of Ca^{2+} across plasma membrane. For example, induction of cytokinin-like mitosis in Funaria (Saunders & Hepler, 1982), induction of Ca^{2+} uptake by Marsilea microspores (Wick, 1978), inhibition of cytoplasmic streaming in Tradescantia stamen hair cells (Dorée & Picard, 1980), inhibition of pollen tube tip growth (Herth, 1978), and artificial induction of red-light induced chloroplast rotation in the dark (Serlin & Roux, 1984a, 1984b). Therefore, it can be suggested that the fundamental mechanism of the action of A23187 in plants is similar to that in animals.

As described above, the ionophore can promote Ca^{2+} fluxes across a plasma membrane without forming channels. However, in situ, a rise in the cytoplasmic calcium concentration in response to an excitatory or stimulatory signal is attributed to a flux of Ca^{2+} mainly via specific channels. Further, Ca^{2+} may also be transported across plasma membrane by the operation of a Ca^{2+} pumping ATPase and the Na^{+}/Ca^{2+} exchanger mechanism (see Carafoli, 1984). As in the case of the divalent ionophore, most of the information as to how calcium ions cross plasma membranes is known for animal systems and great emphasis is given to muscle cells especially of heart, and to neurones. Therefore, any review of calcium movement across plasma membranes must obviously be centred on those discoveries.

Animal physiologists have long known that the signalling and transduction capacity of excitable cells in the nervous system rests on the ability of these cells to regulate ion permeation across their surface membranes, and it is now accepted that ionic channels are the elementary excitable components in, for example, the membranes of

muscle and nerve cells. In fact, ionic channels have a very wide distribution in different animal tissues and are required for the normal functioning of non-nervous cell types (see, for a review, Parsegian, 1984).

Ionic channels are intrinsic macromolecular proteins that are embedded in the lipid bilayer matrix of cell membranes, and they function as pores through which diffusion of ions and molecules takes place. As its function demands, an ionic channel is a transmembrane structure which it is thought needs to contain both a hydrophobic surface (allowing insertion in the membrane lipid) and hydrophilic residues (likely to be arranged as a 'hole' through the membrane, thereby permitting ion permeation) (Takeda et al., 1985). The hole is filled with water and a cation passing through it would bind two or three negatively charged sites on its journey down an electrochemical gradient (Sperelakis, 1984).

It is well known, at least for animal cells, that ionic channels have many diverse, yet well defined roles (Parsegian, 1984) and calcium channels are the most studied type. The movement of calcium across the plasma membrane is usually electrogenic, which means that, in favourable conditions, it can be measured as a membrane current (Reuter, 1983). The driving force is the electrochemical gradient of the ion across the membrane, and the likely structures through which the ions permeate are calcium channels. These channels in excitable membranes are controlled by voltage-dependent gating: that is, their opening and closing kinetics are the result of changes in membrane potential (Reuter, 1983; Sperelakis, 1984). It has recently been suggested that a 'voltage sensor' in the membrane, for example, a protein group with dipole properties, which may be an integral part of an ionic channel, could react to a change in electric field (e.g.

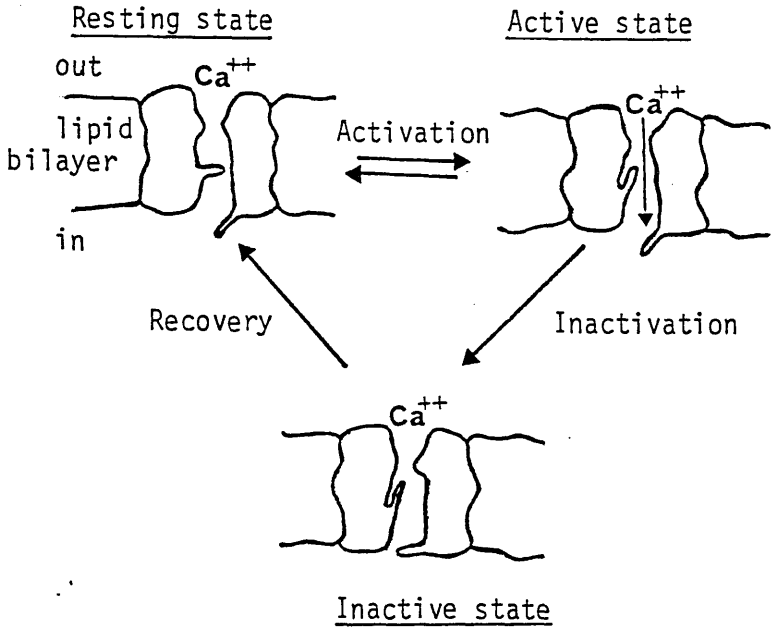


Figure 4.2

Cartoon model for the three hypothetical states of a slow channel. In the resting state, the activation gate (in the middle of the channel) is closed and the inactivation gate (at the end of the channel) is open. Depolarization to the threshold activates the slow channel to the active state, opening the activation gate rapidly, thus making the entire channel open. This activated channel spontaneously inactivates to the inactive state because of the closure of the inactivation gate. The recovery process upon repolarization returns the channel from the inactive state back to the resting state which is again available for reactivation. Calcium ions can pass down an electrochemical gradient when both gates are in the open position (active state of the channel). A channel blocker (see below) can bind to the channel in the active or inactive state which would either block the activated channel or slow recovery process for converting from the inactive state back to the resting state. Modified from Sperelakis (1984).

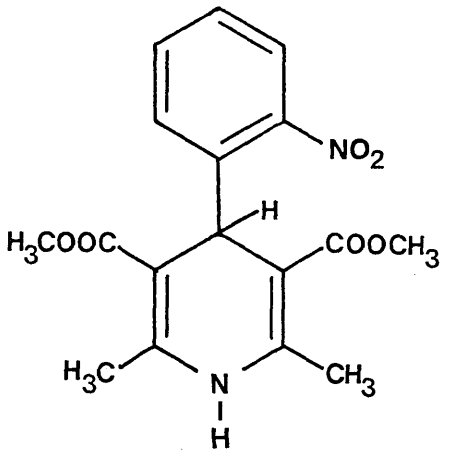
a change in membrane potential) resulting in a reorientation of the charged sensor within the field and, therefore, a change in ion flow through the channel (Reuter, 1983). A three-state sequential model for opening and closing of these channels has been proposed by Fenwick et al. (1982) and Sperelakis (1984) and this is shown in Fig. 4.2. However, it is worth noting that the exact molecular structure of the channels or the precise biophysical interpretation of their gating mechanism is not yet fully understood (Reuter, 1983). The calcium channels are also time-dependent, and are sometimes known as slow channels because they behave kinetically as if their gates open, close and recover (Fig. 4.2) more slowly than those of the other cation channels namely, Na^+ and K^+ (Campbell, 1983; Sperelakis, 1984). The other characteristics of calcium channels which are distinguishable from those for Na^+ and K^+ have been described in detail by Campbell (1983).

One of the most important features of these Ca^{2+} channels is their extreme sensitivity to a variety of compounds variously referred to as Ca^{2+}-channel antagonists or Ca^{2+}-entry blockers or Ca^{2+}-channel blockers (Schwartz & Triggle, 1984). Sometimes these compounds are known as calcium antagonists, but that term is not very precise since it may encompass compounds that potentially inhibit Ca^{2+}-dependent processes or regulatory mechanisms without exerting their primary effects at some other known sites, such as ion channels other than Ca^{2+}-channels (Cohen et al., 1984). Within this definition, Ca^{2+} antagonists can be subdivided into two main categories, namely, calcium availability inhibitors and calcium effect inhibitors. Furthermore, three groups of calcium availability inhibitors can be recognized as follows: Ca^{2+} channel inhibitors, Ca^{2+} efflux stimulants and stimulants of Ca^{2+} uptake into storage sites. The ultimate result

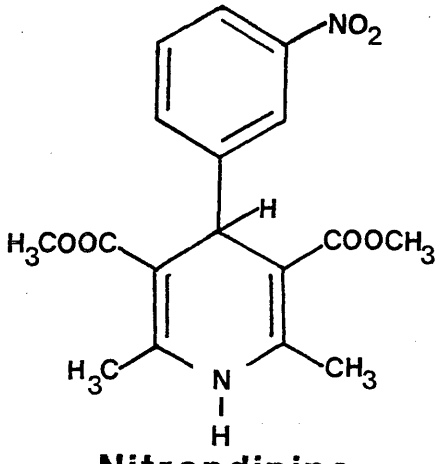
of the action of all these groups is a reduction of available calcium in the cytosol.

A number of subclasses of organic calcium channel inhibitors can be identified based on their chemical nature, for example, (a) dihydropyridines such as nifedipine, nitrendipine and nimodipine (Fig. 4.3), (b) verapamil and gallopamil (D600) (Fig. 4.4) and (c) diltiazem and structurally related compounds (Fig. 4.4). The qualitative differences in pharmacological effects (these chemicals are used as drugs) of these various subclasses suggest that different groups of agents exert their effects by acting at different sites, and/or by different mechanisms of action (Henry, 1980; Millard et al., 1983). Many of the biochemical and pharmacological effects of these compounds have been characterized using animal systems and the importance of the use of them in plant research is now being appreciated.

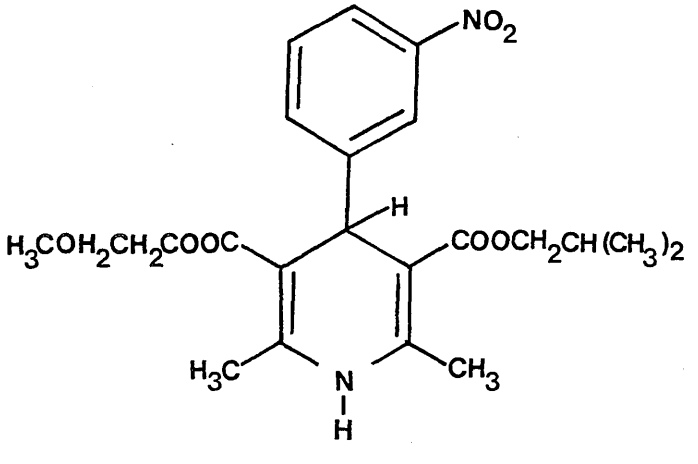
Calcium antagonism as a concept of specific drug action was pioneered by Fleckenstein from his observations that verapamil mimicked the cardiac effect of Ca^{2+} withdrawal, an effect overcome in apparently competitive fashion by increased Ca^{2+} (Fleckenstein, 1971; 1983). Subsequent studies have added many other agents, including the dihydropyridine derivatives and diltiazem and related compounds to this category. Verapamil and D600 have been found to affect a variety of animal tissues, the effects on which are thought to be due to blockage of Ca^{2+} channels. They block slow Ca^{2+} current in squid giant axon, calcium channels in vertebrate heart, K^{+}-induced luminescence in luminescent cells of Obelia and L-glucose-induced contraction in barnacle muscle, and inhibit tension in Guinea pig atrium, excitation-contraction coupling of frog sartorius muscle, Ca^{2+} efflux and release of growth hormones from pituitary, secretion



Nifedipine



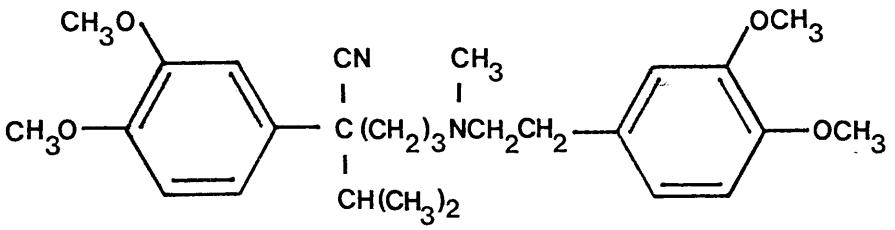
Nitrendipine



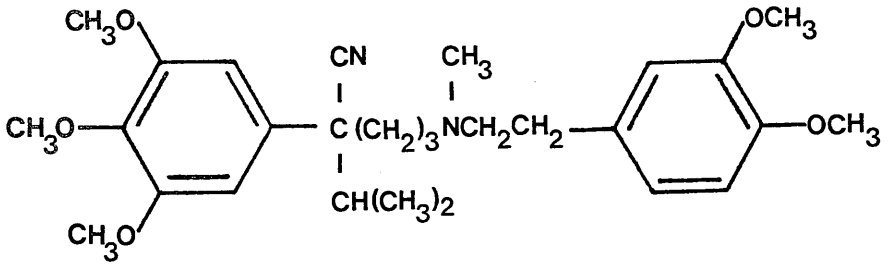
Nimodipine

Figure 4.3

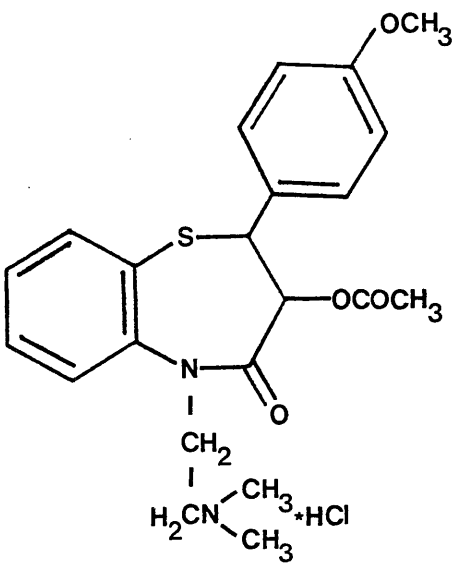
Chemical structures of three dihydropyridine-group calcium channel blockers.



Verapamil



Gallopamil (D600)



Diltiazem

Figure 4.4

Chemical structures of various calcium channel blockers.

of rabbit lacrimal gland and glucose-induced insulin secretion (see for references Campbell, 1983). Apart from their physiological interest, a most important aspect of these drugs is their therapeutic value.

In the United States there are three drugs on the market which have been labelled Ca^{2+} channel-blocking drugs or Ca^{2+} antagonists. They are verapamil, nifedipine and diltiazem (Schwartz, 1984; Schwartz & Triggle, 1984). These agents have been approved for the treatment of cardiovascular diseases. Since the calcium channel blockers dilate peripheral resistance vessels, they may be useful in the treatment of hypertension. There are reports that they may also be effective in the treatments of migraine headache. Since Ca^{2+} is thought to be ultimately involved in all processes that require stimulation, either electrical or chemical, it is not surprising that drugs whose primary effect appears to be an inhibition of the entry of calcium across cell membranes should exhibit a wide spectrum of therapeutic activity (Schwartz, 1984). The pharmacological action of these drugs has been examined in numerous isolated and intact cardiovascular systems (see, for references, Schwartz & Triggle, 1984).

Despite the broad spectrum of pharmacological and therapeutic action exhibited by the Ca^{2+} channel antagonists, there is ample evidence that these agents have specific rather than non-specific actions (Schwartz & Triggle, 1984). Although these agents do not share a single structure-activity relationship within each chemical group, there is clear dependence of activity upon chemical structure, including stereospecificity (Rosenberger & Triggle, 1978; Mannhold et al., 1982). However, this chemical heterogeneity suggests that these agents may antagonise calcium channel function at different sites by different mechanisms (Cohen et al., 1984; Freedman & Miller, 1984; Schwartz & Triggle, 1984). It is evident that the site of action of

these chemicals is at the level of the plasma membrane from the observation that they had no inhibitory effects upon Ca^{2+} responses in skinned cardiac and smooth muscles (Morad et al., 1982; Spedding, 1983). However, one cannot rule out the possibility that there is no effect of these drugs at an intracellular site, and there are some reports that they act at 'free' sarcoplasmic reticulum (Colvin et al., 1982) and calmodulin (Epstein et al., 1982). Nevertheless, such reported effects need not necessarily confuse our understanding of the primary action of these drugs, since it is found that high drug concentrations are needed to affect those systems, and therefore, the relevance of those in vitro effects to the therapeutic action of these drugs is still doubtful (Schwartz & Triggle, 1984).

Considerable progress as to how these calcium channel blockers act at cellular level has been made with the use of specific radioligand binding studies. It has been found that $[H^3]$-nitrendipine and $[H^3]$-nifedipine bind specifically to crude or partially purified membrane preparations from cardiac, skeletal and smooth muscle, and from brain, and also to intact isolated cells (De Pover et al., 1982; Ehlert et al., 1982a, 1982b; Fosset et al., 1983; Gould et al., 1982; Murphy et al., 1982). Furthermore, it has been reported that the binding of these drugs is temperature dependent. Some studies have revealed that in their binding to membranes they are competitive with each other (see Schwartz & Triggle, 1984), but there are important qualitative and quantitative discrepancies in binding of these drugs. Further, it is very likely that several discrete binding sites exist for the different categories of antagonists (Schwartz & Triggle, 1984).

It has now been firmly established that calcium channels play an important role in the regulation of Ca^{2+} fluxes across the membrane of animal cells. These channels, and the effects as well as the

mechanisms of action on them of the channel blockers have been characterized to some extent (Lee & Tsien, 1983; Reuter, 1983; Schwartz & Triggle, 1984). However, very little is known about the calcium channels in plant cells. Voltage-gated specific ionic channels are thought to be involved in calcium fluxes in Characeaen giant algae (Hope & Walker, 1975; Lunevsky et al., 1983). Evidence is now accumulating to suggest that calcium channels do exist in higher plant cell membranes too, and that the putative channel blockers act on them in the same way as in the animal systems. The results of Reiss & Herth (1979) suggested that the localized wall secretion in the tip region of the pollen tube of Lilium longiflorum was due to an asymmetric distribution of channels in opposite sides of the cells. The use of channel blockers, including inorganic cations such as La^{3+}, has been found to arrest or delay a number of processes in which calcium is thought to be involved in plants. In dividing stamen hair cells of Tradescantia, La^{3+} and D600 arrested or greatly extended metaphase (Hepler, 1985). Verapamil and La^{3+} arrest cells of Micrasterias from developing their multilobed morphology (Lehtonen, 1984). Wayne and Hepler (1984, 1985b) showed that La^{3+} blocked the red light-stimulated Ca^{2+} uptake and consequently, the germination of Onoclea spores. Lanthanum is also found to inhibit the light-induced phototactic response in Chlamydomonas and this effect was reversed by addition of Ca^{2+} (Nultsch, 1979). Saunders and Hepler (1983) reported that La^{3+}, Verapamil and D600 inhibited the cytokinin-induced bud formation in Funaria protonema, and this effect was also found to be overcome by addition of Ca^{2+}.

It appears, therefore, that the movement of calcium across cell membranes and the action of calcium channel blockers are basically similar both in animals and plants. Further, these channel blockers

are potentially very useful tools in establishing the existence of such channels in plant cell membranes, and also in substantiating the concept that these channels open in response to a depolarization of the membrane potential so that calcium flows into the cell, raising the internal free calcium ion concentration (Hepler & Wayne, 1985). However, it would not be expected that every channel blocker will behave in the same way both in animal and plant cells, and it is probable that the degree of effectiveness of these blockers may vary from one system to another.

There can be little doubt that, at least in animal systems, an increase in intracellular Ca^{2+} levels in response to a stimulus triggers and/or maintains some biochemical reactions which eventually lead to a response. Thus, calcium acts as a mediator of many cellular responses, and is a regulator of major importance in cellular homeostasis. In order to elucidate the mechanisms of calcium control and the role of calcium in stimulus-response coupling, it is necessary to examine the cellular receptors for calcium. Data from chemical, physical and biological studies strongly suggest that the cytoplasmic receptors (intracellular targets) for calcium acting as a signal transducer or a biological messenger, are a class of calcium-binding proteins referred to as calcium-modulated proteins (Bergess et al., 1983; Kretsinger, 1976, 1980; Watterson et al., 1976). Calcium-modulated proteins reversibly bind calcium with dissociation constants in the micromolar range under relative physiological conditions. Since most calcium modulated proteins are intracellular and have dissociation constants that span the range of intracellular free calcium concentrations, they are postulated to be the major transducers of biological calcium signals (Van Eldik & Watterson, 1985). Most calcium-modulated proteins characterized to date are not enzymes, but are effector

proteins capable of transducing a calcium signal into a cellular response by their ability to regulate or modulate the activity of other macromolecules, including enzymes, in a calcium-dependent manner. These include parvalbumin, troponin C, S100, myosin light chains, vitamin D-dependent calcium binding proteins and calmodulin (Burgess et al., 1983). Of all these, calmodulin is by far the most extensively studied calcium-modulated protein.

The first evidence for an activator protein of 3',5'-nucleotide phosphodiesterase was obtained in the late 1960's by Cheung (Cheung, 1967, 1969, 1970) and this was soon confirmed by Kakiuchi and Yamazaki (1970). This activator protein was found in several tissues (Cheung, 1971) and was shown to be dependent upon the presence of Ca^{2+} (Kakiuchi & Yamazaki, 1970). These early observations are credited as the discovery of calmodulin, and in the years immediately following, the existence and isolation of calmodulin from many sources were documented (reviewed by Dieter, 1984, Marmé & Dieter, 1983 and Walsh & Hartshorne, 1983). As a result of this diversity of experimental systems, a number of different names for calmodulin was generated: these include calcium-dependent activator, calcium-dependent regulator (CDR), modulator protein, troponin C-like protein and several others (see Marme, 1983). Cheung et al. (1978) proposed the term calmodulin to indicate that calcium is involved and also that the protein serves a modulatory function.

Calmodulin has been shown to be ubiquitous in the animal (Cheung et al., 1978; Waisman et al., 1975) and plant kingdoms (Anderson & Cormier, 1978; Anderson et al., 1980; Marmé & Dieter, 1982). It has been identified in all eukaryotic cells so far examined and including vertebrates and invertebrates, plants and higher fungi, several unicellular eukaryotes, and yeasts (Hubbard et al., 1982; Klee & Vanaman,

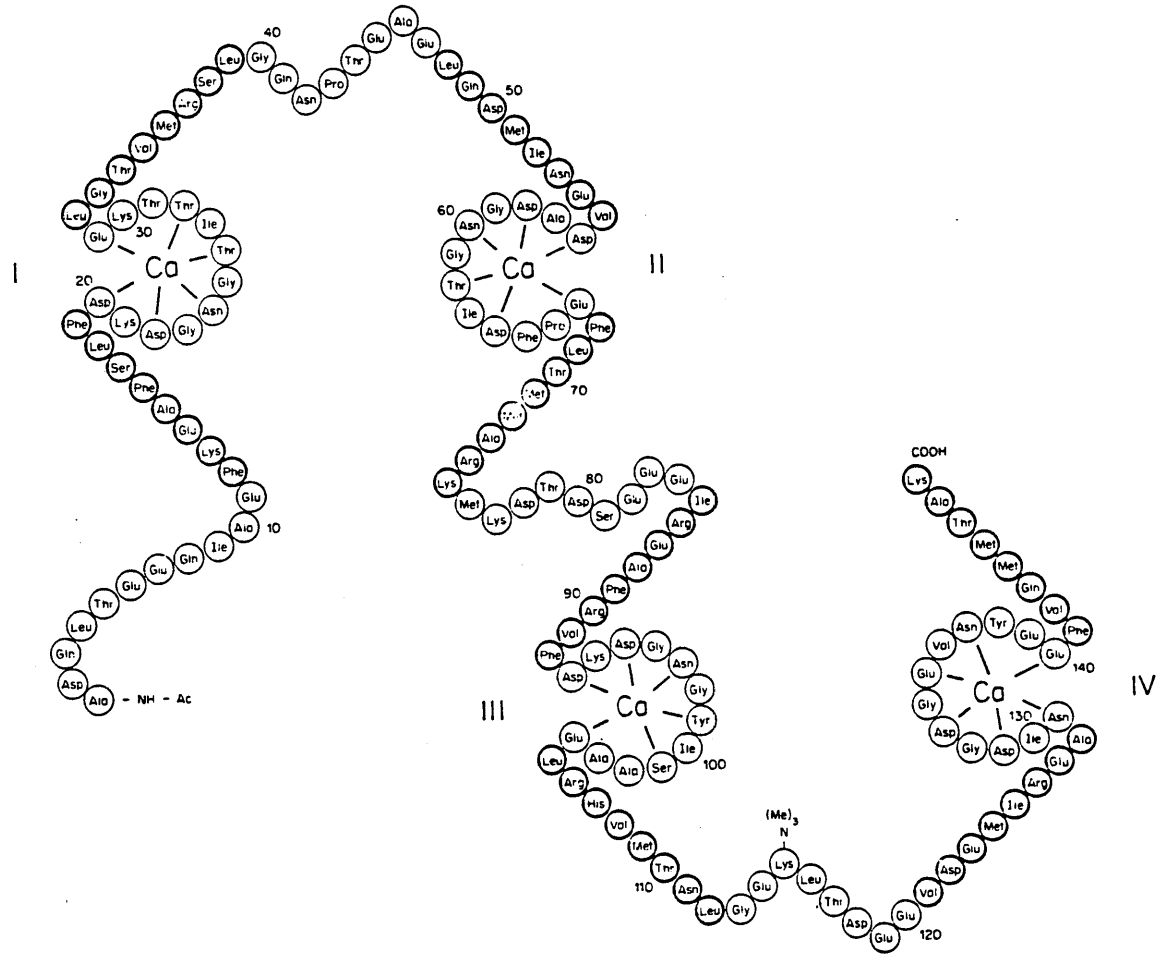


Figure 4.5

Calmodulin, showing the four Ca^{2+}-binding sites. Reproduced from Campbell (1983).

1982). To date, no protein resembling calmodulin has been isolated from a prokaryote (Hartshorne, 1985; Klee & Vanaman, 1982). However, only a limited number of organisms (primarily enterobacteria) appears to have been examined and these organisms are found to contain small acidic proteins that bind calcium (Klee & Vanaman, 1982).

Calmodulin is a heat-stable, monomeric, globular, calcium-binding protein (with dissociation constants between 10^{-6} - 10^{-5} M: Lin et al., 1974) of molecular weight about 17,000. In the majority of cases it contains 148 amino acids with a high proportion of acidic residues (Fig. 4.5), resulting in an isoelectric point of $\sim$4.0 (Cheung et al., 1978; Watterson et al., 1980). It lacks tryptophan and, in most instances, cysteine and hydroxyproline, but it does contain the unusual amino acid, trimethyllysine (Klee & Vanaman, 1982; Klee et al., 1980; Kretsinger, 1976; Watterson et al., 1980). However, evidence is now available that some plant calmodulins contain a cysteine residue (Burgess et al., 1983). The amino acid trimethyllysine is lacking in homologous Ca^{2+}-binding proteins, for example, parvalbumin and troponin C, and an earlier speculation was that this residue may impart some distinctive functional properties to calmodulin. Subsequently, however, it has been shown that this amino acid is absent from some calmodulins (e.g. from barley, fungi and some animals) and further, that the function of Octopus calmodulin was not impaired by its omission (see Hartshorne, 1985). Therefore, the function, if any, of trimethyllysine is not clear. In reflection of the ubiquitous nature of calmodulin and an assumed uniformity of function, it has been shown that the amino acid sequence of calmodulin from a diverse source, is remarkably similar (see Klee & Vanaman, 1982; Van Eldik & Watterson, 1985; Walsh & Hartshorne, 1983). Mammalian calmodulins are essentially the same but, plant and invertebrate calmodulins show

small differences in amino acid sequence (Van Eldik & Watterson, 1985).

Calmodulin has four structural domains that are similar to each other in amino acid sequence and are similar to the four domains of skeletal muscle troponin C (Burgess et al., 1983; Klee & Vanaman, 1982; Watterson et al., 1980). Within each of these domains is a proposed calcium-binding structure with an α-helix:loop:α-helix configuration (Fig. 4.5) that is referred to as an EF-hand, based on homology with the known Ca^{2+}-binding structure in parvalbumin (Klee & Vanaman, 1982; Kretsinger, 1976; Van Eldik et al., 1982a). Comparison of the complete amino acid sequence of calmodulin and troponin C demonstrates unequivocally that these proteins form a subclass of closely related calcium modulated proteins (Burgess et al., 1983). The relative affinities of these four domains towards Ca^{2+} have been interpreted differently by different investigators. Most of them (but not all) think that the four sites are not equivalent (see Hartshorne, 1985 for a discussion). The sequence of Ca^{2+}-binding to these sites may also affect the subsequent function of calmodulin. Binding of Ca^{2+} to calmodulin results in a conformational change in the protein and, therefore, the sequential changes associated with the filling of the Ca^{2+}-binding sites of the Ca_n^{2+}·Calmodulin (n = 1-4) complexes may have distinct conformations (Hartshorne, 1985). Dieter (1984) questioned whether the different conformations of Ca_n^{2+}·Calmodulin complex interact with different proteins in the cell and whether such complexes may thus translate different intracellular free Ca^{2+} concentrations into different responses.

The role of calmodulin in general is to detect the calcium concentration transients that occur during normal cell functioning, and then to reflect the ambient Ca^{2+} concentration via regulation of various processes. In a resting cell the free Ca^{2+} concentration is

in the order of 10^{-4}-10^{-5} mol m^{-3} and an activation of the cell results in an increase in Ca^{2+} concentration to about 10^{-3} mol m^{-3} or slightly higher (Campbell, 1983; Williamson, 1981). Thus calmodulin must be equipped to detect Ca^{2+} at micromolar concentrations, and the calcium-binding constants for calmodulin have been found to possess appropriate affinities (Hartshorne, 1985). The majority of known calmodulin-dependent systems are enzymatic and the general mechanism for the activation (regulation) of any calmodulin (CaM)-dependent enzyme has been proposed as follows (Cheung, 1980; Cheung et al., 1978; Klee & Vanaman, 1982):

$$nCa^{2+} + CaM \longrightarrow Ca_n^{2+}.CaM^*$$

$$Ca_n^{2+}.CaM^* + E \longrightarrow Ca_n^{2+}.CaM^*.E^*$$

where E stands for apoenzyme and the asterisk (*) for a new conformation. The first step is the binding of Ca^{2+} to calmodulin which induces a conformational change of the calmodulin that favours its interaction with the inactive partner-apoenzyme and forms the active ternary complex (holoenzyme). Reduction of the intracellular level of Ca^{2+} causes a dissociation of the ternary complex and a loss of enzymatic activity. With this type of reaction mechanism, the regulation, or activation, of a given enzyme depends on several factors. At limiting concentrations of calmodulin, or Ca^{2+}, the concentration of $Ca_n^{2+}.CaM$ complex will obviously be restricted. An increase in either Ca^{2+} or available calmodulin will shift the equilibrium to favour complex formation and thus the apparent Ca^{2+}-sensitivity of a given calmodulin-dependent system can depend on the calmodulin concentration. The latter in turn is governed by the presence and concentration of other calmodulin-binding proteins and their binding affinities for calmodulin. If the concentration of $Ca_n^{2+}.CaM$ is less than that

required to saturate all the calmodulin binding sites, then whether or not a particular enzyme is activated depends on its affinity for calmodulin. Those with a stronger affinity would be activated before those of lesser affinity. This sort of selectivity might be responsible for specific activation or regulation of certain enzymatic processes in preference to other coexisting calmodulin-dependent systems.

One of the striking features of calmodulin is its lack of tissue or species specificity. Samples of calmodulin from different eukaryotes show biological cross reactivity. For example, brain cyclic nucleotide phosphodiesterase activity was found to be stimulated by calmodulin from pea and squash (Anderson & Cormier, 1978; Anderson et al., 1980; Dieter & Marmé, 1980b), and NAD kinase from pea was stimulated by porcine brain calmodulin (Anderson & Cormier, 1978; Anderston et al., 1980). Further, Dieter and Marmé (1980b) found that bovine brain calmodulin stimulated microsomal Ca^{2+} uptake in squash. The biochemical, biophysical and physiological features of calmodulin both from animals and plants, have been extensively reviewed by a number of authors: e.g. Burgess et al. (1983), Cheung (1980), Dieter (1984), Klee and Vanaman (1982), Marmé and Dieter (1983) and Van Eldik and Watterson (1985).

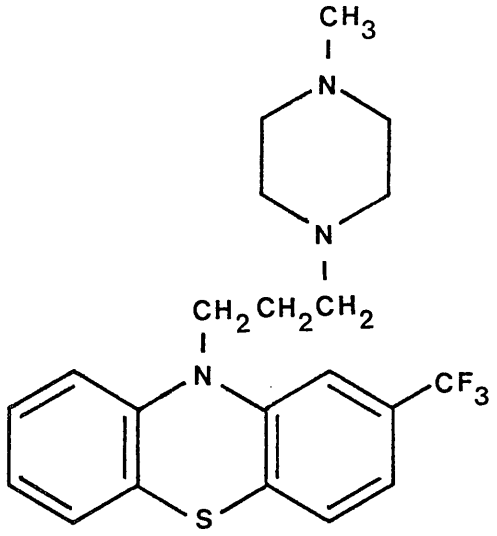
A remarkable number and variety of systems are now thought to be regulated by calmodulin. As mentioned above, the role of calmodulin was first recognized with cyclic nucleotide phosphodiesterase and this was followed by brain adenylate cyclase (Broström et al., 1975; Cheung et al., 1975). It was only in 1978 that Anderson and Cormier presented evidence for a heat-stable plant protein that was able to activate bovine brain cyclic AMP phosphodiesterase and that was identified as calmodulin shortly thereafter (Anderson et al., 1980). Since then a large body of evidence has been accumulated to suggest

that animal and plant calmodulins have very similar biochemical and functional properties and that the molecular mechanisms by which Ca^{2+} transduces its information into the biochemistry and physiology in plant and animal cells are very similar.

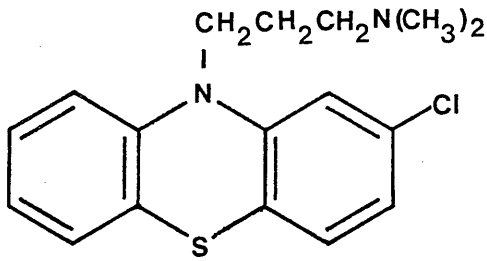
Calmodulin has been shown to regulate a number of physiological processes in plants. The activity of several enzymes is found to be calmodulin-dependent. These include NAD kinase (Anderson & Cormier, 1978; Dieter & Marmé, 1980; Muto & Miyachi, 1977; Simon et al., 1984), membrane-bound protein kinase (Hetherington & Trewavas, 1982, 1984b; Veluthambi & Pooviah, 1984), soluble protein kinase (Polya & Davies, 1982; Polya & Miccuci, 1984; Veluthambi & Pooviah, 1984), quinate: NAD 3-oxidoreductase (Graziana et al., 1983a, 1983b) and calcium transport ATPase (Dieter & Marmé, 1981; Dieter et al., 1984). NAD kinase is the only known enzyme which catalyses the phosphorylation of NAD in the presence of ATP (Marmé & Dieter, 1983). This enzyme is, therefore, an important regulatory protein in higher plants, since many key enzymes involved in the metabolism of sugars, lipids and amino acids are dependent on pyridine nucleotides and have a clear preference for NAD or NADP. Therefore, NAD kinase is a favourite target for many regulatory signals (Dieter, 1984; Marmé & Dieter, 1983). Calcium transport ATPase is also of particular interest since it could function in restoring the cytoplasmic calcium concentration to a resting level after stimulation. Detailed descriptions of all the enzymes that are regulated by calcium and calmodulin seem to be beyond the scope of this introduction and, further, explicit accounts of them have been given by Dieter et al. (1984), Klee & Vanaman (1982) and Marmé and Dieter (1983). There are some indications that calmodulin is involved in cytokinin-mediated physiological processes in plants and this will be dealt with later in this introduction. Calmodulin is also thought

to participate in the following: secretion of peroxidase from cultured spinach cells (Sticher et al., 1981), volume regulation in Poterioochromonas (Kauss, 1983) and light-dependent chloroplast movement of Mougeotia (Wagner et al., 1984).

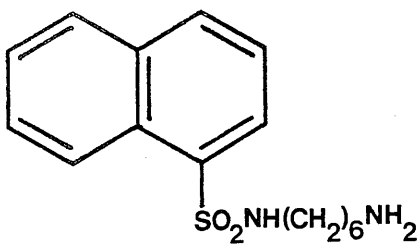
For exploring the mechanisms of some of the cellular processes, the role of calmodulin was established by isolation and recombination of pertinent components, for example, for NAD kinase activity (Marmé & Dieter, 1983), but in many other systems the role of calmodulin was inferred from the use of calmodulin 'antagonists'. The observation that phenothiazines, e.g. trifluoperazine, inhibit some types of phosphodiesterase activity (Uzunov & Weiss, 1972) led to the eventual discovery that these compounds were inhibitory because of their interaction with calmodulin (Levin & Weiss, 1978, 1979). It is now known that the activation of various enzymes by calmodulin can also be inhibited in a calcium-dependent manner by drugs of several other classes, such as naphthalene sulphonamide derivatives (e.g. N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (abbreviated as W-7)) (Hidaka et al., 1979, 1981), diphenylbutylamine derivatives (e.g. compound 48/80: a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde) (Gietzen et al., 1983; Levin & Weiss, 1979) (Fig. 4.6). These drugs are found to bind calmodulin and thereby inhibit a wide range of calmodulin-dependent processes. The interaction of calmodulin antagonists with calmodulin has a practical value in that these compounds (or suitable derivatives) may be covalently linked to an inert matrix and used for affinity chromatography in the purification of calmodulin (for example, Charbonneau & Cormier, 1979). This class of drugs has been widely used as calmodulin antagonists to study physiological functions of calmodulin in vivo and in vitro. However, some of these compounds do not appear



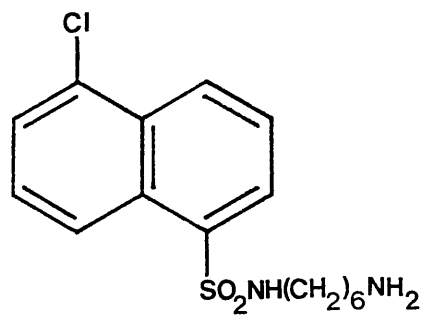
Trifluoperazine



Chorpromazine



W-5



W-7

Figure 4.6

Chemical structures of four calmodulin antagonists.

to be specific for calmodulin and, therefore, should be used with caution in biological studies (see Hartshorne, 1985).

A number of processes in plants has been reported to be inhibited by calmodulin antagonists mainly, trifluoperazine, chlorpromazine and W-7. Polito (1983) found that the germination and the tip growth in pollen of pear were arrested by trifluoperazine and chlorpromazine. This inhibitory effect was thought to be due to an alteration in Ca^{2+} mobilization. In Mougeotia phytochrome-mediated chloroplast re-orientational movement, an effect in which calmodulin is thought to be involved, was inhibited by 20-50 mmol m^{-3} trifluoperazine and 0.1 mol m^{-3} W-7 (Wagner et al., 1984). In Onoclea chlorpromazine, trifluoperazine and calmidazolium (another calmodulin antagonist, also known as R24571) reversibly inhibited red light-stimulated spore germination, a process in which calmodulin is believed to have an intermediary role (Wayne & Hepler, 1984). The involvement of calcium ions in cytokinin-regulated processes has been reported by several workers. LeJohn and coworkers showed that cytokinin stimulated the release of Ca^{2+} from a glycoprotein on the surface of the water mould Achlya (LeJohn & Cameron, 1972; LeJohn et al., 1973, 1974). Ralph et al. (1976) found that Ca^{2+} could substitute for cytokinin in leaf disc expansion. Furthermore, a Ca^{2+}-cytokinin interaction has been noted in several systems where these two factors appear to enhance ethylene production synergistically (Green, 1983; Lau & Yang, 1975) and to delay senescence in Zea leaf discs (Pooviah & Leopold, 1973). That calmodulin may be involved in some of these events is supported by the findings of Elliott and coworkers, who have shown that a variety of calmodulin inhibitors, including trifluoperazine, chlorpromazine, dibucaine and others, blocked betacyanin synthesis in Amaranthus as well as growth and cell division in soybean callus

cultures (Elliott, 1980, 1983; Elliott et al., 1983). Saunders and Hepler (1983) have shown that cytokinin-induced bud formation in Funaria was inhibited by trifluoperazine and chlorpromazine. There is some indication that W-7 and chlorpromazine inhibit the synthesis and secretion of α-amylase (a process which is stimulated by gibberellins) in scutellar tissue of rice grain (Mitsui et al., 1984).

Therefore, it seems certain that calmodulin participates in calcium-involved signal transduction in plants and that the calmodulin antagonists are useful tools in the preliminary establishment of any such process. It has been shown in the previous Chapter that calcium ions are required for the inhibition of stomatal opening by abscisic acid. The underlying mechanism of the action of ABA in this system is not fully understood, and therefore, the involvement of calcium channels and calmodulin has been examined in this Chapter using the putative calcium channel blockers and calmodulin antagonists. Effects of manipulation of intracellular calcium were also investigated using the divalent cation ionophore A23187.

MATERIALS AND METHODS

Abaxial epidermal peels were obtained as described in Chapter 2. In the experiments where the effects, on the action of ABA, of calcium channel blockers (namely, La^{3+}, nifedipine and verapamil) and calmodulin antagonists (namely, compound 48/80, trifluoperazine and W-7) were investigated, the epidermal pieces were pre-incubated for two hours in the test solution containing either a channel blocker or a calmodulin antagonist and then transferred into a fresh test solution of similar composition with ABA in addition, and further incubated for 2 hours before the apertures were measured. The rest of the experiments had an incubation period of 3 hours. All the treatments were carried out under illumination in the absence of CO_2.

The chemicals used in the experiments were all obtained from Sigma Chemical Company, Poole. They (except the ionophore A23187) were dissolved in the 'basic incubation medium' (Chapter 2) in the stated concentrations and combinations required by each experiment. A23187 was first dissolved in absolute methanol (analytical grade) and then dissolved in the basic incubation medium. The final test solution contained 0.02% methanol which was found to have no effect on stomatal opening. Cell viability of the epidermis was examined by the neutral red staining method.

RESULTS AND DISCUSSION

The use of calcium channel blockers or calmodulin antagonists on stomatal studies has not been reported to date, except for one published study on calmodulin binding drugs by Donovan et al. (1985). Therefore, trial experiments were carried out to determine the dose-response relationships for all the drugs used to find the most appropriate concentrations for use in subsequent experiments. From the experiments described in the previous chapter, a passage of calcium ions into the guard cells was implied during the action of ABA on isolated epidermis. As mentioned in the introduction, under normal conditions calcium ions cross the plasma membrane of cells via discrete channels. To see whether these channels are required when ABA inhibits stomatal opening, three channel blockers were incorporated separately in the incubation medium in a pretreatment period of two hours and then the epidermal pieces were transferred into a fresh medium with or without ABA. Controls without a channel blocker were also incubated for the same period before transferring them into ABA. The concentrations of ABA used in these experiments were 10^{-5} and 10^{-4} mol m^{-3}. Figure 4.7 shows the effect of the inorganic channel blocker, La^{3+}, supplied as $LaCl_3$ at 1.0 mol m^{-3}, on the action of ABA on isolated epidermis.

Figure 4.7

Effect of $LaCl_3$ on ABA-induced stomatal closure. Epidermal pieces were preincubated for 2 h in the control medium (50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer, pH 6.15), with or without 1 mol m^{-3} $LaCl_3$ and then transferred into fresh media with or without ABA and incubated for 2 h. All incubations were carried out under illumination and CO_2 free conditions. Means of 90 measurements, with standard errors.

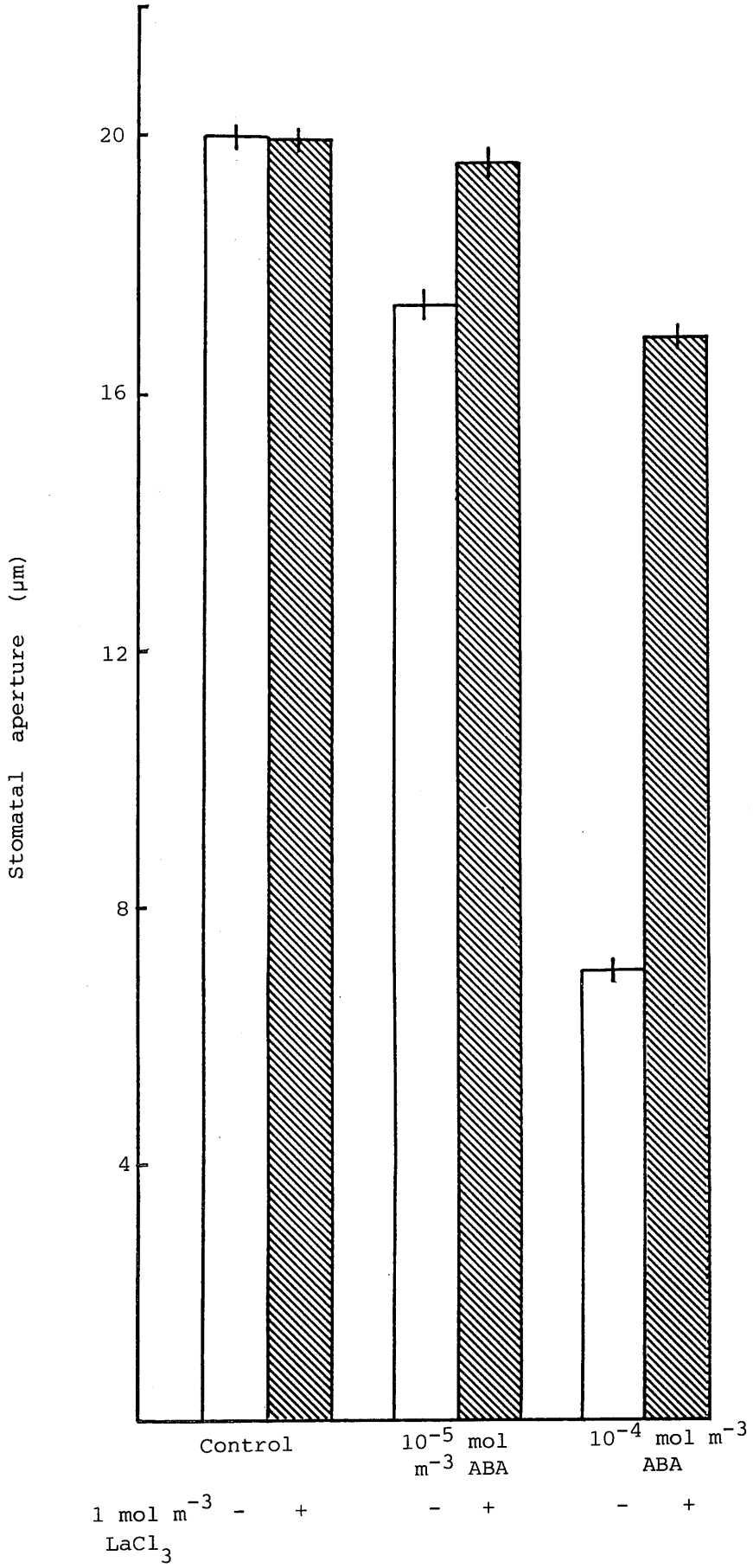

Stomatal aperture (μm)
20
16
12
8
4
Control
10-5 mol m-3 ABA
10-4 mol m-3 ABA
1 mol m-3 LaCl3
-
+
-
+
-
+

Figure 4.7

Figure 4.8

Effect of 0.2 mol m^{-3} verapamil on ABA-induced stomatal closure. The procedure was similar to that in the $LaCl_3$ experiment. Means of 90 measurements of individual stomata, with standard errors.

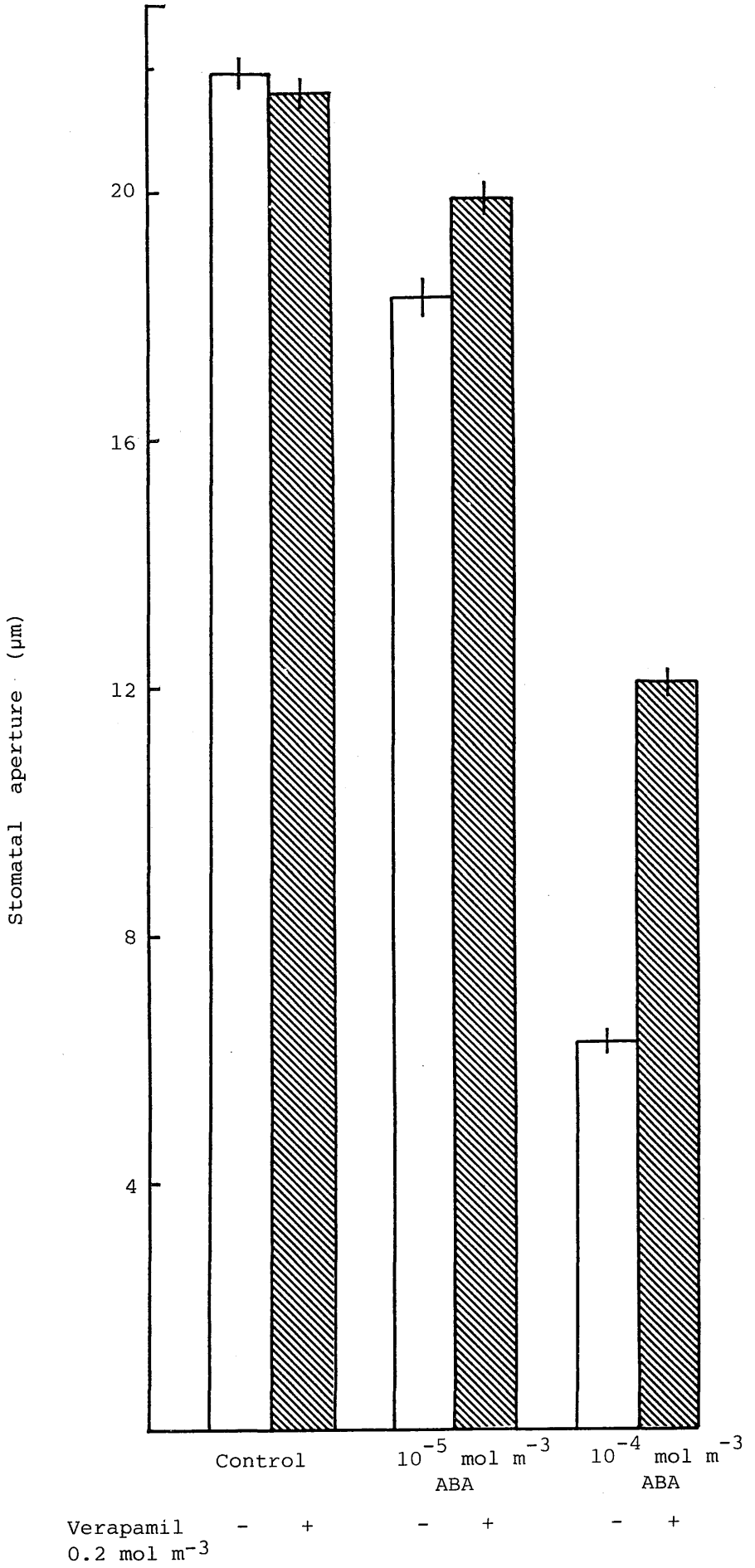

Stomatal aperture (μm)
20
16
12
8
4
Control
10-5 mol m-3 ABA
10-4 mol m-3 ABA
Verapamil - + - + - +
0.2 mol m-3

Figure 4.8

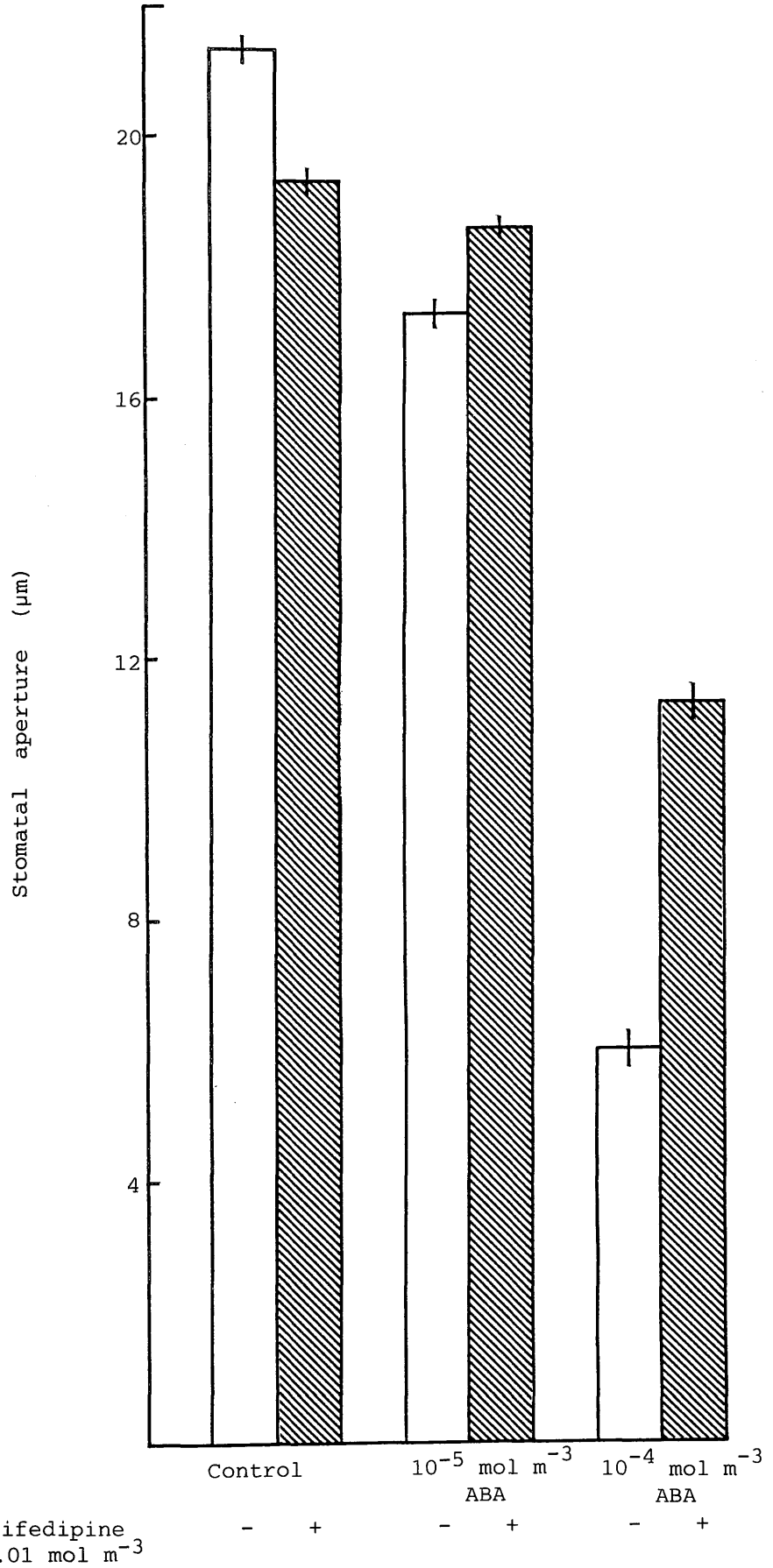


Figure 4.9

Effect of 0.01 mol m^{-3} nifedipine on ABA-induced stomatal closure. The procedure was similar to that in the $LaCl_3$ experiment. Means of 90 measurements of individual stomata, with standard errors.

Lanthanum chloride had no effect on stomatal opening in the controls, but it significantly reduced the suppression of opening by ABA. The observed effect of this inorganic cation cannot be attributed to an osmotic contribution by the ion, since the concentration used here was only 1.0 mol m^{-3}. To confirm that the reversal of the ABA effect was due to a blockage of calcium channels by La^{3+}, similar experiments were conducted using two other chemically different putative channel blockers, namely, verapamil and nifedipine, and the results are shown in Figures 4.8 and 4.9 respectively. As with La^{3+}, 0.2 mol m^{-3} verapamil had no effect on its own on stomatal opening (compared with the controls), but significantly enhanced opening when ABA was present. Nifedipine, on the other hand, at 0.01 mol m^{-3} slightly reduced stomatal apertures in the controls, but again removed the inhibitory effect of ABA in a similar fashion to La^{3+} and verapamil. These results substantiate the conclusions of the previous Chapter, that apoplastic calcium enters the cytosol during the action of ABA. Additional calcium was not provided in the medium used for incubating the epidermis, and so the limited supply of apoplastic calcium would presumably have maximized the effects of the calcium channel blockers.

Although now there are some reports which suggest that certain ions migrate across plant cell membranes through specific channels (Takeda et al., 1985), the existence of such channels for calcium has not yet been established convincingly. Much of the evidence concerning calcium channels is circumstantial, coming from the studies of channel blockers which appear to have the ability to interfere with calcium movement across plasma membranes and thereby arrest a particular Ca^{2+}-mediated process (see Hepler & Wayne, 1985). However, recently, Hetherington and Trewavas (1984) reported a specific binding of labelled nitrendipine to pea shoot membranes. They characterized

this binding, and found that it was very similar to the binding of nitrendipine to animal membranes. Therefore, it seems reasonable to interpret the results of channel blocker-experiments with reference to their well studied effects on animal tissues. However, their effects on other cellular activities should not be overlooked. Von Willert and Kluge (1973) reported that verapamil (10^{-2}-10^{-1} mol m^{-3}) enhanced efflux of malate from vacuoles of leaf cells of Bryophyllum daigremontianum. Furthermore, in animal cells, verapamil and gallopamil have been found to reduce K^+ outward currents under certain circumstances, and an opposite effect was observed with nifedipine (Krebs, 1984). If such a situation occurred in the guard cells, then an alternative interpretation would be possible for the action of verapamil: ABA is known to increase K^+ efflux from guard cells (MacRobbie, 1981a), and if verapamil decreased outward K^+ current then an antagonistic action between these two agents would be expected. However, such an action of verapamil in plant tissues has not yet been reported and a similar explanation for the action of La^{3+} and nifedipine is not likely. Since all three agents behaved in a fashion essentially similar to each other, it can be concluded that the observed effects were most probably due to a blockage of calcium channels.

An increase in intracellular calcium ion concentration thus seems likely to be the primary effect of an external supply of ABA to the guard cells. So far these findings appear to fulfil two of the three criteria proposed by Jaffe (1980) for establishing that a particular process is mediated by Ca^{2+} (see the General Introduction). It was, therefore, interesting to examine the third requirement, that is, whether an experimental generation of an increase in the intracellular Ca^{2+} concentration could stimulate the response. The divalent

Figure 4.10

Effect of A23187 on stomatal opening in light and under CO_2 free air.

A, control

B, 0.1 mol m^{-3} $CaCl_2$

C, 0.05 mol m^{-3} A23187

D, 0.1 mol m^{-3} $CaCl_2$ + 0.05 mol m^{-3} A23187.

Means of 90 measurements of individual stomata, with standard errors.

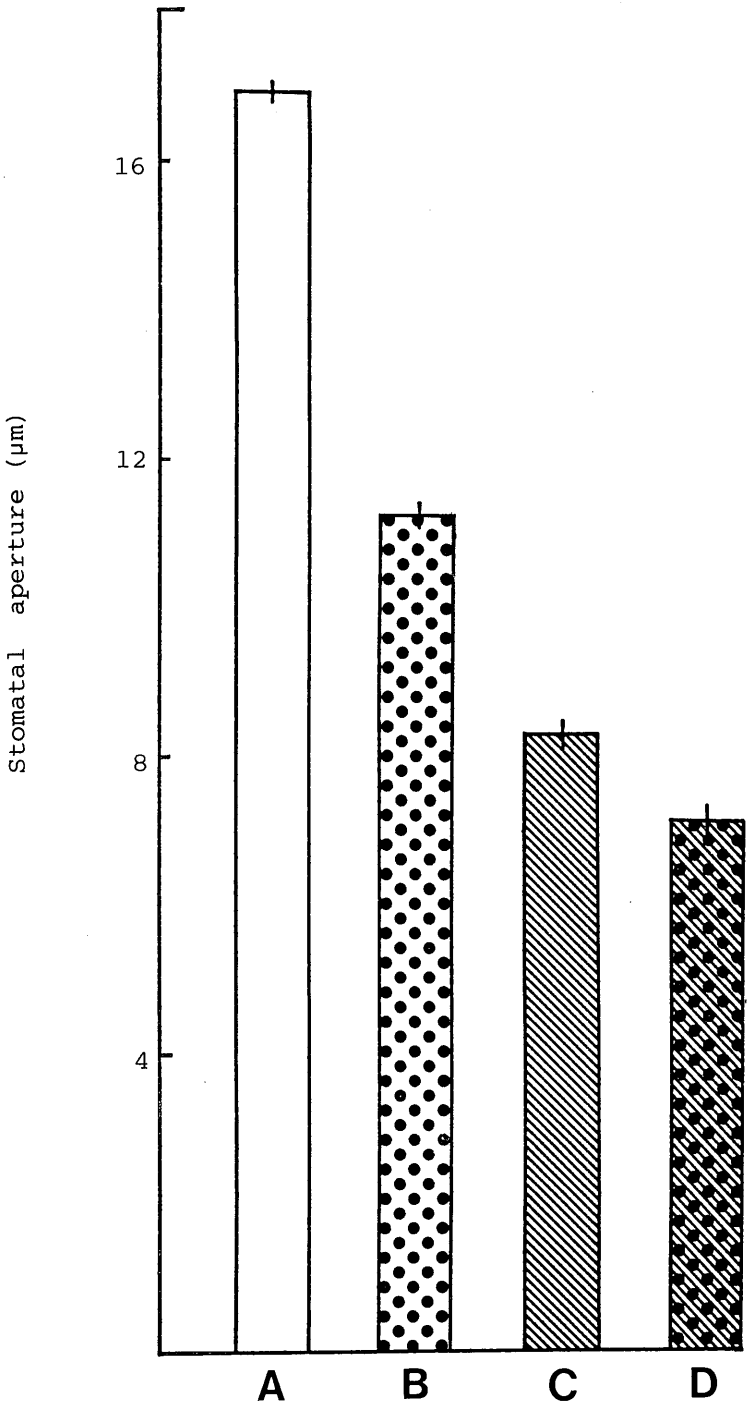


Figure 4.10

cation ionophore, A23187, was employed to manipulate the intracellular calcium ion concentration, and Fig. 4.10 shows that the treatment with the ionophore alone at 0.05 mol m^{-3} reduced stomatal opening by more than 50 per cent, which exceeded the effect of 0.1 mol m^{-3} $CaCl_2$. There was a less-than-additive effect when 0.1 mol m^{-3} $CaCl_2$ and 0.05 mol m^{-3} ionophore were combined.

It can be suggested that when A23187 partitioned into the plasma membrane of the guard cells, it increased the penetration of apoplastic Ca^{2+} into the cytosol. It would thus mimic the proposed action of ABA. However, one must be cautious in interpreting the results because the possibility cannot be ruled out that A23187 renders the membranes permeable to other cations such as H^+ and Mg^{2+} (Hinds & Vincenzi, 1985; Pressman, 1976). Hinds and Vincenzi (1985) argued that A23187 is not a specific calcium ionophore since they found that it also made cell membranes permeable to Mg^{2+}. Hence, it was interesting to see if extracellular Mg^{2+} has any effect on stomatal opening in Commelina communis. Epidermal pieces were incubated for 3 hours in 50 mol m^{-3} KCl in 10 mol m^{-3} MES buffer with a range of $MgCl_2$ concentrations (0.25-2.0 mol m^{-3}) and the results are shown in Table 4.1. It was found that over the concentration range tested, Mg^{2+} had no inhibitory effect, whatsoever, on stomatal opening. In contrast, 0.25 mol m^{-3} $CaCl_2$ inhibited stomatal aperture by about 50 per cent. This strongly justified the employment of A23187 in this study as a calcium ionophore to investigate the effects of experimentally increased cytosolic Ca^{2+}.

The next important question which will obviously be raised is how an increased Ca^{2+} level could couple the stimulus (ABA) to the response (stomatal closure). A probable candidate for participation in this event is calmodulin. Increased calcium levels can activate calmodulin

Treatment	Control	0.25 mol m^{-3} $MgCl_2$	0.5 mol m^{-3} $MgCl_2$	1.0 mol m^{-3} $MgCl_2$	2.0 mol m^{-3} $MgCl_2$	0.25 mol m^{-3} $CaCl_2$
Aperture (μm)	15.98 (0.46)	16.08 (0.43)	17.06 (0.62)	16.27 (0.39)	16.47 (0.40)	8.05 (0.50)

Table 4.1. Effect of $MgCl_2$ on stomatal opening. Means of 90 individual stomata with standard errors in parentheses.

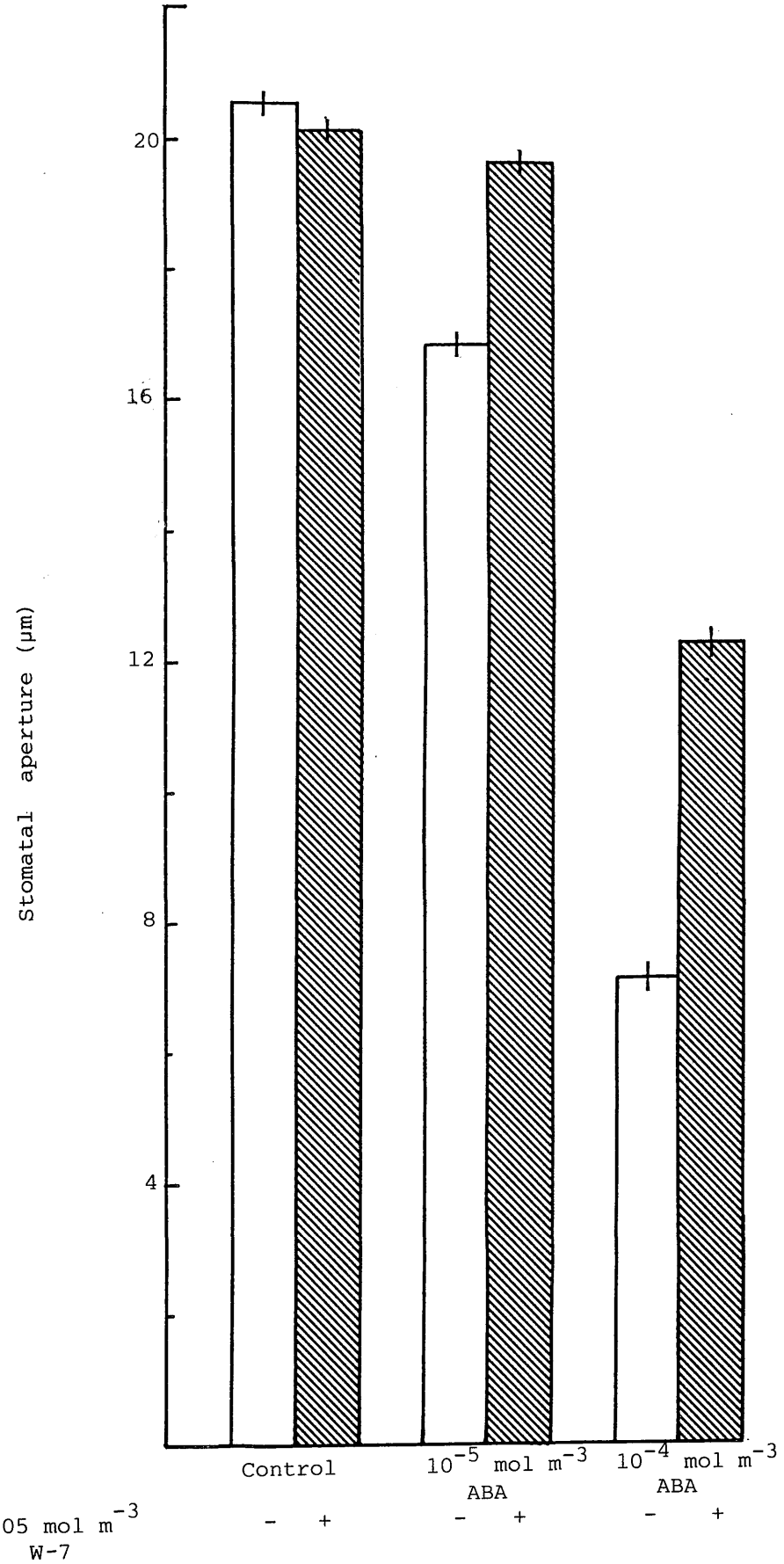


Figure 4.11

Effect of the calmodulin antagonist W-7 (0.5 mol m^{-3}) on the responses of stomata to ABA. The procedure was similar to that in the experiments with calcium channel blockers. Means of 90 measurements of individual stomata, with standard errors.

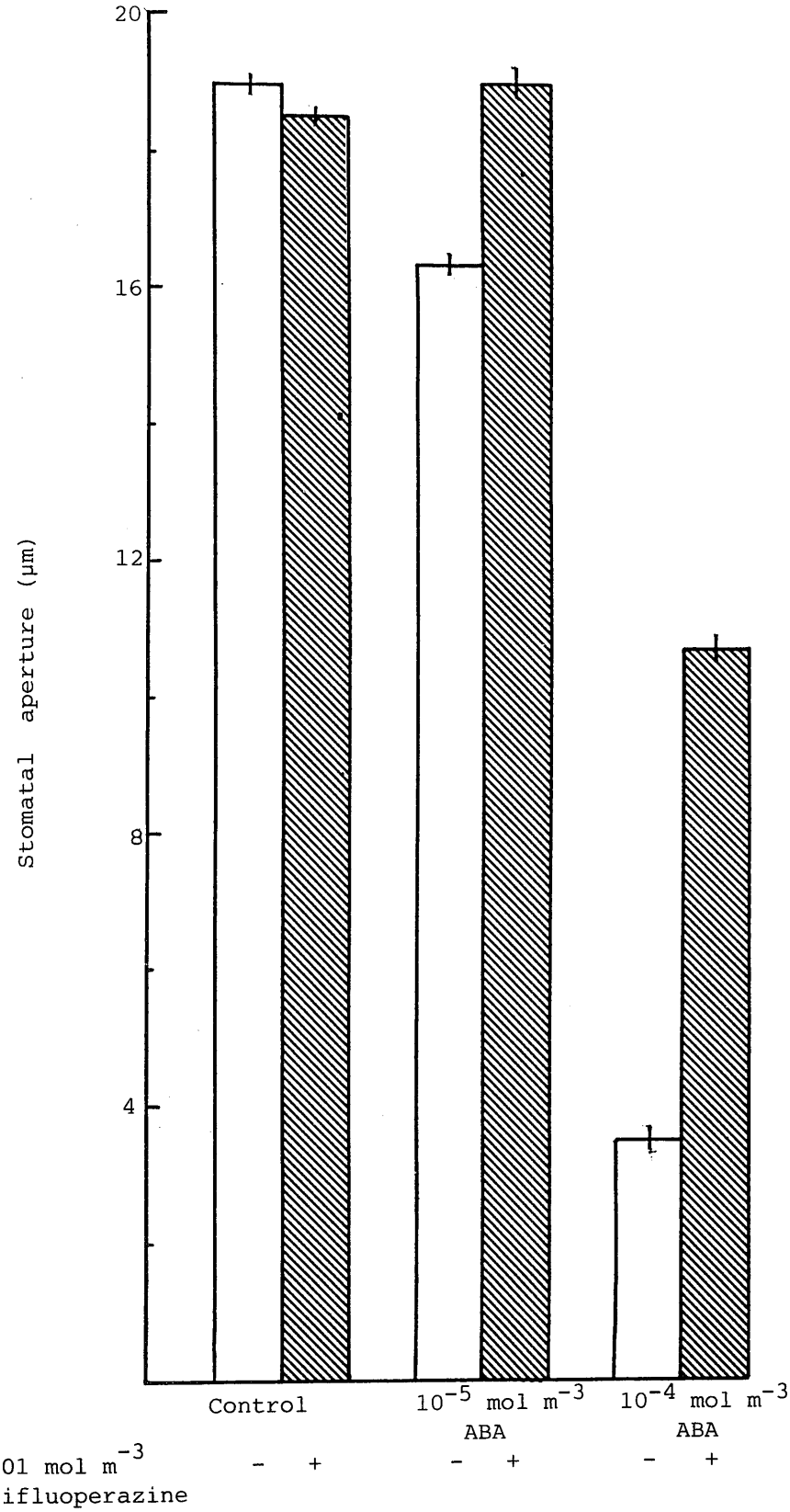


Figure 4.12

Effect of 0.01 mol m^{-3} trifluoperazine on ABA-induced stomatal closure. Means of 90 measurements of individual stomata, with standard errors.

	Control	10 mmol m^{-3} W-7	25 mmol m^{-3} W-7	50 mmol m^{-3} W-7	100 mmol m^{-3} W-7
-ABA	19.12	19.13	18.91	18.85	19.08
	(0.13)	(0.15)	(0.14)	(0.18)	(0.23)
10^{-4} mol m^{-3} ABA	3.48	9.75	11.01	12.33	13.90
	(0.27)	(0.20)	(0.18)	(0.19)	(0.13)

Table 4.2. Dose-response study of effects of W-7. Abaxial epidermal pieces were preincubated for 2 hours in test media without ABA and then transferred into fresh test solutions with or without 10^{-4} mol m^{-3} ABA and incubated for a further 2 hours. Means of 90 individual measurements of stomatal aperture in μm. Standard errors are shown in parentheses.

which would then influence a number of cellular processes, one or several of which could lead to a reduction in net accumulation of K^+ in guard cells and consequently stomatal closure. To explore this possibility, the ability of guard cells to respond to external ABA was examined in the presence of calmodulin antagonists. A procedure similar to the channel blocker-experiments was adopted and the results for W-7 are presented in Fig. 4.11. As in the case of the channel blockers, 0.05 mol m^{-3} W-7 had no effect on stomatal opening in the controls, but significantly reduced the inhibitory effect of 10^{-4} mol m^{-3} ABA. The inhibition caused by 10^{-5} mol m^{-3} ABA was almost totally suppressed by 0.05 mol m^{-3} W-7. The results of a dose-response study using W-7 are shown in Table 4.2, which shows that the suppression of the action of ABA by W-7 occurred even at a concentration of 0.01 mol m^{-3}. The effects on the action of ABA, of 0.01 mol m^{-3} trifluoperazine and 1 µg/ml Compound 48/80 were also examined. As can be seen in Figures 4.12 and 4.13, neither of these had any significant effect on stomatal opening in the controls, but they both greatly reduced the effect of 10^{-4} mol m^{-3} ABA and almost completely suppressed that of 10^{-5} mol m^{-3} ABA.

The report by Donovan *et al.* (1985) suggests that trifluoperazine and Compound 48/80 have some influence on stomatal opening of *Commelina communis*. They found that those drugs significantly stimulated stomatal opening in conditions of light-plus-CO_2 and slightly promoted opening in dark-minus-CO_2. They also found no significant effect in light-minus-CO_2 treatments. They concluded that the gross effects of high external Ca^{2+} concentrations on stomatal responses did not involve calmodulin, and postulated that the observed effects of the drugs were based on Ca^{2+} in the micromolar range. However, a careful scrutiny shows that their results are somewhat confusing; they reported that

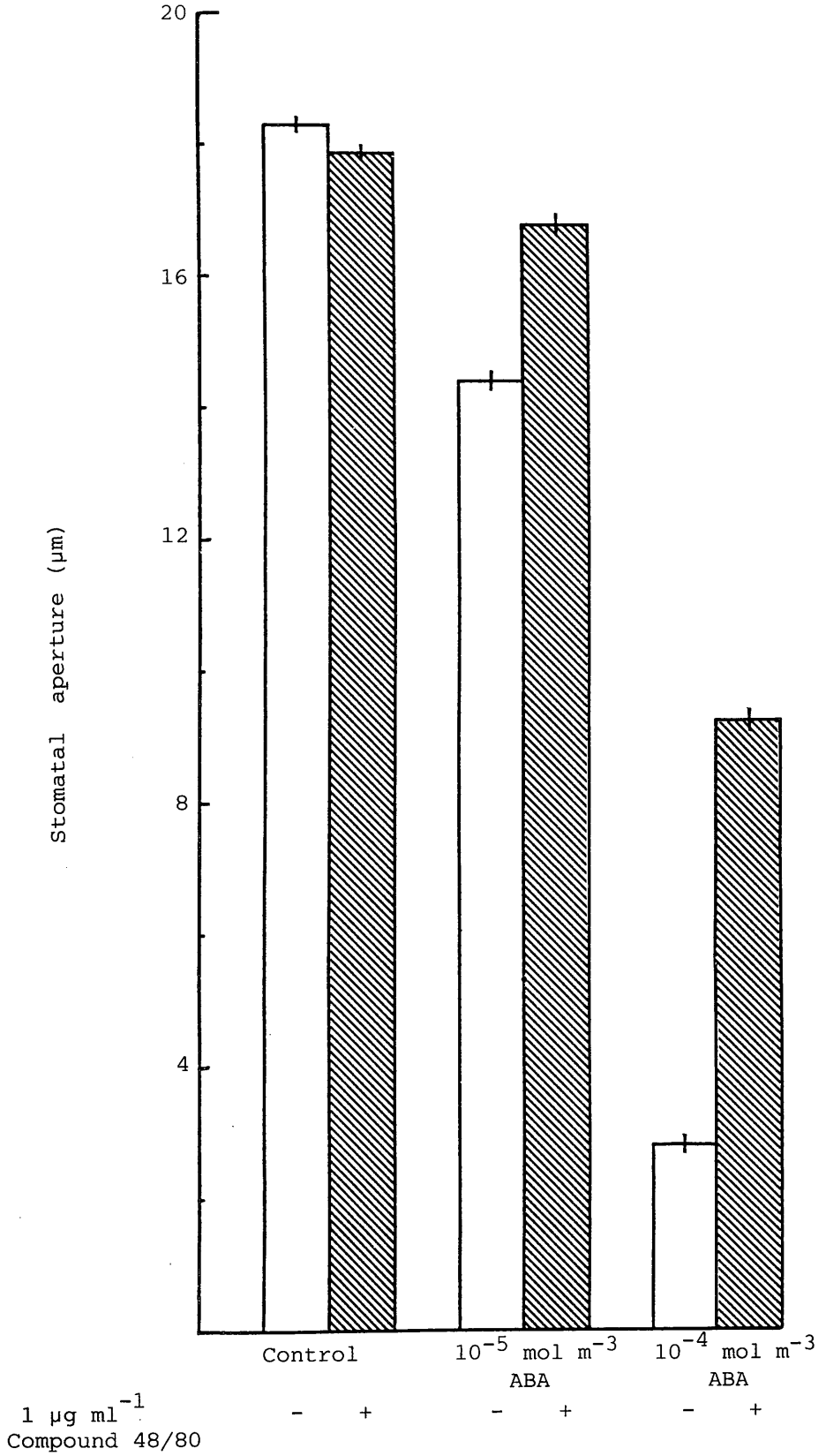


Figure 4.13

Effect of 1 µg ml^{-1} compound 48/80 on the responses of stomata to ABA. Means of 90 measurements of individual stomata, with standard errors.

the stomatal apertures of the epidermal peels immediately taken from the plants from an environmental chamber were 17 ± 4 μm. In our experience, the stomata attained such wide apertures only when the isolated epidermis was incubated under conditions conducive for opening (e.g. a sufficient amount of K^+ in the medium, CO_2 free conditions etc.). Furthermore, the data of Fig. 3 of that report show that the darkening of the isolated epidermal strips apparently had no effect compared with the controls: i.e. the guard cells continued to maintain (?) wide apertures (about 16.5 μm in the dark compared to about 17.5 μm in light with a LSD about 3.3 μm). My own data, however, suggested that when the antagonists were present, the stomata continued to respond to external stimuli such as CO_2 and light. However, it was important to examine the cell viability of the epidermis after each drug treatment. The epidermal pieces were, therefore, stained with neutral red and observed under the microscope. No visible damage to the cells caused by these drugs during the short period of application was detected and the guard cells continued to accumulate the vital stain neutral red (Plate 4.1).

The use of inhibitors on intact cells can lead to complex responses and it cannot be assumed that the primary target is the only one affected. These drugs might have some non-specific actions on cells. Kauss (1983) pointed out that trifluoperazine and chlorpromazine had some non-specific effects especially on membrane transport properties. From their studies on cytoplasmic streaming, Woods *et al.* (1984) showed that trifluoperazine and chlorpromazine, but not W-7 unless the concentration was very high, inhibited the expression of Ca^{2+} sensitivity. Since the W-series inhibitors are thought to be more specific for calmodulin activity than either trifluoperazine or chlorpromazine, they attributed the inhibition observed to effects other than calmodulin

Plate 4.1

Photomicrographs of neutral red-stained epidermal tissues treated with calcium channel blockers and calmodulin antagonists (X.310).

A 1 mol m^{-3} $LaCl_3$

B 0.01 mol m^{-3} nifedipine

C 0.2 mol m^{-3} verapamil

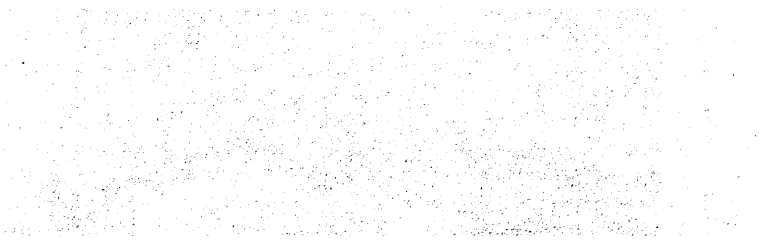
D 0.05 mol m^{-3} A23187

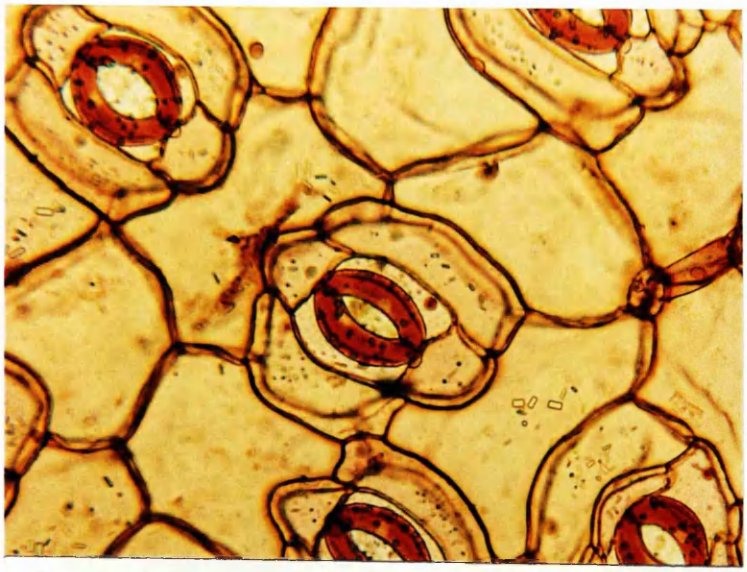
E 0.05 mol m^{-3} W-7

F 0.01 mol m^{-3} trifluoperazine

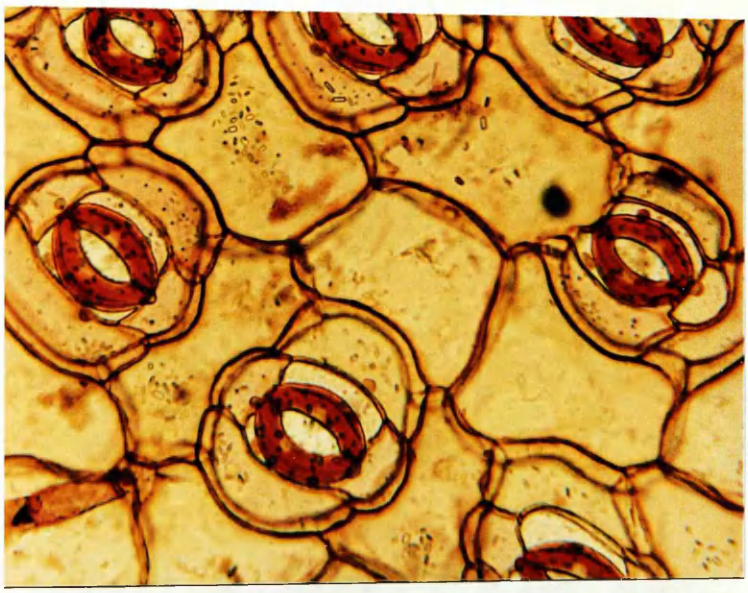
G 1 μg ml^{-1} compound 48/80.

N.B. The real colours of the stained epidermal tissues have not been reflected in the photographs.

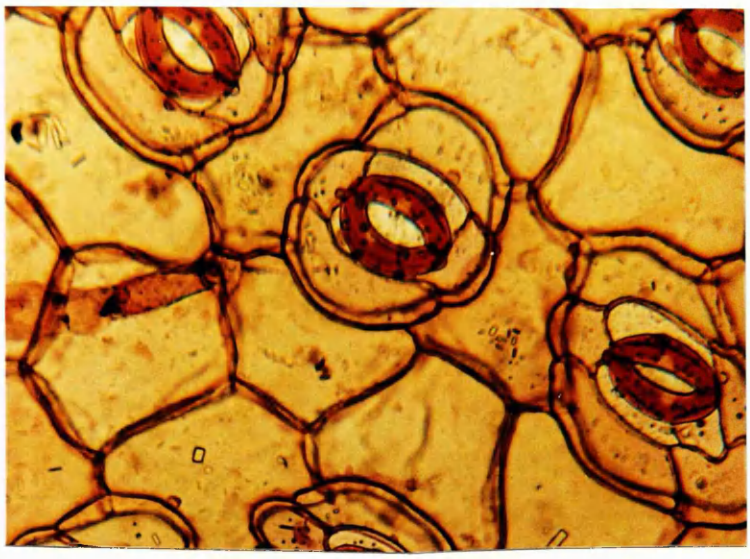




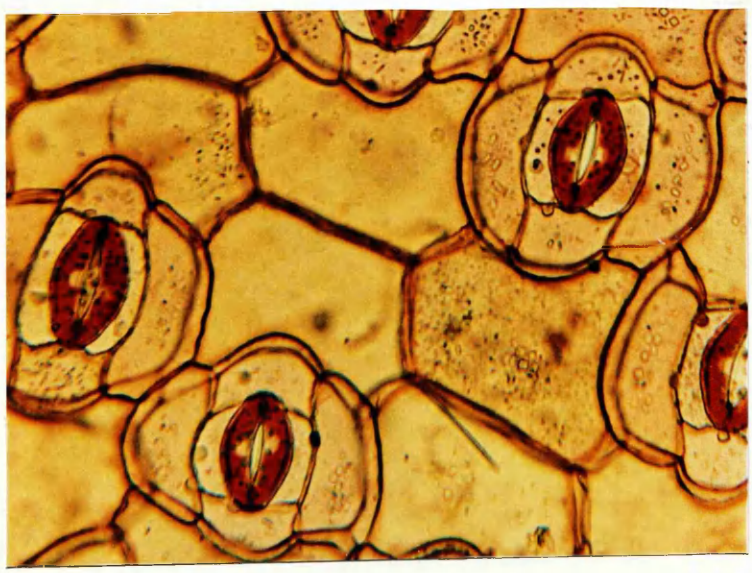
A. 1 mol m^{-3} $LaCl_3$



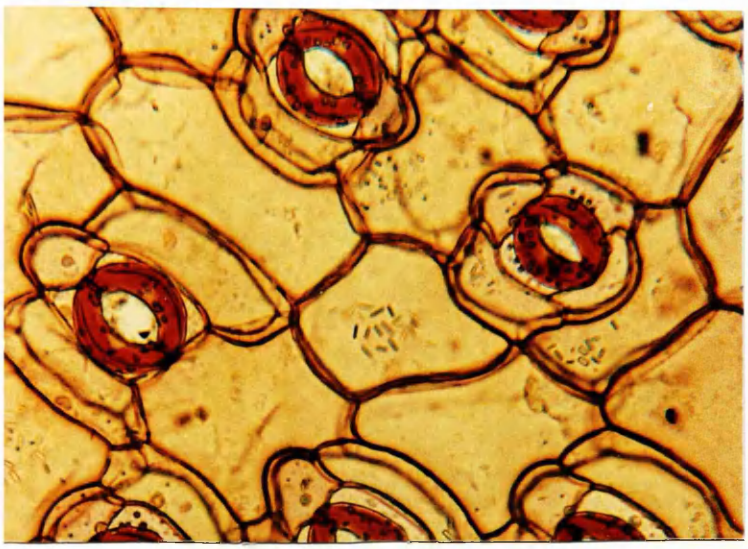
B. 0.01 mol m^{-3} nifedipine



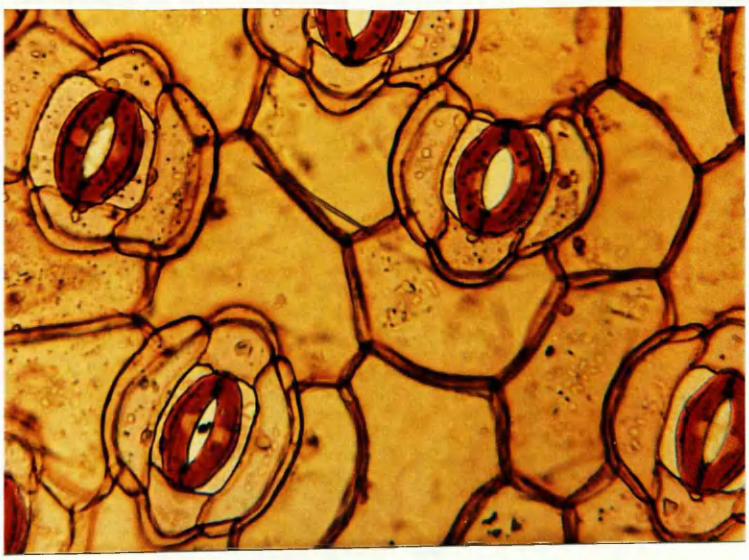
C. 0.2 mol m^{-3} verapamil



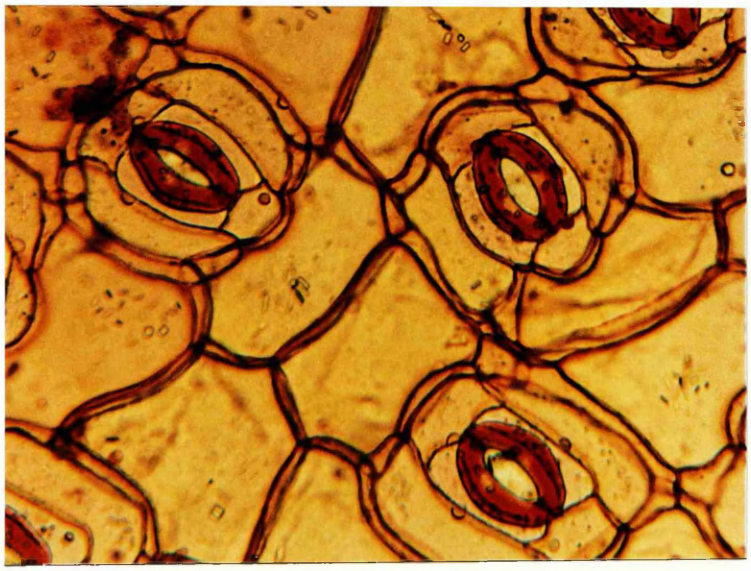
D. 0.05 mol m^{-3} A23187



E. 0.05 mol m^{-3} W-7



F. 0.01 mol m^{-3} trifluoperazine



G. 1 μg ml^{-1} compound 48/80

inhibition. Gietzen et al. (1983) described compound 48/80 as a highly specific calmodulin inhibitor and showed that all the other calmodulin antagonists used in their investigation, apart from compound 48/80, inhibited the basal calmodulin-independent activity of Ca^{2+} transport ATPase. They proposed that its structure possibly hindered incorporation of a compound into the lipid bilayer and thus it did not perturb the lipid environment of the enzyme. It is important to note, however, that the results obtained in this study with all three calmodulin antagonists were essentially similar so that the suppression of the inhibitory action of ABA would probably be a consequence of the antagonistic effects on calmodulin of the drugs used.

The results presented here clearly suggest that the action of ABA on guard cells requires a free passage of calcium ions into the cytosol. They may then act as second messengers interacting with calmodulin to produce the overt stomatal response to ABA. On the basis of these results and previous reports on the involvement of calcium ions and calmodulin in plant hormone action, the scheme shown in Figure 4.14 can be proposed for the stimulus-response coupling of ABA on stomata. According to this the movement of calcium ions from the apoplast seems to be the primary effect of the action of ABA on guard cells. Nevertheless, it would be unwise to rule out the possibility that intracellular redistribution of calcium ions in response to ABA may play a part in the intact plant cell. Intracellular organelles such as chloroplasts, endoplasmic reticulum, mitochondria and vacuole may provide stores of Ca^{2+} that can be released (Fig. 4.15), either through the direct action of the primary stimulus or indirectly through Ca^{2+}-induced Ca^{2+} release (see the General Introduction and also Hepler & Wayne, 1985). It is known that light promotes calcium uptake into chloroplasts (Muto et al., 1982), and if this occurs in

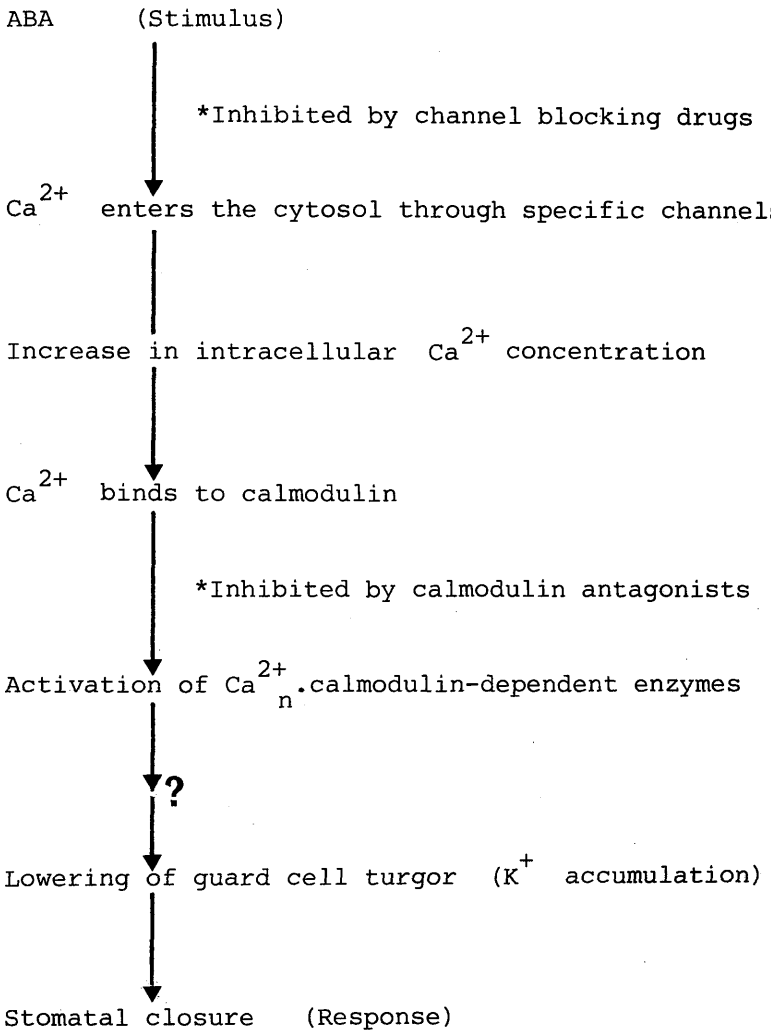


Figure 4.14

Proposed chain of events in the mediation of calcium and calmodulin in the response of stomata to ABA.

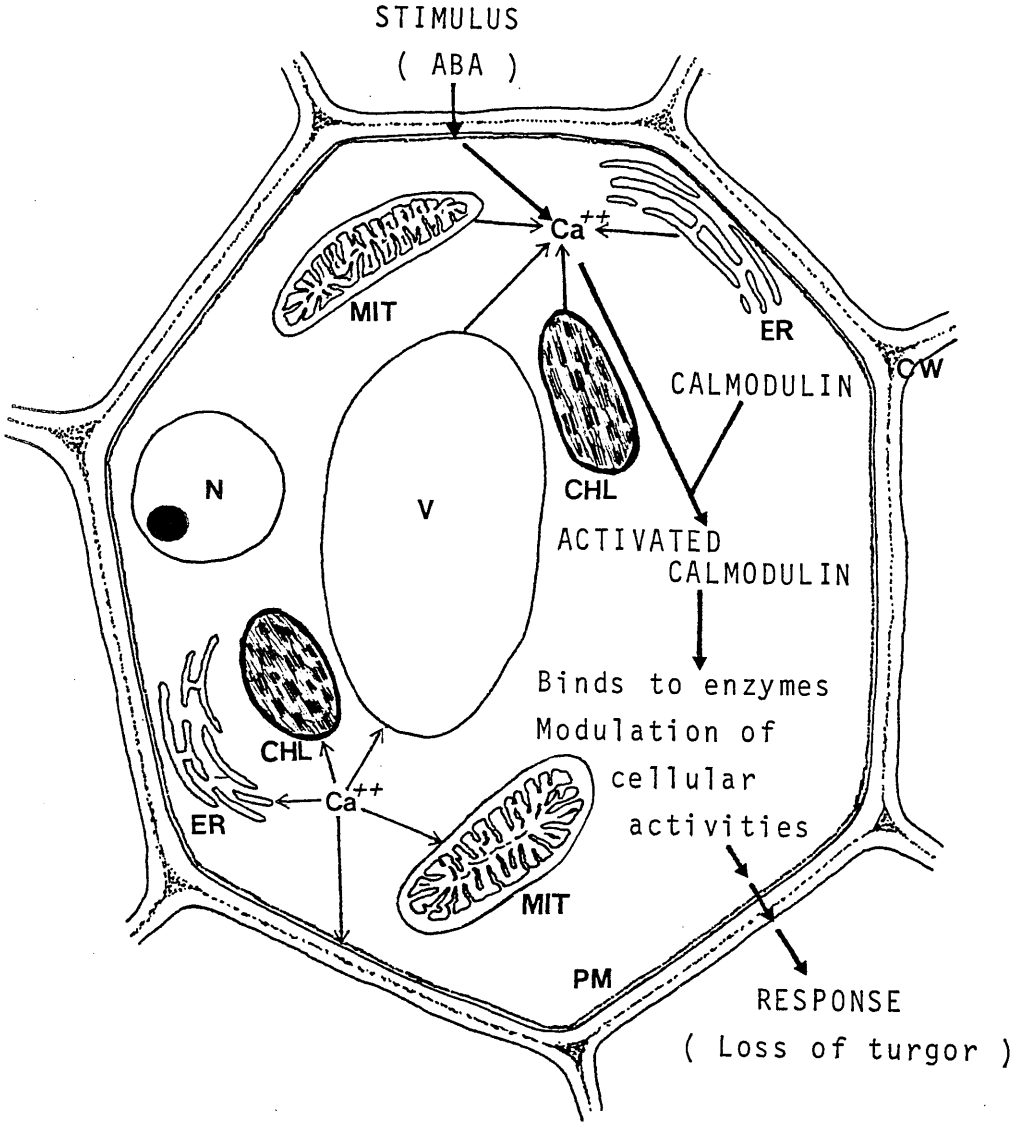


Figure 4.15

Suggested mechanism of stimulus-response coupling of ABA action on a plant cell.

CHL chloroplast

CW cell wall

ER endoplasmic reticulum

MIT mitochondria

N nucleus

PM plasma membrane

guard cell chloroplasts which possess photosystems I and II but may not reduce CO_2 (Mansfield, 1985), it might provide a basis for stomatal opening in light and closure in the dark.

One of the important points of the proposed scheme in Fig. 4.14 is the lack of information about the link between activated calmodulin (Ca_n^{2+}.calmodulin) and the lowering of guard cell turgor. The processes that are involved in bringing about or controlling the ionic fluxes between guard cells and their neighbours are not yet precisely understood, and it has been suggested that plasma membrane ATPases may be involved, and also there is some evidence that ABA affects their activities (Willmer, 1983). The data presented in this Chapter have thrown some light on the underlying mechanism of action of ABA on plant tissues (Fig. 4.15).

CHAPTER FIVE

THE ROLE OF ABSCISIC ACID AND CALCIUM IN DETERMINING THE BEHAVIOUR OF ADAXIAL AND ABAXIAL STOMATA

INTRODUCTION

The amount of gas exchange occurring through a leaf surface depends on both the densities and dimensions of stomata. In general, stomatal densities are higher in abaxial (usually the lower side) than in adaxial epidermis of most species with the exception of members of Gramineae, in which there are more or less equal densities on both surfaces. The ratio of conductance between the adaxial and abaxial surfaces is, however, not precisely related to the ratio of numbers of stomata, because the stomatal conductance is dependent not only upon the number of stomata, but also upon their size.

A well known characteristic of many amphistomatous leaves is a disparity of stomatal apertures on adaxial and abaxial surfaces. Adaxial stomata achieve narrower apertures than abaxial under the same environmental conditions even when these are most favourable for opening. The differences persist when the two epidermes are removed from the leaf, and they cannot be attributed to different conditions of microenvironment, e.g. irradiance or spectral quality of light reaching the guard cells. Leaf inversion studies have shown that this disparity is chiefly a reflection of inherent differences between the two epidermes (Kanemasu & Tanner, 1969; Kassam, 1973; Pemadasa, 1979a; Turner, 1970, 1984). However, Aston (1978) and Raschke *et al*. (1978) working with sunflower and maize respectively, found that adaxial and abaxial stomata did not differ inherently in their responses to light. Turner (1984) also reported that in sunflower there was no inherent difference between the two epidermes. Travis and Mansfield (1981) examined the responses of adaxial and abaxial stomata to light on isolated epidermis of *Commelina communis* and found that stomata of the abaxial epidermis were considerably more sensitive to light, than those on the adaxial surface, supporting the view that the differences in

photosensitivity are inherent rather than a result of differences in the microenvironment within the leaf (Pemadasa, 1981). Pemadasa (1981, 1982a) found that this apparently inherent difference in behaviour could be overcome by the addition of fusicoccin or indol-3-ylacetic acid (IAA) to the incubation medium or application of IAA to the leaf, suggesting that hormones, particularly IAA, play an important role in the differential opening of adaxial and abaxial stomata. Both IAA and fusicoccin are known to influence a cellular process which is thought to be of fundamental importance in the control of turgor of guard cells, namely the stimulation of H^+ extrusion through the plasma membrane, which is associated with the uptake of K^+ (Marrè, 1979). Since IAA-induced adaxial opening was associated with increased K^+ accumulation Pemadasa (1982a) proposed that under normal conditions the proton pump might operate at a lower capacity in adaxial than in abaxial guard cells.

Pemadasa's findings also suggested that the disparity in stomatal opening on the two epidermes might be the result of a gradient of IAA across the leaf. Hayes and Lippincott (1976) observed a higher sensitivity, in terms of nastic movements, to IAA in the abaxial than in the adaxial surface of leaves of Phaseolus vulgaris, and proposed that a geopositive transport system could lead to differential levels of auxin in the two epidermes. Assays to support or refute this suggestion, however, are technically difficult and have not yet been achieved.

Snaith and Mansfield (1982b) found that IAA strongly antagonizes the effect of ABA on stomatal opening. They also showed that the amount of externally supplied IAA could determine the responsiveness to ABA in the incubation medium (Snaith & Mansfield, 1982b). This indicates that the stimulation of opening of adaxial stomata by IAA

(Pemadasa, 1982a) cannot simply be assumed to be the result of a deficiency of IAA: it could occur due to the presence of a higher concentration of ABA in the adaxial epidermis. Furthermore, from the data presented in the previous chapters, it is evident that calcium ions also are a determinant factor for the action of ABA. It was, therefore, interesting to investigate the interplay between ABA, calcium and IAA in the differential behaviour of adaxial and abaxial stomata.

MATERIALS AND METHODS

The preparation of epidermal tissue was similar to that described in Chapter Two. With care, it was possible to obtain epidermal tissue from both adaxial and abaxial surfaces of the same leaf. Epidermal pieces were incubated for 3 ± 0.25 hours in light in the absence of CO_2. The basic incubation medium contained 50 mol m^{-3} KCl and 10 mol m^{-3} MES buffer (pH 6.15) in which the required concentrations and combinations of each additional compound were dissolved. IAA (96% purity) was obtained from Sigma Chemicals Co., Poole.

RESULTS AND DISCUSSION

The mechanisms of action of plant hormones at the subcellular level are not yet fully understood, and the causes of varying sensitivities in different tissues have not been explained. Differences in the sensitivity to specific hormones, or even in the nature of responses, are well-known and are clearly of fundamental importance (Trewavas, 1981). One of the best examples of differential behaviour of apparently similar cell types is the maximum opening that is normally attained by adaxial and abaxial stomata. The involvement of hormones in controlling this situation is strongly suggested by the work of Pemadasa (1982a) who found that a foliar application of IAA

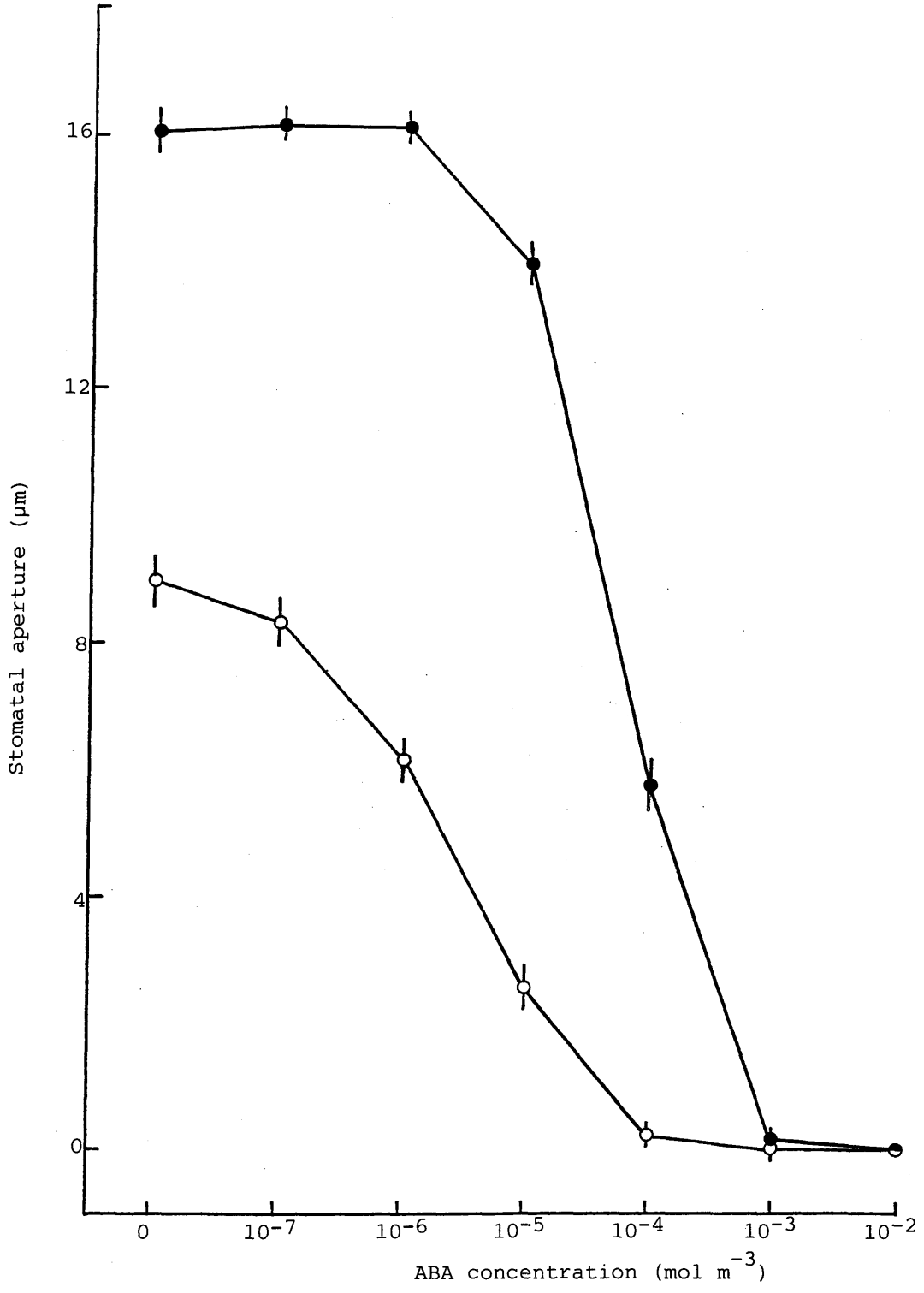


Figure 5.1

Responses of adaxial (O) and abaxial (●) stomata to a range of concentrations of ABA. Means of 90 measurements of individual stomata, with standard errors.

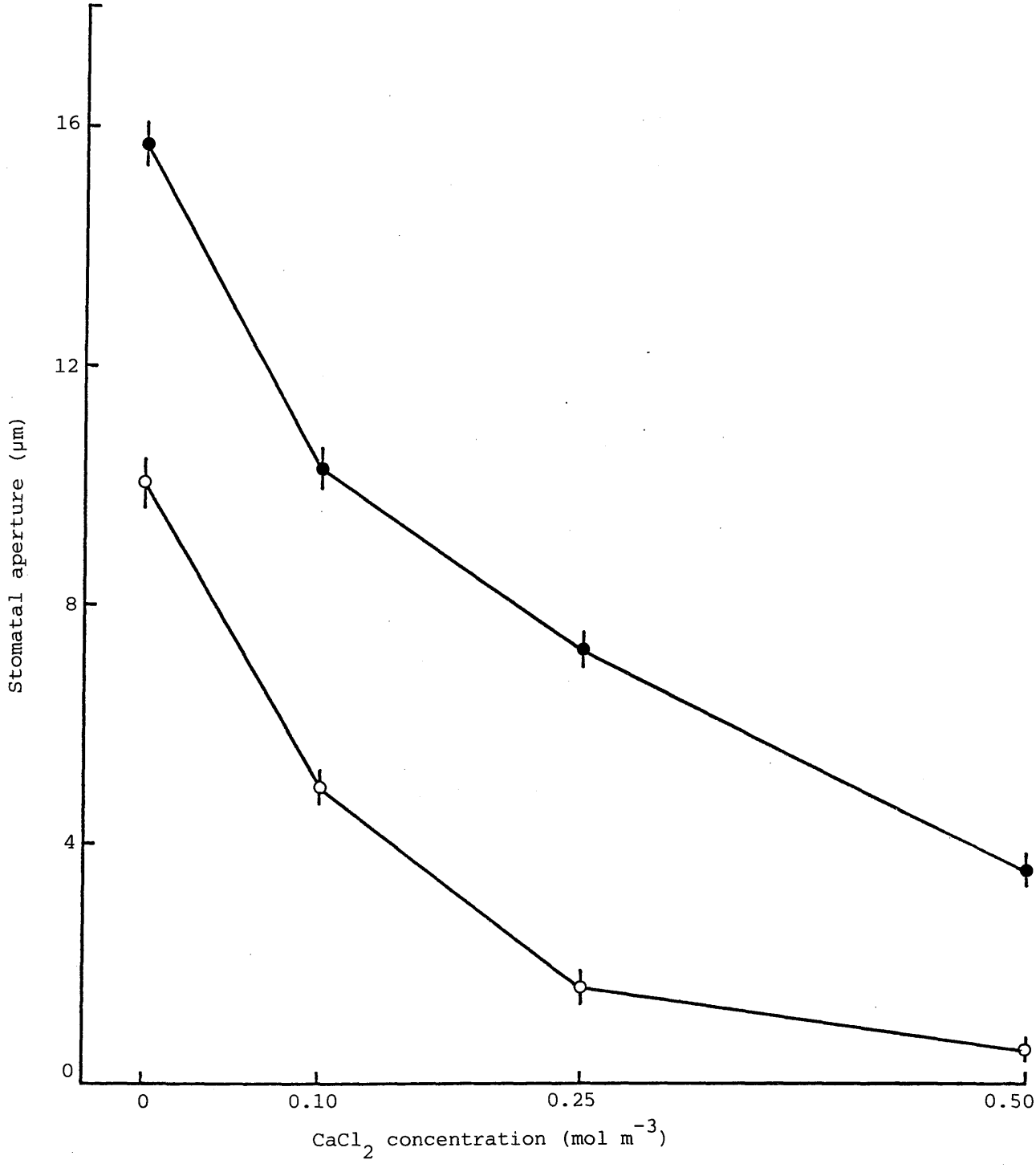


Figure 5.2

Responses of adaxial (O) and abaxial (●) stomata to increasing concentration of calcium. Means of 90 measurements of individual stomata, with standard errors.

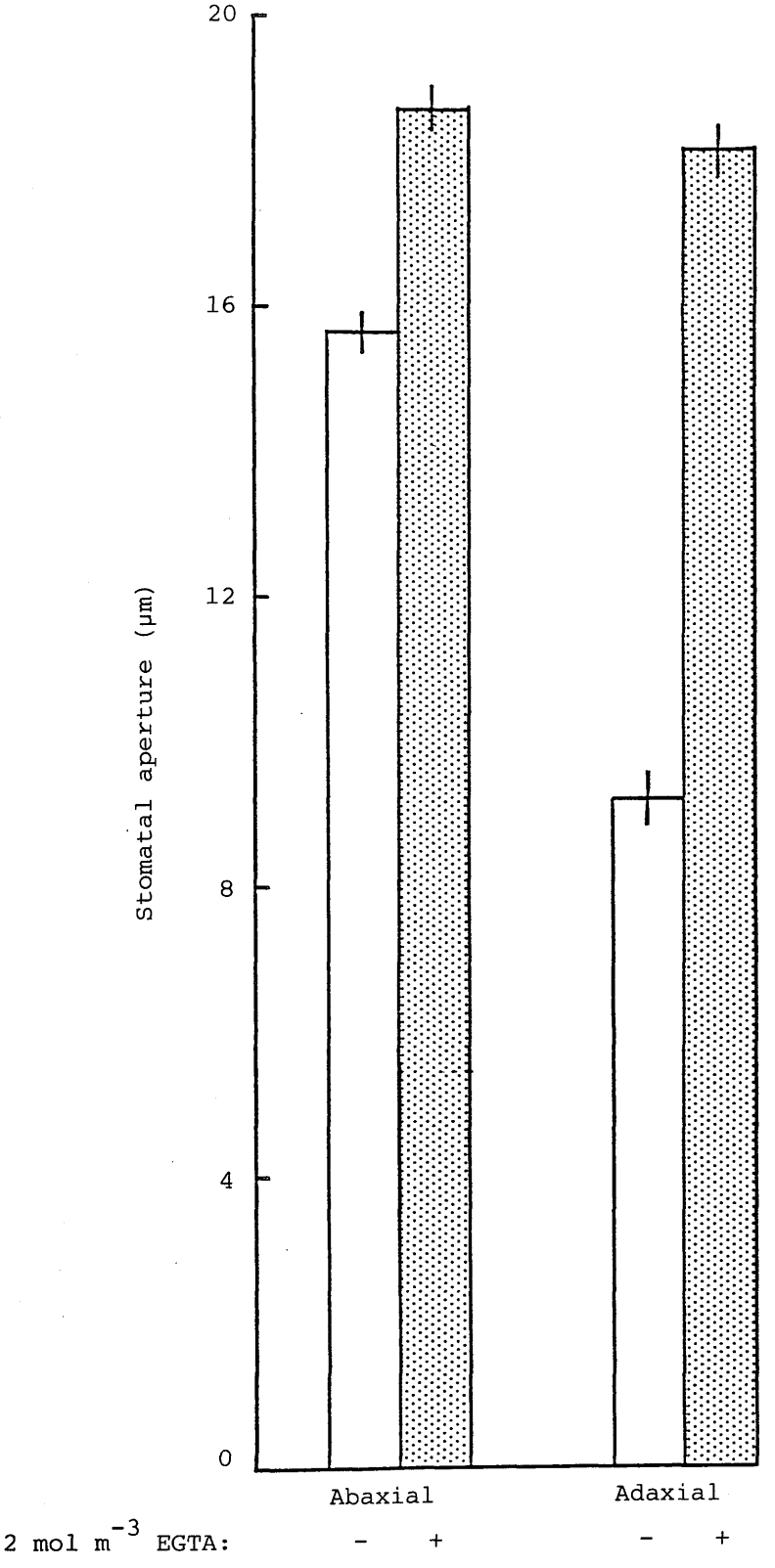


Figure 5.3

Opening of adaxial and abaxial stomata in the presence and absence of 2 mol m^{-3} EGTA. Means of 90 measurements of individual stomata, with standard errors.

enabled adaxial stomata to open as widely as the abaxial. This clearly suggested that the inherent suppression of opening on the adaxial epidermis was the result of a deficiency of IAA. As already explained in the introduction, the situation may not, however, be as simple as this, for there is an interdependence between IAA and ABA in determining stomatal opening in Commelina communis (Snaith & Mansfield, 1982a, 1982b). Thus the suppression of opening of adaxial stomata might be the result of a higher concentration of endogenous ABA in the guard cells which can be counteracted by external application of IAA. To examine whether there is any differential responsiveness to ABA between adaxial and abaxial stomata, epidermal pieces were incubated in a range of ABA concentrations (10^{-7} to 10^{-2} mol m^{-3}) and the results are shown in Figure 5.1. The two curves show a clear difference between the two epidermes. The opening of adaxial epidermis was significantly ($P < 0.001$) depressed in 10^{-6} mol m^{-3} ABA, but there was no response of the abaxial stomata at this concentration. Different responses to exogenous ABA would be expected if the guard cells from the two locations contained different amounts of endogenous ABA. The curves in Fig. 5.1 suggest that the concentration of ABA in the external medium required to produce a comparable response is approximately ten times higher for abaxial than adaxial. This conclusion is reached if one compares (a) the minimum concentration required to cause a small suppression of opening, (b) the concentration required to suppress opening by 50 per cent, and (c) the minimum concentration required to prevent any detectable opening. These results indicate that the sensitivity to external ABA of adaxial stomata is greater than that of abaxial stomata. It has already been shown that Ca^{2+} ions are involved in the action of ABA on guard cells. It was, therefore, interesting to see the effects of an external supply of Ca^{2+} on adaxial and abaxial

stomatal movements. In the next experiment, adaxial and abaxial epidermal peels were incubated for 3 ± 0.25 hours in the presence of Ca^{2+} (0.1, 0.25 and 0.5 mol m^{-3}) and the results are shown in Figure 5.2. The familiar difference in apertures between the two epidermes was evident with no added calcium in the medium, and a difference of approximately the same magnitude persisted as the calcium concentration was increased to 0.5 mol m^{-3}. The difference between these two dose-response curves could be explained in terms of different tissue (or intracellular) concentrations of Ca^{2+}: the adaxial epidermis (or in particular the guard cells) may have a higher concentration of Ca^{2+} which suppresses stomatal opening. Thus suppression would be roughly equivalent to that produced by 0.1 mol m^{-3} calcium when supplied via the external medium to abaxial epidermis. Therefore, the nature of the two curves in Fig. 5.2 suggests that the inherent difference in opening of stomata on the two epidermes could be the result of different endogenous levels of Ca^{2+}, and that either a decrease in the adaxial levels of Ca^{2+}, or an increase in the abaxial levels, might cause the stomata to behave similarly. This possibility was examined by chelating apoplastic Ca^{2+} with EGTA. Adaxial and abaxial epidermal pieces were incubated in the presence and absence of 2 mol m^{-3} EGTA, and the results are shown in Figure 5.3. This shows clearly that EGTA is able to eliminate the differential behaviour of adaxial and abaxial stomata. In the presence of 2 mol m^{-3} EGTA the stomata from both surfaces opened more widely than did abaxial stomata in the controls. Therefore, these results strongly support the view that Ca^{2+} also is involved in the differential opening behaviour. This suggestion is further supported by the results obtained from an experiment where the effects of 1 mol m^{-3} $LaCl_3$ on adaxial and abaxial epidermes were investigated. The results in Figure 5.4 show that

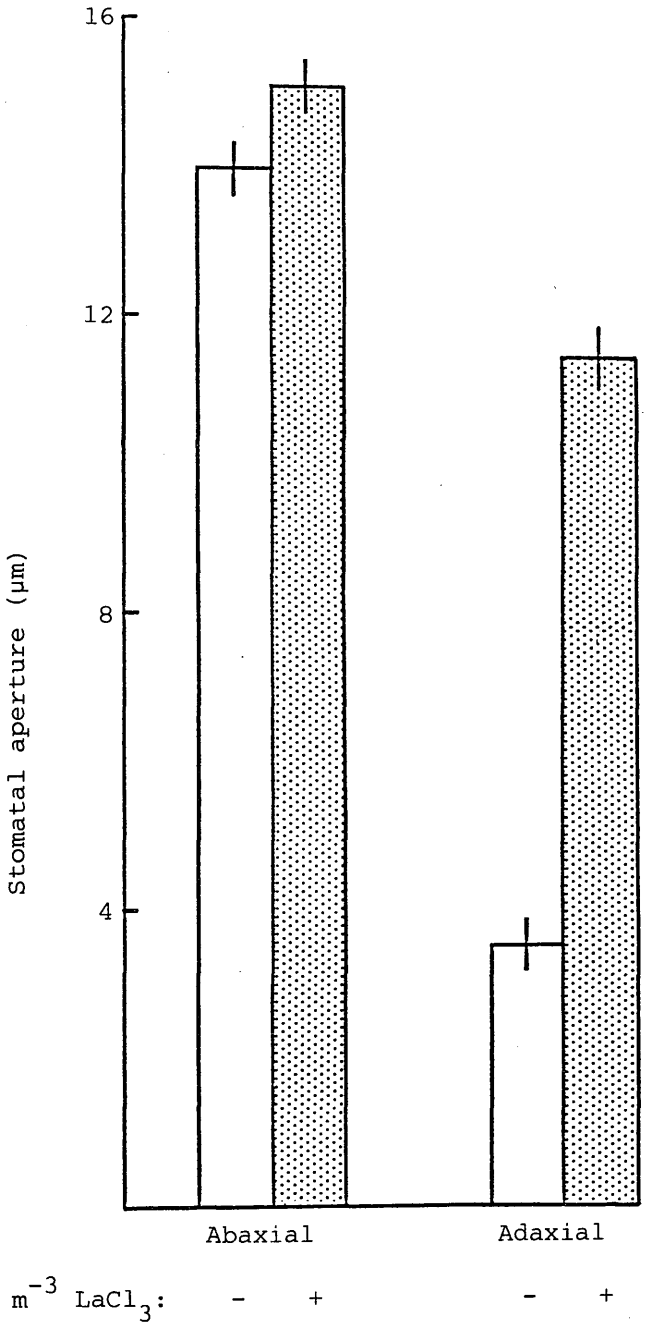


Figure 5.4

Effect of 1 mol m^{-3} $LaCl_3$ on the opening of adaxial and abaxial stomata. Means of 90 measurements of individual stomata, with standard errors.

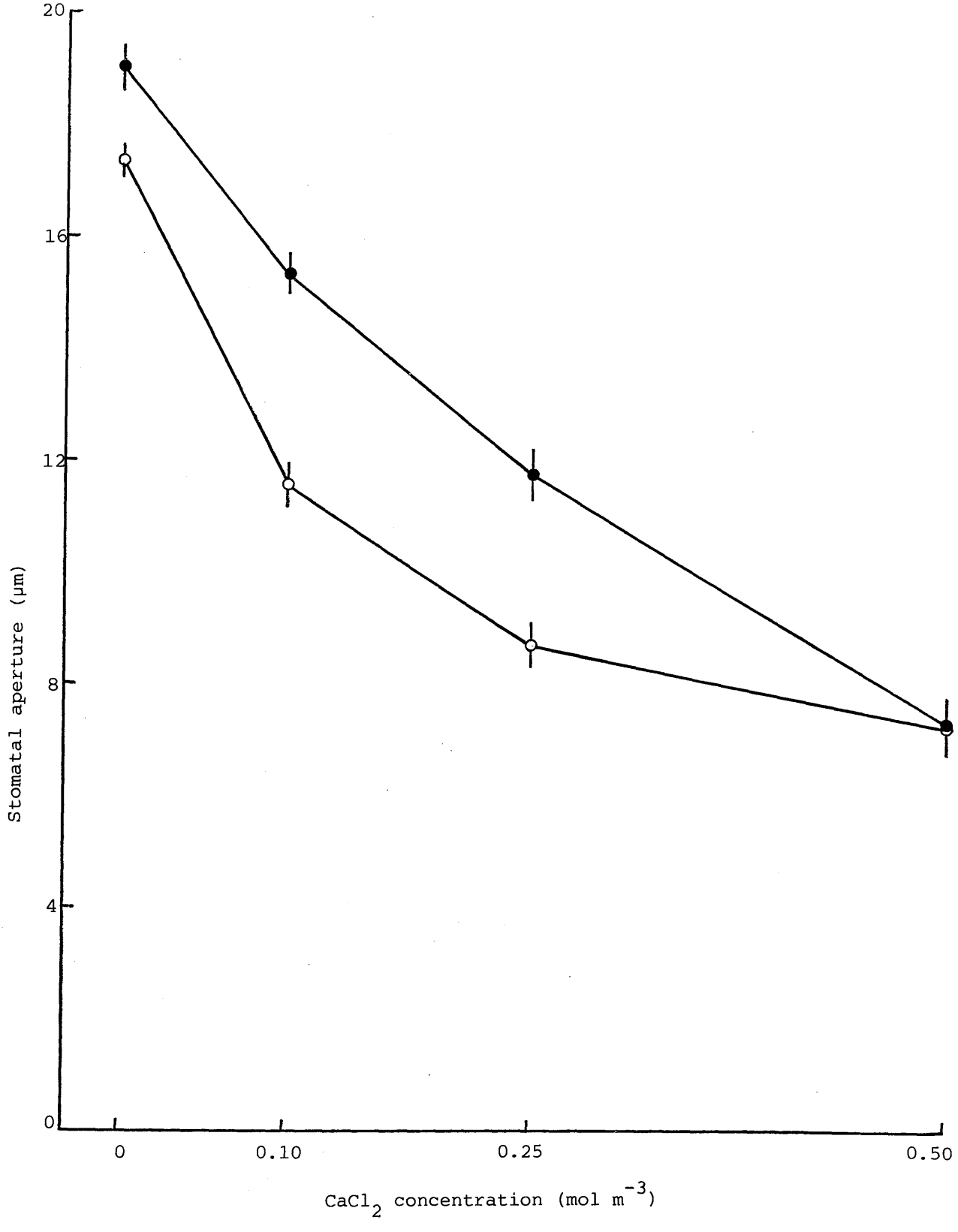


Figure 5.5

Responses of adaxial (O) and abaxial (●) stomata to increasing concentrations of calcium in the presence of 0.5 mol m^{-3} IAA. Means of 90 measurements of individual stomata, with standard errors. See Figure 5.2 for controls.

there was a large difference in the response of stomata from the two surfaces: the opening of the adaxial stomata increased almost 3-fold in response to $LaCl_3$, whereas there was a little effect of $LaCl_3$ on the abaxial stomata. It is important to note that there was no added ABA or calcium in this experiment, and that the effect observed was achieved solely by the added $LaCl_3$. Therefore, the effect seen here could be the outcome of a relatively high content of endogenous ABA and/or Ca^{2+} at their critical locations in the adaxial epidermis (in particular the guard cells). However, one cannot rule out the possibility of the involvement of IAA in this phenomenon which was first suggested by the work of Pemadasa (1982a).

In the next experiment the effects of IAA and fusicoccin, two agents known to cause adaxial stomata to open as widely as abaxial ones, were investigated in the presence of a range of calcium concentrations, and the results are shown in Figure 5.5 and Table 5.1 respectively. IAA at 0.5 mol m^{-3} could partially narrow the gap between the opening on the two epidermes, but it was unable to suppress the effect of externally supplied Ca^{2+} completely. At the highest concentration of calcium (0.5 mol m^{-3}), however, adaxial and abaxial stomata opened to the same extent (Fig. 5.5). Fusicoccin (10^{-2} mol m^{-3}), in contrast, stimulated the adaxial stomata to open nearly as widely as abaxial ones. A comparison between the data in Table 5.1 and Fig. 5.5 shows that there is an essential difference between the effects of fusicoccin and IAA on stomata. Although both of these agents might be expected to enhance the efflux of protons through the plasma membrane of the guard cells, IAA is not able to suppress the effects of calcium to any marked degree. It is known from the studies of other systems that the precise manner in which these two compounds control proton extrusion differs. In maize coleoptiles, IAA may act

$CaCl_2$ concentration (mol m^{-3})	Adaxial	Abaxial
zero	18.31 ± 0.13	19.21 ± 0.17
0.01	17.74 ± 0.10	18.88 ± 0.17
0.25	17.31 ± 0.14	18.95 ± 0.18
0.50	17.70 ± 0.13	18.95 ± 0.16

Table 5.1. Effect of fusicoccin on Ca^{2+}-induced stomatal closure. Pieces of adaxial and abaxial epidermis were incubated for 3 h under CO_2-free conditions in 10^{-2} mol m^{-3} fusicoccin and 50 mol m^{-3} KCl. Mean apertures (μm) of 90 individual stomata are shown with standard errors. See Fig. 5.2 for controls.

by pumping protons into vesicles the contents of which are subsequently released to the exterior of the cell (Ray, 1977). Fusicoccin apparently acts directly on the plasma membrane, stimulating proton efflux via a plasma membrane ATPase (Marrè, 1979). If the two compounds differ in the same manner in their action in guard cells, an explanation for the differential ability to reverse the effect of calcium ions may be more readily found.

The possible involvement of calcium in the differential behaviour of adaxial and abaxial stomata is worthy of further investigation. It is common for adaxial stomata to open less widely than abaxial, and this may represent a state of 'polarity' in the leaf. Polar transport of calcium across maize roots and oat coleoptiles under the influence of gravity has been reported recently (Lee et al., 1983; Slocum & Roux, 1983). In oat coleoptiles there was a marked redistribution of calcium to the upper side within 30 minutes. It has already been shown that there is a strong synergism between ABA and Ca^{2+} in suppressing stomatal opening. A redistribution of calcium along with ABA would thus lead to an amplified response of the stomata, and only very small changes in both agents might be necessary to establish the differential opening between the stomata on adaxial and abaxial surfaces. Further amplification might be achieved if IAA concentrations were to fall while those of ABA increased (Snaith & Mansfield, 1982a).

These interpretations, however, might leave some doubts about the role of endogenous ABA in the differential behaviour of the two epidermes: since the receptors of ABA are apparently located on the outside of the plasma membrane of the guard cells, ABA may exert its effects from outside (Hartung, 1983; Hornberg & Weiler, 1984), and so it might be argued that any ABA within the guard cells will have no effect in this phenomenon. Further, if the ABA located outside the

guard cells (coming from the rest of the epidermis or more likely from mesophyll cells) is the critical factor affecting guard cell turgor, then isolated epidermes may not be expected to exhibit differential stomatal opening. When the epidermal strips are floated on the incubation medium any ABA released from other epidermal cells would presumably be diluted in the bathing medium, and if this is the case a reasonable question to ask is: What causes the observed disparity in opening? Nevertheless, it would be unwise to assume that any ABA translocated to the guard cells from elsewhere may readily be diluted in the incubation medium, before it participates in the proposed mechanism of action since one cannot rule out the possibility that ABA which had already reached the guard cells would become associated with receptors on the plasma membrane (Hornberg & Weiler, 1984) and remain physiologically active during the course of 3-4 hours experimental periods. However, further research is needed to confirm or refute these interpretations. One line of research of immediate importance in this aspect is to measure intracellular Ca^{2+} levels in the two epidermes, but techniques are not available at present to permit the measurement of free calcium in the guard cells, or in the apoplast surrounding them (see the next Chapter). Secondly, determination of ABA levels in the two epidermes may provide useful information relevant to this problem. However, preliminary work carried out in our laboratory to determine the whole tissue contents of ABA has not provided any conclusive evidence; the results were subject to considerable variation and no consistent differences were found (R.C.Cox, unpublished data). Also, it is doubtful if such whole tissue analyses can contribute usefully to unravelling this problem. Attempts were not made to analyse the endogenous ABA content of guard cells because it is unlikely that this could have been achieved satisfactorily for

two main reasons: (a) the initial isolation of intact guard cells or their protoplasts is likely to cause a relocation of ABA from elsewhere in the epidermis, and (b) it is possible that guard cells can manufacture ABA (Weiler et al., 1982) and so ABA newly formed in response to the stress of procedures used to isolate the cells or protoplasts could confuse the picture.

The results presented in this Chapter have illustrated the complexity of endogenous chemical factors that might be responsible for establishing the pattern of stomatal behaviour in leaves, and have highlighted the importance of interactions between these factors in influencing stomatal sensitivity.

CHAPTER SIX

MEASUREMENT OF INTRACELLULAR CALCIUM

INTRODUCTION

The experiments described in the previous Chapters have suggested that the stomatal closure induced by abscisic acid is mediated through processes involving calcium, which are activated and/or accelerated by an increase in the intracellular Ca^{2+} concentration: a blockage of free entry of Ca^{2+} by calcium channel blockers was able to reduce the inhibitory effects of ABA on stomatal opening. As described in Chapter Four, calcium channel blockers have been widely used to examine the stimulus-response coupling of many cellular processes which are thought to be mediated via Ca^{2+} fluxes into the cytosol. Increases in cytosolic Ca^{2+} concentration which followed a stimulation have successfully been detected and estimated in animal systems by a number of methods such as precipitation, bioassay, polarography and methods involving photoproteins, metallochromic indicators, fluorescent indicators and Ca^{2+} specific microelectrodes (see Campbell, 1983). However, evidence of the success of such measurements in plant systems is scanty and the only report known to date is that of Williamson and Ashley (1982) who microinjected the photoprotein, aequorin, into Chara internodal cells and successfully measured changes in calcium concentration following an action potential. The major reason for this is the lack of a suitable technique for measuring calcium in plant cells. The methods used for animal systems are difficult to apply to plants, with their tough cell walls, thin layer of cytoplasm and high turgor pressure (Cork, 1986). Nevertheless, knowing the intracellular calcium concentration and whether it changes following a stimulation (by an extracellular signal or an intracellular regulator) is of immediate importance in obtaining definitive evidence for the regulatory role of intracellular calcium. One of the methods which has not frequently been employed in measuring intracellular calcium concentration is

electron microprobe X-ray analysis. This method allows the measurement of the mass total calcium per unit mass of microvolume within the specimen in a non-destructive fashion (Gupta and Hall, 1978). The procedure used to prepare the tissue quantitatively preserves the elements at their original locations. It also preserves the cellular fine structure and allows subsequent imaging at a suitable resolution. Further, X-ray microprobe analysis in the electron microscope is believed to be the most powerful tool available at present for identification and estimation of total calcium in tissue sections (Campbell, 1983; Gupta and Hall, 1978).

Principles of X-ray spectrochemical analysis

The technique of X-ray emission spectroscopy measures the X-ray fluorescence produced when a specimen is irradiated by an electron beam. Any electron of the beam with energy higher than the binding energy of an electron in an atom of the specimen may transfer all or part of its energy to remove a bound electron from the atom and create a vacancy in a particular orbit. If this vacant site occurs in an inner orbit, or low energy level, it will be immediately filled by an electron from a higher energy level with the emission of an X-ray photon, the energy of which is equivalent to the difference between the two energy levels. If the original ionization causing the vacancy was created in the innermost or K-orbit, the X-ray is identified as a K emission, and, since the replacement of electron could be provided from any one of several outer orbits (L, M, etc.), emitted X-rays of different energies are possible and are designated K_{α}, K_{β} etc. One of the most important characteristics of emission spectroscopy is the fact that, because the binding energy of the electron in an orbit is related to the charge on the nucleus, each element produces its own characteristic emission

spectrum and, since only inner orbits are involved, the X-ray production is independent of the chemical state of the atom (Van Steveninck and Van Steveninck, 1978).

The wavelength/energy of the X-ray emission is characteristic for each element and the intensity is related to the quantity of the element in the part of the section analysed. The physical principles and instrumentation have been described in detail by Gupta and Hall (1978), and Van Steveninck and Van Steveninck (1978). Instruments are now available which can detect down to about 10^{-18}g of calcium. This apparently high sensitivity for Ca^{2+} is, however, not very attractive when expressed in terms of tissue concentration, since in a thin section it is equivalent to about 4 mmol/Kg wet weight (Campbell, 1983). This means that in order to detect Ca^{2+} at different sites in the cell, the local concentration has to be very high. However, this technique has proved useful in the analysis of intracellular precipitates of calcium and has confirmed the presence of intracellular sites of some calcium stores (Campbell, 1983; Gupta and Hall, 1978). An attempt, therefore, has been made to examine the applicability of this technique in the measurement of intracellular Ca^{2+} levels of Commelina communis guard cells after treatments with EGTA, ABA and calcium.

MATERIALS AND METHODS

Epidermal strips of C.communis were incubated for 3 h in light under CO_2-free air in 50 mol m^{-3} KCl buffered with 10 mol m^{-3} MES containing either 2 mol m^{-3} EGTA, 10^{-4} mol m^{-3} ABA or 0.5 mol m^{-3} $CaCl_2$. The controls contained only buffered KCl.

Preparation of the specimens for microprobe analysis

Following the incubation, the epidermal strips were washed and blotted carefully. Using a thin layer of Tissue Tek II O.C.T.

compound (Miles, Naperville, Illinois, U.S.A.) as an adhesive, they were mounted cuticular surface upward onto carbon-coated aluminium specimen carriers which were pre-shaped to present an angle of 45^{o} to an energy dispersive (EDS) detector fitted to the scanning electron microscope (SEM). The mounted specimens were quench frozen in nitrogen slush, temperature 63K, after which they were transferred to the SEM without contact with the atmosphere. The specimens were examined at a temperature lower than 83K. If residual water (from the washing procedure) in the form of ice was present, that was removed by sublimation from the specimen surface. To achieve this, specimens were heated on the specimen stage to 173K after which the stage was rapidly cooled to 83K and the specimens were then ready for coating. They were coated with 22 nm of aluminium before analysis in a JEOL JSM 50A SEM equipped with a Kevex EDS detector and Link System 860 Series II analyser. The accelerating voltage was 25 kV. Analysis was performed by repeated scans across an area of 1 μm^{2} until a standard preset gross integral of 80,000 counts for aluminium was reached. Each guard cell was scanned in three different places (that is, the middle and either end). Both guard cells of four individual stomata were scanned for potassium, calcium and chlorine. The total spectrum counts were in excess of 300,000.

The micrographs shown in the Plates 2.4 and 2.5 (Chapter Two) were taken from epidermal pieces from the controls and those treated with 2 mol m^{-3} EGTA respectively. Plates 2.4a and 2.5a were taken by scanning a stomatal complex with a low beam of electrons. Plates 2.4b and 2.5b are the micrographs of electron emission for potassium taken after 8 and 2 scans respectively.

Quantification

Methods of measuring elemental mass fraction (the proportion of the element in the analysed volume) depend on the type of specimen undergoing analysis, i.e. whether it is a bulk specimen not transparent to electrons of the beam, a moderately thick section or a thin section, in which case X-ray intensity is approximately proportional to thickness. With bulk specimens, the intensity of a characteristic peak for an element (after 'background' subtraction, see Figure 6.1) on comparison with a suitable standard, gives a semiquantitative measure of elemental mass fraction. The background can be assessed in several ways, either by taking a mean of values on either side of the peak, taking care to avoid any characteristic lines in the spectrum, or by extrapolating from the lower energy or longer wavelength side (Van Steveninck and Van Steveninck, 1978). With the Link System 860 Series II analyser, it is possible to obtain both the gross integral (with the background) and net integral (after background subtraction) counts for each element. The local mass fraction (C_x) in bulk specimens is given by the following equation (for further details, see Gupta and Hall, 1978):

$$C_x = K \frac{I_x}{I_w}$$

where, I_x is the characteristic X-ray intensity and I_w is the continuum X-ray intensity observed at a wavelength near that of the characteristic line of the element and K is a constant determined by means of a standard. The ratio of net peak intensity to continuum (I_x/I_w) gives a measure of the local mass fraction of the element and this measure is unaffected by the specimen thickness.

Measurements of elemental ratios in different parts of a specimen or in comparison with a standard may be of value. Thus:

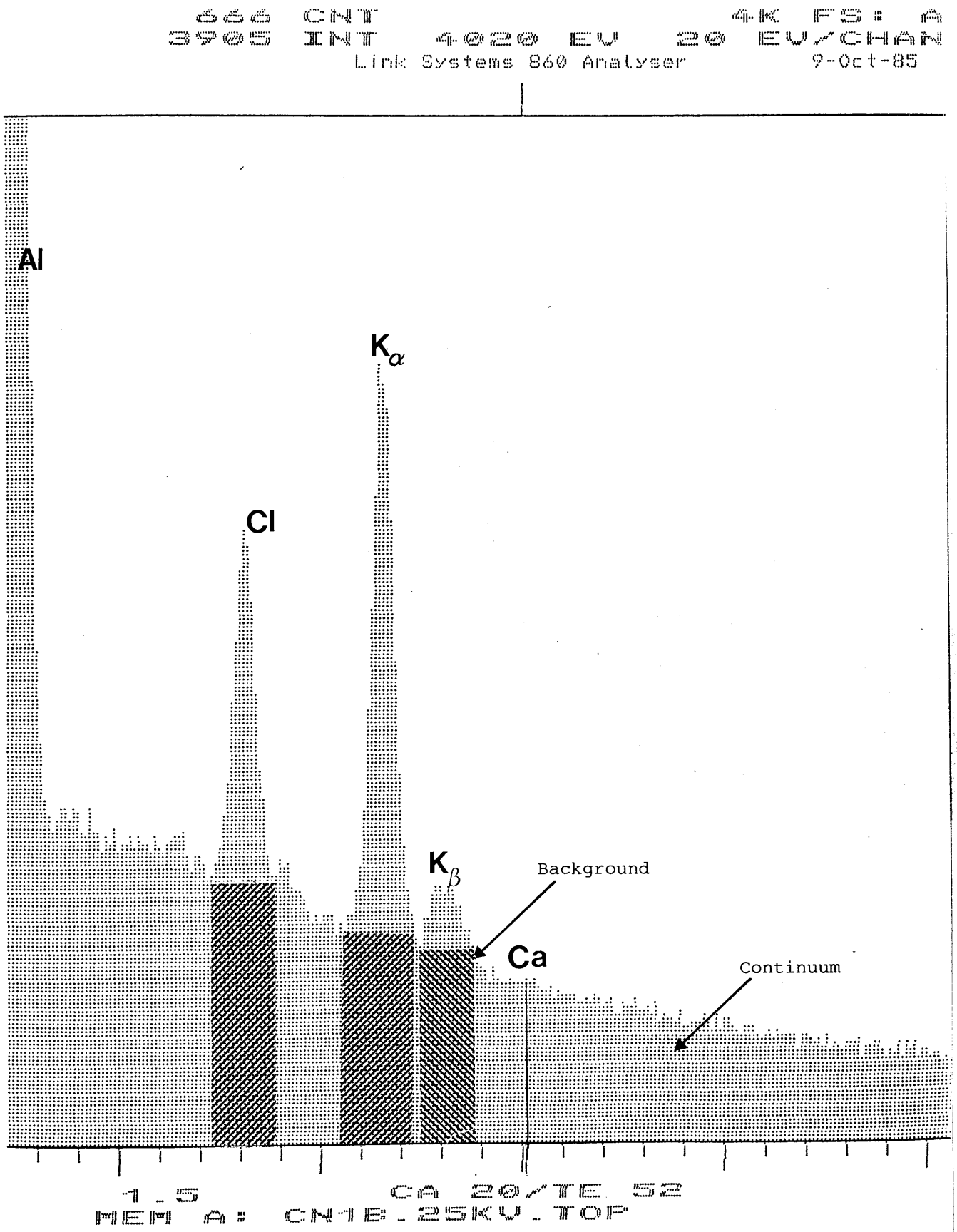


Figure 6.1

Energy dispersive X-ray spectrum from a guard cell of Commelina communis showing characteristic peaks for chlorine and potassium.

$$\frac{(C_A/C_B)_1}{(C_A/C_B)_2} = \frac{(I_A/I_B)_1}{(I_A/I_B)_2}$$

where C and I are mass fractions and peak intensities of two elements, A and B, in places 1 and 2. However, it is now appreciated that the total mass fraction equation is of little applicability for hydrated bulk specimens (Dr. K. Oates, personal communication) and, therefore, an alternative method of comparison was employed in this study. The percentage net integral count for each element recorded by the analyser was compared with that of aluminium as the standard. The rationale for adopting this method was as follows: All the analyses were standardized on 80,000 counts in the window set for aluminium. The aluminium coating had a constant thickness over both a single specimen and from one speciment to another. The take-off angle of X-rays was essentially constant. Thus, relating the net counts acquired for elements of interest to a standard consisting of net aluminium counts should provide comparable, but not fully quantitative data (Robards and Oates, 1986). An important point worth mentioning here is that the range of X-ray energy of calcium K_α emissions is more or less identical to that of potassium K_β emissions, and the EDS detector cannot distinguish between these two. Therefore, the recorded X-ray counts at the calcium K_α region may be an overestimation. With the hope of avoiding this difficulty, the K_β X-ray emission for calcium was also estimated simultaneously.

RESULTS AND DISCUSSION

Table 6.1 shows the means of the percentage counts recorded by the analyser, for calcium, chlorine and potassium in the guard cells of four individual stomata. The treatments were control, 10^{-4} mol m^{-3} ABA, 2 mol m^{-3} EGTA and 0.5 mol m^{-3} $CaCl_2$. Three measurements were

Treatment	Chlorine	Potassium	Calcium K_{α}	Calcium K_{β}
Control	8.18	17.55	1.58	0.09
	(0.42)	(0.69)	(0.45)	(0.18)
10^{-4} mol m^{-3} ABA	1.39	12.01	0.64	0.03
	(0.11)	(0.43)	(0.49)	(0.01)
2 mol m^{-3} EGTA	16.69	32.08	1.42	0.02
	(0.79)	(1.03)	(0.25)	(0.01)
0.5 mol m^{-3} $CaCl_2$	1.94	7.94	3.15	0.13
	(0.16)	(0.66)	(0.30)	(0.02)

Table 6.1. Mean counts for chlorine, potassium and calcium expressed as a percentage of total net counts for aluminium. Standard errors are shown in parentheses.

made on each guard cell and, therefore, each figure in Table 6.1 represents a total of 24 individual counts (replicates). As can be seen in Table 6.1, the percentage X-ray counts for potassium in ABA- and $CaCl_2$-treated guard cells were lower than those in the controls. In EGTA treatments the percentage counts were very high and significantly different from the controls. However, there was no significant difference between percentage calcium K_α counts (also K_β counts) in different treatments. This may be due to the great variability in X-ray counts within treatments (both in different guard cells and in the same guard cell at different locations). The distribution of various elements in a cell is not uniform (Laffray et al., 1982) and this may be a reason for the observed variations within guard cells. For example, if the intracellular organelles, such as chloroplasts, endoplasmic reticulum, mitochondria etc., which are known to store calcium, had been included in the scanned area then heavy counts for calcium would be expected.

Another reason for the lack of success of this investigation is that this method is not specific for measuring the concentration of a free ion. The displacement of electrons of a particular element by the X-ray beam and the consequent emission of characteristic X-rays can occur regardless of the ionic state of that element. Therefore, it measures the X-ray counts of both free and bound forms of that element. Furthermore, X-ray emissions can take place from an element present in the path of the electron beam which passes first through the apoplast and then the symplast. This could also mask the real number of X-ray counts which one would expect to measure from the symplast.

This technique, therefore, proves to be unsuitable for the detection/measurement of an element such as calcium, which is believed to be present in the cytosol at micromolar or submicromolar concentra-

tions. However, it has proved to be of value for the mapping of an element present at high concentrations (e.g. potassium at submolar quantities; see Plates 2.4 and 2.5, and the discussion of Chapter Two).

Another technique which might be of some interest in measuring intracellular calcium concentrations in the stomatal complex is microfluorimetric analysis. Fluorescent dyes such as chlorotetracycline (CTC), quin-2 and fura-2, and the photoprotein, aequorin are known to complex with calcium ions and emit fluorescence which could be measured by using a microfluorimeter. To employ this technique, it is necessary for these agents to gain access into the cytosol. Some compounds, such as chlorotetracycline, can easily penetrate into the cells (Campbell, 1983). However, quin-2 and its acetoxymethyl ester (quin-2-AM) may not readily be loaded into the cells (Tsien, 1981). A very recent report by Cork (1986) describes some difficulties with the application of quin-2-AM to measuring cytoplasmic free calcium in plant cells. Cork has attributed this failure to extracellular hydrolysis of the membrane-permeable ester, quin-2-AM. He has also found that long incubation periods employed in an attempt to overcome that problem had caused another difficulty: the by-product of ester hydrolysis, formaldehyde, was inhibitory to cellular processes. To avoid these shortcomings, those agents can be microinjected into the cytosol, and this method has been successful in experiments with single cells or a tissue with giant cells, e.g. Chara (Williamson and Ashley, 1982), but it has very limited applicability to our experimental system.

A success in the measurement of cytoplasmic calcium activity using ion-selective microelectrodes has recently been reported by Miller (1986). He has used the specific calcium ionophore ETH 1001 as the calcium ion sensor in the microelectrode, and measured a wide range of calcium ion concentrations up to pCa 8 in Chara internodal

cells. However, small cells like guard cells which are only a few micrometers in diameter may be difficult to impale with microelectrodes without damaging the cell. Therefore, this method also may turn out to be of limited practical value for intracellular calcium measurements in guard cells.

CHAPTER SEVEN

GENERAL DISCUSSION

Abscisic acid has now been identified in a great variety of species and found to be of universal occurrence among vascular plants. It is present in mosses, but it appears to be absent from liverworts, all divisions of algae, bacteria and fungi, except for the fungus Cercospora rosicola (see Addicott & Carns, 1983). In liverworts and algae lunularic acid, a C_{15} compound with a structure somewhat similar to ABA, appears to have the same physiological role as ABA in higher plants (Pryce, 1972). The role of lunularic acid as a substitute for ABA will be discussed later in this discussion.

Ever since its discovery, abscisic acid has been considered as one of the several hormones which control plant growth and development [the possible hormonal functions of ABA have been described by Addicott & Carns (1983)]. However, since the turn of this decade, there has been some strong criticism over the use of the term 'hormone' to describe this class of physiologically active phytochemicals, and several alternative names such as plant growth regulators and plant growth substances have been proposed from time to time. However, these names have also seemed inadequate since not all the phenomena affected by those compounds involve growth. In his critical review of the prevailing concepts concerning the action of those phytochemicals, Trewavas (1981) refutes the hormonal concept which suggests that plant growth and development are controlled solely by changes in the concentrations of these phytochemicals. He also argues that the growth substances are not hormones and explains the difficulties in drawing a parallel with mammalian hormones (Trewavas & Cleland, 1983).

The term 'hormone' was initially given to physiologically active organic compounds in animals and it may be defined as an organic substance produced in a minute quantity in one part of organism and transported to another location where it exerts a profound effect when

necessary. This means that a hormone has a localized biosynthesis, and it is released on receipt of an appropriate signal and acts at a site of action some distance from the site of biosynthesis. It is these definition-associated requirements that are provocatively disputed by Trewavas (1981). However, some of Trewavas' arguments have been questioned by Cleland (Trewavas & Cleland, 1983) who supports the concept of hormones in relation to plants. It is not intended to consider Trewavas' arguments in detail here since they have been extensively discussed elsewhere (Blackman, 1984; Trewavas & Cleland, 1983; Weyers, 1984). It is, however, worth mentioning the important differences between plant and animal hormones. Each group of phytohormones is synthesized in more than one part of the plant, for example, ABA can be synthesized in stems, roots, leaves, fruits and seeds (Milborrow, 1983). Each can influence the cells where it is made in addition to its distant site of action, for example, when petals fade ethylene accelerates the senescence of the cells in which it is produced as well as influencing the senescence of neighbouring cells (Potts et al., 1982). Finally, each hormone affects different cells in different ways, rather than exerting one single type of response, for example, ABA is known to be involved in inducing dormancy, stomatal closure and senescence (Addicott & Carns, 1983).

Although an enormous amount of work has been done on the effects of abscisic acid on plant tissues, a little attention has been devoted to elucidating the molecular basis of its action. The scantiness of experimental evidence concerning the mechanism of action of ABA may partly be due to failure in the careful designing of proper experiments and, perhaps, due to the lack of suitable techniques. These facts have inevitably left some plant physiologists wondering whether abscisic acid (and also other groups of phytohormones) have any hormonal role

in plants or even whether true hormones exist in plants (see Weyers, 1984).

The experimental evidence presented in this thesis has provided some answers to such questions. Abscisic acid appears to increase guard cell membrane permeability to calcium ions and the subsequent increase in intracellular calcium levels seems likely to modulate certain enzymatic activities in the guard cells via a calmodulin-involved process(es). The evidence supporting this view has been discussed in detail in the previous Chapters and will not, therefore, be repeated here. The proposed mechanism of action of ABA on guard cells seems to be closely parallel to the well studied action of hormones and neurotransmitters in animal cells and, therefore, has provided vital information about a topic currently in much debate. It is important, however, to mention here that without further evidence it cannot be assumed that ABA controls the activities of all cells in the same manner, but it seems unlikely that the kind of mechanism discovered in guard cells is not present in other locations in plants. Dekock (1979) reported that ABA treatments increased intracellular calcium levels in roots. Further, ABA has also been found to promote both volume flow and ion release to the xylem in sunflower roots (Glinka, 1980).

The results discussed in Chapter Five of this thesis have clarified another aspect of the action of plant hormones covered by Trewavas (1981). Trewavas claimed that 'plant growth substances' are not limiting factors in development, and introduced the notion that the regulatory factor is the 'sensitivity' to growth substances (Trewavas & Cleland, 1983). Nevertheless, he recognized that this concept requires the presence or absence of some factor to which the tissue responds, thereby acknowledging that there might be an 'all or nothing' response to a plant growth

substance. However, as Cleland has pointed out, small changes in concentration in plant hormones regarded by Trewavas as being of no physiological importance, may be important via changes in ratios of hormones (Trewavas & Cleland, 1983). This would be of great importance in systems in which several plant hormones are known to be involved, especially when they have antagonistic effects to each other. For example, it has recently been reported that both auxins (Snaith & Mansfield, 1982a, 1982b) and cytokinins (Blackman & Davies, 1984) can modify the stomatal responses to ABA in an antagonistic fashion. Therefore, the tissue sensitivity for a particular hormone can now be defined in the light of those reports and the evidence presented in Chapter Five. That is, the response of a particular tissue to a particular hormone is not dependent upon the absolute concentration of the hormone present, but it is rather a reflection of the outcome of a complex interaction of that hormone with other hormones and also with non-hormonal factors; e.g. the sensitivity of the stomata of Commelina communis and Zea mays to ABA is found to be dependent on the concentration of IAA, cytokinin and KCl present in the medium (Blackman & Davies, 1984; Snaith & Mansfield, 1982b). The results shown in Chapter Five suggest that the disparity in stomatal opening in adaxial and abaxial epidermes is dependent on the concentration of calcium ions and/or abscisic acid, in addition to IAA (Pemadasa, 1982). Furthermore, the sensitivity to a particular hormone may also be governed by the extent of binding sites (receptors) present in the tissue (Starling et al., 1984). Therefore, the higher sensitivity to ABA observed in adaxial epidermis could also be due to the presence of a greater amount of ABA-receptors in adaxial guard cells than in abaxial ones. Thus, the experiments described in Chapter Five have shown that adaxial and abaxial epidermes provide an accessible system for studying the factors controlling 'tissue sensitivity' to

hormones. It can be suggested that this may be a more favourable system for further study than those involved with structurally complex tissues, for example, hypocotyl segments, aleurone layers, roots, etc.

It seems worthwhile here to give some consideration to a quite different topic namely, ABA and the geotropic responses of roots. There are still many doubts surrounding the role of ABA as the inhibitor from the root tip mediating geotropic responses. The mode of action of ABA which has already been proposed demands a gravi-stimulated polar transport of ABA in the roots, which seems to have little supportive experimental evidence. In the light of our findings it is possible to suggest an alternative explanation for the involvement of ABA. A recent report by Lee and coworkers (Lee et al., 1983) suggests that a polar transport of calcium ions across root tips of maize occurs under the influence of gravity. If this happens in all plants, then we can postulate that the differential distribution of calcium ions may be responsible for a differential activity of ABA on upper and lower sides of roots. In this case the concentration of calcium would be critical for the sensitivity of roots to ABA and for the differential growth in gravi-stimulated roots, and the absolute concentration of ABA might be of lesser importance.

Furthermore, the experimental evidence presented in this thesis suggests that the probable site of primary action of ABA in the guard cells is at the plasma membrane. This is in full agreement with the previous reports of Hartung (1983) and Hornberg and Weiler (1984) who proposed that the site of action of ABA in guard cells is located at the outer surface of the plasma membrane. A very recent report by Behl and Hartung (1986) also supports these conclusions. By compartmental analysis of ABA in 'isolated' guard cells under various treatments, they found that under unstressed conditions the cytoplasm contained high ABA levels (at millimolar range) which decreased by about 80% with the onset

of water stress. In contrast, they found no change in vacuolar ABA levels before and after stress treatments. Therefore, they have suggested that the cytoplasm of unstressed guard cells acts as an effective trap for ABA, and as water-stress develops it may act as a source releasing ABA to the apoplast. These findings, together with those of Cornish and Zeevaart (1985) who pointed out that under reduced water supply the initial increase of apoplastic ABA may be relevant to rapid stomatal closure, led Behl and Hartung (1986) to conclude that guard cells themselves may be a main source for the initial release of ABA to the apoplast under water-stress. Thus it can be surmised that ABA from guard cell-cytoplasm triggers stomatal closure while that from mesophyll cells maintains the response. An ABA redistribution mechanism of this nature may well explain the lack of a synchronous correlation between stomatal closure (with the onset of water-stress) and the increase in the bulk ABA content of leaves. It also stands against some of the 'iconoclastic' (Horgan, 1984) views (e.g. Trewavas, 1981) about the physiological roles of plant hormones.

This study has not only unravelled some doubts concerning the behaviour of ABA as a plant hormone, but also provided very important evidence which supports the concept of a universal calcium messenger system. As mentioned elsewhere in this thesis, evidence for the role of calcium as a second messenger in plants is emerging at a rapid rate. The action of ABA on stomata seems to be a classical example for the involvement of calcium in stimulus-response coupling in plants. Although a variety of physiological processes in higher plants has been reported to be mediated by calcium, firm evidence of a comparable role of calcium in stimulus-response coupling has not been forthcoming. Most of those reports have dealt with one aspect of the multifaceted 'calcium story'; for example, one group of investigations has been carried out only to

explore the effects of the presence or absence of calcium on a particular physiological process, while the objective of another has been to find if there might be any involvement of calcium channels or calmodulin. By contrast, this study has covered most of these areas. Nonetheless, it has also provided knowledge which is far from complete because the mode of action of activated calmodulin in guard cells is not known. Future experiments should, therefore, be designed to elucidate the response element to which Ca^{2+}-CaM binds or the enzymatic process(es) involved in the lowering of guard cell potassium accumulation. Another piece of evidence of crucial importance also needed, is the demonstration of changes in calcium concentration in guard cell cytoplasm following different treatments which bring about stomatal opening and closure. As discussed in the preceding Chapter, the size and the location of guard cells may impose some practical difficulties for this, and a prospective technique to overcome those would be the use of isolated guard cell protoplasts in, for example, microfluorimetric analysis.

One of the most recent excitements in ABA research is the discovery of ABA in mammalian brain by Le Page-Degivry and coworkers. They found relatively large amounts of ABA in the brains of pigs and rats, and smaller amounts elsewhere in the body (Le Page-Degivry et al., 1986). The 'animal ABA' was extracted in the same way as plant ABA and was analysed with a sensitive and accurate radio-immunoassay, mass spectrometry and a biological assay on stomata of Setcressa purpurea (Commelinaceae). They reported that the 'animal ABA' was identical to plant ABA in chemical as well as biological characteristics. From a careful investigation they found that rats eating a diet containing high levels of ABA had less ABA in their brain, and they therefore suggested that the brain manufactured its own ABA. The role of ABA in animal tissues is not clear but it seems reasonable to speculate that it may be involved

in altering membrane properties, and thus regulating ionic movements, particularly potassium (possibly by affecting calcium-regulated potassium channels; Hugues et al., 1982).

Trewavas (1981) suggested that the action of 'plant growth substances' is unique to plant systems, claiming that time there was no evidence of equivalent action on animal tissues. However, for several years there has been evidence that ABA has effects of some kind on animals (reviewed by Visscher, 1983). ABA has been found to inhibit the biosynthesis of the tumour promoter, chorionic gonadotropin (an analogue of the animal hormone gonadotropin) and to have some inhibitory effects on the reproduction performance of mammals. It is also known that ABA has some chemical similarities to several insect hormones and pheromones, and that it affects insect growth and reproduction (Visscher, 1983). Furthermore, it has been suggested that 5-hydroxytryptamine (5-HT) or serotonin, the counterpart of auxin in animals, plays an important part in regulating the permeability of capillary membranes and, hence, blood pressure, in addition to its role as a neurotransmitter. In aphids 5-HT inhibits wing formation, resulting in the production of wingless forms with high rates of reproduction (Harrewijn, 1976). Abscisic acid at high concentrations, on the other hand, promoted the production of winged aphids which suggested that, as in plants, ABA might antagonize the function of 5-HT (auxin counterpart) in insect metabolism (see Visscher, 1983). These findings, therefore, do not support Trewavas' arguments.

Preliminary studies in Lancaster by Dr. H. Huddart and colleagues have indicated that ABA has effects on ionic movements in animal tissues consistent with its being a calcium agonist. Therefore, it seems likely that the proposed mechanism of action of ABA may equally

apply to animals. If so, then abscisic acid would represent a fundamental 'messenger molecule' common to animals and plants. However, noteworthy among several difficulties in suggesting ABA as a universal messenger molecule, is the fact that ABA has been found not to be ubiquitous. As mentioned earlier, some groups of plants do not have ABA. Nevertheless, it is known that in liverworts and algae, lunularic acid (a natural growth inhibitor) can substitute for ABA, and also that ABA can mimic the action of lunularic acid (Pryce, 1972; Schwabe & Valio, 1970; Valio et al., 1969). It has been reported that lunularic acid is produced in the growing tips of Lunularia cruciata thallus under long-day (dormancy inducing) conditions which suggests that it plays an important role in drought resistance (Schwabe & Valio, 1970). The mechanism of action of lunularic acid is not yet known and merits investigation.

The importance of endogenous plant hormones in controlling stomatal movements to optimize water consumption with respect to carbon gain has been discussed in a number of recent publications (Mansfield, 1985, 1986; Mansfield & Davies, 1985) and, therefore, it will not be repeated here. The efficiency of water use in plants can be controlled by artificial means, and it may be of paramount value especially for the management of crops growing in arid or semi-arid regions where the soil water supply may be limited. The use of antitranspirants can be envisaged to reduce the excessive loss of water from plants at critical stages of growth. Many of the prospective antitranspirants tested so far are known to have complicated side effects and, therefore, are of limited practical value. Phenylmercuric acetate proved to be a very powerful antitranspirant causing stomatal closure at very low doses and improving water use efficiency in crops. However, it has been shown to cause a substantial physiological toxicity to leaf epidermis and to inhibit

the photosynthetic activity of the mesophyll. These constraints together with the increasing antipathy towards the application of toxic elements such as mercury to food crops have undermined its use as an antitranspirant. Therefore, the discovery of new antitranspirants is of immediate importance to improve the world's food production, and may eventually be beneficial to the survival of mankind.

Recognition that certain chemicals, e.g. calcium channel blockers, can antagonize the action of ABA may open a new area of investigation in the search for novel antitranspirants. It has been found that some analogues of dihydropyridines (e.g. BAY K 8644 and CGP 28 392) antagonize competitively the action of calcium channel blockers in animal tissues (Freedman & Miller, 1984). Those compounds are believed to open the calcium channels and have been called calcium agonists. These compounds should theoretically mimic the action of ABA and function as antitranspirants and research into this area could yield considerable rewards.

In conclusion, the evidence presented in this thesis substantiates the hormonal nature of ABA, and establishing this has been given great emphasis throughout this discussion. It was not, however, the purpose of it to dispute totally the alternative notions about the influence of plant hormones nor to object to the use of a different terminology. Further, this study has clarified some doubts concerning the molecular basis of the action of ABA and has linked together a number of scattered pieces of evidence to build up a firm hypothesis with calcium as a second messenger in stimulus-response coupling. As we learn more about the cellular mode of action of compounds like ABA, new possibilities for controlling physiological functions in plants begin to emerge. New generations of agricultural chemicals may be one of the benefits of research of this kind. It is also hoped that this study will stimulate future researches and provide new ways of thinking and of approaching old problems.

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APPENDIX

List of publications which include results submitted in this thesis.

1. De Silva, D.L.R., Hetherington, A.M. and Mansfield, T.A. (1985). Synergism between calcium ions and abscisic acid in preventing stomatal opening. New Phytologist, 100: 473-482.

2. De Silva, D.L.R., Cox, R.C., Hetherington, A.M. and Mansfield, T.A. (1985). Suggested involvement of calcium and calmodulin in the responses of stomata to abscisic acid. New Phytologist, 101: 555-563.

3. De Silva, D.L.R., Cox, R.C., Hetherington, A.M. and Mansfield, T.A. (1986). The role of abscisic acid and calcium in determining the behaviour of adaxial and abaxial stomata. New Phytologist, (in press).

4. Hetherington, A.M., De Silva, D.L.R. and Mansfield, T.A. (1985). Abscisic acid, calcium ions and stomatal function. Proceedings of The NATO's advanced workshop on Calcium. In: Molecular and Cellular Aspects of Calcium in Plant Development, edited by A. Trewavas and D. Marmè, Plenum Press.

5. De Silva, D.L.R., Hetherington, A.M. and Mansfield, T.A. (1986). Calcium, calmodulin and the action of abscisic acid on stomata. A paper presented at the first meeting of Plant Transport Group of the Society for Experimental Biology, held at the University of York.