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Measurements of Ca^{2+} fluxes in intact plant cells

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SUMMARY

Owing to the central role of Ca^{2+} in signal transduction processes, it is important to measure membrane fluxes of Ca^{2+} in cells which are as undisturbed as possible, particularly when studying the control of these fluxes. To this end, techniques have been developed to measure Ca^{2+} fluxes in intact, turgid plant cells. The measurements are principally of influx across the plasma membrane where Ca^{2+} transport is likely to occur through cation-selective channels. The most direct method measures tracer fluxes of ^{45}Ca, but special procedures are required to distinguish between influx and extracellular binding of Ca^{2+}. Unfortunately, such techniques are currently only applicable to giant cells where surgical separation of the intracellular contents from the cell wall is possible.

The influx of Ca^{2+} into normal, resting cells of the green alga *Chara corallina* is usually about 0.3 nmol m^{-2} s^{-1} (at an external Ca^{2+} concentration of 0.5 mol m^{-3}). This flux is up to 5 times higher in actively growing cells, 20 times higher in cells depolarized by 20 mol m^{-3} K^+ and 1000 times higher during an action potential. Reducing cell turgor by a wide range of solutes increases Ca^{2+} influx, especially near plasmolysis. Ca^{2+} influx is sensitive to alterations in both external and cytosolic pH, but is inhibited by complete darkness and by low concentrations of La^{3+}. Various organic Ca^{2+} channel antagonists had mixed effects on Ca^{2+} influx into *Chara*. The work described in this paper should enable further study of the control of Ca^{2+} fluxes into intact, turgid plant cells, and their role in signal transduction and the control of cellular activities.

1. INTRODUCTION

Calcium is a divalent cation found in relatively high concentrations inside plant cells (say, 1–10 mol m^{-3}). However, Ca^{2+} is unusual in that at least 99% of the cytosolic ions are chelated in various forms, reducing the free concentration (or 'activity') to well below 1 mmol m^{-3}. This extremely low activity means that small fluxes of Ca^{2+} into the cytosol can rapidly increase this low free concentration; as alterations in cytosolic Ca^{2+} can be easily effected, this makes Ca^{2+} an appropriate ion for signalling changes in the environment of the cell. In addition, the potent chemical properties of Ca^{2+} (notably its penchant for binding to phosphate, a common component of most biological molecules) make Ca^{2+} a suitable ion to control many cellular activities. The combination of these two characteristics means that Ca^{2+} is an ideal ion for the translation of a signal to an alteration in cellular reactions. Examples of such signal transduction by Ca^{2+} are now numerous (see Johannes *et al.* 1991).

As cytosolic Ca^{2+} levels are important in the control of cellular functions, and as alterations in cytosolic Ca^{2+} levels are sensitive to alterations in the transmembrane fluxes of Ca^{2+}, it is of central importance to study the mechanism and control of Ca^{2+} fluxes in the intact cell. Until recently, however, there had been very few such studies. In this paper, we review our recent measurements of Ca^{2+} influx into intact turgid plant cells.

2. ENERGETICS AND MECHANISMS OF Ca^{2+} FLUXES

There is a significant electrochemical potential gradient favouring the passive movement of Ca^{2+} into cells, due to gradients across the plasma membrane of both concentration (external Ca^{2+} often exceeds 1 mol m^{-3}, whereas cytosolic free Ca^{2+} is about 10^{-4} mol m^{-3}) and potential (the cytosol is negative by 100 to 200 mV relative to the outside). The main pathway for the passive, inward movement of Ca^{2+} is most probably through various cation-selective channels (Johannes *et al.* 1991; Schroeder & Thuleau 1991). Therefore, we assume that most of the ^{45}Ca influx measured is moving in through cation-selective channels.

The ability of ion channels to catalyse extremely rapid movements of ions (Tester 1990) makes a high rate of Ca^{2+} influx possible. However, in the intact cell, it seems that the ion channels which allow the inward movement of Ca^{2+} are mostly closed. Evidence for this comes from the fact that Ca^{2+} influx in the resing cell is very low, but during the action potential, influx is about three orders of magnitude higher (see below). In other words, the plasma membrane has a high capacity for Ca^{2+} influx

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(through ion channels), but this capacity is by no means fully used in the resting cell. This is in contrast to the pumps actively removing Ca^{2+} from the cytosol; pumps have a much lower turnover rate than channels, and when there is no influx of Ca^{2+}, removal by pumps must equal influx through channels. This can be achieved by the pumps either being more numerous than the channels, or being active for longer periods of time than the channels. To maintain the low cytosolic activities found in all living cells, Ca^{2+} must be actively removed from the cytoplasm, either to the outside of the cell or to intracellular organelles. In plants, removal from the cell is most likely to be catalysed by an ATP-hydrolysing Ca^{2+}-extruding pump (Evans *et al.* 1991). It seems likely that this pump also allows the movement into the cell of H^+; the passive influx of H^+ provides energy additional to that obtained from the hydrolysis of ATP for the (energetically most unfavourable) removal of Ca^{2+} (Rasi-Caldogno *et al.* 1987; Miller & Sanders 1989). Most efflux from cells measured with ^{45}Ca is likely to be due to the activity of this Ca^{2+}/nH^+ exchanging ATPase.

The endoplasmic reticulum (ER) contains a Ca^{2+}-translocating ATPase which appears to be distinct from that found on the plasma membrane (Evans *et al.* 1991). Although the ER has a large surface area and contains an ATPase which has a high affinity for Ca^{2+} (see Table 2 in Evans *et al.* (1991)), only a small fraction of influx across the plasma membrane into the 'cytosol' would be expected to be sequestered into the ER as the volume inside the ER is small; therefore, the capacity of this compartment would be small, and it would fill up rapidly.

The tonoplast has a solute-coupled antiporter, where, for each Ca^{2+} pumped from the cytosol, at least three H^+ must move from the vacuole (Blackford *et al.* 1990). Transport of ^{45}Ca into the vacuole is probably catalysed by this transporter.

There can also be a passive movement of Ca^{2+} from these organelles, causing an increase in cytosolic free Ca^{2+}. The mechanism of movement from the ER is not known, but Ca^{2+} release from the vacuole occurs through at least two different cation-selective channels, one sensitive to the secondary messenger, inositol-1,4,5-trisphosphate, the other insensitive to this compound (Johannes *et al.* 1991).

3. MEASUREMENT OF Ca^{2+} FLUXES IN PLANTS

(*a*) *Physical constraints*

Many perturbations of the cellular environment will affect the signal transduction system. Due to the central importance of Ca^{2+} in signal transduction, the control of Ca^{2+} influx may well be adversely affected by prolonged environmental perturbation. Therefore, it seems best to minimize manipulation of cells when studying the control of Ca^{2+} fluxes into cells (as well as when studying other components of the signal transduction system). Ideally measurements should be made on intact turgid cells under conditions favourable to normal growth. The major impediment to the use of plant cells under these conditions is the large amount of Ca^{2+} in the cell walls. In many cases, more Ca^{2+} is bound in the cell wall than is contained in all of the intracellular compartments (Cleland *et al.* 1990; Reid & Smith 1992*a*). This is particularly a problem in tracer flux measurements but practically any manipulation which alters the ionic composition of the solution adjacent to the plasma membrane can result in release or increased binding of Ca^{2+} in the cell wall, and potentially bias measurements of Ca^{2+} fluxes across this membrane. Thus a degree of caution needs to be exercised when interpreting flux measurements on walled cells. The complications caused by extensive extracellular binding of Ca^{2+} can be removed by the use of protoplasts. This will improve the accuracy of the flux measurements but at the possible cost of creating conditions which do not relate to the situation faced by a 'normal' plant cell.

There appear to be only three models which have been used to measure short-term Ca^{2+} influx into turgid cells; two electrophysiological techniques and a radioisotopic approach. It should be noted that another non-invasive technique currently being used to study changes in intracellular Ca^{2+} activities, fluorescence ratio imaging, gives an excellent description of the net results of various Ca^{2+} fluxes (influxes and effluxes through the plasma- and endo-membranes). Although fluxes through particular channels can be inferred by various manipulations (see, for example, Gilroy *et al.* (1991)), ratio imaging does not allow the direct study of unidirectional Ca^{2+} fluxes across particular membranes (e.g. the influx through plasma membrane-bound channels).

(*b*) *Electrophysiological methods*

Intracellularly impaled microelectrodes can be used to measure the currents carried by ions as they cross membranes. This is particularly useful where large currents are carried by one or a few ions. However currents which may be attributed to fluxes of Ca^{2+} measured using microelectrodes in the normal ('resting') state of cell would be small, and could not be distinguished from other larger currents, such as those due to movements of K^+ and H^+. In charophyte algae electrical measurements of Ca^{2+} currents are restricted to those made during an action potential, when a large proportion of Ca^{2+} channels are open for about 500 ms (Lunevsky *et al.* 1983). Some electrophysiological studies have been made of Ca^{2+} channels open for this short period (e.g. Lunevsky *et al.* 1983; Tester & MacRobbie 1990), but it is not known how applicable the results are to cells in the normal, resting state, or even to cells responding to an environmental stimulus; the role of the action potential is still the subject of speculation (Tester 1990).

A newer technique which has been used to measure fluxes of a variety of plant nutrient ions involves the use of ion-selective microelectrodes to chart the spatial profile of ionic activities established in the solution adjacent to plant membranes as a result of influx or efflux from the cells (Lucas & Kochian 1986). The

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application and limitations of this technique for measuring Ca^{2+} fluxes are discussed below in relation to the measurement of Ca^{2+} fluxes in higher plants.

(*c*) *Radioisotopic tracer studies*

In comparison with the other major ions found in plant cells, there have been very few published measurements of tracer fluxes of Ca^{2+}. This situation is undoubtedly due to the complications imposed by the high capacity and high affinity of cell walls for binding divalent cations (Dainty & Hope 1959; Stassart *et al.* 1981; Richter & Dainty 1989; Reid & Smith 1992*a*). The main difficulty is in differentiating between plasma membrane influx and extracellular binding of Ca^{2+} in the cell wall. Various strategies have been devised to remove external bound tracer from the cell walls after the influx period so that the amount of tracer which is actually absorbed by the tissue can be measured. The validity of the influx measurements depends very much on the speed and efficiency with which the rinsing procedures remove the external tracer.

A separate but equally complex problem concerns the feasibility of measuring the unidirectional influx of an ion whose cytoplasmic free concentration is very low. Tracer influxes are normally measured over short periods because the internal pool will eventually equilibrate with the labelled material in the external solution and efflux will then cause the influx to be underestimated. In plants the pool of free Ca^{2+} in the cytoplasm is only of the order of 0.1–0.2 mmol m^{-3} (Miller and Sanders 1987; Gilroy *et al.* 1991), which, depending on the sizes of the influx and efflux would be expected to equilibrate with ^{45}Ca in the external solution over a period of seconds or minutes. This is much too rapid to be accurately measured in a system where a relatively long rinse period is required to remove ^{45}Ca from the cell wall. However, this view ignores the fact that the cytoplasmic free Ca^{2+} exchanges with a variety of much larger intracellular pools. The specific activity of free Ca^{2+} in the cytoplasm will be determined by the ratios of the radioactive and non-radioactive fluxes. It seems likely that the free Ca^{2+} in the cytoplasm is buffered by a much larger pool of bound but non-organelle Ca^{2+} which may be as large as 60 mmol m^{-3} (Baker & Dipolo 1984) and which would be expected to exchange relatively rapidly compared with the efflux across the plasma membrane. In addition to this non-organelle pool there will also be exchange with the large pool of Ca^{2+} in the vacuole and with the ER, whose membranes provide a vast surface area to the cytoplasm. Thus even a low area-specific flux across the ER would amount to a relatively large total exchange.

For a simple system with two intracellular pools (e.g. bound cytoplasmic Ca^{2+} and vacuolar Ca^{2+}) in series with a much smaller compartment (free Ca^{2+}), the specific activity in the small pool initially rises as a function of the ratios of the radioactive and non-radioactive fluxes. Where the exchange between the small compartment and another compartment (e.g. bound cytoplasmic Ca^{2+}) is much larger than the other fluxes, the specific activity of the small compartment will rise in close parallel with the specific activity in the larger compartment. There are clearly a variety of Ca^{2+} pools within the cytoplasm which exchange with the free Ca^{2+} so that on balance it seems unlikely that the free cytoplasmic pool of Ca^{2+} equilibrates rapidly with ^{45}Ca in the external solution. Even if it did, the unidirectional influx would only be significantly underestimated if the efflux from the cell was of the same magnitude as the other fluxes from the cytoplasm to the cytoplasmic organelles and vacuole.

4. Ca^{2+} FLUXES IN PROTOPLASTS

There have been some measurements of Ca^{2+} fluxes into the protoplasts of higher plant cells (table 1). Using protoplasts removes the confounding problem of large amounts of Ca^{2+} binding to the cell wall, but it requires a large disturbance to the cell during enzymic digestion of the wall and removal of debris. Most importantly, removal of the wall necessitates a reduction of turgor pressure to zero; as seen below,

Table 1. *Ca^{2+} fluxes into protoplasts*

tissue	Ca_o^{2+} (mol m^{-3})	K_o^+ (mol m^{-3})	flux (nmol m^{-2} s^{-1})	notes	refs[a]
Nicotiana suspension culture	1	1 (?)	3.6	assuming diameter of 30 μm	1
Zea dark-grown leaves				assuming diameter of 20 μm and 10^{-10} g protein/protoplast[b]	2
kept in dark	0.07	0	0.8		
1 min red light			1.8		
Hordeum leaf	0.2	5	6	assuming diameter of 20 μm	3
Daucus cell culture	0.1	5	47	assuming diameter of 20 μm	4
Vallisneria leaf mesophyll	0.1	0.1	60	red light-stimulated influx	5
Amaranthus cotyledons	1	11	9	using diameter of 18 μm given in ref. 6	6
		1	11	$pH_o = 4.5$	7
			15	$pH_o = 5.5$	

[a] Values calculated from data presented in the following papers: 1, Mettler & Leonard (1979); 2, Das & Sopory (1985); 3, Wrona *et al.* (1988); 4, Graziana *et al.* (1988); 5, Takagi & Nagai (1988); 6, Elliott & Yao (1989); 7, Rengel & Elliott (1992).
[b] Ca^{2+} uptake in Das & Sopory (1985) is expressed per milligram protein; conversion to a surface area basis was made by comparison of data from Das & Sopory and Dureja *et al.* (1984) to obtain a ratio of 9 μg protein per 9×10^4 protoplasts.

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this particularly unusual condition for a plant cell may cause a very large increase in Ca^{2+} influx. Therefore, Ca^{2+} fluxes measured on protoplasts should be treated with much caution.

As can be seen from the magnitude of the fluxes in Table 1, there is much variability in the fluxes measured, and all values are much higher than those measured in turgid cells of *Chara* (see below). Some of these differences may be due to tissue-specific effects, but it is likely that much of the variability and of the high rates measured is due to fundamental problems inherent in the use of protoplasts.

5. Ca^{2+} FLUXES IN HIGHER PLANTS

There have been a number of reports of measurements of ^{45}Ca fluxes in whole cells and tissues, in which most of the extracellular tracer was removed by rinsing in increased concentrations of $CaCl_2$ (e.g. Rincon & Hanson 1986; Cramer *et al.* 1987; Tretyn 1987) or $LaCl_3$ (MacRobbie 1989). Most of these studies are based on the implicit assumption that the amount of ^{45}Ca bound in the cell walls following rinsing (desorption) is small in comparison with the plasma membrane influx. In only a very few cases (e.g. Macklon 1975; Cramer *et al.* 1987) have attempts been made to identify apoplasmic pools of ^{45}Ca and to quantify the error that the residual ^{45}Ca exchange with these pools has on the estimated cellular influx of Ca^{2+}. By comparing live and dead roots and using compartmental analysis, Macklon (1975) considered it was possible to distinguish between exchange in the cell wall and tonoplast fluxes. He obtained half-times for exchange of the Donnan free space of around 20 min compared to slower exchanging compartments which he identified as cytoplasm and vacuole with half-times of 55 and 800 min respectively. Cramer *et al.* (1987) used similar methods with cotton roots but they were less confident about their ability to differentiate between apoplasmic and symplasmic ^{45}Ca. The large pool size of Ca^{2+} in the cell wall compared with the cytoplasm means that the (much smaller) cytoplasmic component of the efflux may well be swamped by exchange within the apoplasm. Our pessimistic view of the prospects for measuring short term tracer influxes without physically separating ^{45}Ca in the cell wall from the cell contents is founded on our experience with charophyte cells where Ca^{2+} influx across the plasma membrane is normally very small in comparison with the exchange in the cell wall (see below). Spanswick and Williams (1965) found that in *Nitella*, ^{45}Ca efflux from dead cells (isolated cell walls) was identical to that in intact cells. However, Macklon (1984) feels that there are fundamental differences between charophyte algae and higher plant roots and is more optimistic that reliable tracer influxes can be measured in complex tissues.

Another technique which may have advantages over tracer methods is based on the concentration profile established in the external solution adjacent to the plasma membrane due to net Ca^{2+} influx or efflux. Fluxes are calculated from the concentration profile, which is monitored using either stationary or vibrating Ca^{2+}-sensitive electrodes (Newman *et al.* 1987; Kuhrtriber & Jaffe 1990). The resolution of this technique when applied to charophytes is around 5 nmol m^{-2} s^{-1} (P. Ryan, personal communication) and is similar for roots (Ryan *et al.* 1990). However, as the cell wall will be in series with the plasma membrane, it will be very difficult to distinguish between displacement of Ca^{2+} from cell wall sites by any treatment that alters the ionic composition (including pH) of the cell wall space, unless the membrane Ca^{2+} fluxes are relatively large. This is essentially the conclusion reached by Ryan *et al.* (1992) for *Chara*. Huang *et al.* (1992) considered this problem and examined the effects of Al^{3+} on Ca^{2+} fluxes is isolated cell walls using external electrodes. They obtained null fluxes of Ca^{2+} from isolated cell walls treated with Al^{3+} and net fluxes from intact cells. This result is encouraging, although there must still be some doubt concerning the effect of Al^{3+} on plasma processes such as proton pumping or K^+ channels whose activities may alter the ionic composition of the cell wall and thereby alter binding of Ca^{2+}.

6. Ca^{2+} FLUXES IN CHAROPHYTE ALGAE

(*a*) *Methods*

The long cylindrical internodal cells of charophyte algae have provided the most reliable measurements of Ca^{2+} influxes because it is possible with these cells to perform influxes on intact, turgid cells, and then to separate the cell contents from the cell wall at the end of the influx period. We know of four different approaches to measuring ^{45}Ca fluxes in charophytes, the first three of which are probably unreliable because of significant contamination from ^{45}Ca in the cell wall.

Spanswick & Williams (1965) loaded internodal cells of *Nitella translucens* for long periods then washed them for 15 min in 10 mol m^{-3} $CaCl_2$ to remove most of the wall-bound ^{45}Ca. In order to obtain cell sap with the minimum of contamination from the cell wall, they punctured turgid cells so that the sap was expelled away from the cell wall. It was collected and counted. The values obtained of about 0.46 nmol m^{-2} s^{-1} mostly reflect slow uptake to the vacuole rather than plasma membrane influx.

Measurements of influx over shorter periods were made by Hayama *et al.* (1979) using a different technique. They mounted cells in a 3-compartment chamber with the centre compartment isolated from the ends by grease barriers. The cell portion in the central chamber was loaded with ^{45}Ca, rinsed briefly at the end of the uptake period, and the cell contents were then squeezed from the cell. They obtained plasma membrane influxes over a 15 min uptake period of 0.4 and 1.7 nmol m^{-2} s^{-1} for external Ca^{2+} concentrations of 0.13 and 1 mol m^{-3} respectively. The squeezing procedure to separate the cell contents would almost certainly have resulted in some degree of cross-contamination from ^{45}Ca in the cell wall.

MacRobbie & Banfield (1988) attempted to measure fluxes in *Chara corallina* over periods of less

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than 15 min by rinsing cells in cold La^{3+} solutions for 20 min after exposure to ^{45}Ca, in order to remove most of the extracellular contamination. Without separating the cell contents from the cell wall they reported influxes between 2.5 and 5 nmol m^{-2} s^{-1} for an external ^{45}Ca concentration of 0.1 mol m^{-3}.

Reid & Smith (1992*a*) repeated these experiments but fractionated the cells into cell wall, cytoplasm and vacuole. They found that despite the La^{3+} treatment, which removed more than 98% of the extracellular ^{45}Ca, most of the cell-associated tracer activity was still in the wall and the actual plasma membrane flux was only 0.2 nmol m^{-2} s^{-1}. Reid & Smith (1992*a*) supported this low flux with a detailed investigation of Ca^{2+} exchange properties in isolated cell walls of *Chara*, from which they developed methods for the measurement of both long and short term membrane fluxes of Ca^{2+}. The methods are described in detail in Reid & Smith (1992*a*,*b*) and only a brief outline of the influx procedure will be given here. Isolated internodal cells were mounted in a 3-chambered block, similar to that used by Hayama *et al.* (1979), and only the central portion was exposed to ^{45}Ca. At the end of the influx period the cell was rinsed quickly in five to ten changes of a solution containing 2 mol m^{-3} La^{3+} over 3 to 5 min. The ends of the cell were removed and a hypodermic syringe inserted into one end and clamped in place with forceps. The vacuole was displaced by injecting an air bubble through the cell, then the cytoplasm was flushed out by rapidly injecting distilled water through the lumen, leaving a clear sleeve of cell wall. Ca^{2+} influxes were calculated from the ^{45}Ca activity in the vacuolar and cytoplasmic fractions. Various modifications to the basic procedure have been developed to reduce contamination from the cell wall and to obtain estimates of Ca^{2+} efflux (Reid & Smith 1992*a*,*b*). Our Ca^{2+} fluxes for charophyte cells are much lower than those measured by other techniques. In the previous studies there has obviously been recognition of the possibility of cell wall contamination but the magnitude and the complexity of ^{45}Ca binding has been underestimated, leading to higher apparent influxes.

The following sections give an overview of the conditions which influence Ca^{2+} influx in one particular system, internodal cells of the giant alga *Chara corallina*. Unless referenced otherwise the results quoted below for *Chara* are to be found in Reid & Smith (1992*a*–*c*). It is expected that the major controlling factors will be common to most plant types, but confirmation of this will have to await the development of methods for measuring tracer influx which are appropriate to other tissues.

(*b*) *Resting Ca^{2+} fluxes*

In isolated internodal cells of *C. corallina*, the ^{45}Ca fluxes measured over 30 min with a 3 to 5 min rinse lay in the range 0.2 to 0.7 nmol m^{-2} s^{-1} for external concentrations of 0.5 to 1 mol m^{-3}. This is low in comparison with the fluxes in *Chara* of K^+ (4 nmol m^{-2} s^{-1}; Smith & Walker 1989), Na^+ (5 nmol m^{-2} s^{-1}; Smith & Walker 1989) and Cl^- (5–30 nmol m^{-2} s^{-1}; Reid & Walker 1984) from solutions containing these ions at around 1 mol m^{-3}. Analysis of the timecourse of ^{45}Ca influx into mature internodal cells showed two distinct phases in the tracer content of the cytoplasm. Over the first 15 min the influx to the cytoplasm was approximately linear with an average value of 0.4 nmol m^{-2} s^{-1} after which the influx assumed a new steady rate of approximately 0.1 nmol m^{-2} s^{-1}. The faster phase appeared to have a pool size of less than 20 mmol m^{-3} and may represent the free and bound non-organelle Ca^{2+} while the larger slower phase whose activity was still increasing after 100 min may be Ca^{2+} pools within organelles.

The accuracy of the measured influx will depend on the period over which it was measured. A 30 min influx time was used routinely because at shorter times the fluxes were much more variable, presumably because of the greater influence of the small contamination from residual ^{45}Ca in the cell wall. When the vacuolar influx was included the whole cell influx averaged over 30 min was approximately one third lower than the influx measured in the first 15 min. The values given should therefore be considered as the minimum influxes. As will be seen below, for many of the treatments the changes in influx are indeed very large and the relative rather than absolute fluxes are probably more important.

Influx to the vacuole from the external solution showed an initial lag, which argues against rapid equilibration of free Ca^{2+} in the cytoplasm. After 15 min, influx to the cell was dominated by influx to the vacuole of approximately 0.1 nmol m^{-2} s^{-1}. The actual flux from the cytoplasm to the vacuole will be higher than this value unless the specific activity of the cytoplasmic pool of Ca^{2+} is the same as that of Ca^{2+} in the external solution.

By exposing only half of a cell to ^{45}Ca and measuring the efflux of tracer to the vacuole and to the external solution in the non-radioactive half, the efflux from the cytoplasm across the plasma membrane and across the tonoplast were determined as approximately equal. The similarity of efflux from the cytoplasm to the vacuole and to the external solution is interesting in the light of large differences in K_m reported for Ca^{2+} pumps in the plasma membrane and tonoplast (Evans *et al.* 1991). The vacuolar Ca^{2+} pool in *Chara* is large (100–1000 μmol m^{-2}) and the vacuolar flux small, which means that the vacuole would exchange with a half-time of several weeks rather than hours. Long term fluxes of ^{45}Ca from the external solution to the vacuole can be measured without the complexities imposed by the use of divided influx chambers simply by incubating cells in ^{45}Ca for several hours followed by a long rinse (1 h) in La^{3+} solution to remove all but the very slowly exchanging tracer in the cell wall. Cross-contamination from the cell wall ^{45}Ca during the sap separation is then relatively low. The long rinse period results in the loss of fast-exchanging cytoplasmic compartments and the method is therefore better suited to measuring Ca^{2+} accumulation in the vacuole which dominates the cell flux at influx times greater than 1 h.

Addition of 0.1 mol m^{-3} La^{3+} reduced the Ca^{2+} influx in isolated internodal cells to approximately 0.1 nmol m^{-2} s^{-1}, which suggests that La^{3+}-sensitive Ca^{2+} channels are the principal route for entry of Ca^{2+} to the cell.

These fluxes (0.2–0.7 nmol m^{-2} s^{-1}) are lower than into protoplasts (0.8–60 nmol m^{-2} s^{-1}: see table 1) for reasons discussed elsewhere. On the other hand, the influxes in *Chara* are much higher than those measured in vesicles (0.007 to 0.026 nmol m^{-2} s^{-1}) as calculated by MacRobbie & Banfield (1988). These low fluxes may reflect the sidedness and leakiness of vesicles (MacRobbie & Banfield 1988), but may also be due to inactivation of transporters during the preparation of vesicles, or perhaps to loss of components essential for their activity.

(*c*) *Ca^{2+} influx and growth*

Ca^{2+} influx in the internodal cells of a growing plant is higher than for isolated internodes. The accumulation of Ca^{2+} in rapidly expanding cells close to the top of the plant is up to 1.6 nmol m^{-2} s^{-1}, but falls off as the cells mature (figure 1). Fully expanded cells lower on the plant have smaller influxes, and slow growing plants have lower influxes at each level than in fast growing plants. Isolation of cells from the plant causes the plasmodesmatal connections between adjacent internodal cells to close (Reid & Overall 1992), which may be a factor in the reduction in influx in isolated cells if there is normally polar transport of Ca^{2+} to the growing apex, as occurs for photoassimilates in *Chara* (Ding *et al.* 1991).

(*d*) *Control of Ca^{2+} influx*

In a survey of the effects of a range of treatments likely to alter Ca^{2+} influx, a number of conditions were found to increase Ca^{2+} influx but few conditions were found where influx was inhibited. This may be partly due to the fact that in mature isolated cells, most of the Ca^{2+} channels appear to be closed, so there is greater scope for opening rather than closing Ca^{2+} channels.

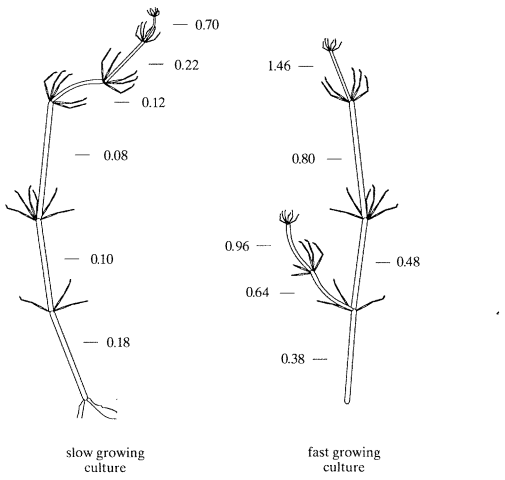

Figure 1. Ca^{2+} influx into internodal cells of intact plants of *Chara corallina*. The rates are in nmol m^{-2} s^{-1}.

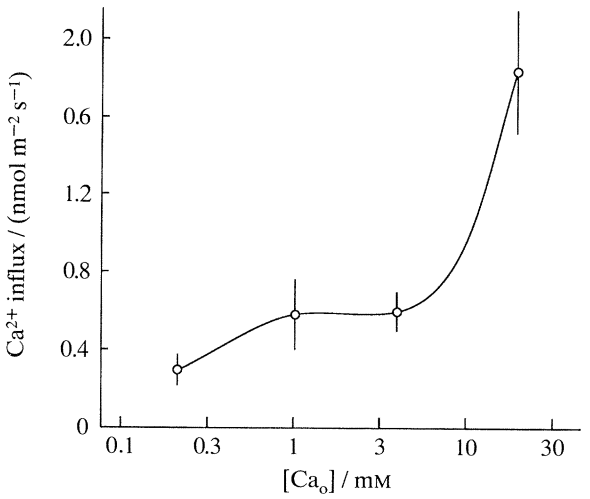

Figure 2. Concentration dependence of Ca^{2+} influx in internodal cells of *Chara corallina*.

(*i*) *Concentration dependence*

Ca^{2+} influx was found to be sensitive to external Ca^{2+} at concentrations less than 0.5 mol m^{-3} or greater than 2 mol m^{-3} but between these values there was little change in influx (figure 2).

(*ii*) *Sodium and potassium*

Increasing concentrations of NaCl between 3 and 100 mol m^{-3} increased Ca^{2+} influx by approximately three-fold (figure 3). This result is interesting given reports of symptoms of Ca^{2+} deficiency in non-halophytic plants grown under saline conditions (Lynch & Läuchli 1985; Maas & Grieve 1987) and, as will be discussed below, the influx response to NaCl may simply be a response to reduced turgor.

The effect on Ca^{2+} influx of changing K^+ concentrations is more complex, and the variation in influx much larger, than for Na^+. In solutions containing K^+ between 0.1 and 5 mol m^{-3}, Ca^{2+} influx was

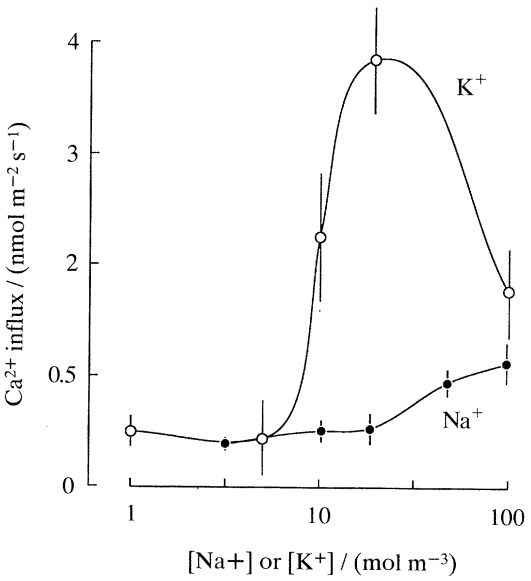

Figure 3. Ca^{2+} influx as a function of the external concentrations of Na^+ and K^+. Ca^{2+} concentration = 1 mol m^{-3}.

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relatively constant, but there was a marked stimulation at 10 mol m^{-3} and a further increase at 20 mol m^{-3} (figure 3). The K^+ concentration at which Ca^{2+} influx begins to increase corresponds roughly to the K^+ concentrations required to depolarize the plasma membrane of *C. corallina* (Keifer & Lucas 1982; Beilby & Blatt 1986) i.e. the transition from a low conductance state dependent on the H^+ pump to a high conductance state dominated by the movement of K^+ through channels. These results suggest voltage gating of Ca^{2+} channels as a major determinant of Ca^{2+} influx, as in animal cell membranes (e.g. Tsien *et al.* 1987). However, the decrease in Ca^{2+} influx between 20 and 100 mol m^{-3} K^+ (figure 3) indicates either that the channels open at moderate depolarizations but close again when the PD approaches zero; or that the Ca^{2+} channels are being blocked by K^+ from the outside; or that K^+ is competing with Ca^{2+} for permeation through the channel; or some other voltage-sensitive factor is operating from the inside, such as voltage-dependent blockade by cytosolic Mg^{2+}, as appears to happen to K^+ channels (Tester 1988; Blatt 1988). With respect to this latter option, the question of competition between Ca^{2+} and monovalent cations for ion channels needs to be investigated further, both electrically and using radioactive tracers.

(iii) pH

Manipulation of the pH on both sides of the plasma membrane failed to produce any large changes in Ca^{2+} influx. Varying the external pH between 5 and 10.4 did not affect the influx, nor did cytoplasmic acidification by butyric acid at concentrations which would normally reduce cytoplasmic pH by 1 unit. The results obtained from experiments in which amines were applied to increase cytoplasmic pH were inconclusive. NH_4^+ (0.2 mol m^{-3}) inhibited Ca^{2+} influx but methylamine at the same concentration was ineffective. Given that both amines have similar effects on cytoplasmic pH (Smith 1980), it seems unlikely that the inhibition of Ca^{2+} influx by NH_4^+ is attributable to a change in pH.

(iv) Light and dark

Bright light (photon flux up to 1000 μmol m^{-2} s^{-1}) did not increase Ca^{2+} influx over the value recorded in dim light (45 μmol m^{-2} s^{-1}), but complete darkness inhibited influx by nearly 50%.

(v) Turgor

C. corallina is termed a 'freshwater' alga; its natural habitat is in ponds and streams where the overall solute concentration is normally very low. Under these conditions it maintains a turgor of approximately 0.68 MPa (equivalent to a difference in osmolarity of approximately 280 mosmol kg^{-1}). When cell turgor was reduced by addition of 100–200 mol m^{-3} mannitol to the external solution, Ca^{2+} influx increased three-fold. Increasing salinity also causes turgor to decrease, and the Ca^{2+} influx response to NaCl shown in figure 3 is indistinguishable from turgor reduction by mannitol (figure 4) over the same range of osmolarity.

In plasmolysed cells, Ca^{2+} accumulation rose dramatically as shown in figure 4. The stimulation of Ca^{2+} accumulation was between 10- and 20-fold for plasmolysis by mannitol, sorbitol, sucrose and betaine. This large increase in Ca^{2+} influx in plasmolysed cells was not caused by a general, non-specific membrane leak since fluxes of other solutes showed only small stimulations or were inhibited. Moreover, the increased Ca^{2+} flux was La^{3+}-sensitive and the cells remained electrically excitable (i.e. electical stimulation generated an action potential which would have allowed a transient Ca^{2+} influx). However, plasmolysis by $Ca(NO_3)_2$ did not follow the same pattern as for the other osmotic solutes and the accumulation rate in 100 mol m^{-3} $Ca(NO_3)_2$ was only slightly higher than in turgid cells, despite the 100-fold increase in the external Ca^{2+} concentration. This is particularly interesting in the light of the observation of Hayashi & Kamitsubo (1959) that cells plasmolysed in $Ca(NO_3)_2$ survive for much longer than cells plasmolysed by other solutes. It seems that the high Ca^{2+} concentration prevents opening of Ca^{2+} channels when turgor approaches zero, although the cells remained electrically excitable in high Ca^{2+}, which indicates the Ca^{2+} channels were still responsive to other stimuli.

The magnitude of the rise in Ca^{2+} influx in cells at zero turgor has disturbing implications for studies of control of Ca^{2+} channels and Ca^{2+} fluxes in protoplasts, patch-clamped membranes and intracellularly perfused cells. There is some evidence that the turgor effect only applies to the plasma membrane, since in *Chara* cells whose vacuoles were perfused with 1 mol m^{-3} ^{45}Ca (open vacuole method of Tazawa *et al.* 1975, which leaves the tonoplast intact) Ca^{2+} efflux was very small (*ca.* 0.03 nmol m^{-2} s^{-1}; R. J. Reid, unpublished data).

Plasmolysis may not affect all cell types in this way, but certainly for *C. corallina* there must be a question regarding conclusions on the control of Ca^{2+} channels derived from studies of cells at reduced turgor. This

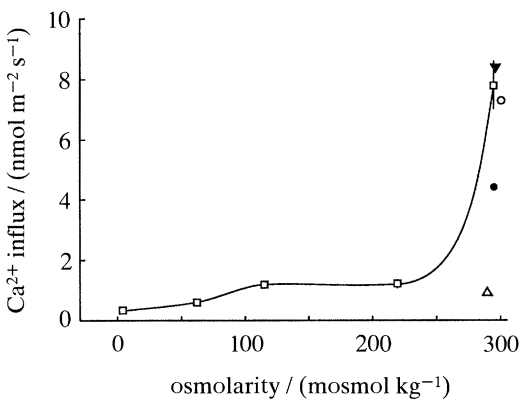

Figure 4. Effects of reduced turgor and plasmolysis on Ca^{2+} influx in *C. corallina*. Solutions contained (mol m^{-3}) Ca^{2+}, 1; Na^+, 3; K^+, 0.2; plus mannitol (open squares), sorbitol (open circles), betaine (filled triangles), sucrose (filled circles) or $Ca(NO_3)_2$ (open triangles). The average osmolarity of the cell sap was 291 ± 3 mosmol kg^{-1} ($n = 6$).

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may account for the high fluxes observed in all studies using protoplasts (table 1). It is also interesting to note that Ca^{2+} fluxes into protoplasts do not appear to be affected by the K^+ concentration (table 1), in contrast to the above results with *Chara*. This may be because it is not possible to stimulate further the low turgor-stimulated flux by addition of high K^+ (i.e. high K^+ will not increase the influx if the channels have already been opened by low turgor).

(*e*) *Action potentials*

Hayama *et al.* (1979) measured ^{45}Ca influxes during action potentials in internodal cells of *Chara* using a three-compartment chamber as described above. Although we were critical of their influx method and attributed their higher basal fluxes to cell wall contamination (§ 6*a*) the large fluxes during a series of action potentials increase the intracellular ^{45}Ca activity to a level at which the extracellular contamination is a relatively minor component of the overall flux. When they stimulated cells 15 times over 15 min, influx rose by a factor of approximately 3 when the external Ca^{2+} concentration was 0.13 mol m^{-3}, with an increase in cellular ^{45}Ca activity of 48 nmol m^{-2} per action potential. Reid *et al.* (1992) have also made measurements of the ^{45}Ca influx during action potentials in *Chara*, using the methods described above. At an external Ca^{2+} concentration of 0.2 mol m^{-3} the influxes were similar to those of Hayama *et al.* (1979) but the background fluxes were much lower (i.e. the relative stimulation of influx by action potentials was much greater). The influx to the vacuole was linearly related to the number of action potentials for frequencies of stimulation of at least two per minute with a slope of 86 nmol m^{-2} per action potential (Reid *et al.* 1992). This flux is too high to obtain accurate estimates of the plasma membrane influx which would be higher than the measured flux to the vacuole if Ca^{2+} were cleared from the cytoplasm after the action potential by transport across membranes other than the tonoplast. In particular, efflux across the plasma membrane would seem likely. If the Ca^{2+} channels remained open during the passage of an action potential for, say 0.5 s (Lunevsky *et al.* 1983) then the influx at the plasma membrane would be at least 170 nmol m^{-2} s^{-1}. Compared with the normal influx of 0.2–0.7 nmol m^{-2} s^{-1}, this represents a considerable capacity for uptake of Ca^{2+}, far in excess of that which normally occurs in a resting cell. This emphasizes the fact that Ca^{2+} channels are normally closed. The increase in cytoplasmic Ca^{2+} concentration due to Ca^{2+} influx occurring during a single action potential can also be calculated. Assuming a surface area/volume ratio of 5×10^3 m^2/m^3 in these cells and a cytoplasmic volume fraction of 5%, the cytoplasmic Ca^{2+} concentration would rise by more than 8.6 mmol m^{-3}, unless a significant amount of Ca^{2+} were removed rapidly from the cytoplasm. This value agrees remarkably well with the peak concentration of free calcium during an action potential in *Chara* measured using aequorin by Williamson & Ashley (1982) of 6.7 mmol m^{-3}.

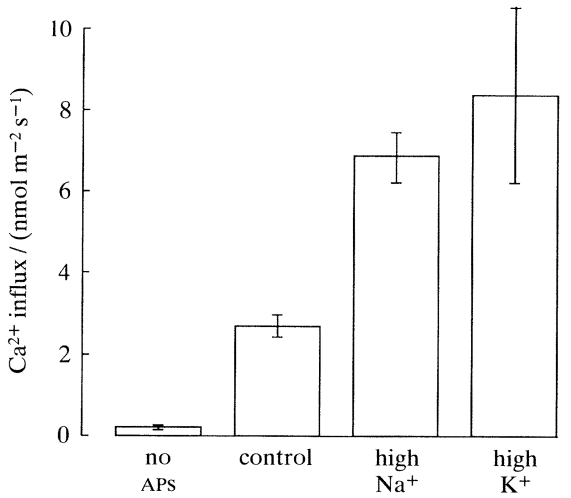

Figure 5. Effects of 15 mol m^{-3} Na_2SO_4 or 10 mol m^{-3} K_2SO_4 on Ca^{2+} influx during action potentials in internodal cells of *C. corallina*. Ten action potentials were generated during an influx period of 10 min.

^{45}Ca influx during action potentials is stimulated by increasing the concentrations of Na^+ and K^+ in the bathing solutions (figure 5). In the presence of 30 mol m^{-3} Na^+, the influx was more than doubled and at 20 mol m^{-3} K^+ the influx was more than triple the control rate. In the case of K^+ some increase in Ca^{2+} influx would be expected in non-stimulated cells at this concentration of K^+ (see figure 3), but all of the stimulated flux in the presence of high Na^+ is related to the action potential.

(*f*) *Pharmacology*

As mentioned above, La^{3+} was found to be a potent blocker of Ca^{2+} influx. Significant inhibition of Ca^{2+} influx was obtained with as little as 3 mmol m^{-3} La^{3+} and the inhibition was slowly reversible (10–20 h). The organic channel antagonists nifedipine, verapamil and bepridil, which have been shown to block various classes of Ca^{2+} channels in animal cells (Tsien *et al.* 1987), failed to inhibit Ca^{2+} influx in *Chara*. MacRobbie & Banfield (1988) reported inhibition of Ca^{2+} influx in *Chara* by nifedipine whenever control fluxes were high, but unfortunately their methods did not distinguish between fluxes into cells and extracellular binding. However, Tester & MacRobbie (1990) found that nifedipine inhibited the inward current during the action potential in *Chara*, consistent with blockage of calcium channels in the plasma membrane. Reid & Smith (1992*b*) found that nifedipine and bepridil actually increased Ca^{2+} influx (table 2), and that verapamil and bepridil killed cells if applied at 50 mmol m^{-3} for more than about 0.5 h. It is difficult to see how this latter result is related to effects on Ca^{2+} channels, given that La^{3+} inhibition of Ca^{2+} influx is not toxic nor are conditions which cause moderate increases in Ca^{2+} influx. This suggests that not only are these inhibitors not specific in their action, but that their non-specific effects are lethal.

Despite the uncertainty about the specificity of Ca^{2+} channel effectors, it is interesting to compare the

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Table 2. *Effects of channel blockers on Ca^{2+} influx to the vacuole of internodal cells of* Chara corallina
(^{45}Ca influx time in non-stimulated cells was 30 min. ^{45}Ca influx time for stimulated cells was 10 min during which they were electrically stimulated 10 times. Nifedipine was applied in the dark. All other treatments were in dim light.)

	concentration (mmol m^{-3})	pretreatment time (min)	^{45}Ca influx (nmol m^{-2} s^{-1}) control	^{45}Ca influx (nmol m^{-2} s^{-1}) treatment	% control
non-stimulated cells					
verapamil	50	20	0.24 ± 0.04	0.28 ± 0.10	ns
nifedipine	50	0	0.24 ± 0.04	0.62 ± 0.12	258
bepridil	50	0	0.44 ± 0.06	0.88 ± 0.44	200
$LaCl_3$	100	60	0.48 ± 0.14	0.14 ± 0.04	29
stimulated cells					
verapamil	50	30	3.48 ± 0.32	4.87 ± 1.13	140
nifedipine	50	30	3.48 ± 0.32	3.00 ± 0.46	86
bepridil	50	5	3.48 ± 0.32	1.51 ± 0.24	43
ruthenium red	50	60	3.48 ± 0.32	4.11 ± 0.24	118
$LaCl_3$	50	120	3.48 ± 0.32	0.92 ± 0.13	26
	2000	30	3.48 ± 0.32	0.98 ± 0.11	28

pharmacology of Ca^{2+} influx under normal conditions when most of the Ca^{2+} channels are closed with that of Ca^{2+} influx during the action potential when most of the channels appear to be open. The most notable difference was the effects of nifedipine and bepridil, compounds which stimulated influx in resting cells, and inhibted influx during action potentials. The reason for these differences between resting and excited cells is a matter for speculation.

The stimulation of Ca^{2+} influx by verapamil during the action potential is also noteworthy; this may be due to an inhibition by verapamil of K^+ channels, thus prolonging the action potential and keeping the cell depolarised for longer. Although blockade of K^+ by verapamil has been reported in animal cells by Kass & Tsien (1975) and at low micromolar concentrations in plants (B. R. Terry, S. D. Tyerman and G. P. Findlay, pers. comm.), no effect of methoxyverapamil on the action potential or on K^+ conductance was reported by Tester & MacRobbie (1990).

7. CONCLUDING REMARKS

With the techniques developed using ^{45}Ca to measure Ca^{2+} fluxes into giant-celled algae, it is possible to study the control and role of Ca^{2+} fluxes into intact plant cells. It is hoped that the information obtained with *Chara* can be used to develop better methods for measuring ^{45}Ca influxes into higher plant cells; preliminary investigations in our laboratory of possible methods for higher plant roots show promise. It should then be possible to measure tissue-specific controls of Ca^{2+} fluxes, such as by plant growth regulators, as well as to test whether controls such as membrane potential difference and turgor pressure do in fact operate in higher plant cells as in *Chara*.

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