

Cytoplasmic calcium measurements in intact higher plant cells: results from fluorescence ratio imaging of fura-2

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Summary

Roots of seedling tomato (*Lycopersicon esculentum*) and oilseed rape (*Brassica napus*) plants were grown on agarose slopes formed on microscope slides. Root hairs were used for microelectrode insertions, for observations of the rate of cytoplasmic streaming and for measurements of fluorescence of the calcium indicator fura-2.

The resting potential across the plasma membrane of root hair cells was between -140 and -160 mV in both species. Insertion of microelectrodes caused immediate cessation of cytoplasmic streaming; 1–4 min later streaming restarted and quickly recovered its normal velocity of $1\text{--}3\ \mu\text{m s}^{-1}$. Once the electrode had been inserted, current pulses had no obvious effect on streaming. Thus, fura-2 injected by iontophoresis was quickly distributed throughout the length of the hair and the epidermal cell body. Injections were made into the cap of cytoplasm at the tip of the hair. Movement of fura-2 from the cytoplasm into the vacuole was observed; this was marked by a great increase in its fluorescence after excitation at 350 nm and occurred after about 15–30 min in tomato, but more rapidly (5–10 min) in oilseed rape hairs. No fluorescence was associated with the cell walls; this was demonstrated by plasmolysing the cells.

The distribution and concentration of Ca^{2+} was estimated from the ratio of fura-2 fluorescence excited at 350 and 385 nm and from digital image analysis. Resting levels of calcium in the cytoplasm near the tip were in the range 30–90 nM, while much higher levels, which eventually saturated the fura-2 response ($>5\ \mu\text{M}$), were seen in vacuoles once the indicator had crossed the tonoplast. This distribution was rapidly perturbed by the addition of the calcium channel blocker, verapamil, to the outer solution. There was a return to the original distribution as the verapamil was diluted. Separate experiments showed that verapamil inhibited cytoplasmic streaming over a time-course similar to these perturbations in cytoplasmic Ca^{2+} and caused a small depolarization (by approx. 30 mV) of the membrane potential. The application of 3×10^{-5} or 3×10^{-4} M-N-ethyl maleimide to the hairs strongly and irreversibly depolarized the plasma membrane electric potential, caused cytoplasmic streaming to stop or slow down and resulted in a very rapid rise in cytoplasmic Ca^{2+} , which became uniformly distributed with saturating 350:385 nm ratios.

Key words: calcium, cytoplasmic streaming, fura-2.

Introduction

There are few measurements of the calcium ion concentration in the cytoplasm of plant cells and these have been made with internodal cells of characean algae (Williamson & Ashley, 1982; Miller & Sanders, 1987), the rhizoids of *Fucus serratus* (Brownlee & Wood, 1986; Brownlee & Pulsford, 1988), diatoms (Brownlee *et al.* 1987), pollen tubes (Nobiling & Reiss, 1987) and *Haemanthus* endosperm cells (Keith *et al.* 1985). All of these observations confirm the expectation, based on experience with animal cells, that cytoplasmic calcium ions are in the range of $<0.1\text{--}1.0\ \mu\text{M}$. Homologies can be found when different

taxa are compared with respect to their calcium-binding proteins and molecular mechanisms for calcium extrusion/sequestration. These have encouraged plant physiologists in their belief that in plant cells, as in animals, perturbation in the level of free calcium can act as a messenger that conveys information about chemical and environmental signals to the biochemical activities of the cell (Hepler & Wayne, 1985). The mechanism of action of many plant growth regulators has eluded plant physiologists for decades, but opportunities to incorporate cell calcium into the scheme of things have been enthusiastically seized upon (see Trewavas, 1986, and references therein). The most pressing need in such schemes is to

demonstrate that cytoplasmic calcium levels in plant cells do change when signals are received (Hepler & Wayne, 1985; Clarkson, 1986). The first report that this does occur showed that, when *Nitellopsis* cells are transferred from darkness to light, the cytoplasmic calcium ion activity, measured by an intracellular calcium-selective microelectrode, fell from a steady value of 390 nM to 145 nM within 1 min and remained at this value (Miller & Sanders, 1987). Thus, in darkness, the free calcium is more than twice as concentrated as in light.

The application of microelectrode techniques to the small cells of higher plants is difficult (Brownlee, 1987); in particular, there is uncertainty about the location of electrode tips. It has been assumed that the potential recorded by voltage electrodes is the sum of tonoplast and plasma membrane, the electrode tip impaling both membranes. In the present work we have chosen to work with root hair cells, which have, in many species, a well-defined cap of cytoplasm at their tip into which electrodes can be unequivocally inserted. They are relatively tough, resilient structures in which there are two readily observable, calcium-dependent processes, namely tip growth, the polarity of which probably depends on a gradient of cell calcium (Jaffe *et al.* 1975; Reiss & Herth, 1979; Ewens & Leigh, 1985; Hepler & Wayne, 1985) and cytoplasmic streaming (Kamiya, 1981; Tominaga *et al.* 1987). Thus, root hairs offer opportunities to make combined studies of cell calcium, membrane electrical properties and physiological responses.

It has been shown that direct injection of fura-2 (e.g. see Tsien & Poenie, 1986; Brownlee & Pulsford, 1988) and quin-2 (Harvey *et al.* 1985) can be used to quantify cytoplasmic calcium by fluorescence measurements. Fura-2 has the important property that its excitation spectrum shifts when calcium is bound to it; the ratio of the emission following excitation at these two wavelengths effectively measures the calcium ion activity (Ca^{2+}). This has the great advantage that the ratio is independent of the amount of dye injected and minimally subject to interference from any constant autofluorescence of the tissue (Gryniewicz *et al.* 1985; Tsien & Poenie, 1986).

In this paper we describe effects of perturbing Ca^{2+} influx and efflux in root hair cells on cytoplasmic Ca^{2+} levels observed from fluorescence ratio imaging of fura-2, injected by iontophoresis. These effects are correlated with changes in cytoplasmic streaming.

Materials and methods

Plant material and culture

Seeds of *Lycopersicon esculentum* cv. Ailsa Craig and *Brassica napus* cv. Raphael were soaked for 24 h in water and then placed in 4.5 cm Petri dishes on the surface of 0.7% agarose made up with mineral nutrients in the following concentrations (mol m^{-3}): KNO_3 (3.0), KH_2PO_4 (0.3), MgSO_4 (0.3) and CaCl_2 (0.01) plus minor nutrients (mmol m^{-3}) FeEDTA (9.2), H_3BO_3 (5.0), CuSO_4 (0.16), MnSO_4 (3.5), ZnSO_4 (0.77), KCl (14.5), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (0.016).

When the radicle was approx. 3 mm long the seedlings were transferred to slopes of agarose, similar in composition to the

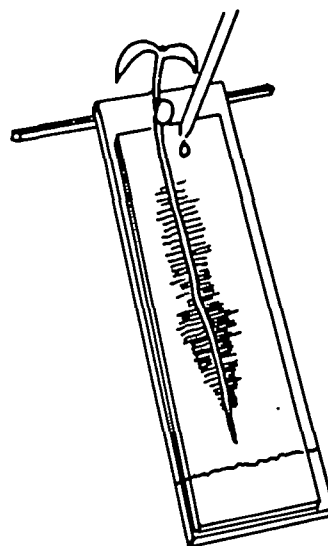


Fig. 1. Apparatus in which seedlings were grown. The surface of glass slides was coated with a layer (approx. 1 mm) of 0.7% agarose made up with a nutrient solution. The agarose was continuously irrigated with culture solution *via* a peristaltic pump (not shown).

above, mounted on glass slides and placed in a transparent box (Fig. 1). A peristaltic pump dripped nutrient solution onto each slope at a rate of approx $0.5 \text{ cm}^3 \text{ min}^{-1}$. The seedlings were arranged in a small slit cut into the agarose surface, so that as the root elongated it followed the interface with the glass slide. When seedlings (2–4 days old) were used for experiments, the whole agarose slice was carefully removed from the slide and inverted so that the flat surface of the root, and numerous partially embedded root hairs, could be readily accessible to electrodes.

Electrophysiology

Preparations were bathed with about 1 cm^3 of the culture solution described above. Glass microelectrodes with tip diameters of $0.5\text{--}1.0 \mu\text{m}$ were either filled with 3 M-KCl for membrane potential (V_m) measurements, or the tip was loaded with 10 mol m^{-3} fura-2 and backfilled with 0.1 M-KCl for iontophoretic injection. The electrodes were connected with silver wire *via* a salt bridge to a microelectrode amplifier and stimulator. After recording stable membrane potentials, current pulses (200 ms, 1 nA, 2 Hz) were given for 8–10 min to load cells with fura-2. During this period diffusion and cytoplasmic streaming distributed the molecule throughout the cytoplasm. Electrodes were positioned and driven into the tips of root hairs at an angle of about 15° to the long axis. Preparations were viewed under a long-working distance $\times 40$ objective attached to a microscope with a fixed stage (Micromanipulation microscope M2, Micro Instruments, Oxford).

Measurement of cytoplasmic streaming

Cytoplasmic streaming was observed using the microscope described above with Hoffman modulation contrast optics. Records were kept by filming events using a video camera (Hitachi, HV 725) attached to a video timer (For A, UGT 33) and a videorecorder with single shot function (Panasonic, AG 6200). On playback of tapes the time was measured for identified particles to move over a set distance on the TV monitor, equivalent to a distance of $10 \mu\text{m}$.

Fluorescence microscopy and image analysis

Fura-2 in cells was excited at 350 and 385 nm as described by Brownlee *et al.* (1987) and Brownlee & Pulsford (1988), using a modified Leitz fluorescence microscope and an image-intensified TV camera (Newvicon CCTV; Panasonic). Subtraction of autofluorescence and video background and 350:385 image ratioing was performed with a Kontron image analyser using IBAS software (Brownlee *et al.* 1987; Brownlee & Pulsford, 1988).

Results

Cytoplasmic streaming

Cytoplasmic streaming was seen in most hairs; small particles moved on defined paths, which were arranged in a loosely helical pattern. The velocity of streaming was greater at a distance of $>30\ \mu\text{m}$ from the tip than in the tip itself where irregular movements of larger masses of cytoplasm were often seen. If streaming was observed at a single location at repeated intervals of 6–10 s, quite wide variations in rates were recorded. In Figs 2 and 5 examples of this behaviour can be seen.

When the tip of the hair was impaled by a microelectrode all streaming ceased; there was an instantaneous

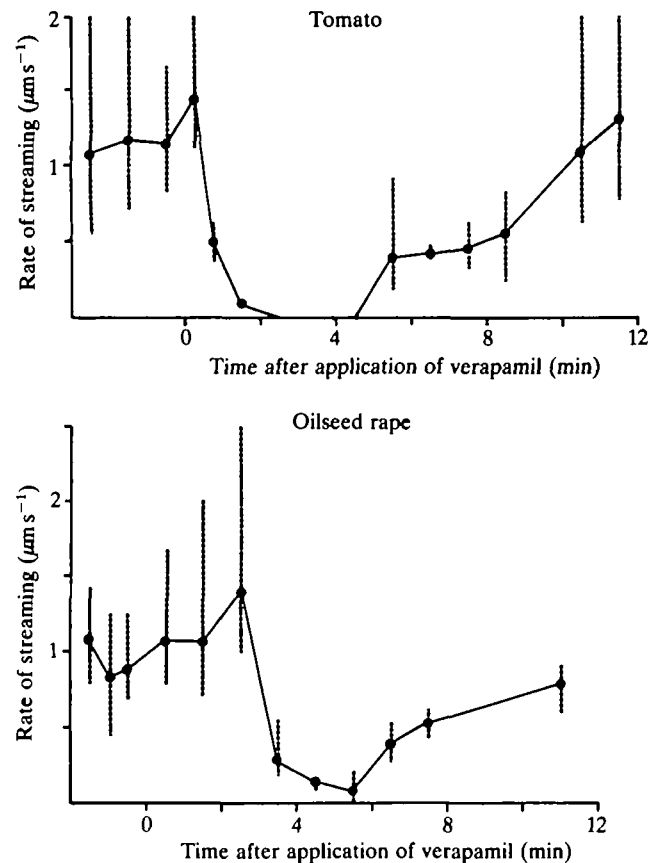


Fig. 2. The effect on cytoplasmic streaming of the addition of a sample of $10^{-3}\ \text{M}$ -verapamil (approx. $0.1\ \text{cm}^3$) to the agarose close to root hairs of tomato and oilseed rape seedlings. The vertical bars indicate the range of rates recorded within a measuring period of 1 min, the points being averages of six measurements.

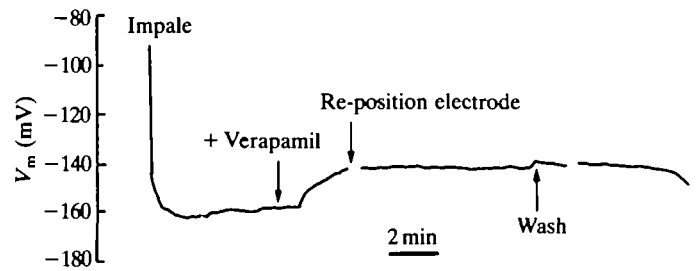


Fig. 3. The effect of verapamil on the membrane potential (V_m) of a tomato root hair.

membrane potential, V_m , of around 90 mV on impalement but this rose with time (Fig. 3). After 1–4 min cytoplasmic streaming re-started, slowly at first but quickly increasing to a rate of $1\text{--}3\ \mu\text{m s}^{-1}$. During this period V_m repolarized quickly and then continued to rise slowly to resting potentials of about $-150\ \text{mV}$ in tomato and $-180\ \text{mV}$ in oilseed rape. Once streaming had started, subsequent injections of current during iontophoresis did not appear to influence streaming (data not shown).

Streaming in both species was influenced in the same way if a drop (approx. $0.1\ \text{cm}^3$) of $10^{-3}\ \text{M}$ -verapamil was placed on the surface of the agarose a few millimetres from the hair being observed (Fig. 2). For a period of up to 2 min the rate of streaming of any given hair speeded up by 20–30% before slowing down very markedly or stopping 3–4 min after the application of the drop. Because of intrinsic variations in the rate of streaming in a given hair, and considerable variation between hairs, it is not possible to show that the apparent acceleration of streaming, after addition of verapamil, is statistically significant. The pattern of behaviour was, however, observed in most instances. During this period there was a small depolarization of the plasma membrane (Fig. 3). If no further drops of verapamil were added to the slide V_m slowly repolarized and streaming restarted and steadily regained its former rate as the external verapamil was either diluted or bound. If cells were irrigated continuously with $10^{-4}\ \text{M}$ -verapamil streaming did not restart (data not shown).

Abrupt, irreversible cessation of streaming followed the application of a drop of $3 \times 10^{-4}\ \text{M}$ -*N*-ethyl maleimide (MalNEt) to the agarose surface (Fig. 5). The membrane potential in tomato root hairs depolarized, over a 2 min period, to a value of $-90\ \text{mV}$. The application of $3 \times 10^{-5}\ \text{M}$ -MalNEt depolarized the plasma membrane to the same extent but did so more slowly (Fig. 4). At both concentrations, V_m depolarization was essentially irreversible. Streaming slowed down within 30 s of the application of the lower concentration of MalNEt but did not cease completely; washing the surface with nutrient solution brought about a partial recovery in the rate (Fig. 5).

Continuous irrigation of the root surface by buffered (Pipes, pH 7) or unbuffered solutions of abscisic acid, ranging in concentration from 10^{-5} to $10^{-3}\ \text{M}$, had no effect on cytoplasmic streaming even after treatments continued for 5 h (data not shown).

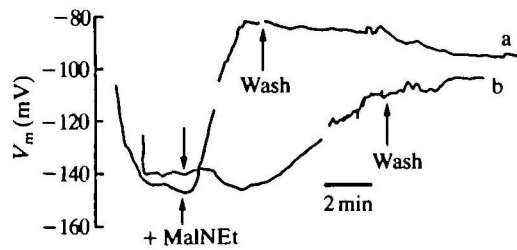


Fig. 4. The effect of addition and removal of 3×10^{-4} M (trace a) or 3×10^{-5} M (trace b) MalNEt on the membrane potential of tomato root hairs.

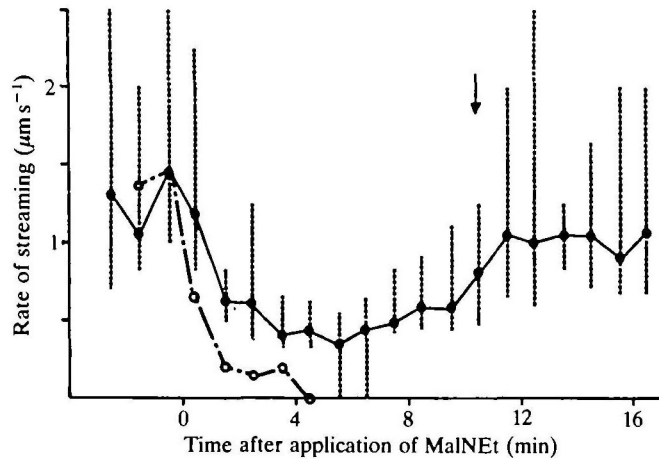


Fig. 5. The effect of adding samples (approx. 0.1 cm^3) of 3×10^{-5} M (—) or 3×10^{-4} M (---) MalNEt on cytoplasmic streaming rate in a root hair of tomato. MalNEt was replaced by culture solution at the time indicated by the arrow. Vertical bars indicate the range of rates recorded within a measuring period of 1 min; the points are averages of six measurements.

Fura-2 fluorescence

Fig. 6 displays three images of the same root hair from tomato. The bright-field image shows an apical cap of cytoplasm above a vacuolated region around which there are peripheral strands of cytoplasm that join with a larger mass of cytoplasm. The two images of fura-2 emission at 510 nm were obtained after excitation at either 350 nm or 385 nm. The corresponding ratio image, which is equivalent to the resting distribution of calcium ions, can be seen in Fig. 8A. To test the possibility that fura-2 may leak from the cell and become bound in the cell wall, cells were plasmolysed after dye injection. Fig. 7 shows that, in a cell plasmolysed by the addition of a drop of 1 M-mannitol, there was no fluorescence associated with the wall.

The ratio images can be compared with a set of calcium standards made up with other ions at approximately the concentrations found in cytoplasm. Images of the whole cell can be produced in false colour contours (not shown), which are much more immediately informative than the images in Fig. 8. For our present purpose, however, we have made a transect across the ratio image between points A and B. Ratio images taken at 2, 4 and 6 min after

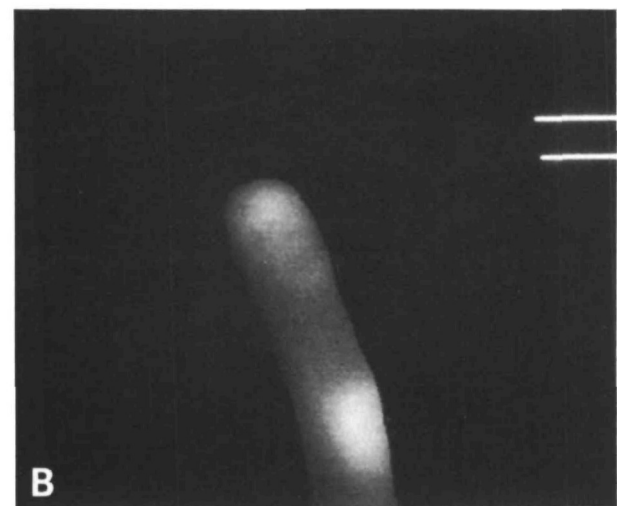
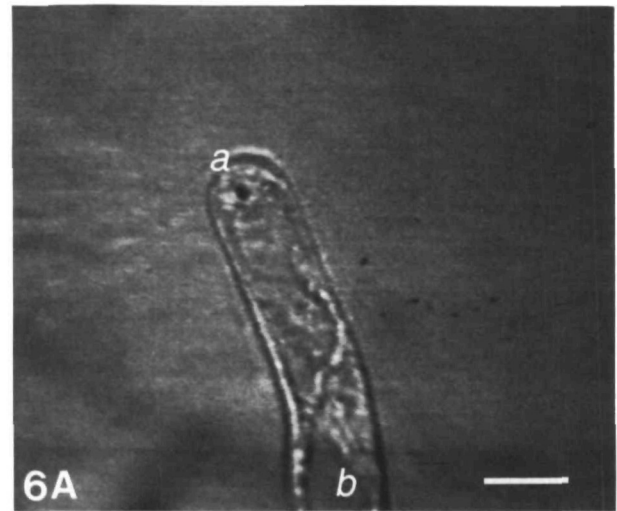


Fig. 6. Transmitted light (A) and corresponding 510 nm fluorescence images, excited by light of 350 nm (B) and 385 nm (C), of a tomato root hair injected iontophoretically with fura-2. *a* and *b* indicate the transect. Bar, $20 \mu\text{m}$.

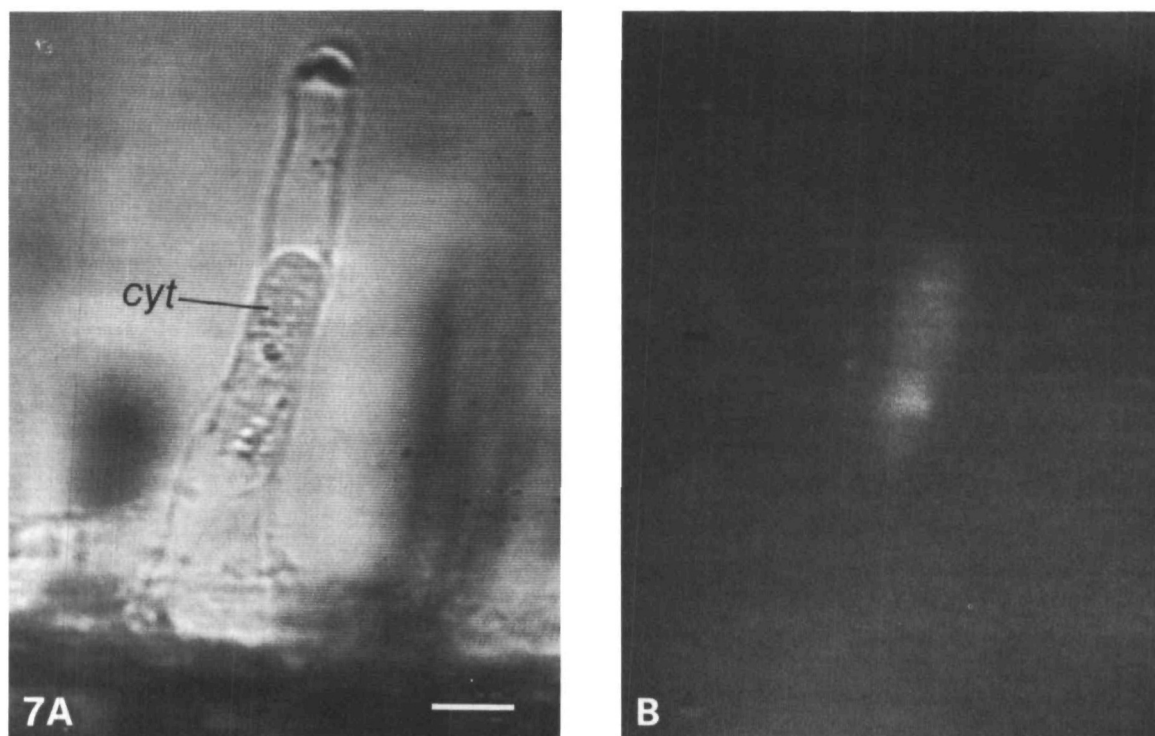


Fig. 7. Transmitted light (A) and corresponding 510 nm fluorescence image excited by light of 350 nm (B) of a tomato root hair that was plasmolysed after injection of fura-2. *cyt*, cytoplasm. Bar, 20 μm .

application of a drop of 10^{-3} M-verapamil are shown (Fig. 8). The cytoplasmic regions had ratio values equivalent to resting cytoplasmic $[\text{Ca}^{2+}]$ of 30–90 nM (Fig. 8A). These regions were characterized by relatively low spatial noise levels in the ratio image in comparison with regions of the cell where there was a vacuole, and probably reflect low dye concentration in this compartment (see Discussion). In such regions the image is composed of signals from vacuolar Ca^{2+} plus the thin overlying layer of cytoplasm. These vacuolar regions had higher mean $[\text{Ca}^{2+}] < 100$ nM. Two minutes after the addition of verapamil, a significant decrease in $[\text{Ca}^{2+}]$ occurred throughout the cell (Fig. 8B), though the vacuolar region remained elevated compared to the cytoplasm at the tip of the hair. Four minutes after the verapamil addition, $[\text{Ca}^{2+}]$ in the cytoplasm had returned to the initial resting levels in the apical region (Fig. 8C); the apparent $[\text{Ca}^{2+}]$ in the vacuolar region increased to a greater extent. After 6 min cytoplasmic $[\text{Ca}^{2+}]$ became slightly higher than had been found initially (cf. Fig. 8A,D), while the ratio signal from the vacuole continued to increase to a value approaching $1 \mu\text{M}$. It must be emphasized that cells were streaming during the first minute, but between minutes 2 and 4 streaming stopped; by 6 min streaming had restarted (see Fig. 2).

Application of a drop of 3×10^{-4} M-MalNEt caused a rapid increase in the 350:385 nm ratio, indicating a rapid rise in cytoplasmic calcium; all the previous patterns of distribution disappeared and a uniform saturating signal was recorded (Fig. 9).

We have not consistently seen a gradient of cytoplasmic

Ca^{2+} in root hair cells. In Fig. 8A, before verapamil treatment, no Ca^{2+} gradient was observed in the apical cytoplasm. During recovery of Ca^{2+} levels 4 min after addition of verapamil, an area of distinctly elevated Ca^{2+} level was observed at the apex of the cell. A similar gradient can be seen in Fig. 9C, where levels at the cell apex are appreciably greater than those 20 μm behind it.

Applications of abscisic acid, ranging in concentration from 10^{-5} to 10^{-3} M, had no effect on the distribution or the level of cytoplasmic calcium (data not shown).

Discussion

The use of fura-2 for measurements of plant cell calcium

The dual wavelength excitation characteristics of fura-2 make it ideal for use with single cells, since, in theory, variations in dye concentration and cell thickness will not affect fluorescence ratio values (see Introduction). However, problems have been identified, which appear especially troublesome in plant cells, when the acetoxymethyl (A/M) esters of quin-2 and fura-2 are used for loading cells. Extracellular hydrolysis of the ester (Cork, 1985) or incomplete internal hydrolysis (Brownlee & Wood, 1986) has limited their use to a few cell types. Furthermore, it has been demonstrated recently that intracellular dye compartmentation can be very significant in animal cells loaded with fura-2 A/M (Malgaroli *et al.* 1987). Electroporation has been used to load plant protoplasts with quin-2-free acid (Gilroy *et al.* 1987) but this method cannot be used with intact cells. We have

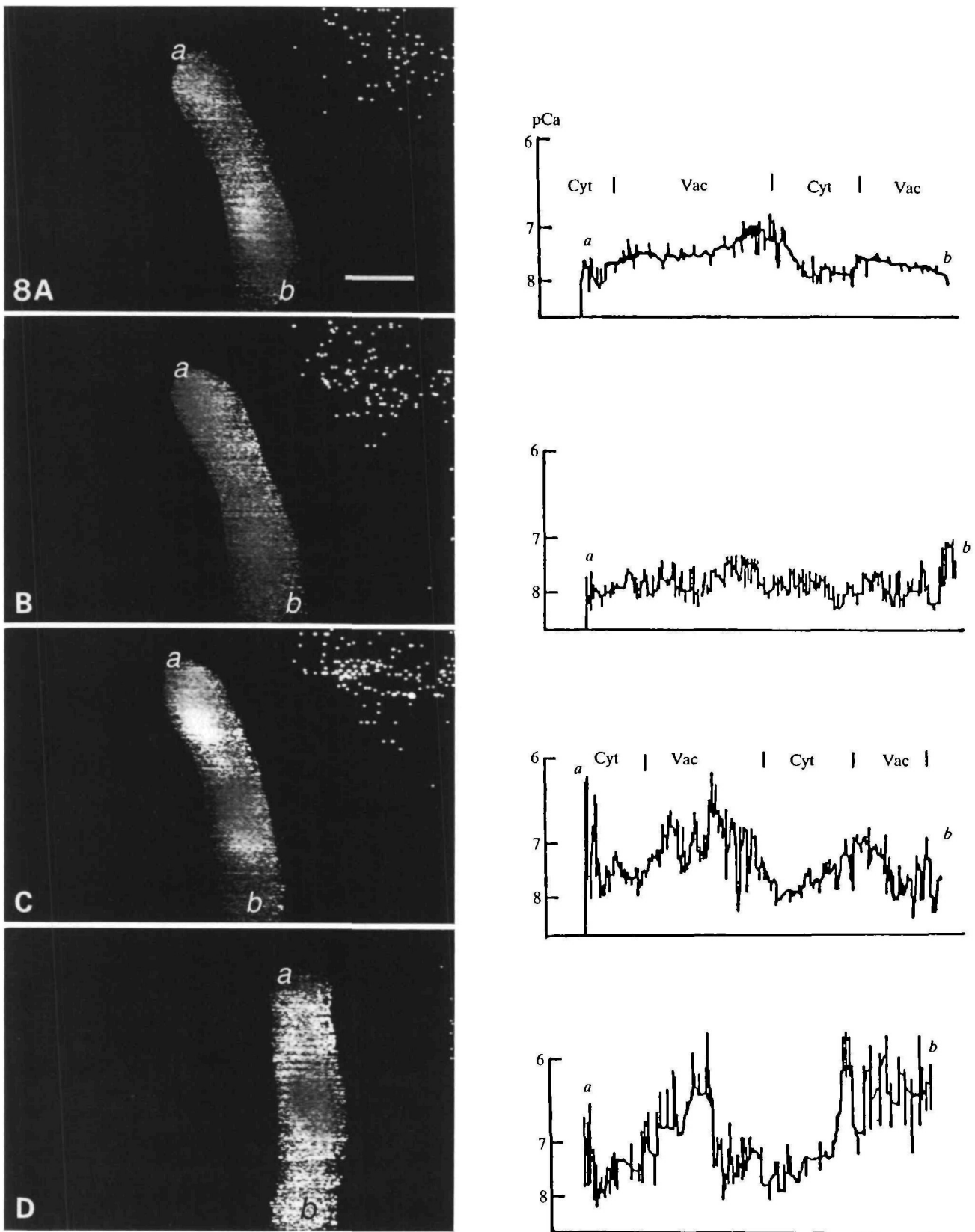


Fig. 8. 350:385 ratio images of the cell shown in Fig. 6 with computed pCa profiles along a transect (*a*-*b*). A. Cell immediately after microinjection; B, 2 min after the addition of 0.1 cm^3 of 10^{-3} M verapamil to the agarose surface close to the hair; C, same cell after 4 min; and D, after 6 min. Bar, $20 \mu\text{m}$.

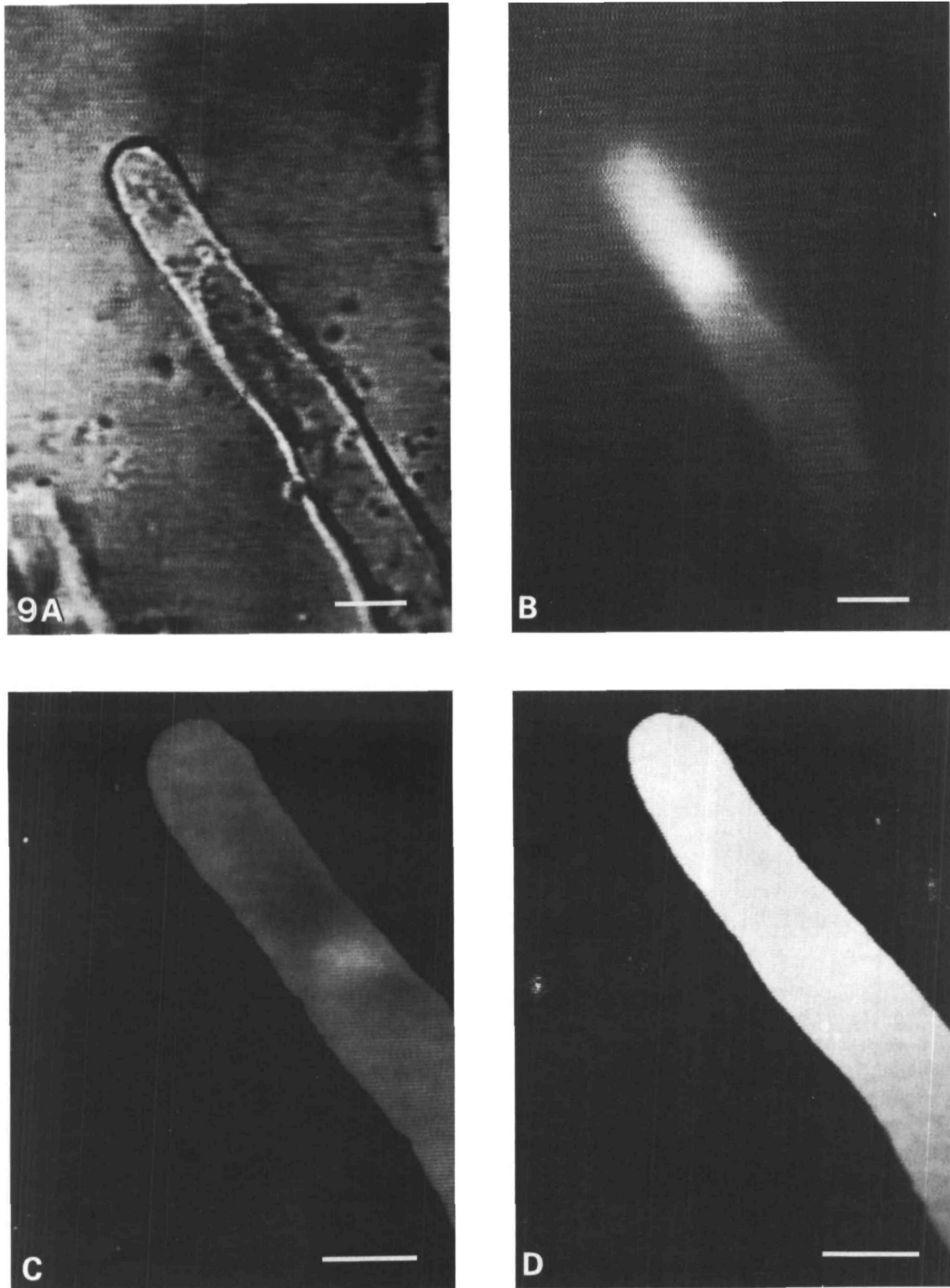


Fig. 9. Transmitted light (A) and 510 nm fluorescence image excited by light of 350 nm (B) of a tomato root hair; corresponding 350:385 ratio image (C). Ratio image 2 min after the addition of 3×10^{-4} M-MalNEt (D). Bars, 20 μ m.

employed direct iontophoretic microinjection of fura-2 into the cytoplasm, a method that appears to have minimal effects on cell function. Only transient effects on cytoplasmic streaming and membrane potential were seen

when cells were first impaled, and streaming restarted during the course of the microinjection. Furthermore, the technique permits the study of intact cells *in situ*. The containment of the dye within the cytoplasm does,

however, remain a problem. The sequence of events shown in Fig. 8 is consistent with the dye gradually moving into the vacuole of root hair cells; the ratio image from such regions is a composite of the vacuole itself and the thin layer of surrounding cytoplasm. Initially, the low signal from the cytoplasm may be a significant contribution to the total, but the signal itself is markedly more noisy than that over the apical areas where there is no vacuole. This problem will be reduced when the time-resolution of fluorescence recording is improved by the use of a more sensitive TV camera. It will then be possible to observe fluorescence much sooner after injection with fura-2 than with our existing set-up. However, when experiments have to be conducted over relatively long time periods the problem remains. With time, sufficient dye enters the vacuole to swamp completely the cytoplasmic signal. Some evidence suggests that fura-2 is transported actively into the vacuole by anion transporters (Brownlee & Pulsford, 1988; Malgaroli *et al.* 1987).

In the root hair cells that we have studied there is a sufficiently large apical area of cytoplasm for unequivocal recording of cytoplasmic Ca^{2+} , but this is rarely the case in fully expanded epidermal or cortical cells in roots. In young cells close to the root apex there would seem to be good prospects for successful measurement, since they lack a conspicuous central vacuole. Our unpublished results show that, at much later times after the injection of fura-2 into root hairs, the dye spreads throughout the epidermal cell body but does not enter adjacent cells in the epidermis. This suggests that movement through plasmodesmata is insignificant and opens up the prospect of making injections into some cells of specific interest, e.g. a developing trichoblast in the epidermis.

Free Ca^{2+} in root hairs compared with other cells

The resting Ca^{2+} values reported here are within the range reported by others using fura-2 with spatial imaging in animal cells (Connor, 1986; Williams *et al.* 1985; Cobbold & Rink, 1987; Malgaroli *et al.* 1987). To date only two reports of fura-2 measurements in plant cells have appeared: the Ca^{2+} levels found in diatoms (Brownlee *et al.* 1987) and *Fucus* rhizoids (Brownlee & Pulsford, 1988) are slightly higher than the 30–90 nM concentrations we have found in root hairs. However, the values for *Haemanthus* endosperm cells (Keith *et al.* 1985) and *Lilium* pollen (Nobiling & Reiss, 1987) using quin-2 are similar to ours.

Free Ca^{2+} measured by ion-selective microelectrodes in *Fucus* rhizoids (Brownlee & Wood, 1986) and *Nitellopsis* (Miller & Sanders, 1987) was markedly higher than we have found with fura-2, being in the range 250–1000 nM. It is difficult to comment on this difference. In the case of *Nitellopsis* the high values were steady over a period of at least 20 min, which would preclude the possibility that the electrode was measuring some locally enriched cytoplasm into which Ca^{2+} had leaked during electrode insertion.

Gradients of cytoplasmic Ca^{2+} have been observed in the growing *Fucus* rhizoid (Brownlee & Wood, 1986; Brownlee & Pulsford, 1988) and in *Lilium* pollen tubes

(Nobiling & Reiss, 1987). Spatial imaging has shown that the gradient in the *Fucus* rhizoid may vary in the same cell at different times, possibly depending on the instantaneous growth rate. Such results are consistent with our findings with root hairs, where marked gradients were seen only on some occasions. Clearly, more work is necessary to correlate short-term dynamics of tip growth with spatial Ca^{2+} changes.

Cytoplasmic streaming

Most evidence suggests that cytoplasmic streaming in higher plant cells is an actomyosin-based mechanism. Immunological evidence shows that both myosin and actin are present in cells from tomato fruits and that polymerized tomato actin activates the Mg-ATPase of both skeletal muscle and tomato myosin at physiological ionic strength (Vahey & Scordilis, 1980). Heavy-chain myosin was identified in Western blots of whole root-tip proteins from *Allium cepa* using a monoclonal antibody to mouse myosin heavy chain. Subsequent immunofluorescence revealed that the myosin co-localized with phalloidin-stained actin (Parke *et al.* 1986).

Cytoplasmic streaming has been studied extensively in internodal cells of *Chara* either in an intact state or in cells in which the vacuole and inner layer of cytoplasm have been removed (Kamiya, 1981; Shimmen & Yano, 1984). In this alga increases in cytosol free $\text{Ca}^{2+} > 10^{-6}$ M, which accompany the action potential in stimulated cells (Williamson & Ashley, 1982), are responsible for the cessation of cytoplasmic streaming. Similarly, streaming ceased in leaf cells of the higher plant *Vallisneria* when permeabilized cells were equilibrated with CaEGTA buffers $< \text{pCa } 6$ (Takagi & Nagai, 1986). In *Chara*, however, no positive Ca^{2+} requirement for streaming has been found (Williamson, 1980); streaming continued in perfused cells with CaEGTA buffers $> \text{pCa } 8$.

Against this background, the effect of the calcium channel blocker, verapamil, which we have described, is difficult to interpret. Plasma membrane vesicles from *Zea mays* roots have been shown to possess a binding site for verapamil that has a dissociation constant about 10 times larger than that of the calcium channel protein in skeletal muscle (H. Harvey & M. Venis, personal communication). The related drug nifedipine also binds avidly to membrane proteins in the tonoplast and plasma membrane of cultured carrot cells (Gillery & Ranjeva, 1986). Nitrendipine applied to germinating pollen grains prevents the formation of a pollen tube, although growth occurs over the whole surface (Reiss & Herth, 1979). Apparently the polarity of calcium distribution was destroyed by the drug, suggesting that *in vivo* calcium channels may be blocked. The addition of verapamil to root hairs initially caused the calcium concentration in the cytoplasm to fall by more than 50%. On the basis of what is known in *Chara*, the inhibition of streaming is contrary to expectation (see above). The present results suggest that cytoplasmic streaming in tomato and rape hairs might have a critical minimum requirement for calcium, but the results could be explained if verapamil treatment had some unsuspected effect on ATP supply.

There was a slight depolarization of the membrane potential after treatment, but the fall was not great and much of the ATP-dependent electrogenic component of the potential must have been in operation.

The addition of MalNEt caused a major depolarization of the membrane potential to a value close to the K^+ -diffusion potential reported in root cells of other species (Higinbotham *et al.* 1970). Cytoplasmic calcium in root hairs increased very rapidly after the addition of 3×10^{-4} M-MalNEt to the external solution and streaming ceased completely. These results are compatible with the known rapid covalent binding of MalNEt to the sulphhydryl groups of membrane proteins. The inactivation of ATPases in the plasma membrane and in the endoplasmic reticulum and vacuole would destroy the basis on which cytoplasmic calcium was kept at its low steady state, without necessarily affecting influx into the cytoplasm *via* calcium channels. The inhibition of streaming might result from a combination of the elevated level of cytoplasmic calcium and direct inhibition of the ATPase, which must be part of the actomyosin system. The partial inhibition of streaming when a lesser concentration of MalNEt (3×10^{-5} M) was added was correlated with a slower depolarization of the membrane potential.

The absence of effects of abscisic acid

Experiments with isolated epidermal strips containing guard cells have indicated a strong positive interaction between the presence of external calcium and the induction of stomatal closure by exogenous abscisic acid (De Silva *et al.* 1985). Such results lead to conclusions about the regulatory effect of abscisic acid being mediated by changes in cytosolic calcium in its second messenger role. Our failure to find any effect, either on cytoplasmic streaming or on fura-2 fluorescence, does not support this idea, nor does it disprove it. Stomatal guard cells are highly specialized and are part of an organ in which abscisic acid levels fluctuate widely in response to environmental changes; by contrast, abscisic acid levels in roots are lower than in leaves and also much less readily perturbed (Chapin *et al.* 1988). If plant growth regulators are to be regarded as analogous to the agonists of mammals, it would not be surprising to find that guard cells possessed an abscisic acid receptor site that was absent in root hairs.

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