PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Spatial characteristics to calcium signalling; the calcium wave as a basic unit in plant cell calcium signalling

Rui Malhó

Phil. Trans. R. Soc. Lond. B 1998 **353**, 1463-1473
doi: 10.1098/rstb.1998.0302

THE ROYAL SOCIETY

Spatial characteristics of calcium signalling: the calcium wave as a basic unit in plant cell calcium signalling

Rui Malhó*, Ana Moutinho, Arnold van der Luit and Anthony J. Trewavas

Plant Biology Department, FCL, University of Lisbon, 1780 Lisboa, Portugal; and ICMB, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, UK

Many signals that modify plant cell growth and development initiate changes in cytoplasmic Ca^{2+}. The subsequent movement of Ca^{2+} in the cytoplasm is thought to take place via waves of free Ca^{2+}. These waves may be initiated at defined regions of the cell and movement requires release from a reticulated endoplasmic reticulum and the vacuole. The mechanism of wave propagation is outlined, and the possible basis of repetitive wave formation, Ca^{2+} oscillations and capacitative Ca^{2+} signalling is discussed. Evidence for the presence of Ca^{2+} waves in plant cells is outlined, and from studies on raphides it is suggested that the capabilities for capacitative Ca^{2+} signalling are also present. The paper finishes with an outline of the possible interrelation between Ca^{2+} waves and organelles and describes the intercellular movement of Ca^{2+} waves and the relevance of such information communication to plant development.

Keywords: calcium; waves; oscillations; signalling; tip growth; aequorin

1. INTRODUCTION

Intracellular or cytoplasmic Ca^{2+}, $[Ca^{2+}]_c$, is a ubiquitous signalling ion controlling turgor pressure, cell growth, division, cell development and secretion. It is an important second messenger that is responsible for mediating the activities of many environmental and endogenous signals. However, $[Ca^{2+}]_c$ seems to be especially significant in plant cell signal transduction. Under resting conditions $[Ca^{2+}]_c$ is maintained between 10 and 100 nM, but on signalling it can accumulate transiently to an average of between 1 and 5 μM. This form of $[Ca^{2+}]_c$ transient is often described as a spike. Single Ca^{2+} spikes have a complex temporal and spatial aspect although the mechanisms that underpin this complexity are only dimly perceived. Downstream events, such as ion channel activation, vesicle fusion, gene expression, and so on, are subsequently activated. That changes in $[Ca^{2+}]_c$ induce critical cellular processes can be termed the 'calcium hypothesis'. Probably the first clear statement of the calcium hypothesis was made by Lionel Jaffe (1980).

2. THE CALCIUM WAVE

(a) *The Ca^{2+} wave underpins Ca^{2+} signalling*

In 1978, Gilkey and co-workers reported that single $[Ca^{2+}]_c$ transients were induced by sperm entry into the *Medaka* egg. By using luminescence imaging technologies, these authors observed the $[Ca^{2+}]_c$ transient to encode a single free Ca^{2+} wave which traversed the egg in about one minute. Initiation of the wave commenced at the sperm entry point. The wave travelled in the cellular domains immediately beneath the plasma membrane. Concomitant with the Ca^{2+} wave, an associated wave of cortical vesicle fusion with the plasma membrane was induced, preventing polyspermy. These first critical observations described some basic spatial and temporal characteristics of $[Ca^{2+}]_c$ signalling.

Certain salient features of a $[Ca^{2+}]_c$ signal in the egg cell can be categorized.

1. The wave is initiated at a defined cellular site.
2. There must be an amplification of local $[Ca^{2+}]_c$ from the initiation site to form the wave.
3. A spatially localized region of high free $[Ca^{2+}]_c$ occurs in the wave itself.
4. As the wave cannot be transmitted by diffusion, it must represent a continuous process of release from internal vesicular stores and uptake back into cell compartments as it moves; this is commonly termed a regenerative increase. Numerous ATPases and Ca^{2+} exchange proteins are responsible for recovering the low cytoplasmic $[Ca^{2+}]_c$.
5. A temporal pattern can be recognized as the wave moves from one side of the egg to the other; a refractory period in Ca^{2+} release from vesicular stores must underpin forward movement.
6. A physiological end-point results with vesicle fusion. In simpler terms, these early results emphasize that localized spatial distributions of $[Ca^{2+}]_c$ are important to elicit physiological responses. These data established that the $[Ca^{2+}]_c$ wave is the spatial correlate of a temporal $[Ca^{2+}]_c$ spike.

(b) *Ca^{2+} signalling can be complex*

The existence of $[Ca^{2+}]_c$ oscillations, waves and various sub-derivatives (Woods *et al.* 1986; Lechletter *et*

*Author for correspondence.

al. 1991) shows the complexity with which $[Ca^{2+}]_c$ signalling can be envisaged in particular cells. A very high frequency of oscillations might simply fuse, creating a permanent $[Ca^{2+}]_c$ gradient. Transmission between cells can generate tissue Ca^{2+} waves (Boitano *et al.* 1992) and pacemaker cells may be hypothesized to have the potential for synchronizing whole tissue Ca^{2+} oscillations. Waves, oscillations, and gradients have been observed in many cell types, and they have to be recognized as primary forms of the temporal behaviour of $[Ca^{2+}]_c$ as a signal.

Most investigators have explored wave initiation and transmission mechanisms. Translation of the spatial information in a $[Ca^{2+}]_c$ signal has received less attention. Transmission and interpretation of a $[Ca^{2+}]_c$ signal may, however, be indissolubly linked. In animals it has been proposed that protein phosphorylation may provide a mechanism for this linkage via the coupled action of a Ca^{2+}-activated phosphatase and a Ca^{2+}-independent kinase (Meyer *et al.* 1992). With changes in Ca^{2+} levels, proteins will be phosphorylated at a constant rate but dephosphorylated in a Ca^{2+}-dependent manner. However, the plant CDPK is Ca^{2+} activated. It was also proposed that cam kinase II may act to maintain the wave once started and act to determine and to measure the possible frequency of subsequent waves (De Koninck & Schulman 1998; Putney 1998).

(c) *Constraints on Ca^{2+} diffusion in the cytoplasm underpin wave construction*

Diffusion of Ca^{2+} in the cytoplasm is much slower than in free solution. Direct measurements in a number of animal cells indicate that the diffusion coefficient of Ca^{2+} is anywhere from tenfold to 1000-fold lower than in water (see, for example, Von Tscharner *et al.* 1986; Allbritton *et al.* 1992; Connor 1993). Many proteins which are attached to the cytoskeleton bind Ca^{2+}. The concentration of these cytoplasmic protein binding sites has been estimated to be nearly 1 mM (Von Tscharner *et al.* 1986). Possible changes in binding site strength and concentration after signalling are almost universally ignored. Mitochondria, endoplasmic reticulum (ER), chloroplasts and vacuoles can actively accumulate Ca^{2+} from the cytoplasm by using specific calcium pumps (ATPases) and thus act as intracellular stores (Liang *et al.* (1997), and references therein). Each of these organelles possesses channels which can release Ca^{2+} upon receipt of specific signals. Further constraints on Ca^{2+} diffusion are therefore exerted by these organelles. In the absence of protein binding and organelle accumulation, any increase in $[Ca^{2+}]_c$ would immediately and uniformly distribute throughout the cytoplasm, particularly in cells with cytoplasmic streaming. Ca^{2+} waves, gradients and oscillations could not be constructed. As waves, gradients and oscillations in $[Ca^{2+}]_c$ have all been detected in plant cells, similar constraints on Ca^{2+} diffusion must exist in the plant cytoplasm.

(d) *Why do cells use Ca^{2+} waves for signalling?*

A complete Ca^{2+} wave should elevate $[Ca^{2+}]_c$ in all parts of the cytoplasm. Why are Ca^{2+} waves used in preference to simple amplitude increases in $[Ca^{2+}]_c$ with no spatial structure? Several reasons for signalling by waves can be suggested.

1. A wave, which occupies only part of the cytoplasm, may alleviate energetic requirements for Ca^{2+} release and uptake.
2. If the frequency information can be interpreted, repetitive waves may be perceived against a background with effectively zero noise.
3. The choice of waves, gradients, oscillations or even simple amplitude changes, greatly increases the diversity of transmitted information available to individual cell types.
4. $[Ca^{2+}]_c$ is toxic at high concentrations. Cells may be able to interpret the peaks of high $[Ca^{2+}]_c$ in oscillations as a continuous high $[Ca^{2+}]_c$ without the necessity of maintaining a high and damaging concentration. In the same way plant cells can interpret skeleton photoperiods as continuous light during the photoperiod.
5. The movement of a wave can be constrained to one region of the cell by spatially regulating factors involved in its production. Different regions of the cytoplasm can then be separately regulated. Distinct physiological processes can therefore be controlled in the same cell at the same time by the same ion. Ca^{2+} responsive proteins (e.g. calmodulin, protein kinases) are not uniformly distributed but may be located in specific cytoplasmic regions in the form of 'transducons' (Gilroy & Trewavas 1994; Trewavas & Malhó 1997). Such structures will greatly improve the selectivity of signal and response. Definite evidence for transducon-like entities has been published recently (Tsunada *et al.* 1997; Hall 1998).
6. A wave may be the most suitable form in which a Ca^{2+} signal can be conveyed into the nucleus so that gene expression can be manipulated (Lipp *et al.* 1997).

(e) *Initiation sites for Ca^{2+} waves and oscillations in plant cells*

Cells use two subcellular locales to initiate Ca^{2+} signals: Ca^{2+} entry across the plasma membrane and Ca^{2+} release from intracellular stores. Both of these sites can act to initiate highly localized signals in the cytoplasm.

We still lack definite information on plant plasma membrane Ca^{2+} channels. Clear indications that such channels exist are derived from observations of several plant cell types. Pollen tubes are highly differentiated and grow rapidly. Growth, generated by fusion of wall-containing vesicles, is confined to the extreme apex. Tube growth requires a relatively narrow range of extracellular Ca^{2+} concentration. Fluorescence ratio imaging studies in a variety of different pollen tubes have shown steep ($3\,\mu M \rightarrow 200\,nM$) tip-high gradients of $[Ca^{2+}]_c$ confined to the apical region 15–20 µm (Malhó *et al.* 1994; Pierson *et al.* 1996). A spatial separation of Ca^{2+} channels from Ca^{2+} ATPases in the plasma membrane is thought to underpin the gradient (Feijó *et al.* 1995). When growth has been transiently interrupted, pollen tubes possess no detectable $[Ca^{2+}]_c$ gradient. Recovery of growth is preceded by the re-establishment of the tip gradient (Malhó *et al.* 1995; Pierson *et al.* 1996). The gradient oscillates in steepness with a similar periodicity to pulsatile growth rates (Messerli & Robinson 1997). Mn^{2+} can enter through putative active Ca^{2+} channels and displace

Ca^{2+} from loaded fluorescent dyes. By imaging the loss of fluorescence in Mn^{2+}, Ca^{2+} entry (and presumably channel distribution) was observed and was confined to the extreme tip region (Malhó *et al.* 1995). It is thought that membrane stretching during turgor-induced growth controls Ca^{2+} channel activity, as Ca^{2+} channel activity is sensitive to osmotic signalling. Very low concentrations of the channel blockers, La^{3+} or Gd^{3+}, slightly diminish growth rates but completely block reorientation of the pollen tube in response to an applied electrical field. Several types of Ca^{2+} channel may thus be present (Malhó *et al.* 1994). This initiation site is an important model for other plant cells.

Taylor *et al.* (1996) used laser microsurgery and patch-clamping on the *Fucus* rhizoid tip plasma membrane. Voltage-regulated and mechano-sensitive channels that carry Ca^{2+} were detected in the plasma membrane of the tip. These channels are activated in response to hypoosmotic shock. A wave of $[Ca^{2+}]_c$ is induced by this osmotic signalling which enables subsequent osmoadaptation. Mn^{2+} as a Ca^{2+} surrogate helped confirm the identity and location of the channels. Growing root hairs contain a Ca^{2+} gradient in the tip and Mn^{2+} treatment confirms the location of active channels here (Wymer *et al.* 1997).

The identity of intracellular channels that act as initiation sites is well established. (1,4,5) $InsP_3$ and cADP-R are second messengers synthesized by enzymes on receipt of signals. On binding to these molecules, intracellular Ca^{2+} channels are activated. Patch-clamp approaches have been used to detect vacuolar Ca^{2+} channels that are sensitive to (1,4,5) $InsP_3$ and blocked by heparin. A second set of Ca^{2+} channels that are sensitive to cADP-R and blocked by ryanodine have been characterized (Allen *et al.* 1995). Furthermore, vacuolar SV channels that preferentially transport Ca^{2+} are also Ca^{2+}-activated (Ward *et al.* 1995). Voltage-activated Ca^{2+} channels have been characterized in the vacuole (Ward *et al.* 1995) and in the endoplasmic reticulum (ER) (Klusener *et al.* 1995). These data imply that Ca^{2+} waves might routinely move over the cytoplasmic surface of the vacuole.

Single Ca^{2+} waves have been observed in pollen tubes but only when induced by global photolysis of caged (1,4,5) $InsP_3$ (Franklin-Tong *et al.* 1996) or photolysis of caged Ca^{2+} (Malhó & Trewavas 1996). Ca^{2+}-induced waves were initiated primarily in the nuclear–rough ER cellular locale, but not in the apical region, suggesting that nuclei–ER are sites of particular sensitivity and/or differential regulation. As the vacuole is confined to the rear of the pollen tube, this organelle cannot easily participate in the initiation of these waves.

Stomata cells are induced to close by increasing the concentration of extracellular Ca^{2+}. Fluorescence ratio imaging of guard cell $[Ca^{2+}]_c$ indicated that the initiation of a truncated wave also occurs again in the vicinity of the nucleus–ER (Gilroy *et al.* 1991). Reductions in extracellular K^+ also caused stomatal closure, but in this case truncated Ca^{2+} waves were initiated from the vacuole membrane.

The initiation of waves may require a concomitant presence of extracellular Ca^{2+}. Takahashi *et al.* (1997) hypoosmotically stimulated cultured cells in the absence of extracellular Ca^{2+}; only when Ca^{2+} was returned to the medium was a $[Ca^{2+}]_c$ spike (wave) detected.

(f) *Propagation of Ca^{2+} waves in the cytoplasm*

The mechanism of wave propagation has attracted considerable analysis in animal cells. One of the most interesting features of Ca^{2+} waves is that despite their travel in a medium of enormous complexity, their characteristics can be well-described by simple mathematical and physical models (Dupont *et al.* 1991; Tang & Othmer 1995). In animal cells, and perhaps in plant cells as well, Ca^{2+} waves are characterized by (i) speed of propagation; (ii) diffusion constant; (iii) minimum propagation radius; and (iv) constant amplitude during propagation.

The formation of a full wave results from the integration of a series of sub-threshold events which separately can be regarded as abortive waves. When cells are either weakly or partly stimulated or are inherently excitable, small local cytoplasmic areas of high $[Ca^{2+}]_c$ have frequently been imaged. These local regions have received picturesque but descriptive names; quarks, blips, bumps, puffs and sparks placed in order of size. All are regarded as elemental units of calcium signalling, capable of releasing a quantal amount of Ca^{2+} when activated (Dawson 1997). Most are of ER origin and may be vesiculate or parts of ER tubules. Quarks are thought to represent single active (1,4,5) $InsP_3$ (or cADP-R)-sensitive channels; the others, increasingly larger channel conglomerates.

Ca^{2+} from the elemental vesicle relies on the binding of two components, (1,4,5) $InsP_3$ and Ca^{2+}, to the (1,4,5) $InsP_3$-sensitive channel. At $[Ca^{2+}]_c$ in the range of 100 nM to 5 μM and with adequate (1,4,5) $InsP_3$, Ca^{2+} release is initiated. Most recent information suggests that (1,4,5) $InsP_3$ binding 'opens' the Ca^{2+} binding site. If Ca^{2+} fails to bind within a limited time period the channel is inactivated. Activation of the channel is thus controlled by a coincidence mechanism and might even be regarded as a coincidence counter (Dawson 1997). Diffusion of released $[Ca^{2+}]_c$ may activate adjacent (1,4,5) $InsP_3$-sensitive channels and recruit them into the elemental Ca^{2+} release unit; but only within a limited cytoplasmic region. However, vesicles vary in the density of (1,4,5) $InsP_3$ sensitive channels and in the sensitivity of the channel to Ca^{2+}. The (1,4,5) $InsP_3$ channel (or receptor) is a family of proteins. Phosphorylation of (1,4,5) $InsP_3$ channels might alter the sensitivity to Ca^{2+} and to (1,4,5) $InsP_3$. The variations in sensitivity and density of channels can ensure a stochastic activation of elemental units.

If an initial plasma membrane signal is small, only low concentrations of (1,4,5) $InsP_3$ are generated. Few vesicles release Ca^{2+} and further recruitment is truncated. Elemental units remain as blips, puffs or sparks. The most recent method for detection of puffs relies on photolysis of loaded caged (1,4,5) $InsP_3$ in cells containing EGTA to restrict free Ca^{2+} movement (Horne & Meyer 1997). When the initial stimulus is large, interaction and coalescence of most active elemental units will drive quarks to sparks and finally coalescence to form a full wave. At (>1 to 5 μM), $[Ca^{2+}]_c$ inhibits the (1,4,5) $InsP_3$-sensitive channel and release becomes self-limiting (Dawson 1997). Thus the wave results from Ca^{2+}-induced Ca^{2+} release. Waves can be induced by an IP_3 analogue, (1,4,5) $InsP_3$-S, which is resistant to phosphatases which normally hydrolyse (1,4,5) $InsP_3$. Concomitant spatial variations in

(1,4,5) $InsP_3$ concentration are not required for wave formation. In animal cells it was found that the feedback regulation of the (1,4,5) $InsP_3$ receptor by $[Ca^{2+}]_c$ at a constant level of (1,4,5) $InsP_3$ represents the minimum requirement for oscillatory Ca^{2+} release (Hajnóczky & Thomas 1997).

This mechanism accounts for some fundamental aspects of Ca^{2+} signalling. First, it explains how the two components (1,4,5) $InsP_3$ and Ca^{2+} can in part compensate for each other. A low concentration of (1,4,5) $InsP_3$ can be offset by a high concentration of Ca^{2+}; conversely, a low Ca^{2+} can be offset by a high concentration of (1,4,5) $InsP_3$. Thus signalling from intracellular channels will normally be initiated by an increased concentration of (1,4,5) $InsP_3$ using resting levels of Ca^{2+}. Conversely, direct opening of plasma membrane-bound Ca^{2+} channels (resulting from membrane potential changes) will increase $[Ca^{2+}]_c$ substantially and initiate signalling with resting levels of (1,4,5) $InsP_3$. This latter mechanism is helped by the presence of plasma membrane-located Ca^{2+}-activated phospholipase C to generate (1,4,5) $InsP_3$. The vesicle recruitment pattern from intracellular and plasma membrane signalling will be different because the signals emerge in different cellular regions (Golovina & Blaustein 1997). Vesicle recruitment patterns will also depend on the particular state of the cell and its previous signalling schedule. Second, the numbers of quantal release units recruited are proportional to the intensity of signalling; amplitude variation in $[Ca^{2+}]_c$ transients is thus explained.

Full $[Ca^{2+}]_c$ waves can be induced in pollen tubes either by photolysis of caged Ca^{2+} or by photolysis of caged (1,4,5) $InsP_3$. In both cases the resting levels of either Ca^{2+} or (1,4,5) $InsP_3$ are sufficient to compensate for changes in the other (Malhó & Trewavas 1996). Substantial resting levels of (1,4,5) $InsP_3$ have been detected in pollen tubes (Franklin-Tong *et al.* 1996). Photolysis of loaded caged (1,4,5) $InsP_3$ in the presence of heparin (to inhibit binding to the (1,4,5) $InsP_3$ channel) induced only truncated waves (Franklin-Tong *et al.* 1996). A decline in membrane potential induced by brief iontophoresis opens voltage-gated plasma membrane Ca^{2+} channels and elevates $[Ca^{2+}]_c$ through extensive regions of the pollen tube (Malhó *et al.* 1994, 1995). Pollen tubes also possess a Ca^{2+}-activated phospholipase C capable of generating (1,4,5) $InsP_3$ (Franklin-Tong *et al.* 1996).

Many fluorescent ratio images of pollen tubes, protoplasts and guard cells have been produced (Gilroy *et al.* 1991; Shacklock *et al.* 1992; Malhó *et al.* 1994, 1995). Small local areas of high $[Ca^{2+}]_c$ are commonly seen which might be equivalent to the quantal release units found in animal cells.

(g) *What parameters of the $[Ca^{2+}]_c$ spike should be measured that might correlate with a physiological response?*

Figure 1 shows a simple $[Ca^{2+}]_c$ transient in tobacco seedlings transformed with the Ca^{2+}-sensitive luminescent protein, aequorin, as a $[Ca^{2+}]_c$ indicator. The transient was induced by irrigating with water at 5° at a 10-s interval. Despite the fact that the signal (the cold temperature) is prolonged, the response is transient. The overall length of the $[Ca^{2+}]_c$ transient of the seedling $[Ca^{2+}]_c$ is about 20 s. Transient $[Ca^{2+}]_c$ responses of many single tobacco cells in intact leaves to cold shock have been imaged by luminescence (H. Page, A. J. Trewavas and N. D. Read, unpublished data). Again the transient length is about 20 s. The resting level of $[Ca^{2+}]_c$ measured with aequorin is about 40 nM. Resting levels measured with fluorescent dyes are routinely 150–200 nM. The difference in these values may result from the much weaker binding of aequorin to Ca^{2+} (estimated K_D, 0.1 mM) compared with fluorescent dyes (K_D *ca.* 100 nM). The use of aequorin may be less perturbing to $[Ca^{2+}]_c$ homeostasis.

A total of three main kinetic characteristics of the transient can be recognized; the rise time, the peak and the decay time. The rise time represents a period of increasing recruitment of vesicles into a release mode; in this phase, Ca^{2+} release is greater than Ca^{2+} uptake. At the peak, release equals uptake. The peak may measure maximum recruitment, but release must equal uptake at this point and therefore more likely measures the changing rate of both processes. In all of the signals we have observed in tobacco seedlings, the decay time is longer than the rise time. While release is obviously continuing during the decay time, the uptake of Ca^{2+} back into vesicles is the dominant event. Some signals that we have investigated result in an increased resting level after the transient and this can be seen in figure 1. The resting level after signalling can be two- to threefold higher and last for considerable periods of time. A change in the concentration of cellular Ca^{2+} binding sites (perhaps induced by phosphorylation) could be responsible. Investigation of a number of signals by us, and others, indicates a $[Ca^{2+}]_c$ refractory period often follows signalling (Gong *et al.* (1998), and references therein). This is obvious in the case of figure 1, because the seedling continues to be stimulated by the cold temperature.

Can any aspect of this $[Ca^{2+}]_c$ signal be related to a physiological event? Much will depend on the complexity of downstream events from the $[Ca^{2+}]_c$ signal, the cellular thresholds and interactions with other signalling pathways. The area under the transient, i.e. $[Ca^{2+}]_c \times$ time, might represent the most useful measurement, particularly if signals have different kinetics. Recent data produced by Dolmetsch *et al.* (1997) suggest that the rise time, peak $[Ca^{2+}]_c$, and the duration of the decay time are crucial elements in signalling. These authors were able to show by mimicking parts of the transient with the ionophore A23187 that different regions of the transient were responsible successively *in vivo* for protein translocation, protein phosphorylation and gene expression. A recent analysis by us (van der Luit *et al.* 1998) suggests that the transient area ($[Ca^{2+}]_c$ × time) in nuclear Ca^{2+} correlates well with changes in the expression of specific calmodulin genes. Transients in cytoplasmic Ca^{2+} failed to correlate well.

Table 1 lists the kinetic characteristics of many of the signals we have observed to influence $[Ca^{2+}]_c$ in transgenic tobacco seedlings expressing aequorin. Each signal induces Ca^{2+} transients which are kinetically distinct from each other. It could be expected that different transients might contribute to aspects of specificity in the physiological and molecular responses to each signal.

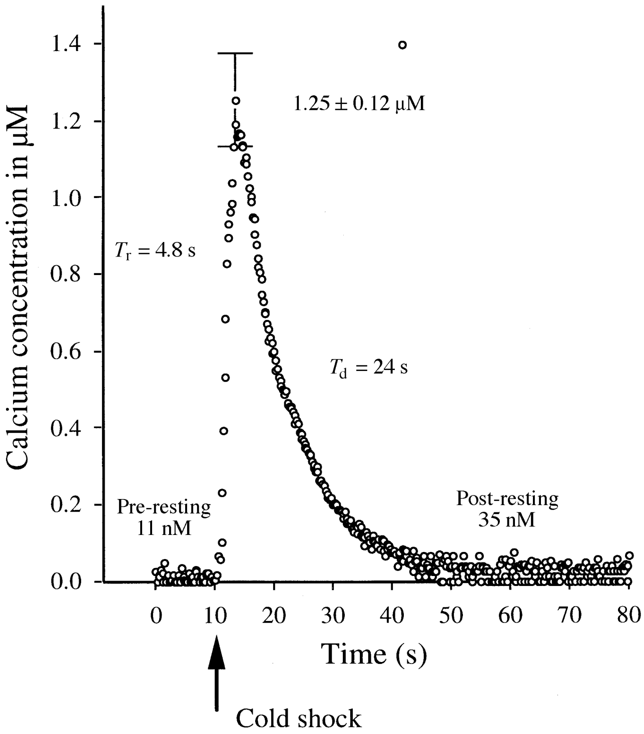

Figure 1. Transient response of $[Ca^{2+}]_c$ to cold shock in tobacco seedlings transformed with aequorin as a Ca^{2+} indicator. The seedling was stimulated by irrigating with water at 5° at the 10-s mark. Luminescence was recorded at 0.2 s intervals and data immediately computed into $[Ca^{2+}]_c$ as described in the rubric. T_r is rise time. T_d is decay time. Peak value is mean ± s.d. of ten different seedlings.

3. THE CALCIUM OSCILLATION

So far, only one type of $[Ca^{2+}]_c$ oscillation—baseline transients or spikes—has been described in plant cells. In this type of oscillation, the integrated $[Ca^{2+}]_c$ elevation is a function of the strength of the stimulus. The mechanisms by which oscillations are generated in animal cells (e.g. opening of plasma membrane channels, release of Ca^{2+} from intracellular stores; see Thomas *et al.* 1996) have already been described in plants (Webb *et al.* 1996). In animal cells, three other types of oscillations have been observed: protein kinase C (PKC) regulated, thapsigargin-activated, and acetylcholine- or cholecystokinin-activated. None of these mechanisms has yet been demonstrated in plants, although recent reports suggest the existence of a PKC-type enzyme (Subramanian *et al.* 1997).

(a) *Ca^{2+} oscillations in plant cells*

Ca^{2+} oscillations were first described in coleoptile cells by Felle (1988) and were induced by auxin. Short-term $[Ca^{2+}]_c$ oscillations in guard cells induced by ABA were reported by Fricker *et al.* (1990) and characterized in much greater detail by McAinsh *et al.* (1995). The frequency was of the order of several minutes. Oscillations (periodicity of several minutes) have been induced in root hairs by nodulation (NOD) factors but they are spatially limited to the cytoplasmic area surrounding the nucleus (Ehrhardt *et al.* 1996). Growing lily pollen tubes maintain a standing gradient of $[Ca^{2+}]_c$ in the tip whose

Table 1. *Characteristics of the Ca^{2+} transient in tobacco seedlings after signalling*

(All data were measured on young transgenic tobacco seedlings containing aequorin, except for the hypoosmotic shock which used transgenic tobacco cells. Signals have been applied as described in Knight *et al.* (1991); Johnson *et al.* (1995); Takahashi *et al.* (1996); Sedbrook *et al.* (1996); Knight *et al.* (1997). Various times have been measured, as indicated in figure 1. The amplitude of the signal can vary with the intensity of the signal, but there is little variance in the kinetics with differing intensities. Note that the kinetics of response to the different signals are unique to each signal.)

signal	lag period	rise time	total transient length
blue light	3–6 s	20–30 s	90 s
cold shock	0	4–5 s	30 s
heat shock	several min	10 min	30 min
wind	0	0.3–04 s	15 s
hypoosmotic shock	30 s	60 s	150 s
hyperosmotic shock	5 s	10 s	30 s
oxidative shock	30 s	10 s	90 s
anoxia	60 s	120 s	300 s
red light	30 min	2.5 h	7 h
auxin-Me	1–2 min	5 min	10–20 min
elicitors	5–10 s	20 s	45–60 s

steepness oscillates vigorously with a periodicity of about 40 s (Pierson *et al.* 1996; Holdaway-Clarke *et al.* 1997; Messerli & Robinson 1997). Using pollen tubes with exaggerated oscillations in growth pattern, Malhó & Trewavas (1996) correlated the oscillation in growth orientation with an oscillation in the ratio of $[Ca^{2+}]_c$ between the two sides of the apical dome. Repetitive $[Ca^{2+}]_c$ spiking was observed by Sedbrook *et al.* (1996) in *Arabidopsis* roots subjected to anaerobic conditions. Robust circadian oscillations in seedling cytoplasmic $[Ca^{2+}]_c$ were induced by light. Oscillations of chloroplast Ca^{2+}, $[Ca^{2+}]_{chl}$, on the other hand were induced by the transfer from light to darkness (Johnson *et al.* 1995). $[Ca^{2+}]_c$ can thus oscillate with variable frequency after chemical or physical signals.

Oscillations increase the diversity of Ca^{2+} signalling available to cells. However, many excitable systems have the ability to respond to perturbation by expressing oscillations in the concentration of key components. Negative feedback, with a delay in information transfer to effect the feedback, is usually responsible for oscillation. Alternatively, interactions between two regulating components (such as Ca^{2+} and (1,4,5)$InsP_3$ in this case) can cause the system to oscillate. Do the oscillations significantly influence cell physiology, or simply reflect the fact that Ca^{2+} can influence its own release and uptake?

Prentky *et al.* (1988) reported cell-specific patterns of oscillating free Ca^{2+}, generating the so-called $[Ca^{2+}]_c$ fingerprint. It is possible then to envisage a mechanism whereby the same stimulus can trigger different signalling responses. Even cells from the same population can have different degrees of sensitivity to the same inducing stimulus (Trewavas & Malhó 1997). Although it may be tempting to propose a unifying theory for Ca^{2+} signalling, the facts which support the theory may not be the same for all cells. Wave curvature and formation of spiral wave patterns depend, among other things, on cell size; spiral waves are not likely to be a widespread phenomenon, although it is possible that they might be present in pollen tubes and account for the frequently observed wandering character of pollen tube growth.

Simple waves and oscillations on the other hand, must be an intrinsic characteristic of all cells because the characteristics of the wave are derived from the cell's biochemical and physiological organization. Experiments photolysing caged-Ca^{2+}, in particular cytoplasmic regions of growing pollen tubes, support this assumption (Malhó & Trewavas 1996). Artificially elevating Ca^{2+} in defined regions of the pollen tube effectively mimics 'nucleation' or 'initiation' sites and catalyses the subsequent influx or the release of Ca^{2+} from an intracellular store. The fact that the cell responds to this stimulus with a transient Ca^{2+} spike is not just a coincidence. It is the proof of a robust, self-regulated system.

(b) *Capacitative Ca^{2+} entry and signalling*

To understand the possible mechanism of oscillation requires the description of two additional components which regulate $[Ca^{2+}]_c$ homeostasis (Berridge 1995). One route for Ca^{2+} entry (through the plasma membrane into the cytoplasm) is regulated by the extent to which the intracellular Ca^{2+} stores are filled. Ca^{2+} entry ceases when the stores are full but recommences as soon as store Ca^{2+} is discharged. This behaviour can be considered analogous to an electrical capacitor. Consequently, Ca^{2+} entry regulated by intracellular store capacity has been described as capacitative Ca^{2+} entry.

The Ca^{2+} channels which are responsible for capacitative Ca^{2+} entry have a measured flux rate orders of magnitude lower than the Ca^{2+} channels which respond to (1,4,5)$InsP_3$ or voltage. The entry channels have been given the name I_{CRAC}; that is current carried by a Ca^{2+} release-activated-Ca^{2+} channel. A variety of ways has been found to induce capacitative Ca^{2+} entry and these include Ca^{2+}-mobilizing agonists, (1,4,5)$InsP_3$, cADP-R, ionophores, thapsigargin or cyclopiazonic acid, or simply placing the cells under Ca^{2+}-free conditions. Basically, any signal which empties the stores sets the process of refilling by capacitative Ca^{2+} entry into operation. The higher resting level of $[Ca^{2+}]_c$, noted in figure 1, after the cold signal could represent capacitative Ca^{2+} entry.

$[Ca^{2+}]_c$ oscillations can be expected if there is a delay between emptying and refilling. The cytosolic $[Ca^{2+}]_c$ oscillations start with the release of Ca^{2+} from the stores into the cytoplasm. Ca^{2+} ATPases in the plasma membrane, ER, and vacuole result in removal of Ca^{2+} and the end of the oscillation before the repeat of the cycle. Ca^{2+} release from ER vesicles or the vacuole is $[Ca^{2+}]_c$ activated and likely to occur explosively once it starts.

What mechanism or signal enables the empty stores to switch on I_{CRAC}? Close association between ER-located (1,4,5)$InsP_3$ receptors and I_{CRAC} channels in the plasma membrane is currently the favoured explanation (Berridge 1995). In plant cells, the close association of the vacuole membrane with the plasma membrane may be a significant pointer to a mechanism.

(c) *Does capacitative Ca^{2+} entry occur in plant cells?*

The study of the behaviour of raphides may indicate the presence of capacitative signalling in plant cells (Trewavas & Malhó 1997). These specialized cells accumulate Ca^{2+} in the form of oxalate crystals, which provides a visible indication of stored Ca^{2+}. The oxalate crystal grows in a separate membrane-bound structure often within the vacuole itself. Raphides have been reported to contain calsequestrin, a high capacity, low-affinity Ca^{2+}-binding protein located in the ER of many cell types (Francheschi *et al.* 1993). Borchert (1990) showed that young mesophyll cells can be converted to Ca^{2+} accumulators by incubation in 4–5 mM Ca^{2+} acetate. Thus raphides and less specialized cells share certain mechanisms for Ca^{2+} homeostasis. Using aequorin-transformed seedlings, we have investigated the influence of solutions of different Ca^{2+} concentrations. Definite effects on $[Ca^{2+}]_c$ were only found at 30 mM Ca^{2+} (M. R. Knight and A. J. Trewavas, unpublished observations). Repetitive spikes with a periodicity of several minutes were observed. At 5 mM only slight influences on the resting level were observed. A slow rate of Ca^{2+} entry at 5 mM exogenous Ca^{2+} is thus indicated.

Franceschi (1989) used roots of *Lemna minor* to investigate oxalate crystal formation. By increasing the exogenous Ca^{2+} concentration to 5 mM, new cells containing crystals were detected within 30 min. However, removal of extracellular Ca^{2+} led to total crystal dissolution within 3 h. In this example the Ca^{2+} store clearly responds to the extracellular Ca^{2+} concentration; slow Ca^{2+} accumulation can be reversed. A total of two calmodulin-binding antagonists were observed to prevent crystal formation and caused dissolution of crystals, even in the presence of Ca^{2+} (Franceschi 1989). Calmodulin antagonists inhibit the ATPases necessary to pump Ca^{2+} back into stores and act like thapsigargin, a known capacitative Ca^{2+} entry signalling molecule (or regulator). Treatment with the Ca^{2+} ionophore A23187 also caused crystal dissolution.

In the pitcher plant, removal of Ca^{2+} from the pitcher fluid led to efflux of Ca^{2+} from the cells forming the pitcher. An equilibrium concentration of 25–50 μM was finally achieved after several days. If, in contrast, excess Ca^{2+} was added to the fluid, the cells removed it until a concentration of about 50–100 μM was again attained (Meir *et al.* 1991). These observations indicate that the Ca^{2+} store is in 'communication' with the extracellular medium as is required for capacitative Ca^{2+} entry. When the stores are empty, they can be filled, and when filled they can be emptied. The raphide would seem to be a specialized version of a much more general phenomenon of Ca^{2+} regulation. McAinsh *et al.* (1995) induced Ca^{2+} oscillations in guard cells by increasing extracellular Ca^{2+} concentration or photolysing caged Ca^{2+}. The conclusion must be that capacitative Ca^{2+} entry is likely to exist in plant cells.

(d) *Do Ca^{2+} oscillations have a cellular function in addition to those of a simple Ca^{2+} wave?*

The proper way to approach this question is to generate artificial Ca^{2+} oscillations inside cells. Gu & Spitzer (1995) investigated the effect of mimicking natural spike oscillations in nerve cells. By re-imposing different frequency patterns of Ca^{2+} elevation it was observed that natural spike frequency was sufficient to promote differentiation including growth. $[Ca^{2+}]_c$ oscillations in pollen tubes are definitely associated with pulses of growth and wall secretion (Li *et al.* 1994; Pierson *et al.* 1996; Messerli & Robinson 1997). While the precise reason for oscillations in the gradient remains unknown, a possible important pointer for future investigation is indicated. McAinsh *et al.* (1995) observed that photolysis of caged Ca^{2+} in guard cells induced $[Ca^{2+}]_c$ oscillations. As release of Ca^{2+} from loaded caged Ca^{2+} can cause closure (Gilroy *et al.* 1990), a possible function in turgor regulation is indicated here. Finally, Goodwin & Brière (1992) have suggested that the production of the whorls in *Acetabularia* might be regulated by a standing oscillatory wave of $[Ca^{2+}]_c$ in the tip. On removal of the old tip, regeneration of a new cap is stimulated. In this single cell system, initiation of tip growth is accompanied by the formation of a Ca^{2+} gradient. Later in development, an annulus of Ca^{2+} forms in the tip, resulting in tip flattening. Computer modelling shows that under perturbation, the Ca^{2+} annulus transforms into a ring of peaks which mimics the symmetries of the whorl (Goodwin & Brière 1992).

4. SPATIAL REGULATION OF Ca^{2+} IN THE CYTOPLASM AND ORGANELLE Ca^{2+}

Waves and oscillations may be regarded as the spatial and temporal manifestations of a single phenomenon, the primary molecular events being responsible for the propagation of the $[Ca^{2+}]_c$ signal to the appropriate subcellular targets. As high levels of relatively immobile cytoplasmic Ca^{2+} buffers limit the range of Ca^{2+} diffusion in the cytoplasm to within a few micrometres, transmission of the signal is achieved either by diffusional distribution of the Ca^{2+} mobilizing the second messenger, or regenerative Ca^{2+} release. Both mechanisms depend on the role of organelles as intracellular stores. Therefore, the subcellular distribution of intracellular Ca^{2+} stores can determine the spatial organization of the $[Ca^{2+}]_c$ signal (Thomas *et al.* 1997).

(a) *The nucleus and regulation of gene expression*

A dynamic and complex nuclear role in Ca^{2+} signalling has frequently been suggested. Lowenstein & Kanno (1963) measured the potential difference across the nuclear membrane and observed a substantial potential difference in the nuclei of some cell types but not others. Thus, in certain cell types, and despite the presence of nuclear pores, the nucleus is unlikely to be freely permeable to Ca^{2+}. More recently, direct measurements of nuclear Ca^{2+}, $[Ca^{2+}]_{nuc}$, have been made after signalling (Badminton *et al.* (1996), and references therein). There are probably as many authors claiming that the cytoplasmic signal can pass unhindered through the nuclear pores, as there are authors claiming that the nuclear membrane is a discrete barrier to cytoplasmic signals. The source of contention is implicit in Lowenstein's early measurements. It may simply depend on cell type. Meyer *et al.* (1995) calculated the diffusion rates of Ca^{2+} in cytoplasm and concluded that if nuclear signals were different from cytoplasmic signals by more than 1 s, the nuclear membrane provided a significant barrier to the free movement of Ca^{2+} ions. A definite criterion for regulation of nuclear Ca^{2+} is thus provided.

Nuclei also contain substantial quantities of calmodulin and a phosphoinositide signalling system located in the nuclear cisterna. In two well-established cases, Ca^{2+} transients in the cytosol are paralleled by oscillations in the nucleoplasm (Gillot & Whitaker 1994; Lin *et al.* 1994; Lipp *et al.* 1997). But the perception and transmission of the signal in the nucleoplasm is initiated by Ca^{2+} release from the (1,4,5)$InsP_3$-sensitive pools located in the nuclear cisternal membrane. A nuclear protein kinase can modify the nuclear cisternal (1,4,5)$InsP_3$ channel to increase its sensitivity to (1,4,5)$InsP_3$ (Matter *et al.* 1993). Nuclear cisternal channels sensitive to cADP-R are also thought to be present (Perez-Terzic *et al.* 1997). After release, uptake of Ca^{2+} into the cisterna is catalysed by a Ca^{2+}-ATPase.

The most interesting observations concern a transit of molecules through the nuclear pore. Depletion of the Ca^{2+} stores in the cisterna or ER dramatically stops the movement of proteins through the pore and the nuclear pore structure is visibly altered (Greber & Gerace 1995; Stehno-Bittel *et al.* 1995; Perez-Terzic *et al.* 1997). Movement of transcription factors such as *cop* or *det* is likely to be an important element in the regulation of gene expression during development by photomorphogenic light (Furuya & Schafer 1996). The recent identification of soluble nuclear pores (so-called vaults) which might bind proteins and move to the nucleus on receipt of a Ca^{2+} signal heightens the interest (Kickhoefer *et al.* 1996). Nuclear vesicle fusion is also thought to be regulated by Ca^{2+} (Sullivan & Wilson 1994). In many ways the nuclear cisterna acts like the ER in wave initiation.

Hardingham *et al.* (1997) injected dextran-linked EGTA directly into nuclei and demonstrated that regulation of gene expression by CREB (cAMP response element-binding protein) is controlled by $[Ca^{2+}]_{nuc}$ but not $[Ca^{2+}]_c$. Regulation of other genes by the serum response element (SRE) system requires changes in $[Ca^{2+}]_c$. Ca^{2+}-dependent phosphorylation of CREB is performed by a nuclear kinase. The same second messenger, cAMP, can then generate diverse transcriptional responses. CREB has been identified in plants, and the recent identification of a plant adenyl cyclase (Ichikawa *et al.* 1997) makes these observations particularly relevant.

We have recently measured $[Ca^{2+}]_{nuc}$ in young tobacco seedlings using nuclear-targeted aequorin. After cold-shock signalling, differences of at least 4 s have been observed between the nuclear and cytoplasmic Ca^{2+} signals (van der Luit *et al.* 1998). On the Meyer *et al.* (1995) criterion, the plant nuclear membrane is a considerable barrier to free Ca^{2+} movement. Further investigation using wind signalling indicated that changes in $[Ca^{2+}]_{nuc}$ correlated with the accumulation of a specific calmodulin mRNA in tobacco. However, a different signal, cold shock, did not exhibit the same simple relation; a cytoplasmic pathway for calmodulin gene expression might be more important in cold-shock signalling.

(b) *Mitochondria and chloroplasts*

The ability of mitochondria to accumulate Ca^{2+} has been known for many years. The uptake depends on the expenditure of energy and anaerobic conditions might be expected to cause release into the cytoplasm (Subbaiah *et al.* 1994). However, it is not clear at the present time whether changes in mitochondrial Ca^{2+} control gene expression required for adaptation to anaerobiosis (Sedbrook *et al.* 1996). Mitochondria have been shown to tune out sustained $[Ca^{2+}]_c$ signals (Hajnózcky *et al.* 1995) and can thus interpret an oscillation rather than a single wave. A synchronization of Ca^{2+} waves by mitochondrial substrates has also been demonstrated (Jouaville *et al.* 1995).

Studies on isolated chloroplasts have suggested that illumination leads to a brief elevation of Ca^{2+} uptake into chloroplasts (inhibited by ruthenium red), leading to activation of certain photosynthetic enzymes (Kreimer *et al.* 1987, 1988). However, illumination also increases the number of Ca^{2+}-binding sites and estimates suggest that steady-state stromal-free Ca^{2+} concentrations very quickly fall below those of chloroplasts in darkness. Targeting of aequorin to chloroplasts as a Ca^{2+} indicator has shown that this is indeed the case (Johnson *et al.* 1995). When lights are switched off there is a very substantive rise in free chloroplast Ca^{2+} which exhibits damped circadian oscillations. Light-induced oscillations in $[Ca^{2+}]_c$ have also been reported. The oscillations may account for an apparent coupling of $[Ca^{2+}]_c$ to circadian variations in photosynthetic reactions and shape changes in *Euglena* (Lonergan 1990).

5. MULTICELLULAR Ca^{2+} SIGNALLING

Ca^{2+} signalling is not confined to single cells. Mechanical stimulation of individual cells embedded in cultured tissues can induce a propagating intercellular wave of increased $[Ca^{2+}]_c$ which is probably carried through gap junctions by (1,4,5)$InsP_3$, (Boitano *et al.* 1992). A similar propagating wave of high $[Ca^{2+}]_c$ moving through a substantial number of root cells has also been induced by simple mechanical stimulation (Legue *et al.* 1997).

In plants, cell-to-cell signalling is likely to occur mainly through plasmodesmata. Changes in Ca^{2+} and (1,4,5)$InsP_3$ levels are known to affect cell-to-cell communication (Robards & Lucas 1990). Plasmodesmata also contain Ca^{2+}-binding proteins in the desmotubule. Connections between the ER and plasmodesmata (Overall & Blackman 1996), can provide the essential elements for active Ca^{2+} signalling in tissues. Filamentous organelles such as the ER may contribute to a faster Ca^{2+} transmission because the signal may be propagated in the plane of the membrane by subtle changes in membrane potential. Multicellular Ca^{2+} signalling can then take place as a sort of Mandelbrot figure. Each cell function in a tissue is like an organelle within the cell; cell functions represent discrete entities with the capability to uptake or release Ca^{2+}.

Using the photoprotein aequorin to report $[Ca^{2+}]_c$ changes, Knight *et al.* (1993) showed that cold shock triggers a multicellular $[Ca^{2+}]_c$ increase in cotyledons which spreads from a point of origin at the cotyledon tip. Communication between tissues was also observed when roots of three-week-old aequorin-transformed seedlings were briefly chilled (Campbell *et al.* 1996). Several minutes later, waves of free $[Ca^{2+}]_c$ were detected in the leaves. When placed under anaerobic conditions, *Arabidopsis* seedlings exhibited consistent $[Ca^{2+}]_c$ spikes at periodicities of about five minutes from the root. Excision of the root caused these spikes to disappear, suggesting that some form of $[Ca^{2+}]_c$ oscillation may be involved in tissue communication (Sedbrook *et al.* 1996). How these oscillations or repetitive spikes travel is not known. The possible relation of this root $[Ca^{2+}]_c$ spiking to stochastic resonance has already been outlined (Trewavas & Malhó 1997).

6. DETECTION OF Ca^{2+} WAVES AND OSCILLATION IN PLANT CELLS

If waves and oscillations are a general mechanism for Ca^{2+} signalling, why are there so few examples in plant cells? One of the reasons may be the presence of large vacuoles in many plant cells. The vacuole often occupies most of the protoplasm, reducing the cytosol to a tiny layer compressed against the wall. This structure significantly complicates the detection of any $[Ca^{2+}]_c$ change. It is unlikely that a signal is propagated through the vacuole as propagation would be extremely slow because it would depend on the rate of diffusion. No regenerative release or amplification of the Ca^{2+} signal by reaction–diffusion mechanisms would occur. The vacuole is an efficient store of Ca^{2+} which can be released in response to specific signals. Strong evidence for this role comes from the identification of several pumps, transporters (Hedrich & Neher 1987) and channels (Alexandre *et al.* 1990; Allen *et al.* 1995) in the tonoplast.

Another reason for the 'missing' waves and oscillations may be technical limitations in current methods for measuring Ca^{2+}. As a high signal-to-noise ratio is needed to make proper measurements, cells are loaded with high concentration of dyes (*ca.* 1–50 μM) which can then act as buffers. The buffering effect of the dyes may not be enough to perturb strong vital processes such as the $[Ca^{2+}]_c$ gradient in pollen tubes, but may be enough to mask small $[Ca^{2+}]_c$ changes. Blumenfeld *et al.* (1992) found that fura-2 diminished the amplitude of $[Ca^{2+}]_c$ transients, and increased the Ca^{2+} diffusion rate and cellular time response without totally compromising cell viability.

Cells are also frequently exposed to very strong 'shock' treatments to induce strong responses that can easily be quantified. This may result in sustained $[Ca^{2+}]_c$ increases with high 'agonist' doses; $[Ca^{2+}]_c$ oscillations may only occur with milder stimuli. In animal cells the interspike period and initial latency decrease as the agonist dose is increased, but the amplitude and kinetics of the individual $[Ca^{2+}]_c$ spikes remain constant over a broad range of agonist doses (Hajnóczky & Thomas 1997).

A different but related problem is to distinguish signal variation between equipment-generated noise from naturally occurring chaotic changes. Recent investigation has suggested that cells generate a background 'noise' that may considerably improve the perception of weak signals (Trewavas & Malhó 1997). To detect and analyse this so-called stochastic resonance is one of the greatest challenges in this area of research.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

7. FUTURE PROSPECTS

Technical improvements continue to provide the researcher with a wide range of tools to investigate the function of waves, oscillations, and other facets of Ca^{2+} signalling in development. There are many caged-compounds (ions, hormones, proteins) that can be used to modulate intracellular concentration and activities and thus mimic activation of different signal cascades. Fluorescence resonance energy transfer (FRET) and fluorescence polarization anisotropy imaging (FAI) are two other technologies of great potential. FRET establishes the proximity between two different fluorophores and it has been used to measure the association between subunits of multimeric proteins (Adams *et al.* 1991) and the imaging of cAMP distributions. Other possible applications are the measurement of Ca^{2+}-dependent binding between different proteins (actin and myosin, for example). More recently, Roger Tsien's group used the principle of FRET to produce a new range of Ca^{2+} indicators (Miyawaki *et al.* 1997). They constructed tandem fusions of a blue- or cyan-emitting mutant of the green fluorescent protein (GFP), calmodulin, the calmodulin-binding peptide M13, and an enhanced green or yellow GFP. Binding of Ca^{2+} makes calmodulin wrap around the M13 domain, increasing the FRET between the flanking GFPs. Calmodulin mutations can tune the Ca^{2+} affinities to measure in the range 10^{-8} to 10^{-2} M.

FAI enables the imaging of cellular distributions of free and bound molecules. By exposing cells to an exciting source of plane polarized light, fluorescent molecules whose movement is hindered will re-emit the exciting light in the same plane which can then be detected and imaged. The distribution of bound and free molecules of any species can be recorded subsequently by imaging the total distribution of the fluorescence-tagged molecules. There are limitations to the technique which so far have prevented its use beyond an enterprising study on free and bound calmodulin during cellular contraction and locomotion (Gough & Taylor 1993). Nevertheless, technical improvements may take this further. Large protein molecules, for example, may act as though they are bound because the speed of rotation is much slower than the lifetime of the fluorescent state. Different fluorophores with long-lived excited states might be the necessary breakthrough required.

Another interesting possibility is to image ion channel distribution with fluorescent probes. These probes are currently available only to animal cells but they may also prove to be useful in plant systems. These studies would be particularly meaningful if combined with lipophilic derivatives for detecting Ca^{2+} near membrane surfaces.

But the most remarkable development of Ca^{2+} research in the past 15 years has been the uncovering of the complexity of the $[Ca^{2+}]_c$ signal. It is most likely that in turn this reflects the complexity of the cellular and cytoplasmic construction. The challenge now is to identify the spatial distribution of Ca^{2+}-regulated downstream proteins. Is it possible that cells adjust their signalling capabilities by using a type of 'pick and mix' of transducon-like entities (Trewavas & Malhó 1997)? As transducons seem to be constructed around a limited number of scaffold proteins, such as Ste5 or Bem (Hall 1998), a simple way of reconstructing the complement of transducons would be to change the basic scaffold protein, thus allowing a reassortment of kinases and other Ca^{2+}-binding proteins into different arrangements and combinations. Wherever we look at Ca^{2+} signalling complexity emerges, but this is no more than a reflection of the fact that life is complex. Perhaps the most useful outcome to any detailed investigations of Ca^{2+} signalling is the insight it has provided into the ultimate question, the nature of life itself.

We thank the Biotechnological and Biological Science Research Council and Junta Nacional de Investigação Científica (grant PBICT/P/BIA/2068/95) for funding this research. We also thank the British Council.

REFERENCES

Adams, S. R., Harootunian, A. T., Buechler, Y. T., Taylor, S. S. & Tsien, R. Y. 1991 Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* **349**, 694–697.

Alexandre, J., Lassales, J. P. & Kado, R. T. 1990 Opening of Ca^{2+} channels in isolated red beet root vacuole membrane by inositol 1,4,5-trisphosphate. *Nature* **343**, 567–570.

Allbritton, N. L., Meyer, T. & Stryer, L. 1992 Range of messenger action of calcium ion and inositol 1,4,5 triphoshate. *Science* **258**, 1812–1815.

Allen, G. J., Muir, S. R. & Sanders, D. 1995 Release of Ca^{2+} from individual vacuoles by both $InsP_3$ and cyclic ADP ribose. *Science* **268**, 735–737.

Badminton, M. N., Campbell, A. K. & Rembold, C. M. 1996 Differential regulation of nuclear and cytosolic Ca^{2+} in HeLa cells. *J. Biol. Chem.* **271**, 31 210–31 214.

Berridge, M. J. 1995 Capacitative calcium entry. *Biochem. J.* **312**, 1–11.

Blumenfeld, H., Zablow, L. & Sabatini, B. 1992 Evaluation of cellular mechanisms for modulation of calcium transients using a mathematical model for fura-2 Ca^{2+} imaging in *Aplysia* sensory neurons. *Biophys. J.* **63**, 1146–1164.

Boitano, S., Dirksen, E. R. & Sanderson, M. J. 1992 Intercellular propagation of calcium waves mediated by inositol triphosphate. *Science* **258**, 292–295.

Borchert, R. 1985 Calcium acetate induces calcium uptake and formation of calcium oxalate crystals in isolated leaflets of *Gleditsia*. *Planta* **168**, 571–578.

Campbell, A. K., Trewavas, A. J. & Knight, M. R. 1996 Calcium imaging shows differential sensitivity to cooling and communication in luminous transgenic plants. *Cell Calcium* **19**, 211–218.

Connor, J. A. 1993 Intracellular calcium mobilization by inositol triphosphate: intracellular movement and compartmentalization. *Cell Calcium* **14**, 185–200.

Dawson, A. P. 1997 Calcium signaling: how do IP3 receptors work? *Curr. Biol.* **7**, R544–R547.

De Koninck, P. & Schulman, H. 1998 Sensitivity of CaM Kinase II to the frequency of Ca^{2+} oscillations. *Science* **279**, 227–230.

Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. & Healy, J. I. 1997 Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* **386**, 855–858.

Dupont, G., Berridge, M. J. & Goldbetter, A. 1991 Signal-induced Ca^{2+} oscillations: properties of a model based on Ca^{2+}-induced Ca^{2+} release. *Cell Calcium* **12**, 73–85.

Ehrhardt, D. W., Wais, R. & Long, S. R. 1996 Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* **85**, 673–681.

Feijó, J. A., Malhó, R. & Obermeyer, G. 1995 Ion dynamics and its possible role during *in vitro* pollen germination and tube growth: a review. *Protoplasma* **187**, 155–167.

Felle, H. 1988 Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. *Planta* **174**, 495–499.

Franceschi, V. R. 1989 Calcium oxalate formation is rapid and reversible process in *Lemna minor*. *Protoplasma* **148**, 130–137.

Franceshci, V. R., Li, X., Zhang, D. & Okita, T. W. 1993 Calsequestrin-like calcium binding proteins is expressed in calcium accumulating cells of *Pistia stratiotes*. *Proc. Natn. Acad. Sci. USA* **90**, 6986–6990.

Franklin-Tong, V. E., Drøbak, B. K., Allan, A. C., Watkins, P. A. C. & Trewavas, A. J. 1996 Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow moving calcium wave propagated by inositol triphosphate. *Pl. Cell* **8**, 1305–1321.

Fricker, M. D., Gilroy, S. G. & Trewavas, A. J. 1990 Signal transduction in plant cells and the calcium message. In *Signal perception and transduction in higher plants. Cell biology*, vol. 47 (ed. R. Ranjeva & A. M. Boudet), pp. 89–102. NATO ASI series H. Berlin: Springer.

Furuya, M. & Schafer, E. 1996 Photoperception and signalling of induction reactions by different phytochromes. *Trends Pl. Sci.* **1**, 301–307.

Gilkey, J. C., Jaffe, L. F., Ridgway, E. B. & Reynolds, G. T. 1978 A free calcium wave traverses the activating egg of the medaka. *J. Cell Biol.* **76**, 448–466.

Gillot, I. & Whitaker, M. 1994 Calcium signals in and around the nucleus in sea urchin eggs. *Cell Calcium* **16**, 269–278.

Gilroy, S. & Trewavas A. J. 1994 A decade of plant signals. *Bioessays* **16**, 677–682.

Gilroy, S., Read, N. D. & Trewavas, A. J. 1990 Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. *Nature* **346**, 769–771.

Gilroy, S., Fricker, M. D., Read, N. D. & Trewavas, A. J. 1991 Role of calcium in signal transduction in *Commelina* guard cells. *Pl. Cell* **3**, 333–344.

Girard, S. & Clapham, D. 1993 Acceleration of intracellular calcium waves in *Xenopus oocytes* by calcium influx. *Science* **260**, 229–232.

Golovina, V. A. & Blaustein, M. P. 1997 Spatially and functionally distinct Ca^{2+} stores in sarcoplasmic and endoplasmic reticulum. *Science* **275**, 1643–1648.

Gong, M., Van de Luit, A. H., Knight, M. R. & Trewavas, A. J. 1998 Heat shock induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. *Pl. Physiol.* **116**, 429–437.

Goodwin, B. C. & Brière, C. 1992 A mathematical model of cytoskeletal dynamics and morphogenesis in *Acetabularia*. In *The cytoskeleton of the Algae* (ed. D. Menzel), pp. 219–238. Boca Raton, FL: CRC Press.

Gough, A. H. & Taylor, D. L. 1993 Fluorescence anisotropy imaging microscopy maps calmodulin binding during cellular contraction and locomotion. *J. Cell Biol.* **121**, 1095–1107.

Greber, U. E. & Gerace, L. 1995 Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal mediated transport into the nucleus. *J. Cell Biol.* **128**, 5–14.

Gu, X. & Spitzer, N. C. 1995 Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature* **375**, 784–787.

Hajnóczky, G. & Thomas, A. P. 1997 Minimal requirements for calcium oscillations driven by the (1,4,5)$InsP_3$ receptor. *EMBO J.* **16**, 3533–3543.

Hajnóczky, G., Robb-Gaspers, L. D., Seitz, M. B. & Thomas, A. P. 1995 Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* **82**, 415–424.

Hall, A. 1998 Rho GTPases and the actin cytoskeleton. *Science* **279**, 509–514.

Hardingham, G. E., Chawla, S., Johnson, C. & Bading, H. 1997 Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature* **385**, 260–264.

Hedrich, R. & Neher, E. 1987 Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. *Nature* **329**, 833–835.

Holdaway-Clarke, T. L., Feijo, J. A., Hackett, G. R., Kunkel, J. G. & Hepler, P. K. 1997 Pollen tube growth and the intracellular cytosolic calcium gradients oscillate in phase while extracellular calcium influx is delayed. *Pl. Cell* **9**, 1999–2010.

Horne, J. W. & Meyer, T. 1997 Elementary calcium release units induced by inositol triphosphate. *Science* **276**, 1690–1693.

Ichikawa, T., Suzuki, Y., Czaja, I., Schommer, C., Lesnick, A., Schell, J. & Walden, R. 1997 Identification and role of adenylyl cyclase in auxin signalling in higher plants. *Nature* **390**, 698–701.

Jaffe, L. 1980 Calcium explosions as triggers of development. *Ann. NY Acad. Sci.* **339**, 86–101.

Johnson, C., Knight, M. R., Kondo, T., Masson, P., Sedbrook, J., Haley, A. & Trewavas, A. J. 1995 Circadian oscillations in cytosolic and chloroplastic free calcium in transgenic luminous plants. *Science* **269**, 1863–1866.

Jouaville, L., Ichas, F., Holmuhamedov, E., Camacho, P. & Lechleiter, J. D. 1995 Synchronization of calcium waves by mitochondrial substrates in *Xenopus*. *Nature* **377**, 438–441.

Kickhoefer, V. A. & Sanjay, K. L. H. 1996 Vaults are the answer, what is the question? *Trends Cell Biol.* **6**, 174–178.

Klusener, B., Boheim, G., Lib, H., Engelberth, J. & Weiler, E. W. 1995 Gadolinium sensitive voltage dependent calcium release channels in the endoplasmic reticulum of a higher plant mechano-senstive receptor organ. *EMBO J.* **14**, 2708–2714.

Knight, H., Trewavas, A. J. & Knight, M. R. 1997 Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Pl. J.* **12**, 1067–1078.

Knight, M. R., Campbell, A. K., Smith, S. M. & Trewavas, A. J. 1991 Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**, 524–526.

Kreimer, G., Surek, B., Woodrow, I. E. & Latzkpoo, E. 1987 Calcium binding by spinach stromal proteins. *Planta* **171**, 259–265.

Kreimer, G., Melkonian, M., Holtum, J. A. M. & Latzko, E. 1988 Stromal free calcium concentration and light mediated activation of chloroplast fructose 1,6, bisphosphatase. *Pl. Physiol.* **86**, 423–428.

Lechletter, J., Girard, S., Peralta, E. & Clapham, D. 1991 Spiral calcium wave propagation and annihilation in *Xenopus laevis* oocytes. *Science* **252**, 123–126.

Legue, V., Blancafluor, I., Wymer, C., Perbal, G., Fantin, D. & Gilroy, S. 1997 Cytoplasmic free Ca^{2+} in *Arabidopsis* roots change in response to touch but not gravity. *Pl. Physiol.* **114**, 789–800.

Li, Y. Q., Chen, F., Linskens, H. F. & Cresti, M. 1994 Distribution of unesterifed and esterifed pectins in cell walls of pollen tubes of flowering plants. *Sex. Pl. Reprod.* **7**, 145–152.

Liang, F., Cunningham, K. W., Harper, J. F. & Sze, H. 1997 ECA1 complements yeast mutants defective in Ca^{2+} pumps and encodes an endoplasmic reticulum-type Ca^{2+}-ATPase in *Arabidopsis thaliana*. *Proc. Natn. Acad. Sci. USA* **94**, 8579–8584.

Lin, C., Hajnóczky, G. & Thomas, A. P. 1994 Propagation of cytosolic calcium waves into the nuclei of hepatocytes. *Cell Calcium* **16**, 247–258.

Lipp, P., Thomas, D., Berridge, M. J. & Bootman, M. D. 1997 Nuclear calcium signalling by individual cytoplasmic calcium puffs. *EMBO J.* **16**, 7166–7173.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Lonergan, T. A. 1990 Steps linking the photosynthetic light reactions to the biological clock require calcium. *Pl. Physiol.* **93**, 110–115.

Lowenstein, W. R. & Kanno, Y. 1963 Some electrical properties of a nuclear membrane examined with a microelectrode. *J. Gen. Physiol.* **46**, 1123–1127.

McAinsh, M. R., Webb, A. A. R., Taylor, J. E. & Hetherington, A. M. 1995 Stimulus-induced oscillations in guard cell cytosolic free calcium. *Pl. Cell* **7**, 1207–1219.

Malhó, R. & Trewavas, A. J. 1996 Localized apical increases of cytosolic free calcium control pollen tube orientation. *Pl. Cell* **8**, 1935–1949.

Malhó, R., Read, N. D., Pais, M. S. & Trewavas, A. J. 1994 Role of cytosolic free calcium in the reorientation of pollen tube growth. *Pl. J.* **5**, 331–341.

Malhó, R., Read, N. D., Trewavas, A. J. & Pais, M. S. 1995 Calcium channel activity during pollen tube growth and reorientation. *Pl. Cell* **7**, 1173–1184.

Matter, N., Ritz, M. F., Freyermuth, S., Rogue, P. & Malviya, A. N. 1993 Stimulation of nuclear-protein kinase-C leads to phosphorylation of nuclear inositol 1,4,5-trisphosphate receptor and accelerated calcium release by inositol 1,4,5-trisphosphate from isolated rat-liver nuclei. *J. Biol. Chem.* **268**, 732–736.

Meir, P., Juniper, B. E. & Evans, D. E. 1991 Regulation of free calcium concentration in the pitchers of the carnivorous plant *Sarracenia purpurea*: a model for calcium in the higher plant apoplast? *Ann. Bot.* **68**, 557–561.

Messerli, M. & Robinson, K. R. 1997 Tip localized Ca^{2+} pulses are coincident with peak pulsatile growth rates in pollen tubes of *Lilium longiflorum*. *J. Cell Sci.* **110**, 1269–1278.

Meyer, T., Hanson, P. I., Stryer, L. & Schulman, H. 1992 Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* **256**, 1190–1202.

Meyer, T., Allbritton, N. L. & Oancea, E. 1995 Regulation of nuclear calcium concentration. In *Calcium waves, gradients and oscillations* (ed. X. Bock & G. R. Ackrill), pp. 252–262. Chichester, UK: Wiley.

Miller, D. B., Callaham, D. A., Gross, D. J. & Hepler, P. K. 1992 Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. *J. Cell Sci.* **101**, 7–12.

Miyawaki, A., Liopis, J., Heim, R., McCafferey, J. C., Adams, J. A., Ikura, M. & Tsien, R. 1997 Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–887.

Overall, R. L. & Blackman, L. M. 1996 A model of the macromolecular structure of plasmodesmata. *Trends Pl. Sci.* **1**, 307–311.

Perez-Terzic, C., Pyle, J., Jaconi, M., Stehno-Bittel, L. & Clapham, D. E. 1996 Conformational states of the nuclear pore induced by depletion of nuclear calcium stores. *Science* **273**, 1875–1877.

Pierson, E. S., Miller, D. D., Callaham, D. A., van Aken, J., Hackett, G. & Hepler, P. K. 1996 Tip-localized calcium entry fluctuates during pollen tube growth. *Devl Biol.* **174**, 160–173.

Prentky, M., Glennon, M. C., Thomas, A. P., Morris, R. L., Matschinsky, F. M. & Corkey, B. E. 1988 Cell-specific patterns of oscillating free Ca^{2+} in carbamylcholine-stimulated insulinoma cell. *J. Biol. Chem.* **263**, 11 044–11 047.

Putney, J. W. 1998 Calcium signalling: up, down, up, down . . . What's the point? *Science* **279**, 191.

Robards, A. W. & Lucas, W. J. 1990 Plasmodesmata. *A. Rev. Pl. Physiol. Pl. Molec. Biol.* **41**, 369–419.

Sedbrook, J. C., Kronenbusch, P. J., Borisy, G. G., Trewavas, A. J. & Masson, P. 1996 Transgenic aequorin reveals organ specific cytosolic Ca^{2+} responses to anoxia in *Arabidopsis thaliana* seedlings. *Pl. Physiol.* **111**, 243–257.

Shacklock, P. S., Read, N. D. & Trewavas, A. J. 1992 Cytosolic free calcium mediates red light induced photomorphogenesis. *Nature* **358**, 153–155.

Steer, M. & Steer, J. 1990 Pollen tube tip growth. *New Phytol.* **111**, 323–358.

Stehno-Bittel, L., Perez-Terzic, C. & Clapham, D. E. 1995 Diffusion across the nuclear envelope inhibited by depletion of the nuclear Ca^{2+} store. *Science* **270**, 1835–1838.

Subbaiah, C. C., Bush, D. S. & Sachs, M. M. 1994 Elevation of cytosolic calcium precedes anoxi gene expression in maize suspension cultured cells. *Pl. Cell* **6**, 1747–1762.

Subramanian, R., Després, C. & Brisson, N. 1997 A functional homolog of mammalian protein kinase C participates in the elicitor induced defense response in potato. *Pl. Cell* **9**, 653–664.

Sullivan, K. M. C. & Wilson, K. L. 1994 A new role for (1,4,5) $InsP_3$ receptors: Ca^{2+} release during nuclear vesicle fusion. *Cell Calcium* **16**, 314–321.

Takahasi, K., Isobe, M., Knight, M. R., Trewavas, A. J. & Muto, S. 1997 Hypoosmotic shock induces increases in cytosolic free calcium in tobacco suspension culture cells. *Pl. Physiol.* **113**, 587–594.

Tang, Y. & Othmer, H. G. 1995 Frequency encoding in excitable systems with applications to calcium oscillations. *Proc. Natn. Acad. Sci. USA* **92**, 7869–7873.

Taylor, A. R., Manison, N. F. H., Fernandez, C., Wood, J. & Brownlee, C. 1996 Spatial organization of calcium signaling involved in volume control in the *Fucus* rhizoid. *Pl. Cell* **8**, 2015–2031.

Thomas, A. P., Bird, G. S. J., Hajnóczky, G., Robb-Gaspers, L. D. & Putney, J. W. Jr 1996 Spatial and temporal aspects of cellular calcium signalling. *FASEB J.* **10**, 1505–1517.

Trewavas, A. J. & Malhó, R. 1997 Signal perception and transduction: the origin of the phenotype. *Pl. Cell* **7**, 1181–1195.

Tsunuda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M. & Zuker, C. S. 1997 A multivalent PDZ domain protein assembles signalling complexes in a G-protein coupled cascade. *Nature* **388**, 243–249.

Von Tscharner, V., Deranleau, D. A. & Bagglionin, M. 1986 Calcium fluxes and calcium buffering in human neutrophils. *J. Biol. Chem.* **261**, 10 163–10 168.

Van der Luit, A. H., Olivari, C., Haley, A., Knight, M. R. & Trewavas, A. J. 1998 Calmodulin gene expression regulated by wind-induced nucleoplasmic calcium levels in *Nicotiana plumbaginifolia*. (Submitted.)

Ward, J. M., Pei, Z. M. & Schroeder, J. I. 1995 Roles of ion channels in initiation of signal transduction in higher plants. *Pl. Cell* **7**, 833–844.

Webb, A. A. R., McAinsh, M. R., Taylor, J. E. & Hetherington, A. M. 1996 Calcium ions as intracellular second messengers in higher plants. *Adv. Bot. Res.* **22**, 45–96.

Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. 1986 Repetitive transient rises in cytoplasmic free calcium in hormone stimulated hepatocytes. *Nature* **319**, 600–602.

Wymer, C. L., Bibikova, T. N. & Gilroy, S. 1997 Cytoplasmic free calcium distributions during the development of root hairs of *Arabidopsis thaliana*. *Pl. J.* **12**, 427–439.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES