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Intracellular Ca<latex>$^{2+}$</latex> Release by InsP<latex>$_{3}$</latex> in Plants and Effect of Buffers on Ca<latex>$^{2+}$</latex> Diffusion

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Intracellular Ca^{2+} release by InsP_3 in plants and effect of buffers on Ca^{2+} diffusion

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SUMMARY

Several evidences favour the idea that InsP_3, a well known second messenger in animal cells, can also be used by plants. The studies supporting this hypothesis are reviewed, with special emphasis on the experiments concerning the release of vacuolar Ca^{2+} by InsP_3. We also report patch-clamp experiments on isolated red beet vacuoles. In some of the patches, the InsP_3-mediated Ca^{2+} channel exhibited a 50 pS conductance state beside the 30 pS state mentioned in a previous work. The InsP_3-mediated vacuolar current was smaller with Ba^{2+} or Sr^{2+} ions replacing Ca^{2+} in both the pipette and external solutions. Heparin (1 mg ml^{-1}) in the extravacuolar medium decreased by 60% only, the InsP_3-mediated Ca^{2+} current. The inhibitory effect of heparin was not specific: with the same concentration used, another current in the tonoplast was reduced by 46%. The coupling by diffusion of Ca^{2+} ions between the InsP_3-mediated Ca^{2+} channel and the Ca^{2+}-sensitive channel in the tonoplast has been studied. The coupling was dependent on the Ca^{2+} buffers (BAPTA or EGTA) in the extravacuolar medium. At least 20 mM BAPTA were necessary to prevent coupling between channels. In the range of concentrations tested (less than 30 mM), EGTA could not prevent coupling. A review of the theoretical models describing diffusion in a buffered medium is presented, and a simplified version from the models is proposed to discuss our results.

1. INTRODUCTION

In a few cases, variations in cytosolic Ca^{2+} have been reported to accompany the transduction of signals in plant cells (Gehring *et al.* 1990; McAinsh *et al.* 1990; Schroeder & Hagiwara 1990; Gilroy *et al.* 1991; Knight *et al.* 1991). There are two basic questions concerning the occurrence of a Ca^{2+} change in the cytosol. The first one deals with the origin of the signal: the ions used to modify Ca^{2+} concentration can be provided by extracellular (Schroeder & Hagiwara 1990) or (and) intracellular (Gilroy *et al.* 1991) Ca^{2+} pools. One of the striking differences between animals and plants is the presence of a large vacuolar Ca^{2+} reservoir in plant cells. At least in principle, the less than μM cytosolic Ca^{2+} could be modified by Ca^{2+} from the vacuolar compartment only. It is now established that InsP_3 can mobilize this Ca^{2+} pool by opening a channel in the tonoplast. In this paper, we review previous studies on the possible use of InsP_3 by plant cells, and we present our last results on the InsP_3-mediated channel from isolated red beet vacuoles.

A second question concerns the propagation of the Ca^{2+} signal itself. Even if it is still common use to speak of 'the' cytosolic Ca^{2+} concentration, it must be kept in mind that the distribution of Ca^{2+} ions may be non uniform in the cytosol. Recent measurements using Ca^{2+} imaging by fluorescent dyes, describe spatial and temporal variations in cytosolic Ca^{2+} during the transduction of a cellular signal (Gilroy *et al.* 1991). When a channel or a Ca^{2+} pump are activated in a membrane delimiting the cytosolic compartment, the ions released near these active sites will eventually spread in this compartment. The propagation of this Ca^{2+} signal depends on the structural properties of the cytosol. There are some evidences for the influence of such a structure in the propagation of 'wave like' signals. However, very near the sites (channels or pumps), it is usually assumed that the movement of Ca^{2+} ions is only due to diffusion: the Ca^{2+} buffer strength of the cytosol is the only 'structural' parameter in this case. We will review the theoretical models used to explain the effect of buffers on the propagation of a Ca^{2+} signal near channels or pumps.

In some experiments, buffers are used to disrupt a stimulus-response coupling. When there is an extended chain of reactions between the stimulus and the response, the buffer is expected to block the step(s) involving a change in cytosolic Ca^{2+}. The isolated red beet vacuole offers a very simple example of coupling between a stimulus and its response at the cellular level. The stimulus (InsP_3) opens a Ca^{2+} channel in the tonoplast, and the Ca^{2+} signal is represented by the diffusion of Ca^{2+} ions in the extravacuolar medium. The response is the change in activity of a Ca^{2+}-sensitive channel, also present in the tonoplast. We report here patch-clamp experiments performed on red beet vacuoles to study the effects of buffers on a

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Ca^{2+} signal. We analyse these effects by using a simplified model for the diffusion of Ca^{2+} in a buffered medium.

2. THE USE OF $INSP_3$ BY PLANT CELLS

(*a*) *Phosphoinositides and inositol phosphates in plants*

Reviews covering the different aspects of phosphoinositide metabolism in plants have been recently published (Boss 1988, Lehle 1990). They insist upon technical difficulties in the identification and quantification of inositol phosphates in different plant tissues. In fusogenic carrot cells, Rincon *et al.* 1988 find no evidence for the existence of Ins(1, 4)P_2 or InsP_3. In contrast, in *Samanea saman* pulvini, a short light treatment elicits a rapid change in phosphoinositide components (Morse *et al.* 1987) and a 30% increase in Ins(1, 4)P_2 plus InsP_3 is detected in this tissue. Since the first report on the existence of phosphoinositides in plants (Boss & Massel 1985), intensive studies have established that plant and animal cells do contain polyphosphoinositol lipids.

(*b*) *Enzymes system*

One of the interesting regulations by Ca^{2+} ions, involving enzymes in the phosphatidylinositol cycle has been found in plants for physiological concentrations of Ca^{2+} (Kamada & Muto 1991). Important differences in the regulation of phosphoinositide metabolism have been established between plants and animals. The phosphatidylinositol bisphosphate level is much lower in plants (Coté *et al.* 1989) than in animal cells (Abdel–Latif *et al.* 1985). A report by Drobak *et al.* (1991) demonstrates that enzymes involved in inositol phosphate metabolism are present in plant tissues. The authors have detected configurations of inositol phosphates which are not mentioned in other mammalian systems. This suggests that metabolism of InsP_3 is complex and very particular, when compared to other eukaryote cells.

(*c*) *Site(s) of action of $InsP_3$*

The InsP_3-mediated Ca^{2+} release has been studied according to various experimental approaches. Most of the protocols use exogenous InsP_3. The first evidence for an InsP_3-mediated Ca^{2+} release has been obtained from experiments performed on microsomes from zucchini (Drobak & Ferguson 1985). Other authors also working on subcellular fractions, have confirmed this result (Reddy & Poovaiah 1987; Canut *et al.* 1989; Lehle & Ettlinger 1990). Ca^{2+} is first loaded into intracellular compartments in the presence of ATP. After accumulation of Ca^{2+} by an ATP-dependent Ca^{2+} pump located in the microsome, the release of Ca^{2+} following addition of InsP_3 is measured. The release is transient and rapid (less than 0.5 min); it is followed by a slower period of Ca^{2+} reuptake. In these experiments, the amount of Ca^{2+} released never exceeds 20% of the Ca^{2+} sequestered into the microsomes (Brosnan 1990). Monovalent ions seem to be required to observe InsP_3-mediated Ca^{2+} release (Canut *et al.* 1989). The data presented suggest that an influx of potassium functions to balance the efflux of positive charges during the Ca^{2+} release. In most of the experiments with microsomes, membrane fractions were not defined with enough accuracy, and it was not possible to determine which of the cell membranes contained the InsP_3 receptor. Recent studies demonstrate that at least *in vitro*, the vacuole responds to InsP_3 by releasing Ca^{2+} in the external medium. Schumaker & Sze (1987) use tonoplast vesicles from oat roots, preloaded with calcium via a H^+/Ca^{2+} antiport system. They demonstrate that InsP_3 specifically releases Ca^{2+} in a concentration dependent manner. Ranjeva *et al.* (1988) confirm the previous data, by monitoring the fluorescence of the Ca^{2+} released in the external medium by intact vacuoles isolated from *Acer pseudoplatanus*. Cornelius *et al.* (1989) also measured a Ca^{2+} release from intact vacuoles (*Neurospora crassa*) with two independent methods (calcium binding to fura-2 and release of ^{45}Ca). In summary, many reports indicate that there is an InsP_3-sensitive site in the vacuolar membrane. However, one cannot discard other sites on intracellular membranes like the endoplasmic reticulum.

(*d*) *Mechanism of the $InsP_3$-mediated Ca^{2+} release*

The data previously reported have strongly suggested that the release of Ca^{2+} by InsP_3 occurs through an ionic channel. Using patch-clamp techniques (Hamill *et al.* 1981) on isolated red beet vacuoles, Alexandre *et al.* (1990) demonstrate that InsP_3 specifically releases Ca^{2+} by activating a Ca^{2+} channel. This channel is activated by voltage over the physiological range (-20 to $+100$ mV, with potential refered to the cytosol). The single channel conductance is 30 pS (5 mM Ca^{2+} on the vacuolar side and 1 mM on the cytoplasmic side). A higher conductance state was sometimes recorded in both vacuole-attached and outside out configurations. In most patches, many flickering events were present for both states. Figure 1 describes an experiment performed in a vacuole-attached patch. Similar records ($n=5$) with substates give values of 30 pS and 50 pS for the two conductance states. The presence of multiple conductance states is found with InsP_3-gated channels in the reticulum of canine cerebellum. The channel exhibits four conductance levels that are multiples of a unit conductance step. The vacuolar current induced by 10^{-6} M InsP_3 was determined as explained in a previous report (Alexandre *et al.* 1990). The other currents were blocked with 10^{-3} M Ca^{2+} and 10^{-4} M Zn^{2+} (Hedrich & Neher 1987, Hedrich & Kurkdjian 1988). Figures 2 and 3 describe experiments performed in whole-vacuole configuration. As shown in figure 2, with mole-for-mole replacement of $CaCl_2$ with $BaCl_2$ or $SrCl_2$ in both external and pipette solutions, the InsP_3-induced currents were smaller for Ba^{2+} and Sr^{2+} than for Ca^{2+} ions. In all cases, these currents increased for positive potentials and were blocked for potentials more negative than -20 mV.

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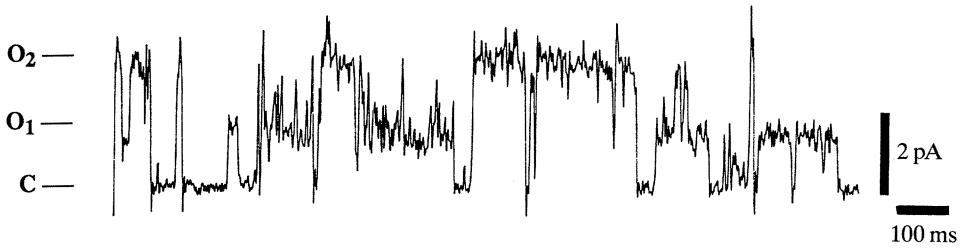

Figure 1. Conductance states in the $InsP_3$-activated Ca^{2+} channel. Single-channel recording for a vacuole attached patch with 10^{-6} M $InsP_3$, 10^{-3} M $CaCl_2$, 10^{-4} M $ZnCl_2$ in the pipette and external solutions (5×10^{-3} M Tris, pH adjusted to 7.5 with MES). Pipette potential: -60 mV. O_1, O_2: conductance states; C: closed state. The large conductance state was obtained in outside–out patches as well, but it was not present in each of the patches tested. For all the experiments described, figure 1 to figure 5, the pipette and the external solutions also contained: 0.1 M KCl, 0.6 M Sorbitol, 2×10^{-3} M $MgCl_2$. The preparation of vacuoles, the current recordings and the analysis of data have been described elsewhere (Alexandre *et al.* 1990; Alexandre & Lassalles 1991).

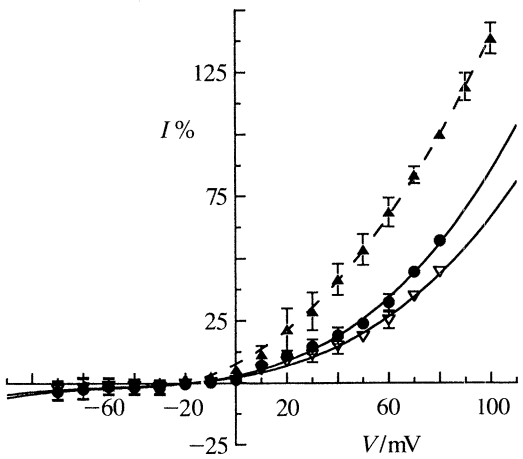

Figure 2. Effect of divalent cations on the $InsP_3$-induced current. The whole-vacuole current was measured either with Ca^{2+}, Ba^{2+} or Sr^{2+} in the pipette and external solutions. The mean currents at different voltages were normalized to the mean current at $+80$ mV with Ca^{2+} (410 pA), to reduce variability between vacuoles. $I\%$ was found by multiplying the normalized values by 100. Symbols: filled triangles, Ca^{2+} ($n=8$); filled circles, Ba^{2+} ($n=3$); open triangles, Sr^{2+} ($n=3$). Pipette medium: 5×10^{-3} M (Ca^{2+}, Ba^{2+} or Sr^{2+}), 5×10^{-3} M MES, pH adjusted to 5.5 with Tris. External medium: 10^{-3} M (Ca^{2+}, Ba^{2+} or Sr^{2+}), 5×10^{-3} M Tris, pH adjusted to 7.5 with MES.

The voltage dependence of the channel for Ba^{2+} and Sr^{2+} was different from the one obtained with Ca^{2+}.

In animal cells, the glycosaminoglycan heparin has been reported to be an inhibitor of the $InsP_3$-induced Ca^{2+} current. In plants, Brosnan & Sanders (1990) report an inhibition of this current in red beet microsomes, by 5 μg ml^{-1} heparin. In contrast, heparin has no effect on the $InsP_3$-mediated Ca^{2+} release of *Neurospora crassa* vacuoles (Cornelius *et al.* 1989). In a set of experiments with red beet vacuoles, we have examined the effect of heparin on the $InsP_3$-induced current in the whole-vacuole configuration, with high extravacuolar Ca^{2+} concentration (10^{-3} M). A 60% decrease in the $InsP_3$-induced current was obtained (figure 3) with 1 mg ml^{-1} heparin (CHOAY, S. A, Paris, France). To verify if heparin was effective specifically on the $InsP_3$-induced current, we looked for a possible effect of heparin on another current in the tonoplast. The tonoplast of red beet vacuoles contains a Ca^{2+} sensitive current. In 10^{-3} M Ca^{2+}, this current exhibits a strong rectification (figure 4, open diamonds), but remains small for positive values of the vacuolar potential. In 10^{-7} M free Ca^{2+} (figure 4, filled triangles), the current reaches values similar in size with the $InsP_3$-induced current. When vacuoles were clamped at $+80$ mV in a low Ca^{2+} solution, the addition of 1 mg ml^{-1} heparin in the external medium reduced the current (figure 4) by $46 \pm 4\%$ ($n=4$). These experiments suggest that heparin decreases in a non specific way the $InsP_3$-induced current. Brosnan & Sanders find that heparin, a widely used inhibitor of $InsP_3$ receptors in animal cells is effective on *Beta vulgaris*. The experiments that we report here show that if heparin inhibits the $InsP_3$ induced current of red beet vacuole, it also inhibits another type of channel of this same vacuole. So even if heparin has already been used to

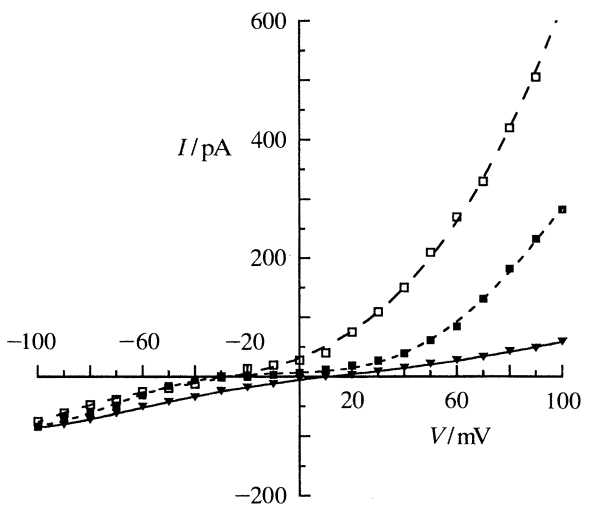

Figure 3. Effect of heparin on the $InsP_3$-induced current. The whole vacuole currents were measured on the same vacuole. *I-V* curves were obtained, first without (filled triangles), then with 10^{-6} M $InsP_3$ (open squares) and finally when 10^{-6} M $InsP_3$ and 1 mg ml^{-1} heparin were added in the external medium (filled squares). Pipette medium: 5×10^{-3} M $CaCl_2$, 5×10^{-3} M MES, pH adjusted to 5.5 with Tris. External medium: 10^{-3} M $CaCl_2$, 10^{-4} M $ZnCl_2$, 5×10^{-3} M Tris, pH adjusted to 7.5 with MES.

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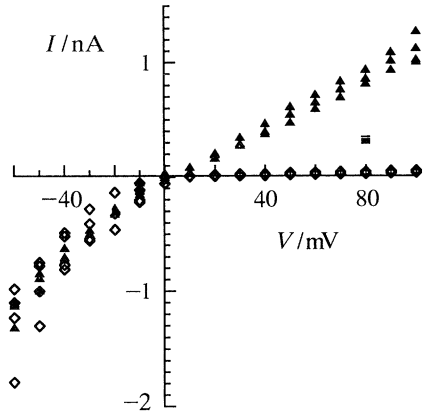

Figure 4. Effect of heparin on the Ca^{2+}-sensitive current. *I-V* curves for four vacuoles were obtained with 10^{-3} M $CaCl_2$ in the external medium (open diamonds). Another set of four vacuoles was used in the next experiments. For each of these vacuoles, an *I-V* curve (filled triangles) was obtained with 10^{-7} M free Ca^{2+} (1.5×10^{-3} M Ca^{2+} and 2×10^{-3} M EGTA at pH 7.5) in the external medium. The vacuole was then clamped at +80 mV and 1 mg/ml^{-1} heparin added in the external medium. A new steady-state value for the current was recorded (dashed line). Pipette medium: 5×10^{-3} M $CaCl_2$, 5×10^{-3} M MES, pH adjusted to 5.5 with Tris. External medium: 5×10^{-3} M Tris, pH adjusted to 7.5 with MES.

purify an $InsP_3$ receptor in rat cerebellum (Supattapone *et al.* 1988), it is doubtful that it can be used for purification of an $InsP_3$ receptor in plant cell membrane. Inhibition of the $InsP_3$-induced Ca^{2+} release by TMB8 also seems to require fairly large amounts of inhibitor (table 1).

(*e*) *Properties of the Ca^{2+} release by $InsP_3$*

The half-maximum $InsP_3$-induced calcium release was obtained for an $InsP_3$ concentration, K_m, varying between 0.2 and 5 μM on the different membranes studied (table 1). As a comparison, values between 0.1 and 1 μM are given for intracellular membranes in animal cells (Berridge & Irvine 1984; Ehrhlich & Watras 1988; Watras *et al.* 1991). A cooperative opening of a Ca^{2+} channel by $InsP_3$ has been mentioned in rat basophilic leukemia cells (Meyer *et al.* 1988). However this is still controversial (Watras *et al.* 1991); in the experiments with plants listed (table 1), there is no report of a cooperative effect of $InsP_3$ on calcium release.

There is no mention of an inhibitory effect of high Ca^{2+} concentration on the $InsP_3$-induced current in plants. In our experiments on red beet vacuole, $InsP_3$ was still effective with millimolar concentrations of Ca^{2+} in the extravacuolar medium (Alexandre *et al.* 1990). In animal cells, the sensitivity of the $InsP_3$-mediated Ca^{2+} release to high Ca^{2+} concentrations is now explained by the regulation of the $InsP_3$ receptor by a Ca^{2+} binding protein, sometimes associated to this receptor. However the $InsP_3$ receptor itself should be Ca^{2+} insensitive (Supattapone *et al.* 1988).

(*f*) *$InsP_3$ and endogenous Ca^{2+}*

In all the experiments quoted above, the Ca^{2+} released by $InsP_3$ was preloaded into the organelle under study. In several experiments, microinjection of $InsP_3$ in a plant cell produces a signal by mobilizing Ca^{2+} from internal stores (vacuole or endoplasmic reticulum). Using caged $InsP_3$ on stomatal guard cells of *Commelina communis*, Gilroy *et al.* (1990) observe an increase in cytosolic Ca^{2+} and the closure of stomata. Blatt *et al.* (1990) also performed experiments with caged $InsP_3$, on guard cells of *Vicia faba*. The effect of $InsP_3$ is detected by a change in activity of K^+ channels in the plasma membrane. In experiments by Thiel *et al.* (1990) and Förster (1990), microinjections of free $InsP_3$ in algal cells results in a change of the plasma membrane conductance. As previously noticed by Irvine (1990), from what is now known on the effect of $InsP_3$ in plant cells, it can be concluded that plants use $InsP_3$, at least for the propagation of fast signals.

3. CALCIUM CHANGE AT THE SUBCELLULAR LEVEL

(*a*) *Localized Ca^{2+} change in the cytosol*

The cascade of events following the opening of a Ca^{2+} channel by $InsP_3$ starts by a change in cytosolic Ca^{2+}, already noticed by several authors (Blatt *et al.* 1990;

Table 1.

material	authors	techniques	K_m	inhibitor
vacuolar membrane vesicles of Oat Roots	Schumaker & Sze (1987)	$^{45}Ca^{2+}$	0.6 μM	TMB8 250 μM
vacuoles *Neurospora crassa*	Cornelius *et al.* (1989)	$^{45}Ca^{2+}$ Fura 2	5.28 μM	dantrolene[a]
vacuoles *Acer pseudoplatanus*	Ranjeva *et al.* (1988)	Quin 2	0.2 μM	TMB8 200 μM
microsomes *Beta vulgaris*	Brosnan & Sanders (1990, 1992)	$^{45}Ca^{2+}$	0.54 μM	TMB8 200 μM
vacuoles *Beta vulgaris*	Alexandre *et al.* (1990)	patch-clamp	0.22 μM	TMB8 100 μM
unicellular green alga *Eresmosphaera viridis*	Förster (1990)	microelectrode		TMB8 200 μM

[a] There is no indication of the concentration used in this experiment

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Gilroy *et al.* 1990; Thiel *et al.* 1990; Förster 1990). Opening an $InsP_3$-mediated Ca^{2+} channel is not the only way to modify cytosolic Ca^{2+}, and other Ca^{2+} channels and Ca^{2+} pumps have already been mentioned in plants (Schroeder & Thuleau 1991; Johannes *et al.* 1991). Modification of the distribution of cytosolic Ca^{2+} in space at a subcellular level has been reported in several experiments. Brownlee & Wood (1986), by use of microelectrode measurements, found higher values for cytoplasmic Ca^{2+} in rhizoid tip, than in sub-tip regions of *Fucus serratus*. Speknejder *et al.* (1989) studied the effect of various BAPTA-type buffers on the development of fucoid eggs. The building of a high calcium region located in the nascent tip seems to be an important step in the development. The authors found that millimolar amounts of the mobile BAPTA buffers seem to prevent a calcium increase in the nascent zone, by facilitating diffusion of Ca^{2+} away from the zone. Gilroy *et al.* 1991 used Ca^{2+}-sensitive indicators and image analysis to detect modification of cytosolic Ca^{2+} on guard cell of *Commelina communis*. Their experiments indicate that spatially localized increases in cytosolic Ca^{2+} occur around the nucleus and the vacuole, and precede stomatal closure.

The calcium signal consisting of spatial gradients and transient changes in Ca^{2+}, is very sensitive to the buffer strength in the cytosol. Blatt *et al.* (1990), showed that exogenous $InsP_3$ reversibly inactivates K^+ channels of the plasmalemma membrane of guard cells. Control is abolished by raising the Ca^{2+} buffer capacity of the cytoplasm with 20 mM EGTA or BAPTA. A similar effect has been obtained in characean algae (Thiel *et al.* 1990): $InsP_3$ is not effective when added with 30 mM EGTA. In experiments on Eremosphaera, Förster (1990) used 20 mM BAPTA to abolish the activating effect of the $InsP_3$-mediated Ca^{2+} release on a Ca^{2+}-dependent K^+ channel. In fucoid eggs, Speknejder *et al.* (1989) used 5 mM BAPTA to damp a spatial gradient.

(*b*) *Ca^{2+} diffusion in an homogeneous buffered medium*

The importance of Ca^{2+} buffers on the propagation of a Ca^{2+} signal in the cytoplasm has been modelled by several authors under various experimental conditions. In the models, a change in cytosolic Ca^{2+}, δCa, initially occurs through opening of calcium channels, or activation of calcium pumps located in a membrane. δCa is then propagated by diffusion in an isotropic medium containing various calcium buffers. The classical equations of simultaneous diffusion and chemical reactions are used (Crank 1967) to study the influence of the buffer parameters on the space and time dependence of δCa. Simon & Llinas (1985) model the effect of a Ca^{2+} channel opening in a medium containing a mobile buffer. The authors indicate that the change in cytoplasmic Ca^{2+} is highly localized and may be effective only immediately near the channel itself. The perturbation around a cardiac Ca^{2+} channel has been studied by Bers & Peskoff (1991). In their model, the Ca^{2+} buffer consists of the fixed Ca^{2+} binding sites of the sarcolemma. The number of sites is about 0.6 site per micrometer, and the binding reaction between calcium ions and the membrane is linear. Under these conditions, the authors demonstrate that the Ca^{2+} binding sites may contribute significantly to the buffering of intracellular Ca^{2+}. However other intracellular Ca^{2+} buffers may also modify the Ca^{2+} spread near a channel mouth. Nunogaki & Kasai (1988) propose a model to describe the effect of a mobile buffer on the diffusion of protons near a H^+ channel. The mobile buffer concentration is regarded as constant with respect to time and uniform with respect to space, because of the large buffer concentration compared to the free H^+ concentration. The authors discuss the steady-state profile for H^+ ions near a channel or in small vesicles. The model can be applied to the opening of Ca^{2+} channels in an intracellular compartment, because of the same basic assumption concerning the diffusion of H^+ or Ca^{2+} ions. The control of cytosolic Ca^{2+} by perfusing a patch pipette with a mobile buffer was studied by Neher (1986) and Mathias *et al.* (1990). The mobile buffer may be considered as a shuttle, which carries Ca^{2+} from the pipette to the cell, then diffuses back when empty. The fixed buffers in the cytosol greatly increase the time for equilibration of the pipette and intracellular Ca^{2+}. In a more complicated model, Sala & Cruz (1990) consider a spherical cell in which three different Ca^{2+} buffers are present. A buffer is characterized by its capacity, mobility and kinetic constants. The Ca^{2+} signal at the cell surface incorporates pump, leak and Ca^{2+} channel. Up to 15 parameters are used in the simulations, to calculate the time dependency of cytosolic Ca^{2+} at several locations in the cell. As underlined by the authors, an adequate combination of the parameters determines the depth reached by the Ca^{2+} signal.

There is a general constraint in a theoretical model: it is difficult to make predictions when the parameters for the model are not determined with enough accuracy. This is the case with the models presented here because the values of the parameters of all the Ca^{2+} buffers in the cytosol have not yet been determined.

(*c*) *Direct coupling between a Ca^{2+} signal and a Ca^{2+}-dependent channel*

We will now present experimental data obtained on a system in which a Ca^{2+} signal is propagated by diffusion between a Ca^{2+} channel and a Ca^{2+}-sensitive channel.

In a previous paper, we reported indirect evidence for a Ca^{2+} change located near the tonoplast membrane of isolated red beet vacuoles (Alexandre & Lassalles 1990). The tonoplast contains several types of channels (Alexandre & Lassalles 1991): among them, an $InsP_3$-mediated Ca^{2+} channel and a Ca^{2+}-sensitive channel. A coupling by diffusion between the two channels was already suggested. To test this hypothesis, new experiments were performed in 10^{-7} M free Ca^{2+} solution. Isolated vacuoles were clamped at +30 mV in different concentrations of

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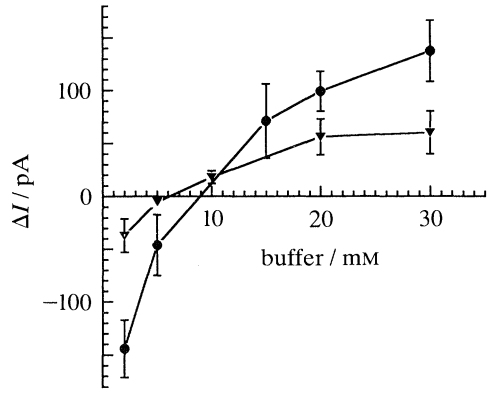

Figure 5. Effect of calcium buffers on the vacuolar current. Vacuoles were clamped at +30 mV and 10^{-6} M $InsP_3$ was added to the external medium. The change in vacuolar current ΔI, was measured for different Ca^{2+} buffer strength in the external medium. The ratio r = total Ca^{2+}/(total buffer − total Ca^{2+}) was kept constant: $r = 3$ for EGTA (filled circles); $r = 1$ for BAPTA (filled triangles). These values correspond to a free Ca^{2+} concentration close to 10^{-7} M for all the solutions used. The apparent dissociation constant for EGTA at pH 7.5 is: 2.6×10^{-8} M (Ringbom 1967), and for BAPTA: 10^{-7} M (Tsien 1980). Pipette medium: 5×10^{-3} M $CaCl_2$, 5×10^{-2} M MES, pH adjusted to 5.5 with Tris. External medium: 5×10^{-2} M Tris, pH adjusted to 7.5 with MES. Filled circles and triangles represent mean values ($n = 4$) ± s.d.

EGTA and BAPTA buffers. The change in vacuolar current induced by 10^{-6} M $InsP_3$ was measured as follows, for each selected buffer solution. The steady state value I_1 was first recorded. When $InsP_3$ was added to the extra-vacuolar medium, the vacuolar current reached a new value, I_2. Variations of $\Delta I = I_2 - I_1$ as a function of buffer concentrations are represented in figure 5. In BAPTA experiments, ΔI reached a plateau for the buffer concentrations larger than 20 mM. For the smaller concentrations and for all the EGTA concentrations used, ΔI strongly depended on the buffer concentration. A plateau is expected for the large values of the Ca^{2+} buffer preventing coupling by diffusion between the channels. Under these conditions, the Ca^{2+}-sensitive current and the $InsP_3$-induced Ca^{2+} current will not be affected by a change in buffer concentrations and ΔI will remain constant. When the buffer concentration is decreased to the point where Ca^{2+} released by $InsP_3$ start closing Ca^{2+}-sensitive channels, ΔI will decrease. Interpretation of ΔI for EGTA is less obvious because the H^+ released by EGTA can modify the Ca^{2+}-sensitive current and the affinity of $InsP_3$ for membrane receptors (Worley *et al.* 1987).

$InsP_3$ is not the only parameter controlling a Ca^{2+} channel in the tonoplast. Johannes *et al.* (1992) report that a voltage gated Ca^{2+} channel is present in the sugar beet vacuole. More generally, other pathways allowing a Ca^{2+} exchange between the vacuole and the cytoplasm are probably still unknown. However, our experiments with the $InsP_3$-gated Ca^{2+} channel suggest that, whenever one of the Ca^{2+} fluxes through the tonoplast is modified, the Ca^{2+}-sensitive channel can be affected.

A coupling by diffusion between channels by Ca^{2+} has already been mentioned by Marty & Neher (1985) in bovine chromaffin cells. The cell membrane contains a type of K^+ channel which is activated by micromolar cytoplasmic Ca^{2+}. According to authors, the capacity of the buffer (EGTA or BAPTA) solutions used, together with the values of the Ca^{2+} flux through the cell membrane, dismiss the possibility of an homogeneous increase of Ca^{2+} over the entire cytosolic compartment. Measurements with various buffer solutions strongly suggest the possibility of a local increase in Ca^{2+} near the K^+ channels. 5.5 millimolar BAPTA nearly suppresses this effect. BAPTA is more efficient than EGTA, but there is no correlation between the activation of the K^+ channel and the value of the equilibrium capacity of the buffer.

(*d*) *Use of characteristic time and characteristic length in Ca^{2+} diffusion problems*

In the last part of this paper, we will try to understand why large amounts of Ca^{2+} buffers are necessary to suppress coupling by diffusion in the above experiments. We will use an approach inspired from Nunogaki & Kasai (1988), to approximate the effect of buffers on Ca^{2+} diffusion. We consider a medium in which the Ca^{2+} buffer properties can be lumped in a single equation:

$$B + Ca \underset{K_d}{\overset{K_b}{\rightleftharpoons}} B\text{–}Ca, \qquad (1)$$

K_b, K_d: rate constants

$K = K_d/K_b$: dissociation constant of the buffer

Ca, B: equilibrium concentrations of free Ca^{2+} and buffer

B–Ca: equilibrium concentrations of Ca^{2+}-bound buffer

Analysis by Crank (1967, p. 121) was first used to solve the problem for the diffusion of Ca^{2+} in a buffered medium, by assuming that the buffering reaction (1) occurs instantaneously and that the buffer is never saturated. Under these conditions, Ca^{2+} appear to be diffusing in a buffered medium with an apparent diffusion constant, D', smaller than the diffusion constant, D, of calcium ions in a free medium. However this approach has been questioned because buffering is diffusion limited and buffer is never of infinite concentration (Simon & Llinas 1985, Irving *et al.* 1990).

Another simplified approach to Ca^{2+} diffusion in a buffered medium is possible if we consider only small perturbations of the free ionic concentration. If B and B–Ca are much larger than Ca, they can be regarded as constant with respect to time and uniform with respect to space (Nunogaki & Kasai 1988). From reaction (1), a homogeneous perturbation, δCa, at time t_o will be damped by the buffer with a time constant t_b

$$t_b = \frac{1}{K_b B}. \qquad (2)$$

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Equation 2 can be considered as a measurement of the temporal buffering power of the solution for small perturbations.

In the following we will consider that for the time smaller than t_b, the buffer has not enough time to react, so Ca^{2+} can freely diffuse over a distance r_b. In many problems of diffusion in homogeneous media, r_b can be approximated by:

$$r_b \approx 2\sqrt{Dt_b}. \quad (3)$$

Equation (3) gives approximatively (i) the distance, r_b, reached by a perturbation (initially located at $r=0$) after a time t_b, and (ii) the time t_b necessary to damp a perturbation initially located in a spherical volume of radius r_b (Crank 1967).

From equations (2) and (3):

$$r_b \approx 2\sqrt{\frac{D}{K_b B}}. \quad (4)$$

Equation (4) gives an estimate of the spatial buffering power of a solution, e.g. the spatial range of a small perturbation in a buffered medium.

For numerical simulations in the cytosol: $D = 6 \times 10^{-6}\ cm^2\ s^{-1}$ (Bers & Peskoff 1991; Speksnijder *et al.* 1989) and K_b is in the range 10^6–$10^8\ M^{-1}\ s^{-1}$, which corresponds to the difference between slow buffers like EGTA and fast buffers like BAPTA (Tsien 1980). Values for B in the cytosol are difficult to appreciate and estimations vary from μM to mM.

We can compare the values for r_b, t_b (table 2) to the corresponding values of the perturbation brought by a channel opening in a non buffered medium. The distribution of ions around a channel mouth has already been described by Crank (1967) and Bers & Peskoff (1991). Calculations indicate the possibility of substantial elevation of Ca^{2+} at μm distance from the channel. We can also use equation (3) to estimate the perturbation brought by the channel. The mean time, t_o, during which the channel is open allows Ca^{2+} to diffuse a distance r_o. For the $InsP_3$-gated Ca^{2+} channel of the red beet vacuole (figure 1), and for the voltage-gated Ca^{2+} channel of sugar beet vacuole (Johannes *et al.* 1992), t_o is a few tenths of ms: this gives for r_o a value in the micrometer range. In most cases, table 2 indicates that r_b is smaller than r_o, so the buffer will damp the perturbation induced by the channel.

In the red beet vacuoles, the mean densities for the Ca^{2+}-sensitive (data not shown) and Ca^{2+}-selective channels (Alexandre *et al.* 1990) are about one channel per square micrometer. If the two-channel populations are randomly distributed on the tonoplast, the mean distance, r_d, between channels will be in the micrometer range. There will be no coupling by diffusion between channels when r_b is much smaller than r_d. Data from table 2 may explain the lack of efficiency of EGTA buffers and the need of at least millimolar concentrations of BAPTA to prevent coupling in the experiments described figure 5.

Table 2. *Values of r_b, t_b for a fast buffer (r_{bf}, t_{bf}; $K_b = 10^6\ M^{-1}\ s^{-1}$) and for a slow buffer (r_{bs}, t_{bs}; $K_b = 10^8\ M^{-1}\ s^{-1}$)*

B	100 μM	1 mM	10 mM
t_{bs}/s	10^{-2}	10^{-3}	10^{-4}
t_{bf}/s	10^{-4}	10^{-5}	10^{-6}
r_{bs}/μm	5	1.5	0.5
r_{bf}/μm	0.5	0.15	0.05

(*e*) *Importance of diffusion for Ca^{2+} signals*

The discussion on coupling by diffusion of Ca^{2+} presented in this paper, depends on the assumption that a Ca^{2+} perturbation never exceeds the capacity of the buffer. This is probably true (except very near the channel mouth) for *in vitro* experiments, in which large amounts of Ca^{2+} are used. It is more questionable *in vivo*, and we cannot predict exactly how far a Ca^{2+} signal can be propagated by diffusion in the cytosol. Regions with a fast buffer will reduce coupling to a distance much shorter than regions with a slow buffer. Variations in the buffer concentration also modify coupling and there are indications that concentration of a Ca^{2+} buffer like calmodulin may not be uniform in the cytosol (Marmé 1989). Differences in kinetic properties and concentrations of the buffer between various parts of the cytosol could then be used to regulate the Ca^{2+} signal propagation.

However it could be necessary to imply other processes than simple diffusion to explain Ca^{2+} changes in the cytoplasm. A rise in Ca^{2+} initially located in a small region can expand as a 'Ca^{2+} wave' (Berridge & Irvine 1989). The wave could be propagated by diffusion on a short distance, triggering a Ca^{2+} release at a further point. A triggering mechanism, the Ca^{2+}-activated Ca^{2+} release, could then enhance the speed of the signal (Neylon *et al.* 1990). If such a trigger is sometimes mentioned in plant cells (Gilroy *et al.* 1990; Schroeder & Thuleau 1991), experimental evidence is still lacking to confirm this hypothesis.

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REFERENCES

Abdel-Latif, A.A., Smith, J.P. & Akhtar, R.A. 1985 Polyphosphoinositides and muscarinic cholinergic and β-adrenergic receptors in the iris smooth muscle. In *Inositol and phosphoinositides: metabolism and regulation.* (ed. J. E. Bleasdale, J. Eichberg & G. Hauser), pp. 275–298, New Jersey: Humana Press.

Alexandre, J., Lassalles, 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, Lond.* **343**, 567–570.

Alexandre, J. & Lassalles, J.P. 1990 Effect of D-myo-inositol 1, 4, 5-trisphosphate on the electrical properties of the red beet vacuole membrane. *Pl. Physiol.* **93**, 837–840.

Alexandre, J. & Lassalles, J.P. 1991 Hydrostatic and osmotic pressure activated channel in plant vacuole. *Biophys. J.* **60**, 1326–1336.

Berridge, M.J. & Irvine, R.J. 1984 Inositol trisphosphate,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

a novel second messenger in cellular signal transduction. *Nature, Lond.* **312**, 315–321.
Berridge, M.J. & Irvine, R.J. 1989 Inositol phosphate and cell signalling. *Nature, Lond.* **341**, 197–205.
Bers, D.M. & Peskoff, A. 1991 Diffusion around a cardiac channel and the role of surface bound calcium. *Biophys. J.* **59**, 703–721.
Blatt, M.R., Thiel, G., Trentham, D.R. 1990 Reversible inactivation of K+ channels of Vicia stomatal guard cells following the photolysis of caged inositol 1, 4, 5-trisphosphate. *Nature, Lond.* **346**, 766–769.
Boss, W.F. & Massel, M.O. 1985 Polyphosphoinositides are present in plant tissue culture cells. *Biochem. biophys. Res. Commun.* **132**, 1018–1023.
Boss, W.F. 1989 Polyphosphoinositide metabolism: its relation to signal transduction in plants. In *Second messengers in plant growth and development* (ed. W. F. Boss & D. J. Morré), pp. 29–56. New York: Alan R. Liss.
Brosnan, J.M. 1990 Opening plant calcium channels. *Nature, Lond.* **344**, 593.
Brosnan, J.M. & Sanders, D. 1990 Inositol trisphosphate-mediated Ca^{2+} release in beet microsomes is inhibited by heparin. *FEBS Lett.* **260**, 70–72.
Brownlee, C. & Wood, J.W. 1986 A gradient of cytoplasmic free calcium in growing rhizoid cells of *Fucus serratus*. *Nature, Lond.* **320**, 624–626.
Canut, H., Carrasco, A., Graziana, A., Boudet, A.M. & Ranjeva, R. 1989 Inositol trisphosphate-stimulated calcium release from Acer microsomal fractions involves the uptake of potassium. *FEBS Lett.* **253**, 173–177.
Coté, G.G., Depass, A.L., Quarmby, L.M., Tate, B.F., Morse, M.J., Satter, R.L. & Crain, R.C. 1989 Separation and characterization of Inositol phospholipids from the pulvini of *Samanea saman*. *Pl. Physiol.* **90**, 1422–1428.
Cornelius, G., Gebauer, G. & Techel, D. 1989 Inositol trisphosphate induced calcium release from *Neurospora crassa* vacuoles. *Biochem. biophys. Res. Commun.* **162**, 852–856.
Crank, J. (ed.) 1967 The mathematics of diffusion. Oxford: Clarendon Press.
Drobak, B.K. & Ferguson, I.B. 1985 Release of Ca^{2+} from plant hypocotyl microsomes by Inositol-1, 4, 5-Trisphosphate. *Biochem. biophys. Res. Commun.* **130**, 1241–1246.
Ehrlich, B.E. & Watras, J. 1988 Inositol 1, 4, 5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature, Lond.* **336**, 583–586.
Förster, B. 1990 Injected inositol 1, 4, 5-trisphosphate activates Ca^{2+}-sensitive K^+ channels in the plasmalemma of Eremosphaera viridis. *FEBS Lett.* **269**, 197–201.
Gehring, C.A., Williams, D.A., Cody, S.H. & Parish, R.W. 1990 Phototropism and geotropism in maize coleoptiles are spatially correlated with increases in cytosolic free calcium. *Nature, Lond.* **345**, 528–530.
Gilroy, S., Read, N.D. & Trewavas A.J. 1990 Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphophate initiates stomatal closure. *Nature, Lond.* **346**, 769–771.
Gilroy, S., Fricker, M.D., Read, N.D. & Trewavas, A.J. 1991 Role of calcium in signal transduction of *Commelina* guard cells. *Pl. Cell* **3**, 333–344.
Hamill, O.P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F.J. 1981 Improved Patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pflügers Arch. Eur. J. Physiol.* **391**, 85–100.
Hedrich, R. & Neher, E. 1987 Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. *Nature, Lond.* **329**, 833–836.
Hedrich, R. & Kurkdjian, A. 1988 Characterization of an anion-permeable channel from sugar beet vacuoles: effect of inhibitors. *EMBO J.* **7**, 3661–3666.
Irving, M., Maylie, J., Sizto, N.L. & Chandler, W.K. 1990 Intracellular diffusion in the presence of mobile buffers, Application to proton movement in muscle. *Biophys. J.* **57**, 717–721.
Irvine, R. 1990 Messenger gets the green light. *Nature, Lond.* **346**, 700–701.
Johannes, E., Brosnan, J.M. & Sanders, D. 1991 Calcium channels and signal transduction in plant cells. *BioEssays* **13**, 331–336.
Johannes, E., Brosnan, J.M. & Sanders, D. 1992 Parallel pathways for intacellular Ca^{2+} release from the vacuole of higher plants. *Pl. J.* **2**, 97–102.
Kamada, Y. & Muto, S. 1991 Ca^{2+} regulation of phosphatidylinositol turnover in the plasma membrane of tobacco suspension culture cells. *Biochim. biophys. Acta* **1093**, 72–79.
Knight, M.R., Campbell, A.K., Smith, S.M. & Trewavas, A.J. 1991 Transgenic plant aequorin reports the effect of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature, Lond.* **352**, 524–526.
Lehle, L. & Ettlinger, C. 1990 Phosphoinositides and cell cycle control in plants. In *Inositol metabolism in plants* (ed. D. J. Morré, W. F. Boss & F. Loewus), pp. 211–220. New York: Alan R. Liss.
Lehle, L. 1990 Phosphatidyl inositol metabolism and its role in signal transduction in growing plants. *Pl. molec. Biol.* **15**, 647–658.
Marmé, D. 1989 The role of calcium and calmodulin in signal transduction. In *Second messengers in plant growth and development.* (ed. W. F. Boss & D. J. Morré), pp. 57–80. New York: Alan R. Liss.
Marty, A. & Neher, E. 1985 Potassium channels in cultured bovine adrenal chromaffin cells. *J. Physiol.* **367**, 117–141.
Mathias, R.T., Cohen, I.S. & Oliva, C. 1990 Limitations of the whole cell patch clamp technique in the control of intracellular concentrations. *Biophys. J.* **58**, 759–770.
McAinsh, M.R., Brownlee, C. & Hetherington, A.M. 1990 Abscisic acid-induced elevation of guard cell Ca^{2+} precedes stomatal closure. *Nature, Lond.* **343**, 186–188.
Meyer, T., Holowka, D. & Stryer, L. 1988 Highly cooperative opening of calcium channels by inositol 1, 4, 5-trisphosphate. *Science. Wash.* **240**, 653–656.
Morse, M.J., Crain, R.C. & Satter, R.L. 1987 Light-stimulated inositolphospholipid turnover in *Samanea saman* leaf pulvini. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7075–7078.
Neher, E. 1986 Concentration profiles of intracellular calcium in the presence of a diffusable chelator. *Expl. Brain Res.* **14**, 80–96.
Neylon, C.B., Hoyland, J., Mason, W.T. & Irvine, R.F. 1990 Spatial dynamics of intracellular calcium in agonist-stimulated vascular smooth muscle cells. *Am. J. Physiol.* **259**, C675-C686.
Nunogaki, K. & Kasai, M. 1988 The H^+/OH^- flux localizes around the channel mouth in buffered solution. *J. theor. Biol.* **134**, 403–415.
Ranjeva, R., Carrasco, A. & Boudet, A.M. 1988 Inositol trisphosphate stimulates the release of calcium from intact vacuoles isolated from Acer cells. *FEBS Lett.* **230**, 137–141.
Reddy, A.S.N. & Poovaiah, B.W. 1987 Inositol-1, 4, 5-Trisphosphate induced calcium release from corn coleoptile microsomes. *J. Biochem.* **101**, 569–573.
Rincon, M., Chen, Q. & Boss, W.F. 1989 Characterization of inositol phosphates in carrot (Daucus carota L.) cells. *Pl. Physiol.* **89**, 126–132.
Ringbom, A. (ed.) 1967 *Les complexes en Chimie Analytique.* Paris: DUNOD.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Sala, F. & Hernandez-Cruz, A. 1990 Calcium diffusion modeling in a spherical neuron. Relevance of buffering properties. *Biophys. J.* **57**, 313–324.

Schumaker, K.S. & Sze, H. 1987 Inositol 1, 4, 5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. *J. biol. Chem.* **262**, 3944–3946.

Schroeder, J.I. & Hagiwara, S. 1990 Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of nonselective Ca^{2+} permeable channels. *Proc. natn. Acad. Sci. U.S.A.* **87**, 9305–9309.

Schroeder, J.I. & Thuleau, P. 1991 Ca^{2+} channels in higher plant cells. *Pl. Cell* **3**, 555–559.

Simon, S.M. & Llinas, R.R. 1985 Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys. J.* **48**, 485–498.

Speksnijder, J.E., Miller, A.L., Weisenseel, M.H., Chen, T.H. & Jaffe, L.F. 1989 Calcium buffer injection block fucoid egg development by facilitating calcium diffusion. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6607–6611.

Supattapone, S.N., Worley, P.F., Baraban, J.M. & Snyder, S.H. 1988 Solubilization, purification, and characterization of an Inositol Trisphosphate receptor. *J. biol. Chem.* **263**, 1530–1534.

Thiel, G., MacRobbie, E.A.C. & Hanke, D.E. 1990 Raising the intracellular level of inositol 1, 4, 5-trisphosphate changes plasma membrane ion transport in characean algae. *EMBO J.* **9**, 1737–1741.

Tsien, R.Y. 1980 New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404.

Watras, J., Bezprozvanny, I. & Ehrlich, B.E. 1991 Inositol 1, 4, 5-trisphosphate-gated channels in cerebellum: presence of multiple conductance states. *J. Neurosci.* **11**(10), 3239–3245.

Worley, P.F., Baraban, J.M., Supattapone, S., Wilson, V.S. & Snyder, S.H. 1987 Characterization of inositol trisphosphate receptor binding in brain. Regulation by pH and calcium. *J. biol. Chem.* **262**, 12132–12136.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES