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Ions and Pollen Tube Growth

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Abstract Ions play a crucial role in the control of pollen tube growth. In this review we focus on four that seem especially important: calcium (Ca^{2+}), protons (H^+), potassium (K^+), and chloride (Cl^-). Ca^{2+} in the extracellular medium is essential for growth; it forms a steep intracellular tip-focused gradient, and exhibits a prominent extracellular tip-directed Ca^{2+} influx. pH is also essential for growth. H^+ form an intracellular gradient consisting of a slightly acidic domain at the extreme apex and an alkaline band located along the clear zone. H^+ also exhibit an apical influx, but in contrast to Ca^{2+} show an efflux along the clear zone, in the region occupied by the intracellular alkaline band. K^+ and anions (possibly Cl^-) appear to participate in the growth process, as evidenced by the striking extracellular fluxes that are associated with tube elongation. K^+ exhibits an apical influx, while an anion displays an apical efflux. An exciting finding has been the discovery that pollen tube growth oscillates in rate, as do all the ionic expressions noted above. While the ionic activities and fluxes show the same period as growth, they usually do not show the same phase. The exploration of phase relationships, using cross-correlation analysis, reveals that most ion expressions lag growth. Thus, intracellular Ca^{2+} activity follows growth rate by 1–4 s, whereas extracellular Ca^{2+} influx follows growth rate by 12–15 s (130°). These observations suggest that Ca^{2+} is a follower rather than a leader in growth. Despite the knowledge that has been gained, several aspects of ionic expression and function remain to be determined. Their elucidation will contribute greatly to our overall understanding of the control of pollen tube growth.

1
Introduction

It is well known that ions play a central role in the control of pollen tube growth. Over 40 years ago Brewbaker and Kwack (1963) revealed that in vitro culture of pollen tubes required calcium (Ca^{2+}). This initial finding prompted considerable further work on Ca^{2+}, with the establishment of the necessary limits (10 μM to 10 mM) (Steer and Steer 1989), the discovery of local gradients and fluxes, and the characterization of numerous targets in both the cytoplasm and cell wall through which this ion can influence pollen tube growth. But Ca^{2+} is not the only ion that directly participates in pollen tube growth; there are others including notably protons (H^+), potassium (K^+),

and possibly chloride (Cl^-) that contribute to the growth process (Holdaway-Clarke and Hepler 2003).

As we ponder the ways in which ions contribute to growth it becomes immediately apparent that many processes are involved. For example, turgor pressure, which drives growth, is dependent on the regulation of certain ions, especially K^+ and anions (possibly Cl^-). H^+ participate in many fundamental processes; perhaps most central are the transmembrane pH gradients, driven by the H^+-ATPase that contribute to energetic and membrane transport processes. Ca^{2+}, widely recognized as a mediator in signal transduction for all eukaryotic cells, has no less of a role in pollen tubes. Potential targets include cell motility and the cytoskeleton, exo- and endocytosis, and cell wall structure, all of which are central to the growth of the pollen tube (see the chapters by Malhó, Yokota and Shimmen, and Geitmann and Steer, this volume).

Our understanding of ions has improved enormously as a result of key technical developments. The use of intracellular reporters has allowed us to observe both temporally and spatially the intracellular activities of these ions during the pollen tube growth. Secondly, the development of extracellular probes, especially those that possess ion selectivity (Kühtreiber and Jaffe 1990), has allowed us to detect the location, direction, and magnitude of extracellular ion fluxes for Ca^{2+}, H^+, K^+, and Cl^-. Our goal in this chapter is to provide an overview of what is known about the relationship between ions and pollen tube growth, and attempt to place this knowledge within a wider context.

2 Ion Gradients and Fluxes

2.1 Ca^{2+}

An exciting and compelling result that emerges from the use of ratiometric indicator dyes is the presence of a striking "tip-focused" gradient in Ca^{2+} activity that is located in the apical domain of the growing pollen tube, immediately adjacent to the region of maximal elongation (Rathore et al. 1991; Miller et al. 1992) (Fig. 1a). Studies with fura-2-dextran indicate that the gradient extends from $\sim 3\ \mu M$ at the extreme apex of the tube to a basal level of $0.17\ \mu M$ within $20\ \mu m$ of the apex (Pierson et al. 1994, 1996). Because of its pK_a for Ca^{2+}, which is $0.57\ \mu M$, fura-2-dextran is close to saturation at $3\ \mu M$, and as a consequence the dye may under-report the true apical $[Ca^{2+}]$. Results using the Ca^{2+}-sensitive photoprotein, aequorin, which is capable of reporting elevated $[Ca^{2+}]$, indicate that the concentration at the apex may be as high as $10\ \mu M$ (Messerli et al. 2000). An important recent advance has been the introduction of cameleon, a transfectable Ca^{2+} indicator that allows observa-

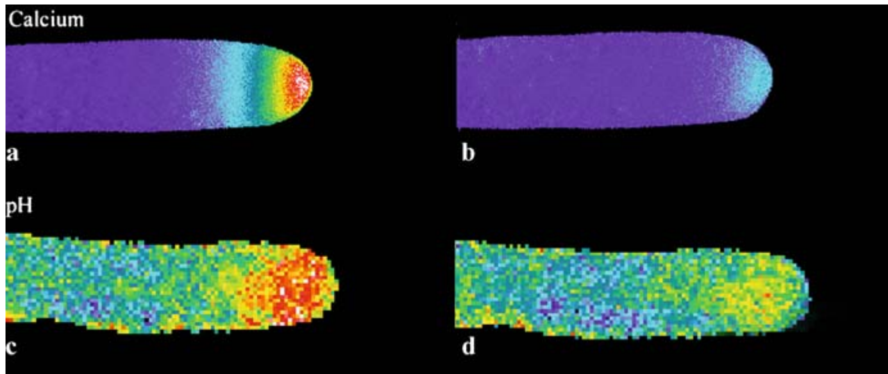

Fig. 1 Calcium and pH. **a**,**b** Changes in magnitude of the tip-focused Ca^{2+} gradient during oscillatory growth. The cytosolic $[Ca^{2+}]$ oscillates between high (**a**) and low (**b**) levels. **c**,**d** Changes in magnitude of the apical pH gradient, which oscillates between high (**c**) and low (**d**) levels

tion of the gradient in pollen tubes including those of *Arabidopsis* growing in vivo (Iwano et al. 2004; Watahiki et al. 2004). The tip-focused gradient can be viewed as a standing wave at the apex which carries important information. Thus Ca^{2+}-responsive proteins, such as calmodulin, will be saturated (and active?) at the extreme apex, whereas these same factors will lack this ion just 20 μm away from the tip and be inactive. Within this framework it is easy to envision how the Ca^{2+} gradient spatially regulates a host of activities at the apex of the pollen tube.

The extracellular flux of Ca^{2+} in many ways mirrors the intracellular gradient. Studies with a Ca^{2+}-selective vibrating electrode reveal an extracellular influx of Ca^{2+} that is focused toward the tip of the tube (Kühtreiber and Jaffe 1990) and is of substantial magnitude (20 pmol/cm^2/s) (Holdaway-Clarke et al. 1997). No efflux of Ca^{2+} has been observed. Both the apical influx of extracellular Ca^{2+} and the expression of the intracellular tip-focused gradient are dissipated by several experimental conditions that inhibit pollen tube elongation. Injection of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) Ca^{2+} buffers simultaneously reduces the gradient, blocks extracellular influx, and inhibits tube elongation (Pierson et al. 1994). But other agents, some of which do not seem related to either intracellular or extracellular Ca^{2+}, achieve the same effects. Thus, inhibition of growth with increased osmoticum, application of a mild thermal shock, or incubation in caffeine, all similarly reduce the tip-focused gradient and eliminate the extracellular influx (Pierson et al. 1996). These effects can be reversed by a return to normal growth conditions with the reappearance of growth together with the reemergence of the intracellular gradient and the extracellular influx. Taken together these results suggest that there is a close coupling between the intracellular gradient and extracellular influx.

A question of ongoing interest concerns the source of Ca^{2+}. The simplest explanation is that the gradient derives directly from the influx of extracellular Ca^{2+} (Fig. 2). This view is supported by studies in which Mn^{2+}, added to the medium, quenched the indicator dye fluorescence; presumably Mn^{2+} gained access to the cytoplasm by passing through Ca^{2+} channels (Malhó et al. 1995). It has long seemed plausible that the deformation of the plasma membrane at the tip, which presumably occurs during turgor-dependent cell elongation, would be sufficient to open mechanosensitive Ca^{2+} channels, allowing the rapid influx of this ion down its electrochemical gradient. Using patch-clamp electrophysiology, Dutta and Robinson (2004) have identified the postulated stretch-activated Ca^{2+} channels; these are associated with the plasma membrane on both the grain and tip of lily pollen tubes. In studies on the grain, the membrane containing the stretch-activated Ca^{2+} channels came from the region where the tube will emerge during germination. To access the apical membrane on pollen tubes, cells were first plasmolyzed, then the apical wall was digested with pectinase, and finally the plasmolysis conditions were reversed, which led to the extrusion of a protoplast that could be subjected to patch-clamp analysis (Dutta and Robinson 2004). Here, they detected a stretch-activated Ca^{2+} channel (conductance $\sim$ 15 pS), similar in properties to those on the grain. Of further pertinence, these channels, as well as elongation of the pollen tube, could be blocked by application of crude spi-

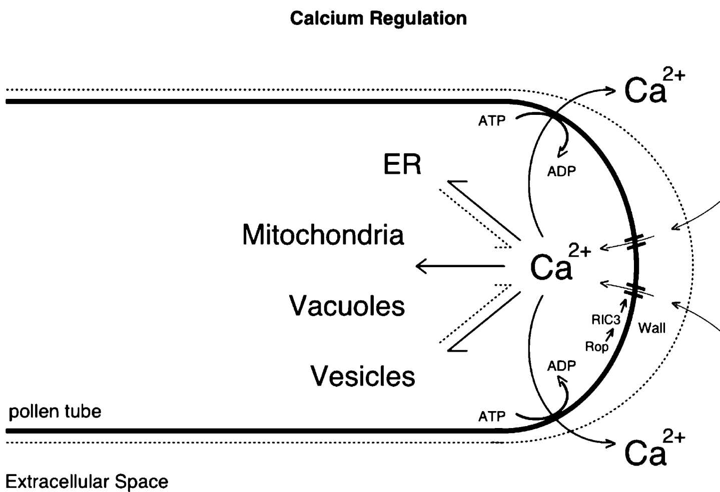

Fig. 2 Likely pathways for the regulation of cytosolic Ca^{2+} in the growing pollen tube. The influx is shown as an *interrupted line* suggesting that Ca^{2+} interacts with cell wall components. Extrusion and sequestration of the ion from the cytosol is presumably accomplished by pumps on the plasma membrane (see the chapter by Sze et al., this volume), the ER, vacuole, mitochondria, and vesicles (*solid lines*). *Dashed lines* represent postulated pathways from cytoplasmic organelles, such as the ER and vesicles, toward the Ca^{2+} gradient

der venom, previously reported to block stretch-activated channels in other membranes (Dutta and Robinson 2004).

While the participation of an extracellular influx seems likely, other sources of Ca^{2+} may contribute to the gradient. Given the presence of the endoplasmic reticulum (ER) and especially the inverted cone of secretory vesicles in the apical domain (Lancelle and Hepler 1992), it is possible that release from these stores could contribute to the intracellular gradient (Fig. 2). Inositol 1,4,5-trisphosphate (IP_3) has been shown to cause an increase in the intracellular $[Ca^{2+}]$ (Franklin-Tong et al. 1996; Malhó 1998), but whether or not this is a natural regulator still requires experimental verification. On balance, the position of the gradient with its maximal level immediately adjacent to the plasma membrane, together with new evidence showing the presence of stretch-activated Ca^{2+} channels in the apical membrane, argue persuasively for the idea that Ca^{2+} influx from the extracellular space is the primary source of this ion during pollen tube growth.

Although we cannot be certain that Ca^{2+} release occurs from the ER and other organelles, it is likely that uptake of Ca^{2+} by these intracellular components, and/or the extrusion of Ca^{2+} at the plasma membrane, play a major role in governing the profile and extent of the tip-focused gradient (Fig. 2). It is generally well appreciated that Ca^{2+} pumps on the plasma membrane, ER, mitochondria, and central vacuole actively participate in the removal of excess Ca^{2+} from the cytosol (Sze et al. 2000). Exactly how these various uptake processes contribute to the conditions observed in the pollen tube is not known in detail, but recent studies provide evidence for a Ca^{2+}-ATPase (ACA9) that is required for pollen tube growth and for proper fertilization (Schiøtt et al. 2004). ACA9, which is a member of the family of autoinhibited Ca^{2+}-ATPases, is expressed predominantly in the male gametophytic tissue, and in pollen tubes is located on the plasma membrane (Fig. 2; chapter by Sze et al., this volume).

A further important issue to consider in our attempts to understand Ca^{2+} regulation concerns the contribution of the cell wall. While the cytoplasmic indicator dyes specifically report on ion activities within that compartment, the identity of the compartment that influences the signal from the Ca^{2+}-selective vibrating electrode is less clear. Although it has been assumed that the signal is due to movement of ions across the plasma membrane (Speksnijder et al. 1989), it must be kept in mind that influx of Ca^{2+} from the extracellular medium must first cross the cell wall. If there is any binding to cell wall components this will contribute to the signal. Since the cell wall at the apex is composed of pectins, which following de-esterification contain carboxyl residues, there are numerous potential Ca^{2+} binding sites. Indeed, calculations suggest that the Ca^{2+}–cell wall interaction dominates the apparent extracellular influx of Ca^{2+}, with the number of ions crossing the plasma membrane at the apex being less than 10% of the total signal (Holdaway-Clarke and Hepler, 2003). A Ca^{2+}–cell wall interaction is thus important; it is

a major activity that must be considered in any model of pollen tube growth and its control.

2.2 H^+

In addition to Ca^{2+}, pollen tube growth is regulated by pH (Fricker et al. 1997; Feijó et al. 1999). Acidic conditions are necessary, with pH 7 being unable to support tube elongation (Holdaway-Clarke et al. 2003). Studies on the intracellular distribution of H^+ reveal that pollen tubes possess a unique intracellular pH gradient (Feijó et al. 1999). However, because of the much greater mobility of H^+ when compared to that of Ca^{2+}, it has been difficult to observe these pH gradients (Fricker et al. 1997; Parton et al. 1997; Messerli and Robinson 1998). The indicator dyes, especially when used at more elevated levels, appear to locally buffer the pollen tube cytoplasm and dissipate the activity that is being sought. When low concentrations of the indicator BCECF–dextran are used (0.3–0.5 μM) a gradient in pH becomes evident, which consists of a slightly acidic (pH = 6.8) apex, with an alkaline band (pH = 7.5) toward the base of the clear zone (Feijó et al. 1999) (Fig. 1c). We presume that the alkaline band is confined to the cell cortex, where it is governed by plasma membrane H^+-ATPases (see the chapter by Sze et al., this volume). In parallel with these intracellular studies, experiments with a H^+-selective vibrating electrode indicate an extracellular influx at the apex of the tube, with a distinct efflux at the edge of the cell near the clear zone. Thus, in marked contrast to Ca^{2+}, the flux pattern for H^+ reveals a current loop, which might be effective as a polarizing factor, especially in the confined spaces of the pistil during fertilization in vivo.

Inhibition of pollen tube growth eliminates the acidic tip but the alkaline band persists, and even extends more closely to the apex (Feijó et al. 1999). These observations are consistent with the influx of H^+ at the tip being dependent on growth and the attendant deformation of the apical plasma membrane. Indeed, it is plausible that H^+ enter through the same stretch-activated channel as Ca^{2+}, thus explaining the marked dependence on growth for the entry of both of these ions. The alkaline band, by contrast, presumably derives from the activity of a plasma membrane H^+-ATPase, which throughout plants is recognized as a key regulatory enzyme for energizing transport processes (Palmgren 2001; chapter by Sze et al., this volume). It is pertinent that inhibitors of the H^+-ATPase, including vanadate, azide, and *N*-ethylmaleimide, block pollen tube growth, while its stimulation by fusicoccin enhances growth (Rodriguez-Rosales et al. 1989; Feijó et al. 1992; Fricker et al. 1997; Pertl et al. 2001). Cytological evidence for a plasma membrane ATPase has thus far been mixed. Feijó et al. (1992) showed ATPase activity on the plasma membrane of both the grain and pollen tube, whereas Obermeyer et al. (1992) only demonstrated strong activity in association with the

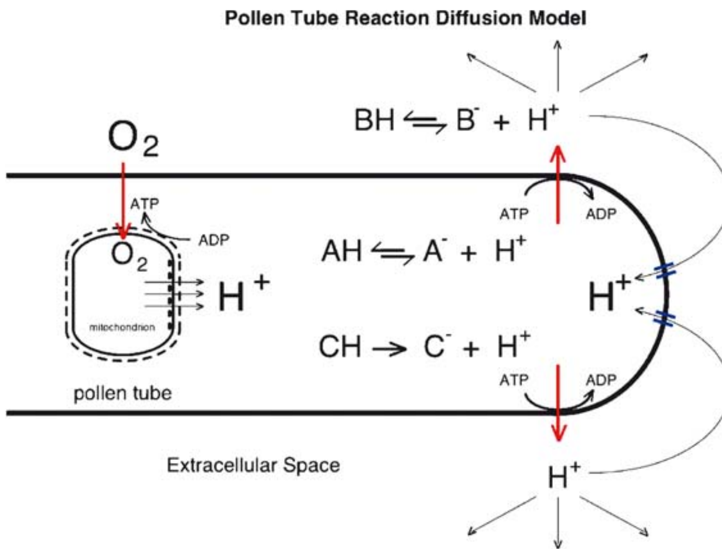

Fig. 3 A nonlinear, reaction–diffusion model for pH regulation [adapted from a model first published by Feijó et al. (1999)]. A principal component is a plasma membrane ATPase that pumps H^+ into the cell wall space leaving behind a domain of OH^-. Mitochondria play a pivotal role as a prime source of ATP; they also generate NAD(P)H and H^+. An important feature of this model is the current loop, in which H^+, extruded by the H^+-ATPase, enter the tip possibly through growth-dependent cation channels. This current loop may serve an important role in defining and controlling pollen tube growth polarity

grain. However, the marked H^+ efflux observed along the clear zone strongly suggests the presence of a H^+-ATPase.

H^+ emerge as potential key regulators of pollen tube growth and polarity (Fig. 3). In an attempt to explain pH regulation, Feijó et al. (1999) put forth a reaction–diffusion model, which emphasizes the contribution of several important factors in the control of local H^+ activity, including intra- and extracellular metabolic reactions, the cytosolic buffering capacity, and the small spatial separation of influx and efflux. When these nonlinear conditions are factored together, a model emerges that supports a stable spatial pattern (Fig. 3) that belongs to a family of processes described by Turing (1952). Simultaneous H^+ and O_2-electrode measurements concur that a key energy insertion point in this model would be the region basal to the inverted cone and alkaline zone that is rich in mitochondria (Kunkel et al. 2005). This area would be a source of both ATP and H^+ which are needed to feed the process. H^+ continue to be dissipated as they are pumped out of the pollen tube's alkaline zone, which in the model is rich in the H^+-ATPase responsible for ejecting H^+. This H^+ ejection feeds a local external low pH, which in turn provides the protons that rhythmically enter at the pollen tube tip

(Fig. 3). Dissipation of the H^+ depends on their order of magnitude higher diffusion coefficient compared to those of other ions as well as the local buffer capacity (Kunkel et al. 2001). The self-regulatory nature of the pollen tube oscillating system was exhibited using phase state attractor diagrams of pollen tube oscillations that once perturbed return to their earlier attractor behavior (Feijó et al. 2001). This modeling approach, which has yet to be fully understood in its application to pollen tube physiology, illustrates the need for novel ways of viewing the complex nonlinear processes that underlie pollen tube tip growth.

2.3 K^+

Early studies on total currents associated with growing pollen tubes came to the conclusion that K^+ was a key ion involved in carrying the observed current (Weisenseel and Jaffe 1976). Furthermore, K^+ entered the apex and exited through the base of the shank or the grain. This question has been readdressed more recently using K^+-selective electrodes. Unfortunately the K^+-selective electrodes often exhibit poor performance (Messerli et al. 1999). The K^+-selective liquid ion exchanger (LIX), which differs in its vehicle from most others, requires a differently shaped and better silanized glass micropipette filled with a longer column of LIX. Nevertheless, with suitable electrodes, it has been possible to document a prominent K^+ influx at the apex of growing pollen tubes.

Considerable evidence exists for the presence of K^+ channels associated with pollen grain or tube plasma membranes. Three different K^+ channels have been detected in lily pollen protoplasts, with the most common exhibiting a conductance of 19 pS (Obermeyer and Kolb 1993; Obermeyer and Blatt 1995). Also, using pollen protoplasts, an inward K^+ channel in *Brassica* has been identified by patch-clamp analysis that is increased in its activity by extracellular Ca^{2+} (10–50 mM), and by an acidic extracellular pH (4.5) (Fan et al. 1999, 2001). By contrast, an outward K^+ channel has been reported that responds to an acidic internal pH (Fan et al. 2003). An inward K^+ channel has also been identified in *Arabidopsis*, both in plasma membranes from the grain and from the apical region of the tube (Mouline et al. 2002). Its conductance of 14 pS suggests that this channel from *Arabidopsis* may be similar to the 19-pS K^+ channel found in lily (Obermeyer and Kolb 1993). It is additionally important to note that a mutation in the *Arabidopsis* K^+ channel caused reduced ion uptake and correspondingly reduced growth (Mouline et al. 2002). Finally, both stretch-activated and spontaneous K^+ channels have been identified by patch-clamp analysis (Dutta and Robinson 2004). The stretch-activated channels are observed in the groove on the protoplast derived from the pollen grain, whereas the spontaneous K^+ channel occurs over the whole surface. Curiously, neither channel was detected in

membranes derived from pollen tube protoplasts. Like the stretch-activated Ca^{2+} channel, the stretch-activated K^+ channel, but not the spontaneous K^+ channel, is inhibited by spider venom (Dutta and Robinson 2004).

2.4 Cl^-

The data and conclusions concerning the participation of K^+ in pollen tube growth are unambiguous, whereas the status of Cl^- is less clear. Zonia et al. (2002) reported a marked efflux of this ion from the apex of tobacco pollen tubes, with an influx occurring along the flanks of the tube starting 12 μm back from the tip. Thus, like H^+, Cl^- fluxes form a current loop in the apical domain, and may play a key role in growth polarity. The inhibition of tube growth together with the initiation of apical swelling through the application of IP_4, a putative Cl^- channel blocker, are consistent with the conclusion that transport of this anion is crucial to pollen tube growth, where presumably it would play a role in turgor regulation. However, these results have been contested by Messerli et al. (2004) who assert that the LIX used by Zonia et al. (2002) is not specific for Cl^-. They further question the idea that Cl^- channels or transport are necessary for pollen tube growth and survival.

The cautions of Messerli et al. (2004) are to be taken seriously. No LIX used for ion-probe measurement is absolutely specific, and there are many instances of interactions of LIX within electrodes with the media in which they are operated. The published relative affinities of a given LIX for related ions were determined for static macroelectrodes and are often dramatically different from the relative affinities measured with microelectrodes, as demonstrated by Messerli et al. (2004). In the case of the Cl^--LIX, Messerli et al. (2004) reported that nitrate provides a substantial relative signal. This type of interference in which the LIX itself carries another ion is a serious conflict that must be overcome. A more serious assertion about the Cl^--LIX is that it reacts with 2-(*N*-morpholino)ethanesulfonic acid (MES), the zwitterionic buffer (Good et al. 1966) commonly used in pollen tube studies because of its favorable pK. MES has a different interaction with the LIX in its protonated vs unprotonated form that potentially makes the LIX a proton sensor in the presence of MES as well as a Cl^- electrode. In the given example, in which Zonia et al. (2002) measure the efflux of Cl^- from the tip of the pollen tube, it is asserted that they are actually measuring the previously reported oscillating proton current (Messerli et al. 2004).

This discrepancy in the interpretation of data may have technical reasons. Messerli et al. (2004) examined the responsiveness of static electrodes to ions using direct-coupled potentiometers, which provide more accurate estimates of relative sensitivities to different ions (including a tenfold greater sensitivity to nitrate and a greater sensitivity of the protonated form of the buffer MES). The direct-coupled potentiometers are reported to give a rapid rise in

the response over a negligible time interval (0.25 ms). However, the voltage measurement in live cells was not done with the direct-coupled potentiometer but rather with self-referencing capacitative electronics, which are not as stable as direct-coupled electrodes. This results in less-linear calibrations and low, voltage-dependent efficiencies [85% efficiency in direct-coupled oscillating mode (Kunkel et al. 2005)]. As a result the conclusions of Messerli et al. (2004) must be examined with caution. Clearly, further experiments in a buffer that does not interfere with the Cl^--LIX are required before we can draw conclusions about the role of Cl^- and other anions in tip growth.

3 Oscillations in Pollen Tube Growth and Associated Ion Expression

An important finding has been the discovery that the rate of pollen tube growth oscillates (Pierson et al. 1995). In vitro, lily pollen tubes longer than 600–700 μm exhibit a change in growth rate from 100 to 500 nm/s over a period of 15–50 s (Pierson et al. 1996). Additionally, the intracellular activities of both Ca^{2+} and H^+, as well as the extracellular fluxes of Ca^{2+}, H^+, K^+, and Cl^-, all exhibited oscillatory profiles which possessed the same period as that of growth, but usually not the same phase (Holdaway-Clarke and Hepler 2003). These observations allow us to decipher the phase relationship between an ion activity or its extracellular flux and the underlying rate of growth. Further, by determining whether an ion expression precedes or follows growth, information can be gained about those events or processes that anticipate and possibly regulate growth, as opposed to those that follow and appear to be governed by the preceding growth event.

When two processes oscillate, but not with the same phase, it is not immediately obvious which one precedes the other. To solve this problem, cross-correlation analysis has been used. Cross-correlation analysis of simultaneously collected time series processes, e.g., tip growth and process "*x*", allows the strength of correlation and the lag between two processes to be established (Brillinger 1981).

3.1 Ca^{2+} Oscillations

Examination of the intracellular [Ca^{2+}] during oscillatory growth indicates that the changes, like those in the growth rate, are substantial, with values from 750 to 3500 nM being observed (Pierson et al. 1996) (Fig. 1a,b). Although initial inspection suggested that intracellular Ca^{2+} and growth were in phase (Holdaway-Clarke et al. 1997; Messerli and Robinson 1997), further studies at higher temporal resolution revealed surprisingly that the maximum [Ca^{2+}] in the tip-focused gradient peaked 1–4 s, or $\sim 20°$, after the peak in

growth rate (Messerli et al. 2000). These observations indicate that Ca^{2+} activity is not a leader of growth but a follower; its changes are dictated by growth rate changes, rather than vice versa. Of further interest, the extracellular influx of Ca^{2+}, which also oscillates, is delayed in phase from the intracellular Ca^{2+} activity. Thus Ca^{2+} influx lags growth by 135°, and intracellular Ca^{2+} activity by 115° (Holdaway-Clarke et al. 1997). These data put constraints on the popular idea that Ca^{2+} is regulating the process of growth; rather it would appear that the growth process determines the subsequent expression of both the intracellular gradient and the extracellular influx.

A further outcome of these data has been the necessity to rethink the relationship between the extracellular Ca^{2+} influx and the intracellular gradient. The influx measurements are a composite that unavoidably includes information about Ca^{2+} entry into the cell wall domain, as well as Ca^{2+} entry into the cytoplasm (Holdaway-Clarke et al. 1997). However, there is not a full consensus on this issue, as others believe that intracellular stores, such as the ER or the secretory vesicles, take up significant amounts of Ca^{2+} and thereby may account for the large influx signal (Malhó and Trewavas 1996; Messerli and Robinson 2003). Whatever the mechanism, it is clear that some form of Ca^{2+} storage is required to account for the marked phase separation in the expression of the intracellular Ca^{2+} gradient and the influx of extracellular Ca^{2+}.

3.2 H^+ Oscillations

In the initial studies on H^+ imaging in pollen tubes it was noted that the intracellular pH oscillates, with the alkaline band being out of phase with the growth rate (Feijó et al. 1999) (Fig. 1c,d). Due to low signal levels, and the tendency for the dye to bleach following rapid sequential measurements, it was not possible at the time to gain the necessary temporal resolution that would allow the definitive measurement of the phase relationship. This question is currently under investigation using more sensitive equipment. Preliminary results indicate that formation of the alkaline band peaks before growth by 3–7 s ($\sim 45°$), whereas the acidic tip follows growth by 3–6 s ($\sim 40°$) (Hepler et al. 2005; Lovy-Wheeler et al. 2005a). These findings point to H^+ pumping and the generation of the alkaline band as an anticipatory event that may serve as a key regulatory event in the control of cell growth.

The extracellular influx of H^+ oscillates, but thus far an oscillatory efflux along the sides of the clear zone, close to the intracellular alkaline band, has not been reported, despite the observation that the alkaline band oscillates. The oscillatory apical influx, when subjected to cross-correlation analysis, lags growth by $\sim 100°$, and consequently is out of phase with the oscillation of the intracellular acidic domain at the pollen tube tip (Messerli et al. 1999). Again, cell wall generation or binding of H^+ might explain these seeming dis-

continuities. For example, when methylated pectins are de-esterified, H^+ are released. Also H^+ fluxes will be associated with the binding of cations to cell wall components.

3.3
K^+ and Cl^- Oscillations

Thus far we do not have information concerning the oscillations, if any, in the intracellular activities of these ions, but the extracellular fluxes of both ions oscillate in relation to the changes in growth rate. The extracellular influx of K^+ presents a very similar phase relationship to growth as do H^+, and is also not significantly different from that of Ca^{2+}; thus, the influx of K^+ lags growth by about 100° (Messerli et al. 1999). Cl^-, in contrast to these other ions, exhibits a marked efflux from the apex, and an influx along the sides of the clear zone (Zonia et al. 2002). Similarly to H^+, only the activity at the apex has been observed to oscillate. In tobacco pollen tubes, Cl^- efflux occurs in exact phase with the growth rate (Zonia et al. 2002). However, as noted earlier, these results have been challenged by Messerli et al. (2004) raising the hypothesis that it may be another anion or H^+ that are being measured by Zonia et al. (2002).

4
Targets for Ion Action

4.1
Cytoskeleton and Motile Processes

The cytoskeleton, especially that composed of actin, plays a crucial role in the control of pollen tube growth. Actomyosin is responsible for driving cytoplasmic streaming, and the transport of the secretory vesicles to the apical domain. But separate from its role in streaming, actin polymerization/turnover in the apical domain is required for pollen tube growth (Gibbon et al. 1999; Vidali et al. 2001; chapter by Yokota and Shimmen, this volume). Thus, agents that block polymerization or turnover, including profilin, DNAse, latrunculin-B, and cytochalasin-D, all inhibit cell elongation at a significantly lower concentration than that needed to stop cytoplasmic streaming (Vidali et al. 2001). Although there has been controversy over the structure of actin, especially in the apical domain, the recent work of Lovy-Wheeler et al. (2005b), using rapid freeze fixation coupled with antibody labeling, provides high-quality images of the actin cytoskeleton in fixed cells. The results revealed the presence of a prominent collar or cortical fringe of F-actin in the apical domain (see the chapter by Yokota and Shimmen, this volume). Starting 1–5 μm back from the tip, this system of longitudinally aligned filaments extends basally through the cell cortex for another 5–10 μm. Thereafter, actin

microfilaments are finely articulated and evenly dispersed throughout the thickness of the tube and extend throughout the shank.

Since ions, especially Ca^{2+} but also H^+, can modulate the actin cytoskeleton in other systems, it seems likely they will do so in pollen tubes. It has been known for years that injection of Ca^{2+} into pollen tubes causes fragmentation of F-actin (Kohno and Shimmen 1987). More recently, different actin binding proteins have been identified, notably myosin, profilin, villin/gelsolin, and ADF, which in response to Ca^{2+} or H^+ exhibit an altered activity toward actin to control its structure or activity. These interactions are described in detail in the chapter by Yokota and Shimmen (this volume) and in Fig. 4. Here we introduce only a few comments on their spatial location and significance for pollen tube growth. Firstly, the tip-focused Ca^{2+} gradient, acting together with profilin and villin/gelsolin, will prevent polymerization and/or fragment existing microfilaments. Together they account for the marked reduction of F-actin in the extreme apex of the pollen tube. In addition, the high $[Ca^{2+}]$, which is sufficient to inhibit myosin, can explain the absence of cytoplasmic streaming in this extreme apical domain. Secondly, the alkaline band can be expected to activate ADF, a pH-sensitive actin binding protein (Allwood et al. 2002; Chen et al. 2002). The colocalization of the alkaline band with the actin fringe invites speculation about a functional interaction. Spe-

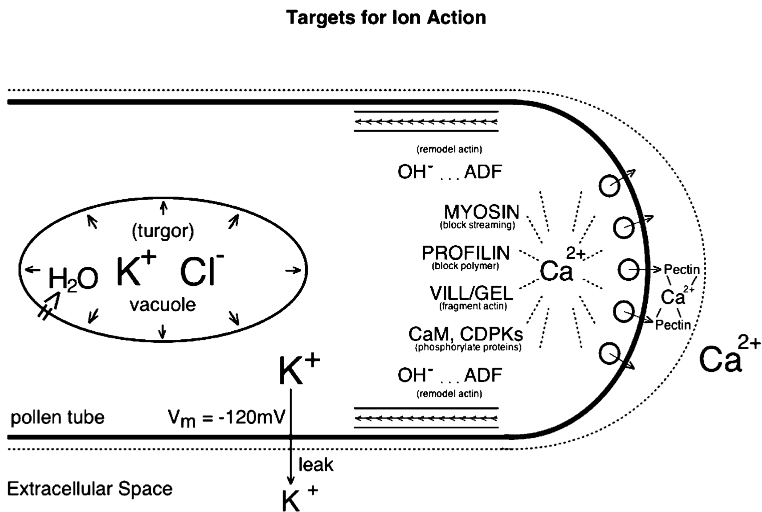

Fig. 4 Several targets and associated pathways for ion-modulated events that appear to play a crucial role in the control of pollen tube growth. The tip-focused Ca^{2+} gradient stimulates secretion, affects the structure and activity of the actin cytoskeleton, and regulates protein phosphorylation. The alkaline band is depicted as specifically modulating the cortical actin fringe through stimulation of actin remodeling by ADF. K^+ and anion (possibly Cl^-) accumulation in the vacuole, together with water uptake, regulate turgor pressure. An outward leakage of K^+, coupled with the retention of fixed negative charges, controls the membrane potential

cifically, the alkaline band through the activation of ADF could contribute to the turnover of the cortical fringe, and contribute importantly to growth polarity. These observations resonate closely with recent work from animal systems (Bernstein and Bamburg 2004). Studies on fibroblasts show that cells lacking the Na^+/H^+ antiporter, and unable to generate localized pH gradients, also lack cytoskeletal anchoring and polarity (Denker and Barber 2002). Among the key cytoskeletal proteins, ADF/cofilin emerges as the most likely candidate to control cell polarity (Ghosh et al. 2004). Bernstein and Bamburg (2004) enlarge this relationship into a comprehensive model for the generation of polarity in animal cells. They suggest that local pH changes at the plasma membrane are the key factor in tipping the balance for F-actin remodeling, and thus in defining the origin of polarity.

4.2 Endocytosis/Exocytosis

It is commonly accepted in plant and animal cells that Ca^{2+} facilitates secretion (Battey et al. 1999). Thus, a strong candidate function for the tip-focused Ca^{2+} gradient would be the stimulation of exocytosis of the apically accumulated vesicles, which contain the cell wall precursors needed for cell elongation (Fig. 4; chapter by Malhó, this volume). Experimental probing of this idea shows that elevation of the Ca^{2+} levels, brought about by local uncaging of a light-sensitive Ca^{2+}-containing reagent, stimulates exocytosis, with the latter being inferred from a reduction in FM1-43 fluorescence (Camacho and Malhó 2003). However, in parallel studies, increasing the concentration of GTPγ S, a nonhydrolyzable analog of GTP, increased exocytosis concomitant with a slight decrease in Ca^{2+}. While these results may seem inconsistent with the idea that Ca^{2+} facilitates secretion, it must be realized that the pollen tubes still exhibit an apical gradient, and thus at the point of secretion the local concentration of Ca^{2+} is well above basal levels. Studies in maize coleoptiles have shown that the secretory process saturates above 1.5 μM, with half maximal stimulation at $\sim$ 0.9 μM (Sutter et al. 2000). If the pollen tube is similar, then even when the gradient is at its low point ($\sim$ 0.75 μM), there is still sufficient Ca^{2+} to stimulate the secretory process (Holdaway-Clarke and Hepler 2003).

The connection between Ca^{2+} and secretion is also supported by studies using Yariv reagent to block pollen tube growth (Roy et al. 1999). Under these conditions, elongation stops, whereas secretion continues with irregular wall thickenings arising, which are accompanied by elevated levels of Ca^{2+} in the adjacent cytoplasm (Roy et al. 1999). It is possible that stretch-activated channels are involved, with the deformation resulting from secretion causing mechanical strain that opens a Ca^{2+} channel. The recent work of Pickard and Fujiki (2005) supports this contention. In cultured BY-2 cells, they show by patch-clamp analysis that inactivation of the wall-associated arabinogalactan

proteins with Yariv reagent deregulates the activity of the stretch-activated Ca^{2+} channels, allowing uncontrolled ion influx. These authors promote the idea of a cortical plasma membrane-associated reticulum, in which a complex of cytoskeleton and arabinogalactan proteins creates a force-focusing system that spatially confines and regulates the activity of stretch-activated channels (Pickard and Fujiki 2005).

4.3 Ion Binding Proteins

A widely accepted function for Ca^{2+} is the ability to activate a process through an intermediary binding protein. Calmodulin (CaM) emerges as a likely candidate protein that can transmit the Ca^{2+} signal to a response element such as a protein kinase (Snedden and Fromm 2001). A second factor is the large family of Ca^{2+}-dependent protein kinases (CDPKs) (Harmon et al. 2001; Harper et al. 2004). These proteins, which are directly regulated by Ca^{2+}, are uniquely found in plants and a few protists, but not in yeast or animal cells.

4.3.1 Calmodulin

Current studies derived from live cells injected with fluorescently labeled calmodulin reveal that this protein is evenly distributed throughout the pollen tube cytoplasm (Moutinho et al. 1998b). Whereas total calmodulin may not accumulate in the apex, that which is activated by binding to Ca^{2+} is elevated in a pattern that is similar to the tip-focused gradient (Rato et al. 2004). These findings have been gained through the use of TA-CaM, a fluorescent analog of calmodulin that changes its quantum yield when bound to Ca^{2+}. These results are not surprising since presumably the tip-focused gradient is sufficient to saturate appropriate binding molecules such as calmodulin. However, what are the interacting proteins to which calmodulin binds? One example would be the actin binding proteins that are modulated by Ca^{2+} and calmodulin, including myosin and villin. Taken together it can be appreciated why there are not organized bundles of actin in the extreme apex and why streaming is markedly suppressed. A second example is ACA9 (Schiøtt et al. 2004), an autoinhibited, plasma membrane-localized Ca^{2+} pump, which is thought to be regulated by calmodulin, an observation consistent with the well-known role of calmodulin in the regulation of ion pumps in other systems (Snedden and Fromm 2001) (Fig. 4). We suspect that there are many other response elements that respond to Ca^{2+}/calmodulin, and that their identity will emerge from future work.

Recent work also supports the idea that calmodulin may interact with cyclic AMP in the regulation of pollen tube growth (Moutinho et al. 2001; Rato et al. 2004). Agents that either substitute for cAMP (8-Br-cAMP) or ac-

tivate adenylyl cyclase (forskolin) cause an increase in activated calmodulin, while inhibition of adenylyl cyclase (dideoxyadenosine) induces a decline in activated calmodulin. Rato et al. (2004) suggest that these interacting pathways participate in the regulation of apical secretion.

4.3.2 CDPKs

CDPKs comprise a large family of proteins in plants, and are prime candidates as possible Ca^{2+} response factors (see the chapter by Malhó, this volume). Curiously, thus far there has been relatively little work on presumptive pollen-specific forms of this protein. Estruch et al. (1994) provided the first evidence for a pollen-associated CDPK isoform that is necessary for pollen germination and tube growth. These authors favored the idea that this CDPK specifically participated in the regulation of the actin cytoskeleton. Subsequently, a CDPK from *Nicotiana alata* was identified that participates in the phosphorylation of stylar RNAses, and provisionally is involved in the incompatibility response in this species (Kunz et al. 1996). Moutinho et al. (1998a) used a fluorescent probe to spatially localize CDPK to the apical domain of the pollen tube. The pattern was similar to that of the tip-focused Ca^{2+} gradient, and was able to change its position in response to stimuli that cause reorientation of the pollen tube. A target for this presumptive CDPK is not known, but it is attractive to imagine that it participates in secretion (Moutinho et al. 1998a). Given the large size of the CDPK family, it seems likely that other pollen-specific members exist. This topic therefore deserves attention in future studies.

4.4 Small G-Proteins

Considerable excitement surrounds the idea that small G-proteins, namely Rops (Rho-related proteins of plants), regulate pollen tube growth, with an important aspect of that control mechanism involving the modulation of Ca^{2+} influx. Rops localize to the apex of the pollen tube where they appear to associate with the plasma membrane (Kost et al. 1999; Li et al. 1999; chapter by Hwang and Yang, this volume). Overexpression of these proteins causes the apex of the pollen tube to swell into balloon-shaped structures (Kost et al. 1999), whereas dominant negative forms of the protein or the injection of function-inhibiting antibodies to Rop block pollen tube growth (Li et al. 1999). Because these antibodies were shown to also eliminate the tip-focused Ca^{2+} gradient, the conclusion has been made that Rop regulates Ca^{2+} influx (Li et al. 1999). Despite this provocative conclusion, we hasten to note that the connection between Rop and Ca^{2+} may not be as compelling or direct as suggested. Several experimental conditions that block pollen tube elongation,

such as injection of BAPTA buffers, mild thermal shock, culture in caffeine, or treatment with elevated osmoticum, also eliminate the tip-focused Ca^{2+} gradient (Pierson et al. 1994, 1996). The fact that Rop inactivation blocks growth is indeed interesting, but the data thus far do not establish that it does so by first blocking Ca^{2+} influx. The results from the growth/Ca^{2+} relationship in oscillating pollen tubes indicate that growth defines the subsequent Ca^{2+} influx, and not the reverse (Messerli et al. 2000).

Despite these reservations, recent work establishes a firmer connection between Rop and Ca^{2+}, and also with the control of the actin cytoskeleton (Gu et al. 2005). In *Arabidopsis* pollen tubes, Rop1 seems to control tube growth through the coordinate activity of two interacting CRIB (Cdc42/Rac-interactive binding) proteins, called RIC3 and RIC4 (Gu et al. 2005). Experimentation suggests that RIC3 promotes Ca^{2+} influx, which may affect pollen tube growth through the modulation of F-actin dynamics. Support for these conclusions stems from the observation that cells expressing RIC3 germinate and express a tip-focused Ca^{2+} gradient at a lower extracellular [Ca^{2+}] than the untransformed controls. In addition, the results show that overexpression of RIC3 causes an apparent degradation of the fine actin filaments in the apical domain.

4.5 Turgor Regulation

Studies showing that the application of an increased level of osmoticum in the medium inhibits pollen tube growth suggest that osmotic regulation and the generation of turgor pressure are essential for cell elongation (Pierson et al. 1996). Messerli and Robinson (2003) have shown that an abrupt increase in turgor pressure, brought about by decreasing the osmolarity of the medium, can generate a brief increase in the growth rate. However, it does not follow that changes in turgor pressure underlie changes in growth rate, since studies using a pressure probe failed to detect a correlation between turgor pressure and growth rate, even during oscillatory growth (Benkert et al. 1997). Given the importance of maintaining turgor pressure, it is attractive to imagine that both K^+ and Cl^- play a key role (Fig. 4), since substantial fluxes of both ions have been measured. As noted by Zonia et al. (2002), the large efflux of Cl^- observed in pollen tubes could contribute to salt extrusion and turgor regulation, as has been shown in other systems, notably guard cells (Cosgrove and Hedrich 1991).

4.6 Cell Wall

Ions, especially Ca^{2+} and H^+, play important roles in the cell wall. The primary focus has been on Ca^{2+}, where its ability to cross-link carboxyl

residues on de-esterified pectins becomes an important factor in determining the structure and yielding properties of the cell wall (Fig. 4; chapter by Geitmann and Steer, this volume). Based on modeling in which the rate of growth was taken into consideration together with published data on the Ca^{2+} content of immature and mature cells walls, Holdaway-Clarke and Hepler (2003) calculated that the flux would amount to 35 pmol/cm^2/s. By contrast, the amount of influx needed to satisfy the intracellular tip-focused gradient was calculated to be only 1.7 pmol/cm^2/s. Based on this large difference and the observation that the measured flux could be as high as 20 pmol/cm^2/s, Holdaway-Clarke and Hepler (2003) reasoned that the cell wall requirement for Ca^{2+} dominated the influx detected by the ion-selective vibrating electrode.

H^+ can also have a profound effect on the cell wall structure and yielding properties. Generally in plants, while increased Ca^{2+} reduces growth, increased H^+ facilitate the process (Cassab and Varner 1988). During pollen tube growth, the action of pectin methyl esterase (PME), which has been secreted into the cell wall, causes the de-esterification of the methoxy residues on pectin (Bosch et al. 2005). This process also releases H^+, which can then downregulate PME because of the enzyme's sensitivity to lowered pH. It has been proposed that the balance between H^+ production and PME activity constitutes a key factor in controlling the oscillation of cell wall yielding properties and thus the oscillatory cell growth (Holdaway-Clarke et al. 1997). But other factors are also regulated by pH. For example, certain acidic PME isoforms are stimulated by lowered pH (Li et al. 2002), as are pectin hydrolases. In addition, there are wall-bound exo-α-glucanases, which have been identified in lily pollen tubes, that are regulated by pH (Kotake et al. 2000; chapter by Geitmann and Steer, this volume).

5 Perspectives

Ions occupy a central position in the control of pollen tube growth. Ca^{2+} attracts most attention, especially given that the tip-focused gradient resides precisely at the place where maximal growth is known to occur. It seems evident that this ion contributes to the localized secretion of cell wall components and cytoskeletal dynamics. We must also consider the likely possibility that future work will uncover a role for this ion in many other processes, especially as other factors such as pollen-specific CDPKs, are identified and characterized. H^+ also emerge as prime growth-controlling ions. Gradients in pH, established through the activity of H^+-ATPases and other H^+-pumping enzymes, serve as the basic energy source for transmembrane transport. But H^+ can also affect the actin cytoskeleton, and in addition modulate the structure and yielding properties of the cell wall. Finally, K^+ and Cl^- are

recognized for the central role they may play in turgor regulation and in determining the membrane potential.

The pollen tube continues to be one of the very best objects for examining ion gradients and fluxes. Its rapid growth probably accounts for the fact that the underlying ionic expressions are amplified and as a consequence easier to recognize than in slower growing cells. Continued work should markedly enlarge our understanding of ion regulation, and provide ideas that may apply broadly to other growing plant cells.

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