RESEARCH PAPER

Calcium deprivation disrupts enlargement of Chara corallina cells: further evidence for the calcium pectate cycle

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Abstract

Pectin is a normal constituent of cell walls of green plants. When supplied externally to live cells or walls isolated from the large-celled green alga Chara corallina, pectin removes calcium from load-bearing cross-links in the wall, loosening the structure and allowing it to deform more rapidly under the action of turgor pressure. New Ca$^{2+}$ enters the vacated positions in the wall and the externally supplied pectin binds to the wall, depositing new wall material that strengthens the wall. A calcium pectate cycle has been proposed for these sub-reactions. In the present work, the cycle was tested in C. corallina by depriving the wall of external Ca$^{2+}$ while allowing the cycle to run. The prediction is that growth would eventually be disrupted by a lack of adequate deposition of new wall. The test involved adding pectate or the calcium chelator EGTA to the Ca$^{2+}$-containing culture medium to bind the calcium while the cycle ran in live cells. After growth accelerated, turgor and growth eventually decreased, followed by an abrupt turgor loss and growth cessation. The same experiment with isolated walls suggested the walls of live cells became unable to support the plasma membrane. If instead the pectate or EGTA was replaced with fresh Ca$^{2+}$-containing culture medium during the initial acceleration in live cells, growth was not disrupted and returned to the original rates. The operation of the cycle was thus confirmed, providing further evidence that growth rates and wall biosynthesis are controlled by these sub-reactions in plant cell walls.

Key words: Cell walls, chelation, growth, pectin, turgor pressure.

Introduction

The cell walls of green plants contain pectin (Ridley et al., 2001), and it was shown that newly-supplied pectin can accelerate wall extension, leading to larger cells than otherwise would occur (Proseus and Boyer, 2006a,b). Because pectin is released to the wall by the protoplast, this finding indicates that the rate of pectin release is an important controller of the rate of plant growth (Proseus and Boyer, 2006b; Boyer, 2009). The experiments were done in Chara corallina, a large-celled green alga that is one of the closest relatives to the algal progenitors of terrestrial plants (Chapman, 1985; Graham, 1985; Niklas, 1992; Scherp et al., 2001). Probably as a result, charophyte wall structure resembles that in terrestrial plants (Anderson and King, 1961; Preston, 1974; Morrison et al., 1993; Popper and Fry, 2003).

The new pectin acted as a chelator to remove calcium from pectins already in the wall (Proseus and Boyer, 2006b). Turgor pressure ($P$) was required for growth to accelerate, suggesting that the new pectin acted preferentially on calcium cross-links distorted by tension in the wall (Proseus and Boyer, 2007). The molecular structure of calcium pectate cross-links suggests that tension might distort and weaken them (Proseus and Boyer, 2007). When the distorted bonds lose their calcium, the wall would loosen and account for the accelerated growth rate of the cell.

Further experiments indicated that after calcium was removed from the wall cross-links, the calcium in the new pectate could form new cross-links to the wall, adding new wall material that strengthened the wall and controlled acceleration (Proseus and Boyer, 2006b). However, the strengthening required additional calcium to occupy the previously vacated calcium sites and thus to maintain the density of cross-linkages holding the wall together (Boyer, 2009). This implies that new
Calcium had to enter the wall from the external medium in order to complete the process. The nature of these sub-reactions is basically cyclical with a stoichiometry proposed in Fig. 1. When the cycle begins by removing calcium from distorted cross-links, it sets in motion accelerated wall extension, relaxation of the distorted bonds, and binding of the new undistorted pectin to the wall, leading to new wall deposition. The increased extension of the wall distorts other wall pectin and starts the cycle over again, making the cycle auto-catalytic (Proseus and Boyer, 2007). Over the long term, the cycle would be maintained by new pectate supplied internally from the cytoplasm (left side of Fig. 1) and new Ca\(^{2+}\) entering from the medium (right side of Fig. 1) at normal P (about 0.5 MPa in *Chlora*).

Proseus and Boyer (2008) tested this cycle by decreasing P briefly in live cells. Because it was shown that the deposition of new cell wall required normal P (Proseus and Boyer, 2006c), the low P blocked deposition and indicated that new wall materials (including pectin) must be accumulating unused (Proseus and Boyer, 2008). When P was restored to normal, growth accelerated and made up for the brief exposure to low P as though the accumulated pectin had been ‘stored’ while P was low. In isolated walls, supplying pectin while P was low simulated the accumulated but unused pectin in live cells. When P was returned to normal in the isolated walls, growth fully recovered the lost amount during exposure to low P and confirmed the action of the cycle.

A further test of the cycle would involve blocking the Ca\(^{2+}\) supply to the wall. Without Ca\(^{2+}\), the sub-reactions would be blocked that lead to wall strengthening (Fig. 1, red X). Lacking new wall cross-links, the extending wall would spread existing cross-links over a larger and larger area. Eventually, the wall would become too weak to withstand the tensions from P, and cell growth should be disrupted.

In the following work, this test was conducted with the alga *C. corallina*. By supplying excess polygalacturonic acid (PGA) to the growth medium around living cells, the Ca\(^{2+}\) in the medium was chelated by the PGA and no longer was a source for the cells. The PGA also acted on calcium in the wall, removing cross-links and setting the pectate cycle in motion. It was found that, without Ca\(^{2+}\) from the medium, the action of the cycle initially accelerated growth but eventually disrupted it.

**Materials and methods**

**Plant material**

The experiments were conducted with single internode cells of the alga *C. corallina* (Klein ex. Willd., em. R.D.W.) grown at 22–23 °C as described by Proseus and Boyer (2006a). All experimental manipulations were done without removing the cells from the culture medium in which the plants were grown. Single internode cells were excised from apical portions of the algal thallus, where the cells were elongating and possessed primary walls. One end of the cell was fastened in a gate in a trough containing the culture medium. The light intensity (10–15 μmol m\(^{-2}\) s\(^{-1}\)), temperature (23 °C), and pH (7–8.0) were essentially the same as in the parent cultures. After excision, the cells grew at rates similar to those in the intact plants for many hours (Zhu and Boyer, 1992). P was measured, changed, and controlled with a pressure probe (Steudle and Zimmermann, 1974). The probe removed or added cytoplasmic solution without altering the external medium (Zhu and Boyer, 1992; Proseus et al., 1999, 2000). Cytoplasmic streaming continued and the cytoplasm entered the large opening in the microcapillary of the probe. The microcapillary thus acted as a reservoir to decrease or increase cell volume with corresponding alterations in P. Wall extension was measured simultaneously in the same cells using a position transducer attached to the free end of the cell by the methods described in Proseus et al. (1999).

For isolated walls, the isolation was carried out in the culture medium according to Proseus and Boyer (2005, 2006a,c) and the open end of the wall was attached to the microcapillary of the pressure probe with ‘Zap-A-Gap CA+’ glue (Super Glue Corp, Rancho Cucamonga, CA, USA) without removing the wall from the medium. P was controlled as in the live cells except the lumen previously occupied by the cytoplasm was filled with mineral oil, and the oil was removed or added to the lumen with the probe. P in the isolated walls was comparable to that in the live cells. Wall extension was measured simultaneously in the same walls as above. The experiments were conducted at 22–23 °C.

**PGA and EGTA**

Polygalacturonic acid (poly-α-(1,4)-D-galacturionate, 80% potassium salt, denoted as PGA) was used for most of the experiments. Obtained from citrus albedo (Sigma, St. Louis, MO, USA), its molecular weight was 170 kDa (about 945 saccharide residues) measured by size exclusion chromatography at the Complex Carbohydrate Research Center, Athens, GA, USA. For experiments,
this PGA was added to culture medium taken directly from the *Chara* cultures and filtered to 0.2 μm to remove cells and debris. The pH of the solution was measured, and if necessary, adjusted to pH 7 before use. In similar fashion, the calcium chelator EGTA (ethylene glycol-bis(beta-aminoethyl ether) N,N',N,N'-tetraacetic acid) (Sigma) was prepared with filtered culture medium and adjusted to pH 7 with NaOH before use. All solutions were stored at 4 °C and used within 3 d or frozen at –20 °C and used within 2 weeks.

**Statistics**

Instead of adding or removing cell solution or mineral oil manually, an automatic controller was sometimes used to maintain $P$ in the cells or isolated walls, as described by Proseus and Boyer (2005). A datalogger (CR7X, Campbell Scientific, Logan UT, USA) and strip chart recorder monitored the output every 5 s from the pressure probe, position transducer, and a thermocouple immersed in the medium around the cell or isolated wall. A computer continually displayed the rate of change in length of the cell. The pressure, length, and temperature data in the datalogger were downloaded to a computer for processing. Each experiment was repeated at least three times and representative data are shown.

**Results**

PGA (35 μM, i.e. 6 mg ml$^{-1}$) caused growth to accelerate in live cells when added to the culture medium containing
0.61 mM Ca$^{2+}$ (Fig. 2B, arrow). This amount of PGA was more than 10 times the amount needed to bind all the calcium in the culture medium, and it remained in solution when added to the medium. After 1–2 h of accelerated growth, deceleration began because $P$ began to fall as the accelerated uptake of water diluted the cell contents ($P$ was uncontrolled in this experiment and allowed to respond to the osmotic potential of the cell contents). Eventually, growth ended completely and the link to the position transducer began to slip from the shrinking cell (Fig. 2B, arrowhead). $P$ then fell to zero abruptly (Fig. 2A). In similar fashion, EGTA (2.5 mM) added to the culture medium first accelerated, then decelerated and disrupted cell enlargement when $P$ dropped to zero (Fig. 2C and D).

In contrast, only acceleration was observed if the PGA- or EGTA-containing culture medium was drained away after a short time and replaced with fresh culture medium (Fig. 3, upward arrows). Because the fresh medium contained 0.61 mM Ca$^{2+}$, it would replace the calcium vacated during the PGA or EGTA exposures. The growth rate returned to the original rate before the exposures. $P$ was controlled in this experiment and kept at normal levels (Fig. 3A and C). Monitoring these cells for many hours showed that growth was maintained essentially at the original rate (data not shown).

In order to determine whether the cell wall had been disrupted when the exposure to PGA was prolonged, the walls were isolated and exposed to 35 μM PGA. The wall grew for 15 h without disruption under these conditions (data not shown, but see Fig. 8 of Proseus and Boyer, 2006a for an example). On the other hand, if instead of adding PGA a concentrated EGTA (25 mM) solution was added to the culture medium, the wall grew for several hours but after about 8 h ceased growing (Fig. 4B). $P$ abruptly decreased to zero at the same time (Fig. 4A) and mineral oil droplets could be seen exiting the wall into the medium.

**Discussion**

These experiments support the operation of the calcium pectate cycle (Fig. 1). Cell enlargement was disrupted by prolonged exposure of live cells to PGA or EGTA in the culture medium, as predicted by the cycle. These externally supplied chelators bound Ca$^{2+}$ in the medium at the same time they partially removed calcium from the wall, denying the live cells a source of Ca$^{2+}$. The calcium cycle thus

**Fig. 4.** As for Fig. 2 except that a wall was isolated from a live Chara cell and exposed to more concentrated EGTA (25 mM instead of 2.5 mM) than was used in the live cell.

**Fig. 5.** Data from Fig. 2 showing critical turgor above which the cells enlarge ($P_c$, horizontal dashed line). According to Proseus et al. (2000), $P_c$ was an average 0.4 MPa with a range ± 0.07 MPa in Chara. When the $P$ fell to $P_c$, enlargement should have ceased (predicted $dL/dt = 0$, vertical dashed lines).
operated and accelerated the enlargement but without new Ca\textsuperscript{2+} entering the wall. After a few hours of acceleration, growth slowed because \( P \) began to fall as the entering water diluted cell contents. Because growth occurs only when \( P \) is above about 0.4 MPa in Chara (Proseus et al., 2000), the decrease in \( P \) had a large effect. Growth eventually ceased at \( P \) of 0.4 MPa (Fig. 5, vertical dashed lines). The complete loss of \( P \) that followed indicated either that the wall had lost integrity or the plasma membrane had been deleteriously affected. The latter possibility is favoured because the same experiment with isolated walls showed no loss in wall integrity even for a prolonged time (e.g. 15 h). Much stronger chelation (concentrated EGTA) was required for integrity to be lost. Consequently, it appeared that the wall structure in live cells remained intact with PGA but eventually became unable to support the plasma membrane, or the membrane itself required a direct source of calcium.

Although external chelators have these effects because they block the external source of Ca\textsuperscript{2+} at the same time that they remove calcium from the wall, the same chelators supplied internally would act differently, without chelating the external supply of free Ca\textsuperscript{2+}. For example, PGA from the protoplasm would remove calcium from the wall without blocking Ca\textsuperscript{2+} entry from outside. Thus, this source would allow the full calcium pectate cycle to operate without disrupting growth, which would continue indefinitely.

If exposure to external PGA or EGTA was not prolonged, the disruption of cell enlargement did not occur. Replacing these chelators with fresh culture medium appeared to generate wall cross-links and provide a continued source of new Ca\textsuperscript{2+} to the wall. With the calcium source renewed, the rate of cell enlargement returned to the rate before exposure to PGA or EGTA presumably because the density of wall cross-links had been restored.

In terrestrial plants, there is also suggestive evidence for a pectate cycle. Ezaki et al. (2005) reported increased extensibility of soybean hypocotyls exposed to a calcium chelator. A previous report that breakage of calcium cross-links did not affect wall extensibility (Virk and Cleland, 1990) was repeated by Ezaki et al. (2005) and found to have used tissue tensions too low for growth to occur and thus too low for calcium effects to be observed. Although it is difficult to be sure, similar arguments may apply to results of Rayle (1989) with Avena coleoptiles because even lower tensions were used. Further support of a role for wall calcium is reported by Thompson (2005) who observed increased wall extensibility when wall calcium was removed with a chelator in tomato. Zhao et al. (2008) used EGTA to remove calcium from the maturing walls of cucumber hypocotyls and found that the mature region resumed elongating, and expansin resumed its activity. McKenna et al. (2009) found an oscillatory release of pectate by exocytosis in tip-growing pollen tubes, which could explain their oscillating growth activity.

In contrast with charophytes whose wall pectins are mostly non-esterified (Anderson and King, 1961; Morrison et al., 1993), the pectins of terrestrial plants are often substantially esterified and limited in the number of cross-links that can be formed with calcium. It may be noted that because turgor pressure is force per unit area, there is less total force on the end walls of small cells than large cells. It thus may be necessary for terrestrial plants to block some of the potential cross-links by esterifying the carboxyl groups in pectate in order to make walls more extensible with the diminished total force. Accordingly, an over-expression of a pectin methylesterase would increase calcium cross-links, enhance wall strength, and diminish growth in tissues having small cells. Derbyshire et al. (2007) found that over-expressing a pectin methylesterase in the terrestrial plant Arabidopsis thaliana (L.) Heynh caused growth to decrease in the hypocotyls, as though the esterase unblocked some of the carboxyl groups in the pectins and increased the cross-linking, tightening the wall. These findings suggest a major role for pectate and wall calcium in the growth of terrestrial plants.

The present work indicates that prolonged exposure of live cells to external chelators will cause transient growth acceleration followed by growth inhibition and disruption. This is likely to make the activity of externally supplied PGA difficult to detect in tissues where prolonged exposure may be required for adequate penetration of the pectate to all of the cells. If the pectate is supplied internally by the cytoplasm, however, the disruption seems unlikely to occur.

References


