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Reevaluation of the Effect of Calcium Ions on Auxin-induced Elongation[1]

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ROBERT E. CLELAND
Department of Botany, University of Washington, Seattle, Washington 98195
DAVID L. RAYLE
Department of Botany, San Diego State University, San Diego, California 92115

ABSTRACT

The mechanism by which calcium ions inhibit cell elongation has been reinvestigated. Growth-inhibiting levels of calcium, when applied to isolated walls (*in vitro* treatment) do not decrease cell wall extensibility as measured by the Instron technique. Thus, the hypothesis that calcium inhibits growth by forming wall-stiffening calcium bridges must be abandoned. Treatment of living auxin-treated sections with calcium (*in vivo* treatment) does cause a decrease in the subsequently measured wall extensibility, but this decline appears to be simply a consequence of the growth inhibition rather than its cause. Growth-inhibiting levels of calcium do not appreciably reduce the rate of auxin-enhanced H^+ excretion. Pretreatment with calcium does not reduce the capacity of walls to undergo acid-activated wall loosening in the absence of calcium. High concentrations of $CaCl_2$ (0.02 M) cause an initial elastic shrinkage of *Avena* sections comparable to that caused by the same osmolarity of mannitol, but the subsequent growth inhibition is too great to be explained by an osmotic inhibition. Calcium ions do inhibit H^+-induced extension of frozen-thawed sections under tension. The growth-inhibitory effects of calcium, then, may be ascribed to a direct inhibition exerted by calcium ions on the H^+-induced wall-loosening process.

The ability of high concentrations of calcium ions to inhibit cell elongation has long been recognized (1, 13, 20, 21). This inhibitory effect has been rationalized (2, 3, 13, 19) by assuming that calcium forms bridges between pectic carboxyl groups and that these bridges result in a mechanical stiffening of the wall which in turn prevents wall extension. The principal evidence for this theory consists of the demonstration by Tagawa and Bonner (19) that the ability of live *Avena* coleoptile sections to be deformed by an applied weight is reduced by pretreatment of the sections with $CaCl_2$. This bending technique, however, is not a reliable test of the wall's mechanical properties, as it is sensitive to changes in turgor, water permeability, and the activity of wall-loosening enzymes as well as to changes in the mechanical properties of the walls (7). The initial objective of this research, then, was to determine if calcium had a direct effect on the mechanical properties of cell walls, using a more definitive test, the Instron technique (5). Failing to find any significant effect of calcium on the mechanical properties of the walls, we then investigated four alternative mechanisms by which calcium might inhibit cell elongation: reduction in turgor pressure, reduction in auxin-induced H^+ excretion, reduction in the capacity for the walls to undergo acid-induced wall loosening, and direct interference by calcium with the biochemical wall-loosening process. It will be shown that the growth-inhibitory effect of high calcium concentrations is most probably due to an inhibition by calcium of the biochemical wall-loosening process.

MATERIALS AND METHODS

The plant material used in this study consisted of 10- or 14-mm sections cut from deleafed coleoptiles of *Avena sativa*, cv. Victory. Methods for growing the seedlings and harvesting the sections have been described (9). In some experiments the cuticle and epidermis were removed (peeled) from the sections with the aid of fine forceps (16). Elongation of live sections and acid-induced extension of frozen-thawed sections were measured by the procedure and apparatus of Rayle and Cleland (17). Since preliminary experiments confirmed earlier reports (13, 19) that 0.02 M $CaCl_2$ causes a maximal inhibition of cell elongation in nonpeeled sections and 0.002 M $CaCl_2$ terminates growth in peeled sections (data not shown), these concentrations were used in all subsequent experiments unless otherwise noted.

Hydrogen ion excretion[2] experiments were carried out using groups of 10 peeled 14-mm segments. The sections were pretreated for 30 min in 1 mM K-phosphate buffer (pH 6.15), containing 1 mM KCl and 0 to 0.02 M $CaCl_2$. The sections were then transferred to 2 ml of the same solution, with or without 10 μM IAA. At 30-min intervals the pH was monitored with an Ingold flat surface electrode, and in some experiments the pH was maintained at 6.15 by titration with 5 mM KOH.

The following procedure was used in experiments designed to test the effect of calcium on the mechanical properties of cell walls. Live sections were first incubated for 120 to 210 min in K-maleate buffer (2.5 mM, pH 4.7), ± 0.02 M $CaCl_2$ and with or without 10 μM IAA. In some experiments sections were harvested at this point by boiling in methanol or by freezing and thawing. In other experiments sections were incubated as above and then transferred to additional solutions containing buffer, ± IAA ± $CaCl_2$ or ± KCl (0.02 M) for various periods of time prior to harvest. Some of the boiled or frozen-thawed sections were incubated for 3 to 4.5 hr in water or in water containing 0.02 M $CaCl_2$ or 0.02 M KCl. The extensibility of all sections was determined using the Instron technique (5), and expressed as plastic (DP) and elastic (DE) compliance values or as per cent extension/100-g load. In some experiments the

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[2] The term hydrogen ion excretion is used here to denote an energy-requiring process which acidified the external medium. Whether the acidification is due to H^+ export, OH^- uptake, or some other mechanism is discussed elsewhere (18).

sections were deproteinized after harvest but before the *in vitro* incubations (5), while in others no deproteinization was used. In each experiment duplicate sets of 12 sections were run for each treatment. All experiments were repeated at least three times.

RESULTS

Calcium and Mechanical Properties of Cell Walls. The effect of calcium on the mechanical properties of *Avena* coleoptile cell walls was examined using the Instron technique to measure the plastic and elastic extensibility. In the first series of experiments calcium was added directly to isolated cell walls. Live sections were first pretreated with auxin in order to insure that the wall extensibility was high, before disruption of the protoplasts by boiling in methanol. The resulting cell wall matrices were then treated with $CaCl_2$, KCl, or water. The extensibility of these isolated cell walls was not reduced by treatment of the sections with 0.02 M $CaCl_2$ (Table I), nor was it increased by treatment with 0.02 M KCl (data not shown). The inability of calcium to stiffen cell walls *in vitro* is unlikely to be a consequence of the method of preparation of the walls as wall material prepared by freezing-thawing show a similar insensitivity to calcium ions (Table I). This insensitivity is not altered by the removal of protein with pronase prior to the calcium treatment (data not shown). Likewise, the presence of the cuticle, which could be a barrier to the entry of ions, does not seem to be a factor, as sections from which the epidermis had been peeled prior to isolation of the walls were similarly insensitive to Ca^{2+} or K^+ (data not shown). We have not found any condition under which addition of calcium ions to isolated cell walls reduces their plastic or elastic extensibility as measured by the Instron technique, and we must therefore question the possibility that calcium inhibits cell elongation via a direct physical stiffening effect on the wall.

In contrast to the *in vitro* data, treatment of live sections with Ca^{2+} or K^+ does alter the wall extensibility as measured by the Instron technique. $CaCl_2$, added to auxin-treated sections, causes a reduction in DP (Fig. 1) and DE (data not shown). This effect is partially reversed by transfer of the sections to fresh medium containing IAA and KCl. KCl causes no increase in the DP of auxin-treated sections in the absence of a calcium pretreatment. In the absence of auxin neither $CaCl_2$ nor KCl affects the extensibility of live sections to any appreciable extent (Fig. 1). The fact that calcium causes wall stiffening only when added to live sections and not when added to isolated walls suggests that this *in vivo* calcium-induced stiffening is an indirect effect (see under "Discussion").

Calcium and Mechanism of Auxin-induced Cell Elongation. If calcium does not inhibit auxin-induced cell elongation by interacting directly with the wall it must interfere with some other aspect of cell elongation. We have examined four possibilities, using as our starting point the hypothesis (7, 16, 18) that auxin causes coleoptile cells to excrete hydrogen ions and that the resulting lowered cell wall pH activates wall-loosening enzymes. As a result of the biochemical wall loosening, turgor-driven wall extension can occur. The possibility that calcium interferes with the first step in this process, the H^+ excretion, was tested and eliminated. The data in Table II show that 2 mM $CaCl_2$, which inhibits elongation of peeled coleoptiles, actually stimulates auxin-induced H^+ excretion. Higher concentrations of calcium (0.02 M) do cause a slight inhibition of H^+ excretion, however.

A second possibility is that calcium might be inhibiting growth osmotically. Previous studies (4, 14) have shown that only a slight reduction in turgor is needed in order to cause a severe inhibition of cell elongation in *Avena* coleoptiles. With osmotic inhibition there is an immediate decrease in growth rate followed by a slow recovery to a new lower growth rate. Often sections show a slight transitory shrinkage immediately after addition of the osmotic agent (4, 11). As can be seen from Figure 2, 0.02 M $CaCl_2$ appears to cause a slight osmotic shrinkage of sections extending rapidly in response to an acidic solution. Similar results were obtained with sections extending rapidly in response to auxin (data not shown). Equal osmolar levels of mannitol cause identical transitory shrinkages. Following mannitol treatment coleoptile sections recover to a new positive growth rate, while calcium-treated sections do not recover. These data taken together with the fact that in peeled sections $CaCl_2$ concentrations as low as 2 mM severely inhibit elongation strongly argue against calcium inhibiting growth by reducing turgor pressure.

A third possibility is that calcium causes the walls to lose their capability to undergo wall loosening in response to hydrogen ions. For example, calcium ions, which are known to cause desorption of wall-associated proteins (15), might desorb the wall-loosening enzymes thus causing them to be nonfunctional; or Ca^{2+} might prevent the export of new wall-loosening enzymes to replace those lost by normal turnover. To test this possibility, live, auxin-treated sections were pretreated for 5 hr with or without a growth-inhibitory calcium concentration. They were then peeled (to facilitate H^+ entry), frozen-thawed, and the ability to undergo *in vitro* acid-induced elongation was measured (Table III). The pretreatment with calcium was found to have no effect on the segments capacity for acid-induced wall loosening, indicating that calcium does not inhibit cell elongation via reduction in the capacity for wall loosening.

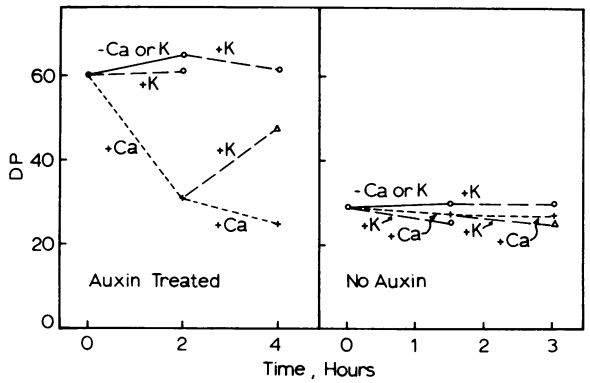

FIG. 1. Effect of *in vivo* calcium and potassium treatment on the plastic compliance values (DP) of *Avena* cell walls. After incubation segments were boiled 5 min in methanol, treated 20 hr in 2 μg/ml pronase, and then subjected to Instron analysis. Left: segments preincubated 150 min in 10 μM IAA (2.5 mM K-maleate buffer, pH 4.7) prior to transfer to fresh solutions containing IAA ± the indicated chloride salts (0.02 M). Right: segments preincubated 120 min in buffer before transfer to the indicated chloride salts (0.02 M).

TABLE I. Effect of calcium on the Instron-measured extensibility of Avena cell walls.

Live sections incubated 3 (A) or 3.5(B) hrs in 2.5 mM K-maleate + 10 μM IAA ± 0.02 M $CaCl_2$, then killed in boiling methanol (A) or by freezing and thawing twice (B). After rehydration some sections were analyzed immediately, while others were incubated for 2(A) or 4.5(B) hrs in water ± 0.02 M $CaCl_2$ or KCl.

Treatments		Expt.A. MeOH-killed		Expt.B. Frozen	
		% Extension/100 g load			
In vivo	In vitro	Elastic	Plastic	Elastic	Plastic
IAA	———	17 ± 1	43 ± 3	14 ± 1	37 ± 2
IAA	H_2O	16 ± 1	37 ± 3	14 ± 2	39 ± 3
IAA	$CaCl_2$	16 ± 2	38 ± 3	14 ± 2	39 ± 2
IAA + Ca	———	12 ± 1	16 ± 3	13 ± 1	21 ± 2
IAA + Ca	H_2O	13 ± 2	17 ± 2	13 ± 4	22 ± 2
IAA + Ca	KCl	13 ± 1	17 ± 3	13 ± 2	21 ± 4

TABLE II. Effect of calcium on IAA-induced H^+ excretion by peeled Avena coleoptile sections.

Conditions described in text. pH maintained at 6.15 by titration every 30 min to 180 min.

Calcium conc.	μMoles H^+ excreted/180 min	
	+IAA	-IAA
0	0.625	0
0.002 M	0.85	0.35
0.02 M	0.525	0.15

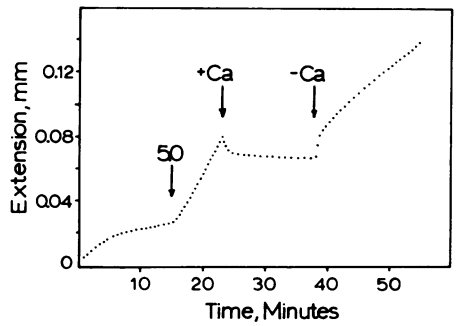

FIG. 2. Time course for inhibition of elongation of live sections. Peeled sections incubated in 10 mM K-Pi (pH 6.5) and at 1st hr solution changed to 1 at pH 5. At subsequent arrows solutions changed to pH 5 solutions + 0.02 M $CaCl_2$ and then back to Ca-free solution. Note immediate shrinkage and total inhibition of acid-induced growth caused by calcium.

TABLE III. Effect of in vivo calcium treatment on ability of frozen-thawed Avena coleoptiles to undergo an in vitro acid growth response.

Unpeeled sections pretreated 150 min in 1 mM K-Pi + 10 μM IAA, then transferred to similar solutions ± 0.02 M $CaCl_2$. After 5 hr sections were peeled, frozen-thawed, and placed in 10 mM K-Pi-citrate, pH 7.0, under 15 g stress. After 1 hr solution changed to 10 mM K-Pi-citrate and extension rate determined at 0, 10 and 60 min.

	Extension rate, %/hr		
	0	10 min	60 min
No $CaCl_2$	0.7	5.8	2.8
+ $CaCl_2$	0.4	5.8	2.4

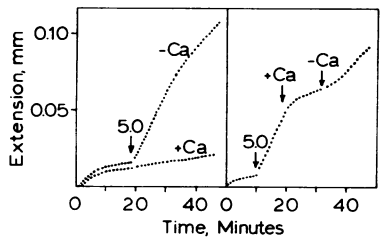

FIG. 3. Inhibition of *in vitro* acid-induced cell wall loosening by calcium ions. Peeled, frozen-thawed sections initially incubated in 1 mM HEPES-NaOH (pH 6.5) under 15-g load, then solution changed at first arrow to 1 mM HEPES-NaOH (pH 5.0) ± 0.003 M $CaCl_2$. Note reversibility of calcium inhibition of acid-induced extension (right).

The final possibility is that ealcium interferes with the acid-induced wall-loosening process itself. To test this, peeled, frozen-thawed coleoptiles were placed in a pH 6.5 buffer, ± 3 mM $CaCl_2$, and under 15-g constant applied load. After approximately 20 min the solution was changed to similar ones at pH 5 and the extensibility was measured (Fig. 3). The acid growth response of the sections was dramatically inhibited in the presence of $CaCl_2$. This inhibition is rapid and readily reversible. It seems that calcium inhibits growth by interfering with the biochemical wall-loosening process.

DISCUSSION

The growth inhibition caused by high calcium concentrations has been explained (2, 13, 19) by assuming that calcium cross-links the polysaccharides of the wall, and that these bridges stiffen the walls, thus preventing turgor-driven wall extension from occurring. The principal evidence for this arises from reports (1, 19) that calcium pretreatment of live coleoptile sections reduced the ability of the sections to be subsequently deformed by an applied weight. This was not a direct test of the calcium wall-stiffening idea (7), however, as the bending test is not necessarily a measure of the mechanical properties of the walls, and the effect of calcium could have been indirect (see below).

We have used the Instron technique to measure the mechanical properties of cell walls after treatment of isolated walls with calcium, and we find that calcium has no effect on these properties. However, before discarding the concept that calcium can directly stiffen cell walls we must consider two questions. First, is the Instron technique a valid measure of the mechanical properties of cell walls? Mechanical properties can be assessed by stress relaxation, creep or stress strain (Instron) techniques. Comparison of the rheology of coleoptile walls as measured by stress relaxation (10, 22), creep (8) and the Instron technique (5, 6, 8) shows that all three provide comparable information about the mechanical properties. If the walls are stiffened by calcium it should result in a decrease in plastic extensibility as measured by the Instron technique. Second, could the apparent insensitivity to calcium be due to some artifact associated with the use of isolated, *in vitro* walls for the measurements rather than live tissues? This is unlikely, as comparisons of the Instron extensibility of live sections with sections killed by methanol boiling or by freezing-thawing indicate that the condition of the tissue has a quantitative but not a qualitative effect on the mechanical properties (5). We conclude that the present technique provides a valid measure of the mechanical properties of the cell walls, and that calcium, although it may form calcium bridges in the walls, simply does not stiffen cell walls.

We have confirmed the observation of Tagawa and Bonner (19) that addition of calcium to live coleoptiles does result in a decrease in the extensibility of the walls, but this effect of calcium would appear to be indirect. It could be a response to a calcium-mediated change in the cell wall metabolism, or it could simply be a consequence of the growth inhibition; inhibition of auxin-induced growth by respiratory inhibitors such as KCN, DNP, and N-ethylmaleimide has been shown to result in a stiffening of the cell wall (6).

Since calcium does not stiffen cell walls directly, its inhibition of growth must be ascribed to some other cause. We have eliminated the possibility that calcium sufficiently reduces turgor pressure or alters the capacity of the walls to undergo acid-induced wall loosening so as to cause the growth inhibition. Furthermore, calcium inhibits cell elongation at concentrations at which it actually promotes H^+ excretion (12). The one major effect of calcium is to prevent acid-induced wall loosening. It should be noted that calcium must be present simultaneously with the hydrogen ions; a pretreatment with calcium is without effect on a subsequent acid-induced extension. Thus, calcium may be acting as a competitive inhibitor for the hydrogen ions, competing for the sites to which the hydrogen ions bind. Alternatively, calcium may simply alter either the protein or the polysaccharides so that an H^+-enhanced enzymic wall-loosening reaction cannot occur. A kinetic analysis of the calcium-

hydrogen ion interaction on wall extension may help to distinguish between these possibilities. In any case, the inhibition of growth by calcium would appear to be a consequence of the inhibition of biochemical wall-loosening process rather than a direct stiffening action of calcium through calcium bridge formation.

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