Differential Effects of Divalent Cations on Plant Membranes[1]

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Abstract

Membrane fractions, including two plasma membrane fractions of differing density, were isolated from onion stem and treated in suspension with varying concentrations of calcium. At calcium concentrations above 1 millimolar, heavy plasma membranes showed an increase in optical density followed by precipitation. Other fractions, including the light plasma membrane prepared in a similar manner, responded by an increase in optical density but did not precipitate. The optical density change, but not the precipitation, was elicited by magnesium ions. The results show a direct interaction of calcium ions with plant membranes. The precipitation of heavy but not light plasma membranes at calcium concentrations which normally inhibit growth provides evidence for heterogeneity of plant plasma membranes and suggests a growth regulatory role for the "heavy" fraction.

Calcium, an essential element for plant growth (23), stimulates or inhibits a variety of cell processes depending on its concentration (9). In the absence of toxic metals, many plants thrive in the presence of only micromolar levels of the ion. When calcium is absent (9) or at supraoptimal (1 to 10 mM) concentrations of calcium (1), growth ceases.

Concentrations of calcium up to 1 mM appear to be necessary to maintain the selectivity for potassium of the low K_m system of monovalent ion uptake (4). At concentrations above 1 mM, calcium begins to inhibit uptake of both sodium and potassium by the high K_m system (6, 18). Above 10 mM, calcium ions exert even more pronounced inhibition of monovalent ion uptake. The effect of calcium on uptake of monovalent ions clearly implicates the plasma membrane as a primary site of calcium interaction. This view is encouraged by the electron-microscopic observations of Marinos (14) on calcium-deficient barley tissue which showed extensive disintegration of cellular membranes. Similarly, animal plasma membranes require calcium for their integrity. Punctured cells do not reseal in the absence of calcium (20); blastula cells disperse when calcium is removed (3); and a protein component of rat liver plasma membrane is solubilized when calcium is removed with ethylenediaminetetracetic acid (EDTA) (15).

Although calcium is clearly implicated as a functional constituent of plasma membranes, the nature of the calcium-membrane interaction has received little attention. Changes of membrane conformation and swelling or shrinking of membrane vesicles give rise to changes in light absorbance by suspensions (5, 8, 21). By measuring absorbance changes and specific precipitation when calcium ions are added, we show a direct interaction between plant membranes and calcium.

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Materials and Methods

Stems of green onions (11) were homogenized for 2 min with a Polytron 20ST homogenizer (Kinematica, Lucerne, Switzerland), operating at approximately 9,000 rpm. The homogenizing medium consisted of 0.1 M K_2HPO_4 (pH 7.4), 20 mM Na_2EDTA, and 0.5 M sucrose in coconut milk (centrifuged at 100,000 x g for 90 min to remove particles) (11). The resulting homogenate was filtered through Miracloth (Chicopee Mills, New York), and centrifuged at 6,000 x g for 10 min. The supernatant was layered on the gradient shown in Figure 1, and centrifuged at 100,000 x g for 1 hour. Fractions were collected from the interfaces of the layers, resuspended in homogenization medium and pelleted for 45 min at 100,00 x g.

Protein was determined by the method of Lowry *et al.* (12). Succinate dehydrogenase (16) was used as a mitochondrial marker.

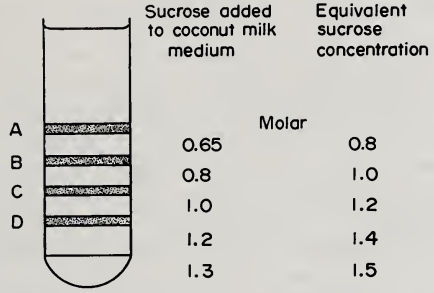

FIGURE 1. *Procedure for sucrose-coconut milk gradient centrifugation.*

For comparative spectrophotometric assays among fractions, suspensions, adjusted to the same protein concentration, were prepared in 0.25 M sucrose, 80 mM Tris, 25 mM MES, and 14 mM mercaptoethanol, adjusted to pH 6.0 with acetic acid. The diluted suspension (1.5 or 3.0 ml) was placed in a cuvette, and absorbance at 450 nm was monitored continuously (Fig. 2) using a Beckman DB spectrophotometer with chart recorder. Calcium was added as a concentrated solution in a sufficiently small volume (50 μl) to give a negligible dilution effect on absorbance. To quantitate precipitation, suspensions at the same protein concentration were treated with 10 mM calcium, and after standing for 2 hours at 25°C were centrifuged in a clinical centrifuge. Precipitate and supernatant were then assayed for protein, and the percentage of the total protein accounted for by the precipitate was compared with that of controls treated similarly in the absence of calcium.

Results

Addition of calcium to plant membrane suspensions resulted in a characteristic pattern of absorbance change: an initial rapid increase, essentially complete within 2 min, and a slower, almost linear rise which

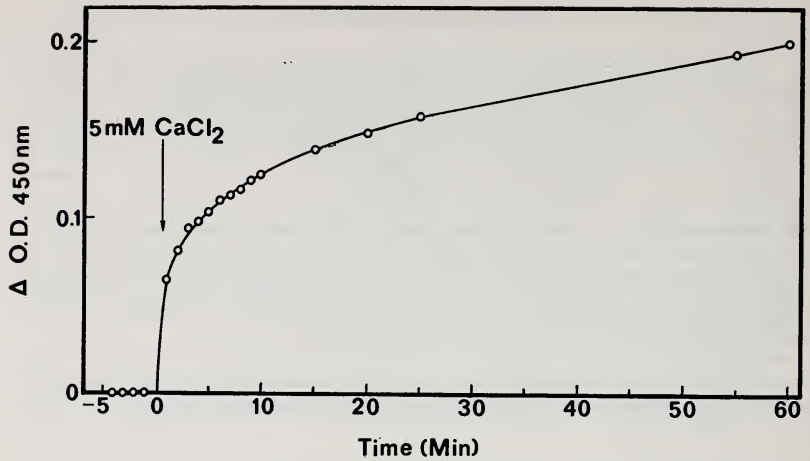

FIGURE 2. *Time course of absorbance increase upon addition of calcium (C fraction).*

continued for at least an hour (Fig. 2). All the fractions described in Figure 1 gave a similar response to calcium concentrations up to 1 mM (Fig. 3). Above 1 mM, the A, B and C fractions increased in absorbance with increased calcium concentration, whereas the D fraction showed a decrease in absorbance due to a clumping of the membranes. The degree of clumping of the D fraction increased with time or increased calcium concentrations. The threshold concentration for precipitation and extent of precipitate formation varied. Fresh preparations precipitated at 1 to 5 mM calcium, whereas membranes stored at 4°C overnight required 5 to 10 mM calcium to precipitate. Only the D fraction was specifically precipitated by calcium (Table 1, Exp. I). Magnesium and monovalent ions were ineffective (Fig. 4). The absorbance rise, however, was elicited by magnesium, but required higher concentrations than with calcium (Fig. 4).

TABLE 1. *Precipitation of membrane fractions by calcium (10mM).*

Experiment	Fraction	% Total Membrane Precipitated	Succinate INT Reductase Activity
I	A	6	---
	B	1	---
	C	8	---
	D	68	---
II	D top	23	4.4μM/mg/hr.
	D bottom	6	7.4μM/mg/hr.

Electron microscopy, using the specific staining reaction of Roland *et al.* (19), showed plasma membrane and mitochondria in the D fraction. When a suspension of this fraction was centrifuged at 100,000 x g for 45 min, the pellet consisted of two layers. The lower layer was light brown and opaque, whereas the upper layer was pale

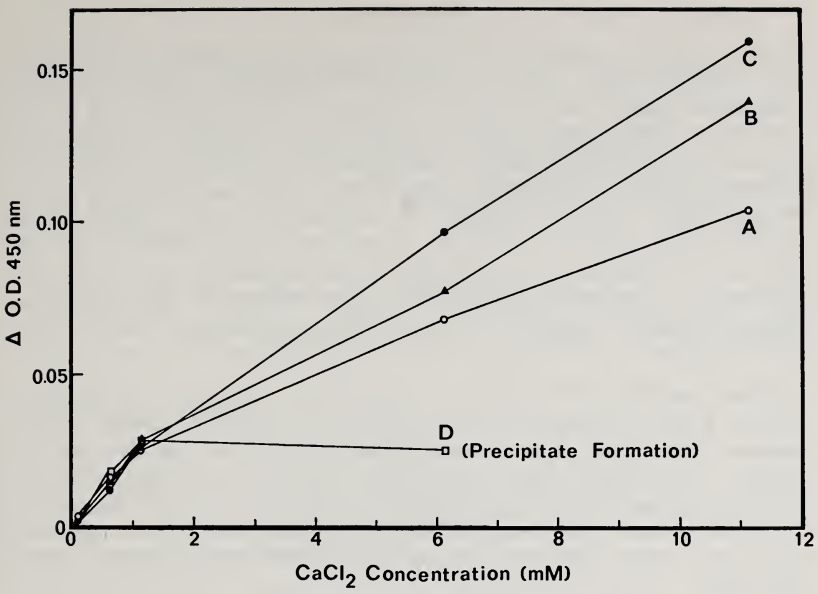

FIGURE 3. *Effect of calcium concentration on initial (2 min) absorbance increase.*

and translucent. Table 1 (Exp. II) shows that the upper layer had a lower mitochondrial activity, but showed a greater precipitation with calcium.

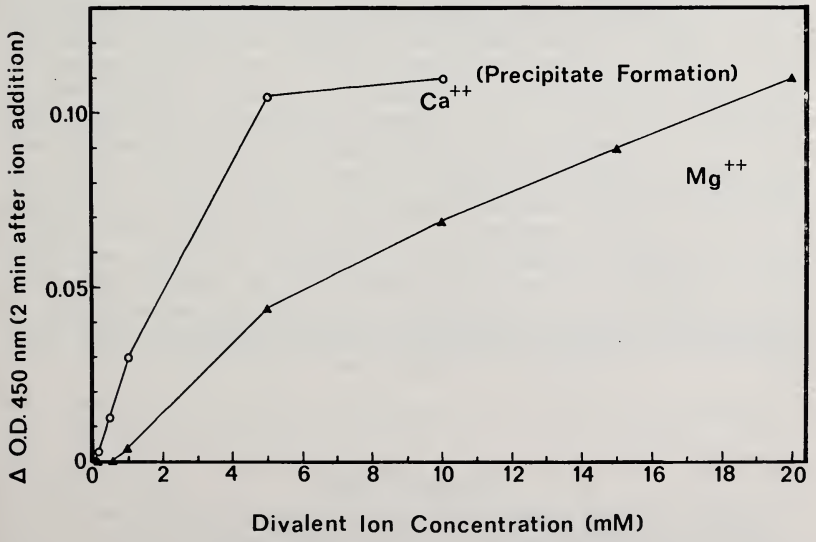

FIGURE 4. *Initial (2 min) absorbance increase comparing varying concentrations of calcium and magnesium ions (D fraction).*

Discussion

The biphasic increase of absorbance by plant membranes with time after adding calcium (Fig. 2) indicates two separate interactions. The initial rapid phase may represent a binding on the divalent ions to the membranes with a concomitant conformational change. It has been suggested that calcium causes a condensation of membranes with a subsequently reduced permeability (4, 13). The slow extended rise in absorbance may represent uptake of calcium into the vesicle lumen (5, 21). Both phases of the rise in absorbance may result from ion influx, with a change in rate occurring as the membrane assumes a more condensed configuration. Regardless of the nature of the absorbance change, a direct interaction between plant membranes and divalent cations is shown.

The precipitation effect is more easily explained. Membranous vesicles carry a negative surface charge (2, 17) which causes mutual repulsion. When the charge is neutralized by positive ions, the vesicles approach each other, and the divalent ions form bridges between adjacent vesicles (22). Such bridging is probably a normal occurrence in animal tissues where adjacent cells juxtapose without the intervention of cell walls. Calcium is essential for cell to cell adhesion in rat liver (10).

In pea epicotyls, auxin stimulated growth is virtually eliminated as the calcium concentration in the external medium is raised from 1 to 10 mM. The results presented here show that a portion of the plant plasma membrane (the D or heavy plasma membrane fraction) is specifically precipitated by calcium concentrations similar to those which inhibit growth. Precipitation occurs only in the "heavy" or D fraction whereas a similar amount of plasma membrane occurs in the "light" or C fraction. Such heterogeneity of plant plasma membrane has been noted for other parameters associated with growth regulation. The regulatory plant pigment phytochrome has been identified only in heavy plasma membrane (F. A. Williamson, M. J. Jaffe, and D. J. Morré, unpublished data), and heavy plasma membranes specifically bind auxins (F. A. Williamson, D. J. Morré, and A. C. Leopold, unpublished data). Hertel, Thomson, and Russo (7) have shown that IAA binds only to fractions of density approximating that of our D fraction. In contrast, the auxin transport antagonist, N-1-naphthylphthalamic acid (NPA), shows a positive correlation of its binding with plasma membrane content, independent of membrane density (11). We conclude that plant plasma membranes are heterogeneous, and that this heteorogeneity is expressed by a number of parameters, including precipitation by calcium ions.

Literature Cited

1. COARTNEY, J. S. 1967. Physical and chemical analysis of auxin-induced cellwall loosening. Unpublished Ph.D. Dissertation, Purdue University, Lafayette, Ind. 72 p.

2. CURTIS, A. S. G. 1962. Cell contact and adhesion. Biol. Rev. 37:82-129.

3. DAN, K. 1960. Cyto-embroyology of echinoderms and amphibia. Int. Rev. Cytol. 9:321-367.

4. EPSTEIN, E. 1961. The essential role of calcium in selective cation transport by plant cells. Plant Physiol. 36:437-444.

5. FAIRHURST, A. S., and D. J. JENDEN. 1966. Spectrophotometric monitoring of calcium uptake by skeletal muscle particles. Anal. Biochem. 16:294-301.

6. HANDLEY, R., A. METWALLY, and R. OVERSTREET. 1965. Effects of Ca upon metabolic and non-metabolic uptake of Na and Rb by root segments of *Zea mays*. Plant Physiol. 40:513-520.

7. HERTEL, R., K. ST.-THOMSON, and V. E. A. RUSSO. 1972. *In vitro* auxin binding to particulate cell fractions from corn coleoptiles. Planta 107:325-340.

8. INESI, G., S. EBASHI, and S. WATANABE. 1964. Preparation of vesicular relaxing factor from bovine heart tissue. Amer. J. Physiol. 207:1339-1344.

9. JONES, R. G. W., and O. R. LUNT. 1967. The function of calcium in plants. Bot. Rev. 33:407-426.

10. LEESON, T. S., and H. KALANT. 1961. Effects of *in vivo* decalcification on ultrastructure of adult rat liver. J. Biophys. Biochem. Cytol. 10:95-104.

11. LEMBI, C. A., D. J. MORRÉ, K. ST.-THOMSON, and R. HERTEL. 1971. N-1-naphthylphthalamic-acid-binding activity of a plasma membrane-rich fraction from maize coleoptiles. Planta 99:37-45.

12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193:265-275.

13. MANERY, J. F. 1966. Effects of Ca ions on membranes. Fed. Proc. 25:1804-1810.

14. MARINOS, N. G. 1962. Studies on submicroscopic aspects of mineral deficiencies. I. Calcium deficiency in the shoot apex of barley. Amer. J. Bot. 49:834-841.

15. NEVILLE, D. M., JR. 1968. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. Biochim. Biophys. Acta. 154:540-552.

16. PENNIGTON, R. 1961. Biochemistry of dystrophic muscle. Mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. Biochem. J. 80:649-654.

17. POSTE, G., and A. C. ALLISON. 1971. Membrane fusion reaction: A Theory. J.Theor. Biol. 32:165-184.

18. RAINS, D. W., and E. EPSTEIN. 1967. Sodium absorption by barley roots: Its mediation by mechanism 2 of alkali cation transport. Plant Physiol. 42:319-323.

19. ROLAND, J.-C., C. A. LEMBI, and D. J. MORRE. 1972. Phosphotungstic acid-chromic acid as a selective stain for plasma membranes of plant cells. Stain Tech. 47:195-200.

20. SICHEL, M., and A. C. BURTON. 1936. A kinetic method of studying surface forces in the egg of *Arbacia*. Biol. Bull. 71:397-398.

21. STEIN, W. D. 1962. Spontaneous and enzyme-induced dimer formation and its role in membrane permeability. III. The mechanism of movement of glucose across the human erythrocyte membrane. Biochim. Biophys. Acta 59:66-77.

22. STEINBERG, M. S. 1958. On the chemical bonds between animal cells. A mechanism for type-specific adhesion. Amer. Natur. 92:65-81.

23. TRUE, R. H. 1922. The significance of calcium for higher green plants. Science 55:1-6.