Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2065–2069, April 1984
Botany

Calmodulin inhibitor in senescing apples and its physiological and pharmacological significance

(calcium/calmodulin activity/senescence/aging/plants)

GOPINADHAN PALIYATH AND B. W. POOVAIAH*

Department of Horticulture and Landscape Architecture, Washington State University, Pullman, WA 99164-6414

Communicated by Kenneth V. Thimann, November 7, 1983

ABSTRACT **While assaying calmodulin activity in senesced apple extracts by using its property of promoting the activity of activator-deficient 3′,5′-cyclic AMP 5′-nucleotidohydrolase (phosphodiesterase, EC 3.1.4.17) from bovine heart, we detected a heat-stable, dialyzable, low molecular weight component that inhibited calmodulin activity. Specific activity of calmodulin as calculated from the linearly increasing portion of the activity curve was in the range of 150 to 160 units/mg of protein in crude extracts from apples stored at 2°C for a period of 6 months with or without calcium treatment. Apple extract that was passed through a phenothiazine-Sepharose affinity column did not promote phosphodiesterase activity, whereas the EGTA eluate of the column promoted phosphodiesterase activity similar to the original extract. The inhibition of calmodulin activity appeared to be lower in extracts from apples stored at 2°C after calcium treatment. The inhibition was found to increase after storage of apples at room temperature for 30 days. Activity of purified bovine brain calmodulin was also inhibited by the inhibitor present in apple extracts, which indicated that the inhibition was not specific to plant calmodulin alone and could have wide applications. The importance of the inhibitor in relation to senescence/aging and its possible pharmacological applications are discussed.**

Biochemical action of calcium in general has been shown to be mediated by calmodulin, a calcium-binding protein of ubiquitous occurrence (1). This protein has been found to modulate the action of many enzymes and proteins after binding with and being activated by calcium (1–3). Addition of exogenous calmodulin has been found to reduce increased lipoxygenase activity associated with senescence in pea leaves (4). Therefore, the modulation of the activity or quantity of endogenous calmodulin could have an important role in the regulation of developmental processes. Calmodulin activity has been reported to be modulated in animal systems by proteins (5) and by a variety of compounds (6), including peptide hormones (7). Though the occurrence of calmodulin has been well established in many higher plant systems (3, 8, 9), no such cases of modulation of its activity by endogenous compounds in regard to its physiological role have been studied. In our study to understand the role of calmodulin in the biochemical action of calcium in delaying senescence, we could also detect the presence of an inhibitor of calmodulin at physiological concentrations. Such an inhibitor could have a wide-ranging physiological role in the developmental processes of the plant and could also serve as a specific inhibitor of calmodulin action with pharmacological applications.

Calcium and cytokinins have been known to interact in delaying senescence in corn leaves (10). Calcium status of the cell could depend on the activity of Ca^{2+}-ATPase, the activity of which is promoted by activated calmodulin (3). Calcium–calmodulin-mediated protein phosphorylation has been recently demonstrated in corn coleoptiles (11). Calcium has also been observed to interact with membranes, conferring structural and functional integrity to them (12, 13). Senescent breakdown in postclimacteric apples has been found to be retarded by the application of calcium (14, 15). Because of these responses signifying the role of calcium and because of the world-wide importance of apples as a food source, we have used this system for studying calcium–calmodulin-mediated regulation of biochemical processes in plants.

MATERIALS AND METHODS

Postclimacteric Golden Delicious apples were collected and stored at 2°C for 6 months. For calcium treatment, apples after collection were dipped in calcium chloride solution under reduced pressure (4% $CaCl_2$, 0.7 kg/cm^2) briefly, rinsed with water, and stored as specified above.

All operations for extraction were done at 4°C. Apple pulp was isolated and homogenized in a mortar with pestle in 50 mM Hepes buffer, pH 7, containing 0.5 M sodium chloride, 5 mM calcium chloride, and 28 mM L-ascorbic acid. The homogenate was squeezed through cheesecloth and centrifuged at 100,000 × *g* in a Beckman L-5-50B ultracentrifuge. Supernatant obtained from this step was used for assaying calmodulin (16). Partial purification of calmodulin was achieved by passing the apple extract through a phenothiazine-Sepharose affinity column as described in Bio-Rad bulletin 1088.

Calmodulin activity was assayed essentially as described by Watterson *et al.* (17), using its property of stimulating activator-deficient cAMP phosphodiesterase (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17, which converts cAMP to AMP) isolated from bovine heart (18). Phosphodiesterase was used at a concentration of 0.02 unit/ml. Calmodulin (bovine brain) when used was at 2 units/ml. One unit of phosphodiesterase is defined as the amount that hydrolyzes 0.5 μmol of cAMP to 5′-AMP per min at pH 8.0 and 30°C. One unit of calmodulin is defined as the amount that will stimulate 0.01 unit of phosphodiesterase to 50% of maximal activity when saturated with calcium ions at pH 8 and at 30°C. Protein was estimated by using the Bio-Rad protein reagent assay (19).

AMP was separated from cAMP by a modification of the procedure of Watterson *et al.* (17), using a C_{18} μBondapak column (3.0 mm × 30 cm, Waters Associates) at ambient conditions. The mobile phase was 10 mM KH_2PO_4, pH 2.5, delivered at 1.5 ml/min by a Waters 6000 model solvent delivery pump. The column eluate was monitored by absorbance (0.02 absorbance unit at full scale, 260 nm), using a Waters model 450 variable-wavelength detector. Under these conditions AMP was eluted at 2.8 min and cAMP at 4.5 min. Peak areas were a linear function of AMP concentration up to 2.8 μg in the absorbance mode.

Fruit firmness, chlorophyll content, ethylene production,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed.

and carbon dioxide production in apples were measured as reported earlier (10, 13, 14).

Hepes, Tris·HCl, calcium chloride, EDTA, EGTA, AMP, cAMP, activator-deficient phosphodiesterase (from bovine heart) and calmodulin (bovine brain) were purchased from Sigma. Phenothiazine-Sepharose was obtained from Bio-Rad.

RESULTS

Calmodulin Activity in Apple Extract. In the assay system described, 70–90 nmol of AMP was formed in 20 min by 0.01 unit of the enzyme. Addition of 1 unit of pure calmodulin gave nearly 2.5- to 2.9-fold stimulation of the basal activity. Calmodulin activity in apple extracts increased linearly with increasing volume of the extract up to 50–60 μl, in the initial stages of storage of apples (Fig. 1). Activity calculated from the linear region corresponded to 160 units/mg of crude protein in extract from calcium-treated apples. Phosphodiesterase has also been reported to be nonspecifically activated by many compounds, such as fatty acids and phospholipids (20). To exclude the possibility that the promotion of phosphodiesterase obtained in apple extract is due to the action of agents other than calmodulin, the following experiment was conducted. Apple extract was passed through a phenothiazine-Sepharose affinity column. Since phenothiazines specifically bind to calmodulin (16, 21), all other, nonspecific, promoters of phosphodiesterase activity would pass through the column and be present in the flow-through. Effect of the original extract, the dialyzed extract, the flow-through, the buffer wash of the affinity column, and the EGTA eluate (diluted in proportion to the original extract) in promoting phosphodiesterase activity was checked. It was found that comparable promotion of phosphodiesterase activity could be achieved only in the original extract, the dialyzed extract, and the EGTA eluate (Fig. 2). The flow-through and the buffer wash of the column did not promote phosphodiesterase activity, indicating that the promotion obtained in apple extract indeed is due to calmodulin only and not due to the nonspecific promotion by agents other than calmodulin. During advanced stages of senescence (as occurring when apples are brought from the cold and stored

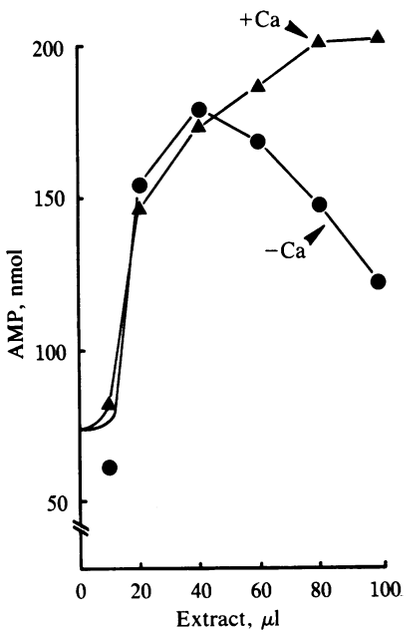

FIG. 1. Promotion of phosphodiesterase activity by extracts from apples stored at 2°C for 6 months with and without calcium treatment.

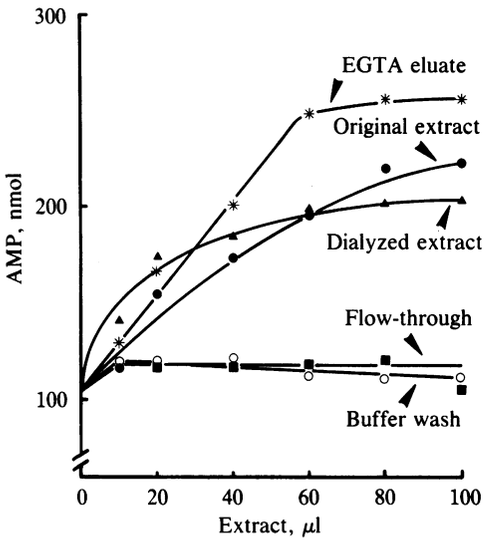

FIG. 2. Purification of calmodulin in apple extract by passing through a phenothiazine-Sepharose affinity column. Phosphodiesterase activity in the presence of apple extract, dialyzed apple extract, flow-through from the affinity column, buffer wash of the affinity column, and EGTA eluate of the column is shown. The tissue was homogenized in 50 mM Tris·HCl buffer, pH 8, containing 0.5 M NaCl, 5 mM $CaCl_2$, 10 mM sodium ascorbate, and polyvinylpolypyrrolidone. The supernatant obtained after 105,000 × *g* centrifugation was extensively dialyzed against extraction buffer before loading on the phenothiazine-Sepharose affinity column. Calmodulin was eluted by using 5 mM EGTA in Tris buffer and EGTA chelated with excess calcium chloride.

at room temperature for 3 weeks or longer) calmodulin activity could not be determined accurately because of an inhibitor that interfered with calmodulin activity.

Inhibition of Calmodulin Activity in Apple Extracts. An interesting observation from our experiments was that when the volume of the extract was increased in the assay mixture beyond 40–60 μl, it tended to inhibit the calmodulin activity. The patterns of calmodulin activity in extracts from apples stored in cold with and without calcium treatment are shown in Fig. 1. It was seen that, in normal apples stored in the cold, increasing the volume of the extract above 40 μl in the assay mixture inhibited calmodulin activity. However, in a similar experiment using extract from apples treated with calcium, such an inhibition did not occur, at least up to 100 μl of the extract in the assay mixture. After storage at room temperature for 15 days, the extract from calcium-treated apples also showed inhibition as in the extract from untreated apples (data not shown).

Therefore, it seemed possible that, with advancing senescence, an inhibitor of calmodulin activity could be formed, and this process could be slower in calcium-treated apples. To check the presence of any low molecular weight inhibitors, the extract from normal apples stored at room temperature was dialyzed against 40 mM Tris·HCl, pH 8, and the effect of the dialyzed sample on the activity of pure bovine brain calmodulin was checked. The assay mixture contained 0.01 unit of phosphodiesterase, 1 unit of calmodulin, and increasing volumes of the extract before and after dialysis. It was seen that, in the 20- to 40-μl volume range, phosphodiesterase activity was stimulated above the normally stimulated level by pure calmodulin (Fig. 3; 220 nmol to 330 nmol with calmodulin and 20 μl of extract). The combined activity decreased and fell even below the normal calmodulin-stimulated activity when the extract volume used was 100 μl. In contrast to this, the dialyzed extract did not show an inhibition with increasing volumes of the extract but reached a saturation level above 40 μl of the extract (Fig. 3).

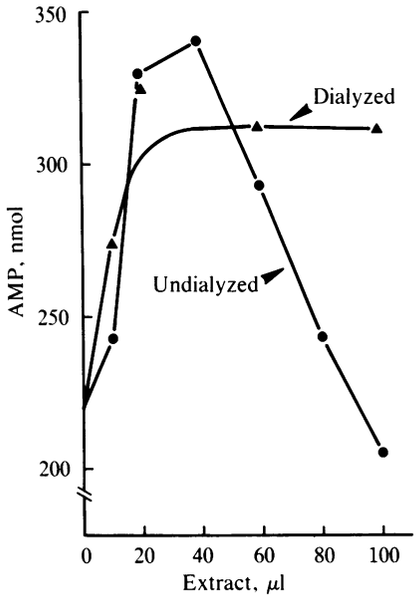

FIG. 3. Phosphodiesterase activity in the presence of 1 unit of bovine brain calmodulin and increasing volumes of apple extract.

Thus, it appeared possible that the inhibitor concentration could be lower in calcium-treated apples as compared to untreated apples. Some of the differences between apples stored with and without calcium treatment are illustrated in Table 1. It can be seen that calcium-treated apples were firmer, possessed lower membrane permeability, and contained more chlorophyll and ascorbic acid. Respiratory CO_2 evolution and ethylene production, which are normally high in senescing systems, were lowered in apples after calcium treatment. These results indicated that senescence-related biochemical processes in apples are delayed by calcium, possibly by acting through calmodulin. And the inhibitor could impair the biochemical activity of calmodulin after binding to and inactivating it.

To confirm whether calcium-treated apples contained less of the inhibitor than untreated apples, the following experiment was conducted. Tissues from calcium-treated and untreated apples were extracted with Hepes buffer and centrifuged at 100,000 × g. The supernatant was collected and 100 ml of the supernatant was dialyzed against 25 ml of Hepes buffer initially to obtain a dialysate with concentrated inhibitor. Small volumes of the extract were further dialyzed against 40 mM Tris·HCl, pH 8, containing 10 mM EDTA and thereafter against the extraction buffer extensively. Stimulation/inhibition of calmodulin-promoted phosphodiesterase activity was studied. Correlation/regression values were calculated between the volume of the extract/dialysate added (from 40 μl onwards) and the amount of 5'-AMP formed during the reaction.

Table 1. Effects of calcium treatment on various parameters of senescence in Golden Delicious apples

Parameter	Control	Ca-treated
Fruit firmness, kg	6.7	8.6
Chlorophyll, mg/100 g of skin	3.37	4.46
C_2H_4, ml/kg·hr	102	55
CO_2, ml/kg·hr	19	12
Hydraulic permeability, $t_{1/2}$ to equilibrium, min	2.4	3.5
Ascorbic acid, mg/100 g of dry wt	14	23

Apples were infiltrated with calcium chloride solution (4%) under reduced pressure* and stored at 2°C, and the given parameters were measured periodically for 12 months. The data were obtained after 3 months of storage at 2°C.

*The calcium infusion process has been patented by B. W. Poovaiah and G. A. Moulton and assigned to Washington State University Research Foundation, U.S. Patent 4,331,961.

The results obtained are summarized in Table 2. Values calculated from the data obtained in an exactly similar experiment using senesced apples are also given for comparison. The inhibition of calmodulin activity was the highest in the extract from senesced apples (slope = −0.4) and the lowest in the calcium-treated apples (slope = −0.1). Extract from untreated apples stored in the cold showed an inhibition intermediate in value (slope = −0.3). Extracts from these apples also inhibited phosphodiesterase activity promoted by bovine brain calmodulin. The magnitude of inhibition was highest in the extract from senesced apples (slope = −1.54). Dialysis could overcome the inhibition in the extract from calcium-treated apples to some extent (slope = −0.07). However, this could not be achieved in extracts from untreated apples and senesced apples. The inhibition was overcome by the addition of excess calmodulin in extracts from apples at an early stage of senescence but not in extracts from senesced apple (Fig. 4). This indicated that at an advanced stage of senescence there could be a higher amount of the inhibitor. Inhibition of calmodulin activity was the highest in the dialysate fraction from senesced apples. In contrast, the dialysate fraction from cold-stored calcium-treated apples did not inhibit calmodulin-promoted phosphodiesterase activity (Table 2). This indirectly confirmed that calcium-treated apples contained lower amounts of the calmodulin inhibitor.

This aspect of inhibition was very clear at an advanced stage of senescence (after storage for 30 days at room temperature). Calmodulin activity could not be detected in the extract from senesced apples, which also inhibited the activity of pure bovine brain calmodulin. The normally stimulated activity (280 nmol of AMP formed in this experiment) was slightly reduced even with 10 μl of the extract (Fig. 5). With increasing volumes of the extract calmodulin activity was reduced further, and there was nearly 65% inhibition of the original stimulated activity at a volume of 100 μl. Stimulation of phosphodiesterase activity was slightly higher in the dialyzed extract as compared to the original extract; still, inhibition was apparent (data not shown). The dialysate did not

Table 2. Correlation between increasing volumes of the apple extract and AMP formed during the reaction

	Untreated apples		Ca-treated apples		Senesced apples*	
Treatment	r	Slope	r	Slope	r	Slope
PDEase + extract	−0.90	−0.30	−0.71	−0.10	−0.93	−0.40
PDEase + CAM + extract	−0.95	−0.58	0.42	0.37	−0.97	−1.54
PDEase + dialyzed extract	−0.92	−0.65	−0.42	−0.07	−0.98	−0.46
PDEase + dialyzed extract + CAM	0.08	0.16	0.60	0.82	−0.68	−0.54
PDEase + dialysate	—	—	—	—	−0.56	−0.06
PDEase + CAM + dialysate	−0.85	−0.67	0.01	0.005	−0.99	−2.02

AMP formed, in the presence of extract, dialyzed extract, and dialysate obtained from untreated apples and calcium-treated apples stored at 2°C and senesced apples stored at room temperature until wrinkles appeared on the surface due to dehydration, was used for the calculations. r is the usual correlation coefficient and the slope is from graphs such as Fig. 5, with units of nmol/μl. PDEase, phosphodiesterase; CAM, calmodulin; —, not assayed.

*Separate experiment, also refer to Fig. 5.

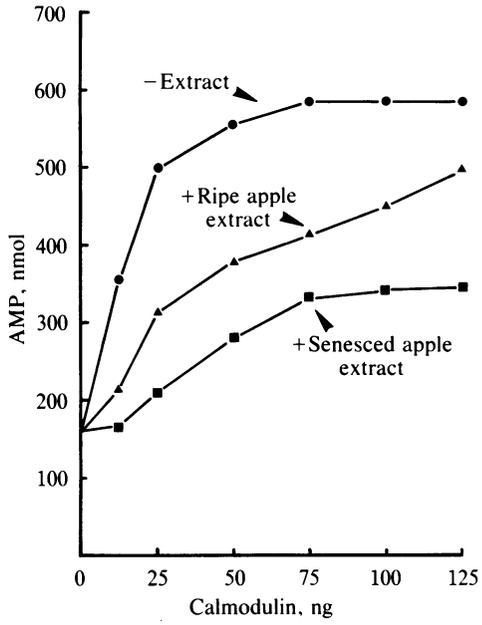

FIG. 4. Effect of increasing amount of calmodulin on the inhibition of calmodulin-promoted phosphodiesterase activity by apple extract. The assay mixture contained 0.015 unit of phosphodiesterase and increasing amounts of calmodulin without the apple extract and with the apple extract (200 μl).

seem to alter the basal phosphodiesterase activity. However, calmodulin-stimulated phosphodiesterase activity was clearly inhibited with increasing volumes of the dialysate (Fig. 5). This experiment showed that the inhibitor is of low molecular weight and does not inhibit phosphodiesterase activity but only calmodulin-stimulated phosphodiesterase activity at physiological concentrations (Fig. 5).

DISCUSSION

Though calmodulin-mediated biochemical processes have been well studied in animal systems, such studies are in their preliminary stages in plant systems. The regulation of plant growth processes by calcium clearly points to the possibility of the existence of such roles in plant growth processes (22, 23). Calmodulin-activated NAD kinase (2) and Ca^{2+}-ATPase (3, 24, 25) have been reported. Calmodulin, after being bound by Ca^{2+} and becoming activated, is known to bind to membrane proteins, eliciting functional changes in them (26, 27). Such binding could lead to localized membrane structure alterations in membranes. Therefore, it appeared possible that calcium could exert its beneficial effects such as delaying senescence through regulation of biochemical events after binding with calmodulin.

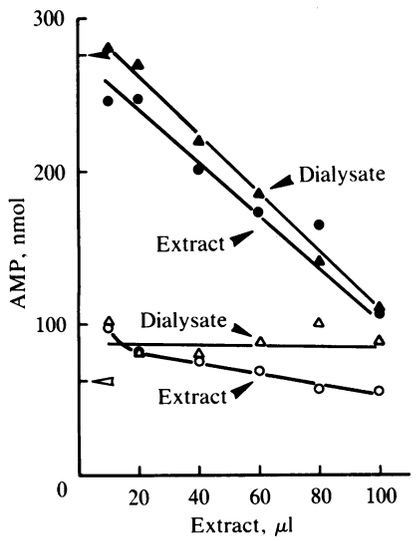

FIG. 5. Phosphodiesterase activity in the presence of undialyzed apple extract from senesced apple (○), and the activity in the presence of 1 unit of added bovine brain calmodulin (●). Values obtained in a similar experiment using dialysate are also shown (△, ▲). The lower arrowhead beside the ordinate indicates the amount of AMP formed in the presence of phosphodiesterase alone and the upper arrowhead indicates the amount of AMP formed by phosphodiesterase in the presence of 1 unit of bovine brain calmodulin.

An interesting result from our study was the presence of an inhibitor of calmodulin action. Since the inhibition was apparent while using apple extract as well as pure calmodulin from bovine brain, the inhibition obtained in apple extract must be due to the action of the inhibitor on apple calmodulin. Calmodulin is an evolutionarily conserved protein (28), and so the inhibition obtained in calmodulin activity from two diverse sources is not surprising. The inhibitor appeared to inhibit only the calmodulin-promoted phosphodiesterase action (Fig. 5). Extracts from calcium-treated apples that showed delayed senescent breakdown also showed lowered inhibition of calmodulin-promoted phosphodiesterase activity. However, after storage at room temperature for 15 days, the extract from calcium-treated apples also showed inhibition as in controls. This is an indication that the inhibitor could by synthesized *in vivo*. When the cytoplasmic concentration of inhibitor increases during senescence, more calmodulin becomes bound with the inhibitor, and it could impair calmodulin-mediated biochemical processes. This could be a key feature in the onset and progress of senescence.

An inhibitor of calmodulin activity showing a similar mode of inhibition of phosphodiesterase was also found in zucchini hypocotyl preparations by Dieter and Marmé (3). The inhibitor from zucchini also reduced the stimulation of microsomal calcium uptake mediated by Ca^{2+}-ATPase, which is a calmodulin-stimulated enzyme. Inhibition of calmodulin activity was also observed in other storage tissues such as strawberry fruits, tomato, and potato that were tested in our laboratory. Therefore, such naturally occurring inhibitor activity appears to exist in many systems and could be a common feature in the regulation of senescence, aging, and possibly other growth processes as well.

Inhibitors of calmodulin activity of wide-ranging nature have been reported in animal systems. In a study involving calmodulin–tubulin interaction in 3T3 cells transformed by simian virus 40, Tash *et al.* (29) found that these cells could be reverted to the normal mode of nucleation and elongation of microtubules by incubation with anti-calmodulin. Peptides having regulatory roles in animals, such as corticotropin and β-endorphin, inhibited calmodulin-promoted phosphodiesterase action (7). Secondary plant products such as papaverine and theophylline also inhibited calmodulin-promoted phosphodiesterase activity (7). However, it was indicated that many of the neuroleptic drugs are nonstereospecific inhibitors of calmodulin-promoted phosphodiesterase activity (30). It is possible that naturally occurring compounds in plants that inhibit calmodulin activity at physiological concentrations could be more stereospecific in their inhibition and therefore could be used as neuroleptic agents with few side effects.

The nature of this inhibitor requires further investigation. It appears to be a small molecule (dialyzable) and to be heat stable. None of the plant growth substances tested (auxin, cytokinin, gibberellic acid, and abscisic acid) appear to have any significant effect on calmodulin-promoted phosphodiesterase activity. L-Serine has been found to be a promoter of senescence (31). However, L-serine did not appear to inhibit calmodulin-promoted phosphodiesterase activity. Com-

pounds other than abscisic acid or ethylene that are known to promote senescence, generally referred to as senescence factors, have been reported to occur in many plants (32). However, the physiological mode of action and chemical structure of these compounds are not known. Taking all these results into consideration, it appears possible that there could be an entirely different class of compounds (referred to as senescins) that mediate senescence processes. More work on other senescing systems would be helpful in this regard.

We thank Dr. K. Veluthambi and Dr. Y. Mizrahi for their careful reading of the manuscript and valuable discussions. The above work was supported by a grant from the Washington State Tree Fruit Commission and partial support from the Washington State Agricultural Experiment Station and National Science Foundation Grant PCM-8208408 to B.W.P. This is technical paper no. 6698 from the College of Agriculture Research Center, Washington State University, Pullman.

1. Cheung, W. Y. (1980) in *Calmodulin*, Calcium and Cell Function, Vol. 1, ed. Cheung, W. Y. (Academic, New York), pp. 1–12.
2. Cormier, M. J., Anderson, J. M., Charbonneau, H., Jones, H. P. & McCann, R. O. (1980) in *Calmodulin*, Calcium and Cell Function, Vol. 1, ed. Cheung, W. Y. (Academic, New York), pp. 201–218.
3. Dieter, P. & Marmé, D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7311–7314.
4. Leshem, Y. Y., Wurzburger, Y., Frimer, A. A., Barness, G. & Ferguson, I. B. (1982) in *Plant Growth Substances*, ed. Wareing, P. F. (Academic, New York), pp. 569–578.
5. Sharma, R. K., Desai, R., Thompson, R. T. & Wang, J. H. (1978) *Can. J. Biochem.* **56**, 598–604.
6. Means, A. R. & Chafouleas, J. G. (1982) in *Calmodulin and Intracellular Ca^{++} Receptors*, eds. Kakiuchi, S., Hidaka, S. & Means, A. R. (Plenum, New York), pp. 141–152.
7. Weiss, B., Prozialeck, W. & Cimino, M. (1980) *Ann. N.Y. Acad. Sci.* **356**, 319–345.
8. Anderson, J. M., Charbonneau, H., Jones, H. P., McCann, R. O. & Cormier, M. J. (1980) *Biochemistry* **19**, 3113–3120.
9. Watterson, D. M., Iverson, D. B. & Van Eldik, L. J. (1980) *Biochemistry* **19**, 5762–5768.
10. Poovaiah, B. W. & Leopold, A. C. (1973) *Plant Physiol.* **52**, 236–239.
11. Veluthambi, K. & Poovaiah, B. W. (1984) *Science* **223**, 167–169.
12. Paliyath, G., Poovaiah, B. W., Munske, G. R. & Magnuson, J. (1982) *Hortic. Sci.* **17**, 530 (abstr.).
13. Poovaiah, B. W. & Leopold, A. C. (1976) *Plant Physiol.* **58**, 182–185.
14. Poovaiah, B. W. & Shekhar, V. C. (1978) *Hortic. Sci.* **13**, 37 (abstr.).
15. Bangerth, F. (1979) *Annu. Rev. Phytopathol.* **17**, 97–122.
16. Charbonneau, H. & Cormier, M. J. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1039–1047.
17. Watterson, D. M., Iverson, D. B. & Van Eldik, L. J. (1980) *J. Biochem. Biophys. Methods* **2**, 139–146.
18. Wallace, R. W., Tallant, E. A. & Cheung, W. Y. (1980) in *Calmodulin*, Calcium and Cell Function, Vol. 1, ed. Cheung, W. Y. (Academic, New York), pp. 13–40.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
20. Wang, J. H. & Waisman, D. M. (1979) *Curr. Top. Cell. Regul.* **15**, 47–107.
21. Jamieson, G. A. & Vanaman, T. C. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1048–1056.
22. Biro, R. L., Hale, C. C., II, Wiegand, O. F. & Roux, S. J. (1982) *Plant Physiol.* **69**, Suppl. 4, 92 (abstr.).
23. Kuzmanoff, K. M. & Evans, M. L. (1982) *Plant Physiol.* **69**, Suppl. 4, 56 (abstr.).
24. Dieter, P. & Marmé, D. (1980) in *Calcium Binding Proteins: Structure and Function*, eds. Siegel, F. L., Carafoli, E., Kretsinger, R. H., MacLennan, D. H. & Wasserman, R. H. (Elsevier/North-Holland, Amsterdam), pp. 211–212.
25. Kubowicz, B. D., Vanderhoef, L. N. & Hanson, J. B. (1982) *Plant Physiol.* **69**, 187–191.
26. Grainstein, S. & Furuya, W. (1982) *Biochim. Biophys. Acta* **686**, 55–64.
27. Vincenzi, F. F. & Hinds, T. R. (1980) in *Calmodulin*, Calcium and Cell Function, Vol. 1, ed. Cheung, W. Y. (Academic, New York), pp. 128–165.
28. Vanaman, T. C. (1980) in *Calmodulin*, Calcium and Cell Function, Vol. 1, ed. Cheung, W. Y. (Academic, New York), pp. 41–58.
29. Tash, J. S., Means, A. R., Brinkley, B. R., Dedman, J. R. & Cox, S. M. (1980) in *Microtubule and Microtubule Inhibitors*, eds. deBrabander, M. & DeMey, J. (Elsevier/North-Holland, Amsterdam), pp. 269–279.
30. Norman, J. A. (1980) *Ann. N.Y. Acad. Sci.* **356**, 415–416.
31. Thimann, K. V. (1980) in *Senescence in Plants*, ed. Thimann, K. V. (CRC, Boca Raton, FL), pp. 85–115.
32. Noodén, L. D. (1980) in *Senescence in Plants*, ed. Thimann, K. V. (CRC, Boca Raton, FL), pp. 219–258.