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Involvement of Calcium and Calmodulin in Membrane Deterioration during Senescence of Pea Foliage[1]

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ABSTRACT

The prospect that Ca^{2+} promotes senescence by activating calmodulin has been examined using cut pea (*Pisum sativum* co Alaska) foliage as a model system. Senescence was induced by severing 17-day-old plants from their roots and maintaining them in aqueous test solutions in the dark for an additional 4 days. Treatment of the foliage with the Ca^{2+} ionophore (A23187) during the senescence-induction period promoted a lateral phase separation of the bulk lipids in microsomal membranes indicating that internalization of Ca^{2+} facilitates membrane deterioration. In addition, microsomal membranes from ionophore-treated tissue displayed an increased capacity to convert 1-aminocyclopropane-1-carboxylic acid to ethylene and an increased propensity to produce the superoxide anion (O_2^-). Treatment of the tissue with fluphenazine during the senescence-induction period, which prevents binding of the Ca:Calmodulin complex to enzymes, delayed membrane deterioration as measured by these criteria. It also proved possible to simulate these *in situ* effects of the Ca^{2+} ionophore on ethylene production and O_2^- formation by treating microsomal membranes isolated from young tissue with phospholipase A_2 in the presence of Ca^{2+} and calmodulin, and these effects of phospholipase A_2 and Ca:calmodulin were inhibited by calmodulin antagonists. The observations collectively suggest that internalized Ca^{2+} promotes senescence by activating calmodulin, which in turn mediates the action of phospholipase A_2 on membranes.

As initially reported by Wong and Cheung (29) and subsequently substantiated by Walenga *et al.* (28) and Stocker and Richter (24), phospholipase A_2 (EC 3.1.1.4), which preferentially hydrolyzes the 2-acyl bond in phosphoglycerides to yield lyso compounds, is a calmodulin:calcium-promoted enzyme. Since phospholipids are a major component of the bilamellar membrane structure, the activation of phospholipase A_2 could play an important role in the loss of membranal integrity and the onset of cell senescence.

According to current theory (11), calmodulin is an intracellular acceptor of Ca^{2+} acting when the internal concentration of Ca^{2+} increases in response to a stimulus. The Ca^{2+} purportedly induces a distinct change in the shape of the calmodulin molecule, and as a result the Ca:calmodulin complex becomes capable of binding to calmodulin-sensitive enzymes and setting in motion the biochemical changes that produce a response to the stimulus. Calmodulin is predominantly cytosol-localized (19), and it is apparent that a key triggering factor for calmodulin activation is the availability of internal Ca^{2+}. Normally, there could be a concentration gradient for Ca^{2+} of several orders of magnitude from outside to inside across the plasma membrane (27). This means that there is relatively little Ca^{2+} inside the cell, and the primary role of such small amounts of Ca^{2+} in the cytosol is presumably to transmit information.

It has also been reported that phosphatidic acid and oxidized di- and trienoic fatty acids act as calcium ionophores in model bilayers and could thus serve as endogenous calcium ionophores in cells as well (23). These compounds are able to translocate Ca^{2+} from an aqueous phase across the intact bilayer into another aqueous compartment, are permselective with respect to the two major divalent cations of physiological fluids (Ca $\gg$ Mg), and do not lyse the membrane. This observation is of particular significance in the context of senescing tissues because there is now evidence that free radical-mediated peroxidation of membrane lipids is a characteristic feature of senescence (3). Moreover, products of membrane lipid peroxidation, which would include oxidized di- and trienoic fatty acids, apparently accumulate in membrane matrices with advancing senescence and contribute to lateral phase separations in the lipid bilayers of senescing membranes (1).

Exogenous Ca^{2+} has been shown to delay senescence of apple tissue slices, in particular ethylene production and the onset of lipid peroxidation (9). However, this effect has been attributed to the ability of Ca^{2+} to rigidify the surfaces of lipid bilayers by acting as a divalent ligand (7) and presumably reflects a stabilizing influence of external Ca^{2+} on the outside surface of the plasmalemma. In the present study, we have examined the prospect that internalized Ca^{2+} promotes senescence in pea foliage by forming a Ca:calmodulin complex that activates phospholipase A_2. The Ca^{2+} ionophore A23187 was applied directly to the intact foliage, and its effects on membrane senescence and free radical production determined. In addition, microsomal membranes isolated from young foliage were treated with phospholipase A_2 in the presence of Ca^{2+} and calmodulin in an effort to simulate the effects of natural senescence on membranes. To gain further insight on the role of calmodulin, the antipsychotic drug fluphenazine, which at low concentrations prevents the binding of the Ca:calmodulin complex to enzymes (8), was also employed.

MATERIALS AND METHODS

Plant growth and treatment. Pea plants (*cv* Alaska) were grown in vermiculite at 23°C with a 16-h photoperiod. Details of the growth chamber environment are provided elsewhere (4). To ensure that Ca^{2+} availability would not be a limiting factor, the growing plants were irrigated with distilled H_2O containing 10^{-3} M $CaCl_2$. Seventeen d after planting, foliar senescence was induced as described previously (4) by severing the plants from their roots and placing them in darkness at 15°C for 4 d in beakers containing 10^{-3} M $CaCl_2$ in distilled H_2O to ensure that

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Ca^{2+} would not be limiting. In the middle of the aging period, stem ends were freshly cut and fresh $CaCl_2$ solutions supplied. Throughout, the beakers were covered to ensure that there was no water loss apart from that attributable to transpiration, and hence no change in the concentration of $CaCl_2$ occurred during the 4-d period.

In another series of experiments, plants were treated with a solution of 50 μM calcium ionophore (A23187). The final 400-ml solution was made up by dissolving the required amount of ionophore in 0.15 ml of absolute ethanol and 0.1 ml of Tween 20, and diluting to 50 μM with 10^{-3} M $CaCl_2$. During growth in vermiculite, the plants were sprayed with this solution every 3 d, and after 17 d, the severed foliage was maintained in darkness at 20°C for an additional 4 d in beakers containing the $CaCl_2$,ionophore solution. Control plants were sprayed with distilled H_2O containing the same amounts of ethanol and Tween 20 and were maintained after cutting in distilled H_2O containing the same levels of ethanol, Tween 20, and $CaCl_2$, but lacking the ionophore. In still other experiments, the growing plants were sprayed every 3 d with 50 μM fluphenazine dihydrochloride (a gift from E. Squibb and Sons) in distilled H_2O and maintained after cutting in the same solution containing 10^{-3} M $CaCl_2$. Since this drug is light-sensitive, it was maintained in darkened glassware, and treatments during the initial 17-d growing period were administered at the beginning of the nyctoperiod.

Microsomal Membrane Isolation. Microsomal membranes were isolated from pea leaves essentially as described previously (25). Twenty g of leaf tissue were homogenized at 4°C in 50 ml of 0.01 M Hepes buffer (pH 7.5) in a Sorvall omnimixer for six periods of 10-s duration with 30-s intervening cooling periods in ice. After filtering through four layers of cheesecloth, the homogenate was centrifuged at 10,000*g* for 20 min and the supernatant centrifuged again at 214,000*g* for 1 h to yield a pellet of microsomal membranes. The membranes were then washed by resuspension in an equal volume of buffer and centrifuged again at 214,000*g* for 1 h. These washed membranes were then dialyzed overnight at 6°C against four changes of 2mM Epps[3] buffer (pH 8.5) in order to remove an inhibitor of the ACC to ethylene conversion that is of cytosolic origin (14) and likely to be present in residual amount despite washing. Protein was determined by the method of Lowry *et al.* (10).

Ethylene and Chl Determinations. Measurements of ethylene production from ACC by isolated membranes were carried out essentially as described previously (14). Reaction mixtures containing membrane, buffer, and ACC as detailed in the table and figure legends were incubated in serological capped test tubes at 30°C and, at specified time intervals, gas samples for ethylene analysis were extracted using tuberculin-needled 1-ml syringes. Ethylene was measured isothermally at 65°C on a Perkin-Elmer model 900 gas chromatograph equipped with an alumina column. The injection temperature was maintained at 120°C. Levels of Chl *a* and *b* were determined as described previously (12).

Electron Spin Resonance. Levels of O_2^- produced by isolated microsomes were determined by ESR measurements of Tiron semiquinone, a radical species that forms when Tiron reacts with O_2^- (13, 18). For measurements of O_2^- production by microsomes alone, 200 μg of microsomal membrane protein were suspended in 1 ml of 2 mM Epps buffer (pH 8.5) containing 10 mM Tiron. After a 15-min equilibration period at room temperature, a sample was drawn up into a 100-μl glass capillary tube, which was then sealed at one end and inserted into the microwave cavity of a Varian E-12 ESR spectrometer. Previous studies (13, 18) have shown that when Tiron is first added to microsomal membranes, the amplitude of the ESR signal increases quickly as Tiron reacts with the O_2^- being produced by the microsomal membranes, reaching a steady state within 5 to 10 min, in which there is an equilibration between formation of the Tiron semiquinone and its breakdown. The signal then remains constant for at least 15 min (13, 18), the equilibration period used in the present study. In some experiments, microsomes (1 mg protein) were incubated for 2 h at 30°C in 1 ml of 2 mM Epps buffer containing 50 μM $CaCl_2$, 10 units of phospholipase A_2, 7 units of calmodulin and, when added, 50 μM fluphenazine hydrochloride before measurements of O_2^- production were made. At the end of the 2-h period, 200 μg of microsomal membrane protein were then mixed with Tiron (10 mM final concentration) and ESR spectra recorded 15 min later.

Tiron solutions were freshly prepared before each assay and kept in darkened glassware. In addition, the prospect that Tiron might be reacting with components of the reaction mixture other than O_2^- produced by microsomes was routinely checked by running blanks consisting of Tiron and all components of the reaction mixture except membranes. When detectable, the amplitudes of spectra obtained from the blank were subtracted from the amplitudes of corresponding test-sample spectra.

X-Ray Diffraction. Wide-angle X-ray diffraction patterns were recorded from centrifuged microsomes as described previously (15). Specimens containing 50 to 75% moisture with respect to final dry weight were placed in a temperature-controlled jacket on a wide-angle camera (Philips, type 1030), and diffraction patterns were recorded for 6 h at room temperature. Densitometer tracings of the patterns were made on a model DU Beckman spectrophotometer equipped with a Gilford gel scanner attachment to which strips of the x-ray film were attached and scanned at 500 nm.

RESULTS AND DISCUSSION

Senescence of pea foliage was induced by severing 17-d-old plants from their roots and maintaining them in aqueous test solutions in the dark for an additional 4 d. This system has been used previously to study senescence, and during the induction period typical symptoms of leaf senescence including loss of Chl and protein and an increase in lipoxygenase activity develop (4). Changes with advancing senescence in the phase properties of microsomal membranes from pea leaves were monitored by wide-angle x-ray diffraction. Gel phase lipid gives rise to a sharp reflection centered at a Bragg spacing of 4.2 Å in diffraction patterns, and it is clear that even by day 17, before senescence had been induced by severing the foliage, small proportions of gel phase lipid were present in the membranes (Fig. 1A). The appearance of gel phase lipid has previously been correlated with the onset of membrane senescence (15), and its presence in the membrane fraction from 17-d-old foliage presumably reflects the fact that, even by this early stage, the lower leaves of the plant have begun to senesce. During the period of induced senescence, the proportion of gel phase lipid increased as indicated by the increased intensity of the 4.2 Å reflection in the diffraction pattern recorded for membranes isolated from leaves after 4 d in darkness (Fig. 1B).

Tratment of the foliage with 50 μM Ca^{2+} ionophore (A23187) during the induction period caused even more pronounced changes in the phase properties of the membranes as well as a 40% reduction in total Chl relative to levels for foliage aged in the absence of ionophore. The 4.2 Å reflection representing lipid in the gel phase was still more intense in patterns recorded for membranes from the ionophore-treated foliage, indicating an

[3] Abbreviation: EPPS, *N*-2-hydroxyethylpiperazine propane sulfonic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ESR, electron spin resonance; O_2^-, superoxide anion; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; W-5, [*N*-(6-aminohexyl)-1-naphthalene sulfonamide; R24571, 1-[bis-(*p*-chlorophenyl)methyl;]-3-[2,4-dichloro-β-(2,4-dichlorobenzyl-ozy)phenethyl]-imidazolium-chloride.

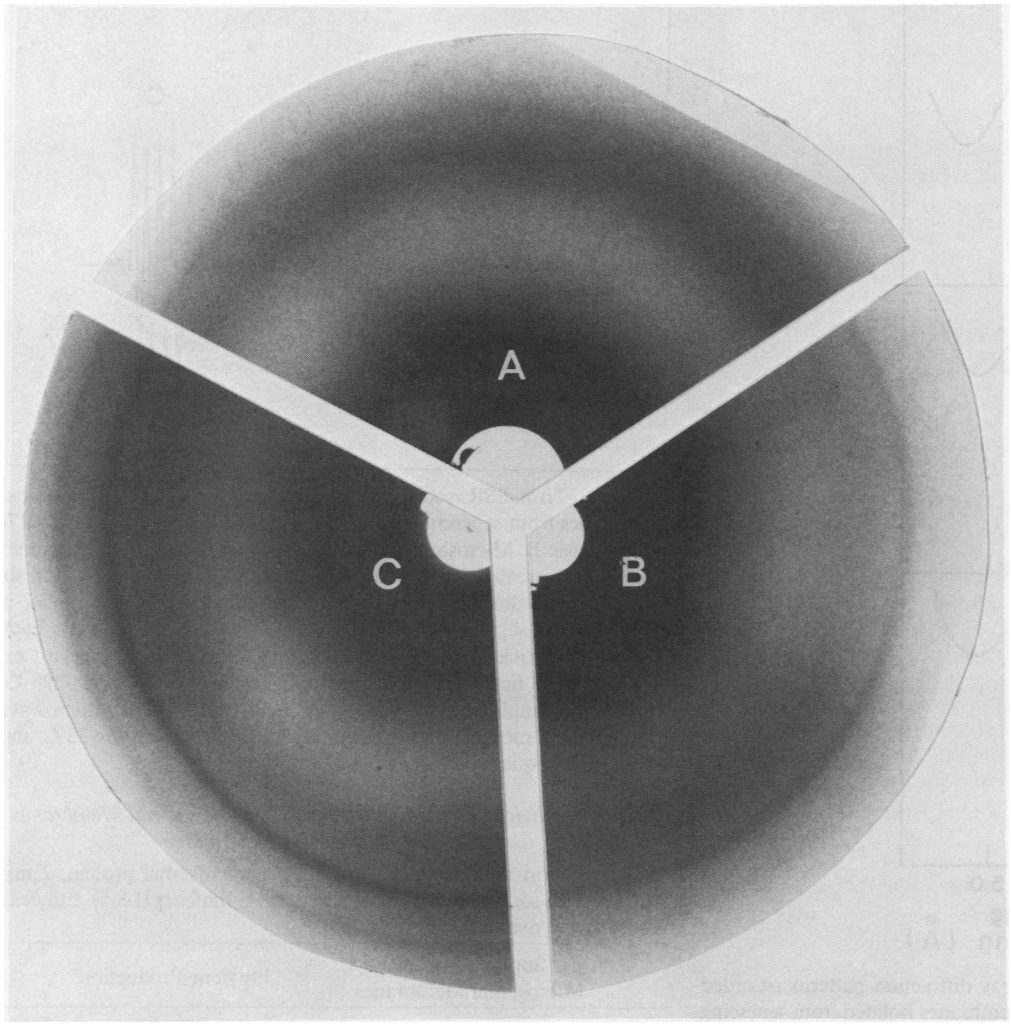

FIG. 1. Wide-angle x-ray diffraction patterns recorded at room temperature for microsomal membranes isolated from senescing pea foliage. A, Pattern for membranes from young unaged foliage featuring (from outside to inside) a sharp ring at a Bragg spacing of 4.2 Å and broad diffuse bands at Bragg spacings of 4.6 and 10 Å, respectively. B, Pattern for membranes from senesced foliage featuring (from outside to inside) a sharp ring at a Bragg spacing of 4.2 Å and broad diffuse bands at Bragg spacings of 4.6 and 10 Å, respectively. C, Pattern for membranes from foliage treated with ionophore (A23187) featuring (from outside to inside) two sharp rings at Bragg spacings of 3.75 and 4.2 A and broad diffuse bands at Bragg spacings of 4.6 and 10 Å, respectively.

increased proportion of gel phase lipid (Fig. 1C). This intensity change in the 4.2 Å reflection is more clearly apparent in the densitometer tracings of the x-ray diffraction patterns (Fig. 2). As well, an additional sharp x-ray reflection at a Bragg spacing of 3.75 Å, which represents an alternative crystalline state of lipid in which there is an orthorhombic rather than hexagonal pattern of the hydrocarbon chains (15), was also evident in the pattern for membrane from the treated foliage (Figs. 1C and 2C). The appearance of both the 3.75 and 4.2 Å reflections representing two distinguishable packing modes of crystalline lipid has been previously correlated with an advanced state of membrane senescence (15, 17), and thus it seems reasonable to interpret these diffraction data as indicating that treatment with the Ca^{2+} ionophore has accelerated membrane senescence. The concentration of ionophore used in these experiments (50 μM) is within the range that is known to cause physiological effects (22).

Electron spin resonance studies have indicated that microsomal membranes from plant tissue produce $O_2^{\cdot-}$ (18), and further, that in senescing tissue the ability of microsomes to form $O_2^{\cdot-}$ increases with advancing age of the tissue. For example, $O_2^{\cdot-}$ production by microsomes from senescent carnation flowers has proved to be about 3 times higher than the level produced by microsomes from young freshly cut flowers (13). Formation of this radical species by microsomes is heat-denaturable indicating that it is enzymically mediated, and is also inhibited by radical scavengers and superoxide dismutase (13, 18). From the present study, it is clear that microsomal membranes from pea foliage also produce increased levels of $O_2^{\cdot-}$ with advancing senescence (Fig. 3). Levels of superoxide were quantified using Tiron, which reacts with $O_2^{\cdot-}$ to form the Tiron semiquinone that is detectable by ESR as a four line first derivative spectrum. The amplitude of the spectrum is proportional to the amount of $O_2^{\cdot-}$ being formed (13, 18). Comparatively low levels of $O_2^{\cdot-}$ were produced by microsomes from control 17-d-old foliage (Fig. 3A). However, microsomes isolated from foliage in which senescence had been induced over a 4-d period showed an increased capability to produce $O_2^{\cdot-}$; indeed, the amplitude of the Tiron ESR signal produced by senescent microsomes was approximately 2.5-fold greater than that of the corresponding signal for control microsomes (Fig. 3, A and B). Levels of $O_2^{\cdot-}$ produced by microsomes from foliage that had been treated with Ca^{2+} ionophore during senescence-induction were approximately three times control levels (Fig. 3, A and C) indicating that, by this criterion as well, application of the ionophore accelerates senescence.

Microsomal membranes from both vegetative and senescing plant tissues have also been shown to be capable of converting ACC to ethylene (14, 18). This microsomal conversion must still be regarded as a model system, although it does have features in common with the conversion of ACC to ethylene *in situ* including a dependence upon O_2 and sensitivity to free radical scavengers (18). Moreover, there are indications that the $O_2^{\cdot-}$ produced by microsomes is involved in the conversion of ACC to ethylene by these membranes; for example, scavenging $O_2^{\cdot-}$ completely inhibits the formation of ethylene (6, 18).

The effects of senescence on the ability of microsomes from pea foliage to convert ACC to ethylene are illustrated in Table I.

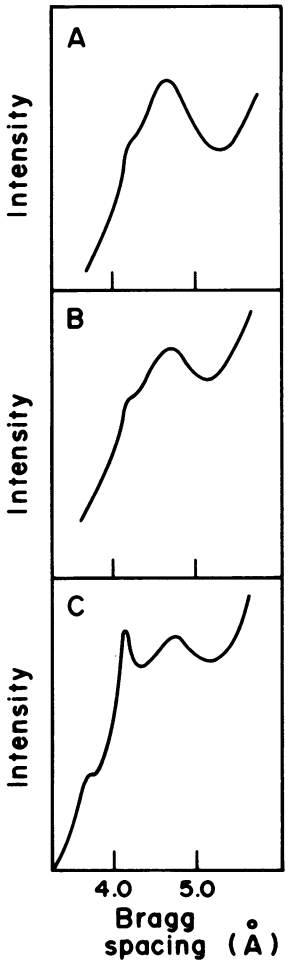

FIG. 2. Densitometer tracings of x-ray diffraction patterns recorded at room temperature for microsomal membranes isolated from senescing pea foliage. A, Membranes from young unaged foliage. B, Membranes from senesced foliage. C, Membranes from foliage treated with ionophore (A23187).

Over a 20-h incubation period, reaction mixtures containing membranes from control 17-d-old foliage produced 0.17 nl ethylene/h, whereas those containing membranes from foliage that had been subjected to induced senescence produced 0.5 nl of ethylene/h. Microsomes from foliage that was treated with Ca^{2+} ionophore during the period of induced senescence produced 1.02 nl ethylene/h over the 20-h incubation period (Table I); this represents an increase of approximately 6-fold relative to the unaged control and approximately 2-fold relative to levels produced by foliage senesced without ionophore treatment. Of particular interest is the finding that treatment of the foliage with fluphenazine during the senescence induction period reduced the capability of microsomes to convert ACC to ethylene by about 26% relative to the conversion capability of microsomes from foliage aged without treatment (Table I). Fluphenazine at concentrations within the range used in these experiments (50 μM) prevents binding of the Ca:calmodulin complex to enzymes, and hence this observation suggests that the ionophore treatment promotes senescence by allowing influx of Ca^{2+} into cells, which in turn activates calmodulin.

There are reports that phospholipase A_2 is a Ca:calmodulin-promoted enzyme (24, 28, 29). Moreover, during senescence there is a pronounced loss of membrane phospholipid that has been attributed to fatty acid deesterification (2, 3, 16). It is, therefore, conceivable that internalized Ca^{2+} activates phospholipase A_2 by forming a Ca:calmodulin complex and promotes senescence by facilitating deesterification of membrane phospholipids. To test this, microsomal membranes isolated from unaged 17-d-old foliage were treated with phospholipase A_2 in the presence of Ca^{2+} and calmodulin in an effort to simulate the increased capabilities to produce O_2^- and ethylene that are acquired during the period of induced senescence in darkness. The time course for conversion of ACC to ethylene by microsomes in the presence and absence of phospholipase A_2 and Ca:calmodulin is illustrated in Figure 4. Microsomal membranes alone possess ethylene-producing capability, but this was increased in the presence of phospholipase A_2 and increased still further when Ca:Calmodulin was present as well (Fig. 4). Moreover, this stimulatory effect of phospholipase A_2 and Ca:Calmodulin was largely inhibited by fluphenazine (Fig. 4).

The effects of additional calmodulin inhibitors—R24571 (26), W-7 (5), and W-5 (5)—on ethylene production by microsomal membranes are illustrated in Table II. Addition of R24571 to the reaction mixture containing phospholipase A_2 and Ca-calmodulin eliminated the stimulatory effect of phospholipase A_2/calmodulin and decreased ethylene production to a level that

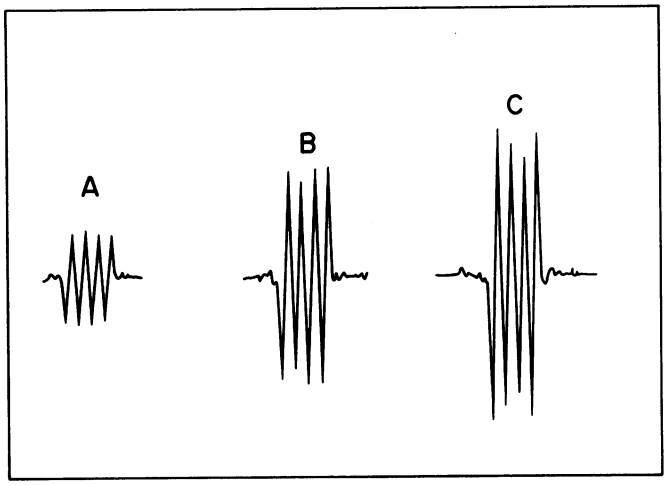

FIG. 3. Tiron ESR spectra reflecting production of O_2^- by microsomal membranes from senescing pea foliage. A, Microsomes from young 17-d-old foliage. B, Microsomes from senesced pea foliage. C, Microsomes from foliage treated with ionophore. Amplitude means ± SD for six replicates from the same membrane preparation were 1.3 ± 0.17, 3.2 ± 0.5, and 4.0 ± 0.16 relative units for A, B, and C, respectively. Each replicate contained 200 μg membrane protein and 10 mM Tiron in 1 ml of 2 mM Epps buffer (pH 8.5). The scan region was 3388 to 3395 G. Modulation amplitude was 1.25 G, receiver gain 8 × 10^3, microwave power 10 mw, modulation frequency 9.545 GHz, temperature 22°C, and microwave frequency 100 KHz.

Table I. *Ethylene Production from ACC by Microsomal Membranes Isolated from Senescing Pea Foliage*

The reaction mixtures contained 300 μg microsomal protein, 2 mM ACC, and 50 μM $CaCl_2$ in 1 ml of 2 mM EPPS buffer (pH 8.5). Ethylene was collected over a period of 20 h.

Source of Foliar Microsomal Membranes	Ethylene Production[a]
	nl/ml·h
A. Young unaged plants	0.17 (0.01)
B. Senescing plants	0.50 (0.09)
C. B + ionophore A23187	1.02 (0.10)
D. B + fluphenazine	0.37 (0.01)

[a] Mean with SD in parentheses for six replicates from the same membrane preparation.

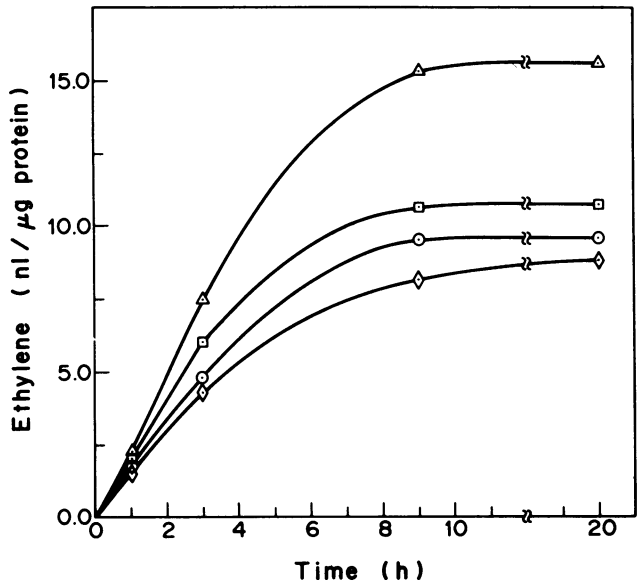

FIG. 4. Effects of calmodulin and phospholipase A_2 on ethylene production from ACC by pea foliage microsomal membranes. Each observation is the mean of six replicates from the same membrane preparation. Standard deviations were less than 0.95. (◇), Microsomal membranes alone; (□), microsomal membranes + phospholipase A_2; (△), microsomal membranes + phospholipase A_2 + calmodulin; (○), microsomal membranes + phospholipase A_2 + calmodulin + fluphenazine. Microsomal membranes were obtained from 17-d-old leaves of pea plants irrigated with 10^{-3} M $CaCl_2$. Each replicate contained 0.2 mg microsomal protein incubated in the dark in 1 ml of 2 mM EPPS buffer (pH 8.5) containing 50 µM $CaCl_2$, 2 mM ACC and, when added, 10 units of phospholipase A_2, 7 units of calmodulin, and 50 µM fluphenazine.

Table II. *Effects of Calmodulin Inhibitors and EGTA on Ethylene Production from ACC by Microsomal Membranes Isolated from Pea Foliage*

The basic reaction mixture contained 200 µg microsomal protein (from 16-d-old foliage), 50 µM $CaCl_2$, and 2 mM ACC in 1 ml 2 mM EPPS buffer (pH 8.5); 250 µM EGTA, 10 units phospholipase A_2, 7 units calmodulin, and 50 µM R24571, W-7, or W-5 were added to the basic reaction mixture in various combinations. Ethylene was collected over a period of 20 h.

Reaction Mixture	Ethylene Production[a]
	pl/ml·h
A. Basic reaction mixture	160 (9)
B. A + phospholipase A_2 + calmodulin	290 (12)
C. A + phospholipase A_2 + calmodulin + R24571	120 (6)
D. A + phospholipase A_2 + calmodulin + W-7	205 (7)
E. A + phospholipase A_2 + calmodulin + W-5	245 (10)
F. A + phospholipase A_2 + calmodulin + EGTA	10 (0.5)

[a] Mean with SD in parentheses for three to six replicates from the same membrane preparation.

was only 75% of that produced by untreated control membranes (Table II, A, B and C). W-7 inhibited the phospholipase A_2/calmodulin stimulatory effect by about 65% (Table II, A, B and D), whereas W-5, a weaker antagonist of calmodulin (5), was less effective (Table II, A, B and E). EGTA almost completely inhibited ethylene production in the presence of exogenous phospholipase A_2 and calmodulin (Table IIF), indicating that both

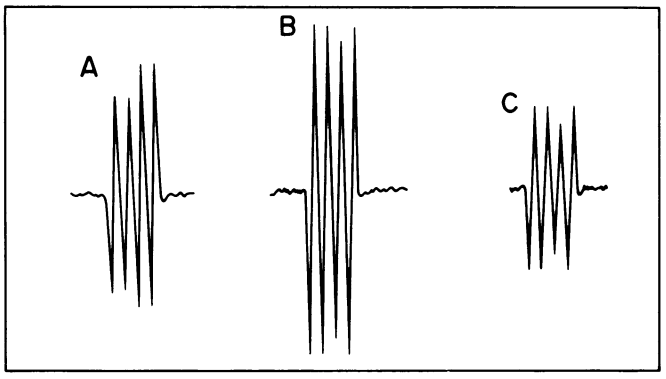

FIG. 5. Effects of 2-h incubations in calmodulin and phospholipase A_2 on the amplitude of the Tiron ESR signal reflecting O_2^- production by pea foliage microsomal membranes. A, Microsomal membranes alone; B, microsomal membranes + phospholipase A_2 + calmodulin; C, Microsomal membranes + phospholipase A_2 + calmodulin + fluphenazine. Amplitude means ± SD for seven replicates from the same membrane preparation were 6.5 ± 0.6, 9.0 ± 1.4, and 4.6 ± 0.5 relative units for A, B, and C, respectively. Microsomal membranes were obtained from 17-d-old leaves of pea plants irrigated with 10^{-3} M $CaCl_2$. The incubation mixtures contained 1 mg microsomal protein in 1 ml of 2 mM EPPS buffer (pH 8.5), 50 µM $CaCl_2$ and, when added, 10 units of phospholipase A_2, 7 units of calmodulin, and 50 µM fluphenazine. At the end of the incubation period, 0.2 ml of the reaction mixture were mixed with 0.8 ml of 10 mM Tiron in the same EPPS buffer for recording of ESR spectra. The scan region was 3385 to 3395 G. Modulation amplitude was 1.25 G, receiver gain 1.26 × 10^4, microwave power 10 mw, modulation frequency 9.545 GHz, temperature 22°C, and microwave frequency 100 KHz.

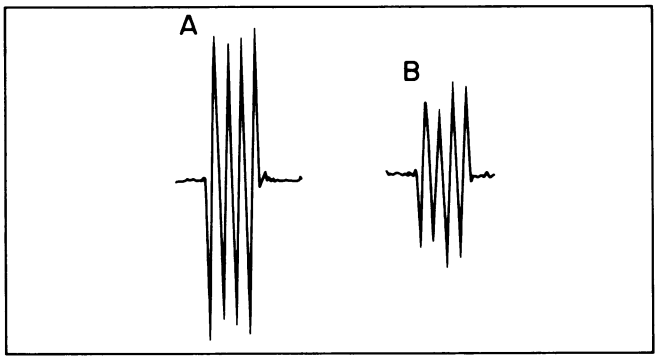

FIG. 6. Effect of superoxide dismutase on the calmodulin/phospholipase A_2 evoked Tiron ESR signal reflecting O_2^- production by pea foliage microsomal membranes. A, Microsomal membranes + phospholipase A_2 + calmodulin; B, microsomal membranes + phospholipase A_2 + calmodulin + superoxide dismutase. Amplitude means ± SD for seven replicates from the same membrane preparation were 8.9 ± 0.4 and 3.6 ± 0.7 relative units for A and B, respectively. Microsomal membranes were obtained from 17-d-old leaves of pea plants irrigated with 10^{-3} M $CaCl_2$. Reaction mixtures containing 1 mg microsomal protein in 1 ml of 2 mM EPPS buffer (pH 8.5), 50 µM $CaCl_2$, 10 units of phospholipase A_2 and 7 units of calmodulin were incubated for 2 h. At the end of the incubation period, 0.2 ml of the reaction mixture was mixed with 0.8 ml of 10 mM Tiron and, when added, 28 units of superoxide dismutase in the same EPPS buffer for recording of ESR spectra. Instrument conditions were as specified in Figure 5.

the endogenous activity and the stimulatory effect of phospholipase A_2 and calmodulin are dependent upon calcium.

After 2 h of incubation, a pronounced effect of Ca:Calmodulin and phospholipase A_2 on ethylene production was apparent (Fig. 4). Measurements of O_2^- production by microsomes after treat-

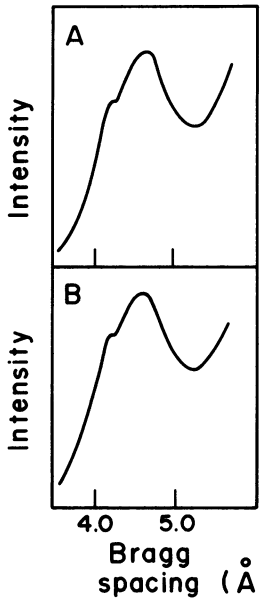

FIG. 7. Densitometer tracings of wide-angle x-ray diffraction patterns recorded at room temperature for microsomes isolated from 17-d-old pea foliage. Microsomes were incubated for 2 h in the absence (A) or presence (B) of phospholipase A_2 and calmodulin. Incubation mixtures contained microsomal membranes (1 mg protein/ml), 50 μM $CaCl_2$ and, when added, 5 units/ml of phospholipase A_2 and 11.5 units/ml of calmodulin in 2 mM EPPS buffer (pH 8.5).

ment with phospholipase A_2 of the same duration indicated that the increased formation of superoxide radical accompanying senescence can also be simulated *in vitro* by inducing lipid deesterification. Microsomes treated over the 2-h period with phospholipase A_2 in the presence of Ca:calmodulin produced 50% more O_2^- than corresponding control microsomes incubated for the same period in buffer and Ca^{2+} (Fig. 5). Moreover, addition of fluphenazine to the reaction mixture containing phospholipase A_2 and Ca:calmodulin decreased O_2^- production to a level that was only 70% of that produced by untreated control membranes (Fig. 5). This suggests that during the 2-h incubation of control membranes in buffer and Ca^{2+} alone there may have been some action by endogenous phospholipase A_2 and calmodulin that had coisolated with the microsomes.

When superoxide dismutase was included in the reaction mixture containing phospholipase A_2 and Ca:calmodulin, O_2^- production was reduced by approximately 60% (Fig. 6). This degree of inhibition by superoxide dismutase is comparable to that reported previously for microsomes (13, 18). Further inhibition is not achieved by adding increased levels of superoxide dismutase, and this has been attributed to the fact that superoxide dismutase, being a macromolecule, may not have as ready access to the site of O_2^- production as the much smaller Tiron molecule (13, 18). Indeed, if the microsomes are both inside-out and outside-in, and the vesicles are largely sealed, one might not expect much more than 50% inhibition of the Tiron signal by exogenously added superoxide dismutase, for the latter would not be able to cross the membrane by reason of its macromolecular size, and the enzyme for producing O_2^- is not likely to have active sites on both sides of the membrane. Previous studies have demonstrated that Tiron does not react with $OH^\cdot$, singlet oxygen, or H_2O_2 (18), and thus the reaction appears to be specific for O_2^-.

Membranes treated for 2 h with phospholipase A_2 in the presence of calmodulin and Ca^{2+} do not acquire increased proportions of gel phase lipid; indeed, the relative intensities of the 4.2 Å reflection deriving from gel phase lipid are closely similar for treated and untreated membrane (Fig. 7, A and B). This is consistent with previous studies indicating that free fatty acids and lysophosphatides do not induce gel phase lipid when added to liposomes of pure phospholipid (Barber and Thompson, unpublished data), and that formation of gel phase lipid appears to be attributable to lipid peroxidation (3, 20). Although free radicals are being produced during the treatment period, their level of production is apparently insufficient to cause peroxidation of lipids on a scale that would induce the formation of additional gel phase.

Taken together, the results support the involvement of intracellular Ca^{2+} in senescence, its role being to activate phospholipase A_2 through calmodulin and thus promote deesterification of membrane phospholipids. Although there is no definitive evidence to date that plant phospholipase A_2 is calmodulin-regulated, the observations that calmodulin antagonists added to reaction mixtures containing exogenous phospholipase A_2 and calmodulin reduce ethylene and O_2^- production to levels below those obtained for untreated control membranes (Table II; Fig. 5) suggest that this is the case, and it is clear from the experiments with EGTA (Table II) that Ca^{2+} is required. In nonsenescent cells, there is a steep gradient of Ca^{2+} across the plasmalemma such that the concentration may be 10,000-fold higher outside the cell than inside (27). However, if during senescence cells become less able to maintain this gradient, Ca^{2+} would become internalized and by reacting with cytosolic calmodulin set in motion phospholipase-mediated membrane deterioration. That this does occur is supported by the finding that treatment of intact foliage with Ca^{2+} ionophore accelerates membrane senescence, whereas treatment with fluphenazine attenuates deterioration. As well, the propensity of microsomal membranes from senescing foliage to produce increased levels of O_2^- and convert ACC to ethylene with greater facility can be simulated by treating isolated membranes with phospholipase A_2 and Ca:calmodulin. The molecular basis for this effect of phospholipase A_2 on microsomes is not immediately clear, but the finding closely parallels the results of a recent study with human neutrophils in which it was shown that calcium, calmodulin, and a plasma membrane-associated phospholipase are involved in promoting increased superoxide production during activation of these cells (24).

Loss of membrane fatty acids is an inherent feature of senescence (2, 3, 16), and thus it is to be expected that phospholipases contribute to membrane deterioration. The products of phospholipase A_2 activity are fatty acids and lysophosphatides. The liberated polyunsaturated fatty acids could serve as a substrate for lipoxygenase and also, by perturbing the bilayer structure, promote lipid peroxidation, which is known to be a characteristic feature of senescence and to initiate lateral phase separation of membrane lipid bilayers (3, 20). It has also been proposed that oxidized di and trienoic fatty acids serve as endogenous Ca^{2+} ionophores (23). These could further promote transport of external Ca^{2+} to the cell interior and, by activating more calmodulin-dependent phospholipase A_2, create a cascade effect. Lysophosphatides, the other product of phospholipase A_2 action, do not accumulate in senescent membranes (16) suggesting that other lipases are involved as well.

It has been reported that application of exogenous Ca^{2+} delays senescence, apparently by preventing the large increase in ethylene production that occurs during the very early stages of senescence (9, 21). However, this has been attributed to an extracellular effect whereby Ca^{2+} associates with the outside surface of the plasma membrane and by acting as a divalent ligand stabilizes the membranes. In fact, parallel ESR studies have shown that the levels of Ca^{2+} found to delay senescence are able to rigidify membrane surfaces (7). Accordingly, inasmuch as the senescence-delaying effect of Ca^{2+} appears to manifest an effect of

external Ca^{2+}, it is not inconsistent with the contention that internalized Ca^{2+} promotes senescence through a calmodulin-mediated effect.

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