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Calcium Stimulation of Ethylene Production Induced by 1-Aminocyclopropane-1-Carboxylic Acid and Indole-3-Acetic Acid

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Abstract. Ca^{2+} stimulates 1-aminocyclopropane-1-carboxylic acid (ACC)- and indole-3-acetic acid (IAA)-dependent ethylene production in mung bean hypocotyls and senescing cucumber cotyledons. This stimulation is much greater in the presence of ACC than IAA. ACC content of hypocotyls also increases markedly with increasing Ca^{2+} concentration. Ca^{2+} stimulation with IAA occurs only after 4 h and may be dependent on new ACC being produced following auxin activation. Stimulation by Ca^{2+} with both ACC and IAA increases with temperature from 20° to 35°C, and also occurs with high internal levels of Ca^{2+}.

Ca^{2+} also stimulated ethylene production in preclimacteric apple fruit tissue and reduced the decline in ethylene in climacteric tissue. ACC-dependent ethylene was also stimulated by Ca^{2+} in this tissue.

The results of this study suggest that Ca^{2+} may stimulate ACC uptake as well as enhance ACC conversion to ethylene.

Calcium can stimulate ethylene production in developing plant tissues such as mung bean hypocotyls. This stimulation has been shown to occur in synergism with cytokinins (Lau and Yang 1974, 1975, Lau et al. 1977), or with cupric salts (Lau and Yang 1976, Yu and Yang 1980), where ethylene biosynthesis is activated by auxin. Ca^{2+} alone does not have a substantial effect on ethylene production.

Calcium also influences ethylene production in senescing tissue, but here the effect is less straightforward. In postclimacteric apple fruit tissue, high concentrations of Ca^{2+} initially reduce, but eventually sustain, ethylene syn-

thesis under conditions in which natural ethylene production is declining (Lieberman and Wang 1982, Ben-Arie et al. 1982). This suggests a role for Ca^{2+} in maintaining membrane-associated, ethylene-synthesizing activity. In support of this, Ca^{2+} has been shown to stimulate ethylene production by particulate fractions from pea epicotyl homogenates (Mattoo et al. 1982), and Ca^{2+} can stabilize microsomal membranes from postclimacteric apple fruit tissue (Legge et al. 1982).

In contrast to these more direct effects on ethylene production, the general effect of Ca^{2+} on plant senescence and fruit ripening is one of delay and depression of the rate of senescence (Poovaiah and Leopold 1973, Bangerth 1979, Ferguson et al. 1983). This may entail a reduction and retardation of ethylene production. For instance, when cucumber cotyledons are excised after 16 days development and floated on water in the dark, there is, after about 8 days, a burst in respiration and ethylene production concomitant with the most rapid decline in chlorophyll levels. Ca^{2+} in the external medium eliminates the respiratory burst, reduces the rate of chlorophyll breakdown, and reduces and retards ethylene production (Ferguson et al. 1983).

These contrasting effects of Ca^{2+} suggest the need for further investigation into the influence of Ca^{2+} on ethylene synthesis. In the present work, the effect of Ca^{2+} on ethylene production stimulated by ACC and IAA in mung bean hypocotyls has been studied. The Ca^{2+} stimulation found has been contrasted with the effect of Ca^{2+} on ethylene production in senescing cucumber cotyledons and apple fruit tissue. In these latter tissues, in contrast with hypocotyls, substantial ethylene production does not require the presence of exogenous stimulators or precursors.

Materials and Methods

Mung Bean Hypocotyls

Mung bean (*Vigna radiata* L. Wilczek) seedlings were grown in vermiculite in the dark, without nutrient supplement, at 25 ± 1°C. When the seedlings were 7 days old (~4 cm high), the hypocotyls were cut into 1–2-cm lengths. Samples of approximately 1 g were placed in 25-ml conical flasks containing 5 ml 0.05M KH_2PO_4 buffer pH 6, 2% (w/v) sucrose, chloramphenicol (50 $\mu g\ ml^{-1}$), plus other solutions where appropriate. In one experiment, Mes buffer (pH 6) replaced the phosphate buffer. A CO_2 trap of 1 ml 12% KOH was included in the flasks, which were sealed with rubber septa and incubated in a shaking water bath at the required temperatures. Ethylene was measured in 1-ml gas samples by gas chromatography with flame ionization detection. Flasks were flushed out with air after each sampling. In one experiment, high concentrations of Ca^{2+} in the hypocotyls were obtained by providing the seedlings with 10^{-1}M $CaCl_2$ 24 h before use; care was taken to avoid contamination of the outside of the hypocotyls with the Ca^{2+} solution.

ACC was measured by grinding 1-g samples of hypocotyls in liquid nitrogen, and extracting in 5 ml of methanol/chloroform/water (12/5/3 v/v/v). After centrifugation and washing, the supernatant was dried under vacuum at 40°C,

taken up in 1 ml water, and 0.3 ml aliquots assayed for ACC by the method of Lizada and Yang (1979).

Senescing Cucumber Cotyledons

Cotyledons of cucumber (*Cucumis sativus* L. cv. Heinz Pickling) seedlings grown for 16 days in vermiculite at 23°C were excised and placed on water or 10^{-4}M $CaCl_2$ in the dark as described by Ferguson et al. (1983). When ethylene was to be collected, four cotyledons (~0.5 g fresh weight) were placed in a 50-ml conical flask in 5 ml of appropriate solution and the flask sealed with a rubber septum. The flasks were incubated at 23°C for 24 h and ethylene measured in 1-ml samples. Ethylene measurements during the experiment indicated that at 8 days the rise in ethylene production (see Ferguson et al. [1983]) was just commencing in the control tissue, although this was occurring later than in experiments quoted in that paper. High concentrations of Ca^{2+} in the cotyledons were obtained by providing the seedlings with 10^{-1}M $CaCl_2$ 24 h before cotyledon excision.

Ca^{2+} was measured in hypocotyls and cotyledons by digesting tissue HNO_3/$HClO_4$ and analyzing the digestate by atomic absorption spectrophotometry.

Apple Fruit Tissue

Plugs of apple (*Malus sylvestris* Mill.) fruit tissue were taken from stored Cox's Orange Pippin fruit (climacteric) or freshly picked Granny Smith fruit (preclimacteric). Longitudinal plugs were taken with a 7-mm-diam cork borer from the cortex of the fruit and the plugs then cut into 0.5-cm segments. Six segments (~1 g fresh weight) were washed in 20 ml 0.3M sucrose for 5 min to stabilize the pH (Ferguson and Watkins 1981), and then transferred to 25-ml conical flasks containing 5 ml 0.3M sucrose plus appropriate additions. A CO_2 trap was added, as for hypocotyls. Incubation was in a shaking water bath in the dark at 25°C. Ethylene was measured as above in 1-ml samples, the gas atmosphere of the flasks being flushed out between samples.

In all experiments, treatments were replicated four times. All weights refer to fresh weight.

Results

Effects of Ca^{2+} on ACC-Stimulated Ethylene in Hypocotyls

Concentrations of Ca^{2+}, 10^{-4}M and above, stimulated ethylene production in the presence of ACC (Fig. 1), the stimulation being greatest during the 4–8 h period, although substantial even during the first 4 h (Fig. 2). In Mes buffer at the same pH, but therefore in the absence of K^+ and phosphate, Ca^{2+} had a similar effect: at 8 h, ethylene production was 26.9 and 60.7 nl $g^{-1}h^{-1}$ in the

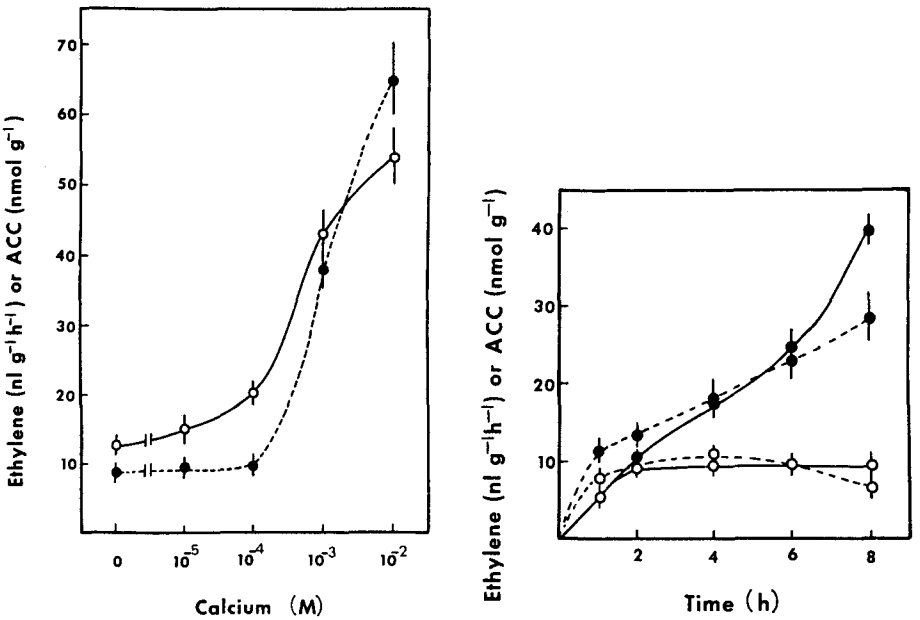

Fig. 1. Ethylene production (○) and ACC concentration (●) in hypocotyls stimulated by 10^{-4}M ACC in solutions of phosphate buffer, sucrose, and different concentrations of $CaCl_2$. Ethylene production is the mean rate during the 4–8-h period; ACC concentration is that in tissue after 8 h. Data are accompanied by SE. **Fig. 2.** Ethylene production (——) and ACC concentration (----) in hypocotyls in solutions of phosphate buffer, sucrose, 10^{-4}M ACC, and presence (●) or absence (○) of 10^{-2}M $CaCl_2$. Ethylene rates are the mean of the preceding time interval. Data are accompanied by SE.

absence and presence of Ca^{2+}, respectively. In the absence of ACC, Ca^{2+} had little effect on ethylene production, the rate being of the order of 0.2 nl $g^{-1}h^{-1}$.

At temperatures tested up to 35°C, ACC-stimulated ethylene production increased in the presence of Ca^{2+} (Fig. 3a). The response of ethylene production to higher temperatures was greater in the presence of Ca^{2+} than with ACC alone.

A stimulation of ethylene production was also found in tissue that had high internal levels of Ca^{2+}. Where the hypocotyl Ca^{2+} concentration had been increased about three times by $CaCl_2$ application, subsequent ethylene production was substantially higher than in untreated tissue (Table 1). The proportional differences were similar to those achieved with Ca^{2+} in the external medium.

Effect of Ca^{2+} on ACC Content of Hypocotyls

Concentrations of 10^{-3} and 10^{-2}M Ca^{2+} increased the levels of ACC in hypocotyls (Fig. 1), the proportional increases being greater than those for ethylene. In the presence of Ca^{2+}, ACC increased rapidly during the measurement period, slightly preceding the rise in ethylene production (Fig. 2). The increases

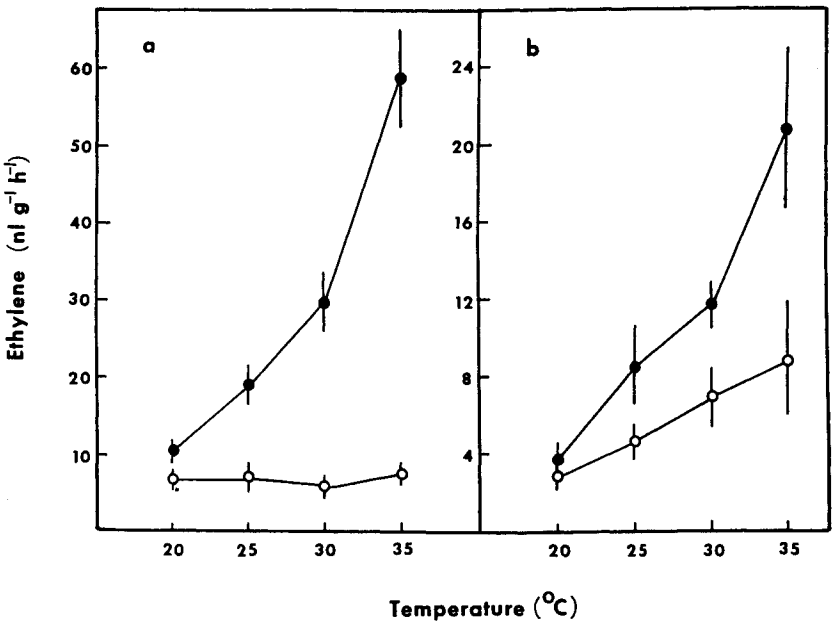

Fig. 3. Effect of temperature on ethylene production by mung bean hypocotyls in solutions of phosphate buffer, and sucrose stimulated by (a) 10^{-4}M ACC, and (b) by 5×10^{-4}M IAA, in the presence (●) or absence (○) of 10^{-2}M $CaCl_2$. Ethylene was collected over the 4–8-h period. Data are accompanied by SE.

Table 1. Effect of high and low internal Ca^{2+} concentrations in mung bean hypocotyls on ethylene production in the presence of 10^{-4}M ACC. Ethylene data are the mean rates for the 0–4-h and 4–8-h time intervals and are accompanied by SE. Hypocotyls were analyzed for Ca^{2+} at the beginning and end of the experiment.

	Ca^{2+} content (μmol g^{-1} fresh wt)		Ethylene (nl g^{-1} fresh wt h^{-1})	
Tissue	0 h	8 h	4 h	8 h
Low Ca	0.52 ± 0.04	0.41 ± 0.03	6.48 ± 0.12	7.90 ± 0.64
High Ca	1.86 ± 0.17	1.10 ± 0.04	9.38 ± 0.55	20.43 ± 2.43

in tissue ACC in response to Ca^{2+} treatment could arise from Ca^{2+}-facilitated uptake and/or feedback stimulation resulting from ethylene accumulation in the reaction vessels. To test these possibilities, two experiments were performed. The first followed ACC levels in hypocotyls in the absence of exogenous ACC, with and without Ca^{2+} and where ethylene (4 μl l^{-1}) was added to the gas atmosphere of the flask. No increase in ACC was found in the tissue under such conditions. The second experiment followed ACC levels in the tissue in the presence of 10^{-4}M ACC ± Ca^{2+}, but under flowing air (500 cc h^{-1}) rather than in a sealed flask. Under these conditions, where there was no ethylene accumulation, Ca^{2+}, particularly at 10^{-2}M, again caused increased ethylene production and ACC accumulation (Table 2).

Table 2. Effect of 10^{-4}M ACC and Ca^{2+} on ethylene production and ACC levels in mung bean hypocotyls. Tissue was subjected to an air flow of 500 cc h^{-1} and analyzed for ACC after 8 h. Ethylene was sampled in the outflow at 8 h. Data are the means of four replicates and are accompanied by SE.

Treatment	Ethylene (nl $g^{-1}h^{-1}$)	ACC (nmol g^{-1})
ACC	36.51 ± 1.34	5.30 ± 0.14
ACC + 10^{-4}M $CaCl_2$	42.00 ± 1.42	6.75 ± 0.66
ACC + 10^{-2}M $CaCl_2$	64.07 ± 10.44	16.95 ± 2.28

Table 3. Effect of 10^{-2}M $CaCl_2$ on ethylene production in mung bean hypocotyls stimulated by 5 × 10^{-4}M IAA. Data are the mean rates for the 0–4-h and 4–8-h time intervals and are accompanied by SE.

	Ethylene (nl g^{-1} fresh wt h^{-1})	
Treatment	4 h	8 h
Control	0.18 ± 0.01	0.16 ± 0.01
Ca	0.25 ± 0.01	0.26 ± 0.02
IAA	4.72 ± 0.55	9.53 ± 0.86
IAA + Ca	4.46 ± 0.50	13.80 ± 1.56

Effect of Ca^{2+} on IAA-Stimulated Ethylene in Hypocotyls

Ca^{2+} also increased IAA-stimulated ethylene production in hypocotyls; this was not apparent until after 4 h and was rarely more than a doubling (Table 3). This stimulation was dependent on Ca^{2+} concentration, with increases after 8 h being 17% at 10^{-5}M, and 80% at 10^{-2}M. As with ACC, there was a distinct temperature response with the Ca^{2+} effect increasing with rising temperature (Fig. 3b).

Effect of Ca^{2+} on Ethylene in Senescing Cotyledons and Apple Fruit Tissue

Detached, senescing cucumber cotyledons responded to stimulation of ethylene production by both ACC and IAA (Table 4). Ca^{2+} also increased ACC-stimulated ethylene both when added only during the period of ethylene collection, and when present during the whole senescence time. Ca^{2+} was only effective with IAA in the latter case, when tissue Ca^{2+} had been increased by incubation in $CaCl_2$ during senescence. Thus the Ca^{2+}-stimulation shown in the data in Table 4 is occurring at the same time as senescence is being retarded by the presence of Ca^{2+} in the external medium.

In preclimacteric apple fruit tissue, ethylene production has been shown to increase over 24 h, and in climacteric and postclimacteric tissue, it declines (Lieberman et al. 1977). In preclimacteric tissue, 10^{-3}M Ca^{2+} in the external medium increased ethylene production (Fig. 4b). In climacteric tissue, Ca^{2+}

Table 4. Effect of 10^{-4}M ACC or 5×10^{-4}M IAA on ethylene production in detached, senescing cucumber cotyledons in relation to the presence and absence of 10^{-4}M $CaCl_2$. Measurements were made after 8 days senescence in water or $CaCl_2$; ethylene was collected during the final 24 h. Data are accompanied by SE.

	Ethylene (nl 4 cotyledons^{-1} h^{-1})	
Treatment during ethylene collection	Senescence in water	Senescence in $CaCl_2$
Water	0.40 ± 0.06	0.09 ± 0.03
ACC	33.44 ± 5.31	
ACC + $CaCl_2$	47.81 ± 6.21	43.16 ± 8.09
Water	0.20 ± 0.04	0.08 ± 0.02
IAA	3.65 ± 0.75	
IAA + $CaCl_2$	3.85 ± 0.72	6.26 ± 0.77

retarded the decline in ethylene production, and in the same tissue (Fig. 4a) increased ACC-stimulated ethylene.

Discussion

In mung bean hypocotyls, in which there is little natural ethylene synthesis, Ca^{2+} alone has little effect on ethylene production. However, in the presence of precursors (such as ACC) or stimulators (cytokinin, IAA), enhancement of ethylene production by Ca^{2+} can be considerable. Yang and his colleagues found that Ca^{2+} stimulated synergistically with both cupric ion and cytokinin (Lau and Yang 1974, 1976). The present results show that in both nonsenescent and senescent tissue a substantial stimulation by Ca^{2+} can be found with exogenous ACC, and lesser stimulation can be achieved with IAA. The difference in extent of stimulation is significant: about 5× with ACC in 4–8 h and about 2× or less with IAA. There is also a stimulatory effect by Ca^{2+} with ACC in the first 4 h, but none over this time interval with IAA. The Ca^{2+}-stimulation with IAA was not found by Lau and Yang (1974). Perhaps the difference lies in the slightly younger tissue and lower IAA concentration used by these authors.

An explanation for the Ca^{2+} response may lie in two areas. First, Ca^{2+} might increase uptake of ACC and IAA into the tissue; second, Ca^{2+} might facilitate the conversion of ACC to ethylene. These possibilities are discussed below.

The increase in hypocotyl ACC content with increasing Ca^{2+} concentration (Fig. 1) is the best evidence for Ca^{2+}-stimulation of ACC uptake. An increase was also found when any effect of accumulated ethylene was removed (Table 2), although the ACC levels achieved were less than expected. The result with IAA might also be explained in this way. Lau and Yang (1974, 1975, 1976) believe that the synergistic stimulation of ethylene production by Ca^{2+} with Cu^{2+} or cytokinin acts prior to ACC in the synthetic pathway. The action of IAA is also believed to be on ACC synthase (Yu and Yang 1979, Yoshi and Imaseki 1981), and any lag in the Ca^{2+} effect with IAA would be compatible with the timing of the buildup of ACC.

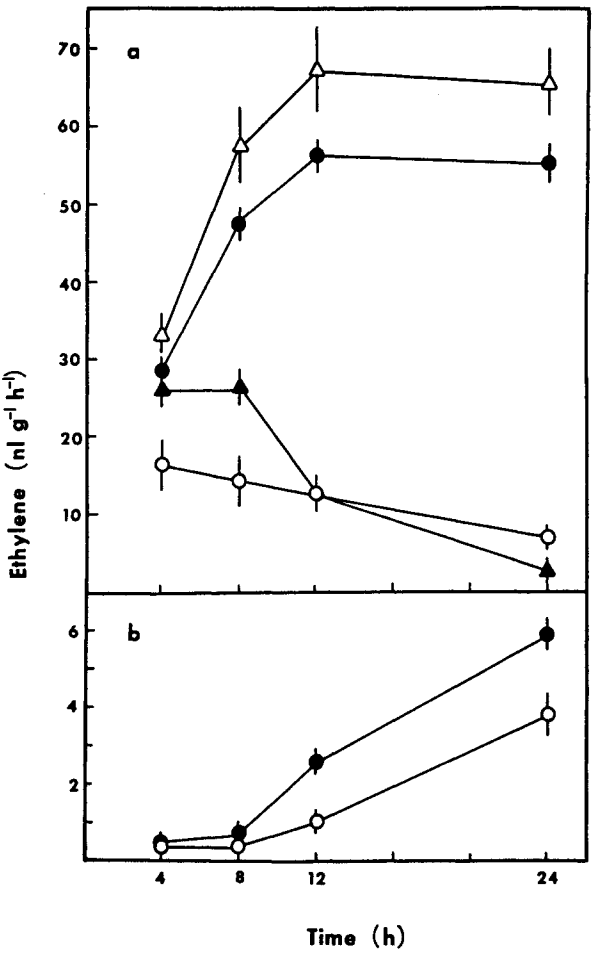

Fig. 4. Effect of 10^{-3}M $CaCl_2$ on ethylene production by plugs of apple fruit tissue. Data are accompanied by SE. (a) Plugs from climacteric fruit in 0.3M sucrose (○), sucrose + 10^{-3}M $CaCl_2$ (▲), sucrose + 10^{-4}M ACC (●), sucrose + $CaCl_2$ + ACC (△). (b) Plugs from preclimacteric fruit in 0.3M sucrose (○), sucrose + 10^{-3}M $CaCl_2$ (●).

There is some less direct evidence, however, that Ca^{2+} might also have an effect on ACC conversion. Fig. 1 shows that an effect on ethylene production can be found at low Ca^{2+} concentrations when there is no apparent effect on ACC levels in the tissue. The Ca^{2+} effect with ACC can also be found in tissue with high internal levels of Ca^{2+}, although it is probable that a large proportion of the Ca^{2+} fed into the tissue is extracellular and may therefore still affect cellular ACC uptake. The greater effect of Ca^{2+} at higher temperatures is possible further evidence. Yu et al. (1980) have shown that the primary site for high temperature inactivation of ethylene synthesis in hypocotyls is the conversion of ACC. In their results, ACC continued to increase in tissue with temperatures up to 35°C, whereas ethylene production declined. The marked effect of Ca^{2+} in stimulating ethylene production at these higher temperatures suggests that Ca^{2+} might protect the system from inactivation. However, these results with temperature are a little at variance with those of Yu et al. (1980) in that ethylene production in the absence of Ca^{2+} did not decline between 30°

and 35°C. Experimental conditions were similar, so variations in tissue may be the explanation.

It has been suggested that the conversion of ACC to ethylene is a membrane-associated step, sensitive to uncouplers and to inhibition by free radical scavengers (Apelbaum et al. 1981a, b, Konze et al. 1980). If ACC conversion is membrane-associated, then a role for Ca^{2+} could be envisaged that involves Ca^{2+} binding to maintain membrane structure and function (Legge et al. 1982, Ben-Arie et al. 1982). Such action might maintain ethylene synthesis in tissue such as postclimacteric apple fruit in which natural ethylene production is declining along with increasing membrane disorganization. The Ca^{2+} effect in hypocotyls could be a combination of such binding and maintenance, along with increased ACC uptake. These results on Ca^{2+} stimulation highlight a distinction between a long-term effect of Ca^{2+} on senescence in general and that of Ca^{2+} on short-term ethylene production. Ethylene synthesis is retarded and depressed by Ca^{2+} in senescing cotyledons (Ferguson et al. 1983), yet in the same tissue, Ca^{2+} can stimulate ethylene synthesis in the presence of ACC and, to a small extent, with IAA (Table 4). This suggests that the effect of Ca^{2+} on senescence *per se* is one of maintaining cell membrane structure and function, generally retarding cell breakdown. Should ACC levels be increased further both exogenously and endogenously, then Ca^{2+} will have an effect in this tissue similar to that in hypocotyls. In apple fruit tissue, the positive stimulation by Ca^{2+} in preclimacteric tissue might be related to conversion of naturally accumulated ACC. In climacteric or postclimacteric tissue, Ca^{2+} can also stimulate ACC-stimulated ethylene production, or maintain production under conditions when it is declining (Fig. 3) (Ben-Arie et al. 1982, Mattoo et al. 1982).

Three actions of Ca^{2+} in senescence and ethylene production can possibly be identified. Ca^{2+} appears able to facilitate uptake of ethylene stimulants (but also senescence retardants) such as cytokinin and auxin, and of the ethylene precursor ACC. Ca^{2+} can retard senescence, probably by maintaining membrane function. In doing so, Ca^{2+} may maintain the potential for membrane-associated enzyme activity such as ACC conversion.

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