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Inhibition of Activity of the Ethylene-Forming Enzyme by α(*p*-Chlorophenoxy)Isobutyric Acid

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Abstract. α(*p*-Chlorophenoxy)isobutyric acid (PCIB) inhibited indole-3-acetic acid (IAA)-induced ethylene production in etiolated mung bean hypocotyl sections. The endogenous level of 1-aminocyclopropane-1-carboxylic acid (ACC) was not significantly affected by PCIB, indicating that PCIB exerted its effect primarily by inhibiting the activity of the ethylene-forming enzyme (EFE). This conclusion was supported by the observations that PCIB inhibited the conversion of exogenously applied ACC to ethylene. The inhibitory effect of PCIB was already evident with 0.05 mM PCIB, and it increased with time after application of the inhibitor. PCIB also significantly inhibited ethylene production in apple fruit tissues, but it only slightly reduced the level of endogenous ACC. Similar to mung bean, EFE activity in apple tissue was significantly inhibited by PCIB. The possibility that PCIB also inhibits auxin-induced ACC synthase activity is discussed.

α(*p*-Chlorophenoxy)isobutyric acid (PCIB) was classified as a substance possessing antiauxin properties (Burstrom 1950). Later its antiauxin properties were studied in detail, particularly in regard to auxin-binding sites in subcellular fractions from maize coleoptiles (Dohrmann et al. 1978) and from *Cucurbita* hypocotyls (Jacobs and Hertel 1978). In all these experiments, it has been shown that PCIB efficiently and specifically competes with indole-3-acetic acid (IAA) for auxin-binding sites in vitro. PCIB has been widely employed to demonstrate the role of endogenous auxin in plant growth processes. Thus, PCIB was first employed by Burstrom (1950) to demonstrate the role of endogenous auxin in the two phases of growth of wheat roots. Subsequently, it was utilized for studying growth of *Avena* coleoptiles (Fransson and Ingestad 1955), elongation of *Avena* leaf sections (Cleland 1964), and curvature of citrus

flower petals (Goldschmidt 1968). Recently, PCIB was found to abolish the geotropic response of roots of cress (Katekar and Geissler 1980). Also, it was used to examine the primary action of IAA in crown gall tumors in potato tuber tissue (Rausch et al. 1984).

Frenkel and Haard (1973) were the first to show that PCIB inhibited ethylene production in pears and attributed it to the antiauxin activity of PCIB. In a recent study, Tsai and Arteca (1984) observed that PCIB inhibited IAA-induced ethylene production in mung bean hypocotyl segments. They suggested that the inhibitory effect of PCIB resulted from the blocking of auxin transport and action. Other investigators also utilized PCIB to examine the involvement of auxin in the regulation of ethylene production (Imaseki et al. 1975) and to study the mechanism of the stimulatory effect of thidiazuron on ethylene production (Suttle 1984, Yip and Yang, 1986).

During studies of ethylene biosynthesis in cucumber growth tips and hypocotyls, we have observed that PCIB also inhibits ethylene-forming enzyme (EFE) activity, a step in the ethylene biosynthetic pathway, which is thought to be independent of auxin control (Yu and Yang 1979). The results of a study on the effect of PCIB on EFE activity are presented in this paper.

Materials and Methods

Plant Material and Treatments

The plant species tested were mung bean (*Vigna radiata* L.) and apple (*Malus silvestris* Mill., var. Golden Delicious). Seeds of mung bean were germinated and grown in Vermiculite for 4 days in darkness at 25°C. Ten 1-cm long hypocotyl segments, 1–3 cm below the hook, were weighed and incubated in 1 ml 50 mM Mes buffer (pH 6.1) in a 22-ml vial.

Apples were obtained from commercial storage houses and stored in our laboratory at 4°C. Peel strips were cut from the equatorial region of apples using a domestic potato peeler and were further cut into 2-cm long sections. Each section was placed with the adhering cortical tissue downward on 2 ml 50 mM Mes buffer (pH 6.1) in a 25-ml Erlenmeyer flask. Plugs (1 cm in diameter and 2 cm long) were excised from the pericarp with a cork borer and further cut into disks, about 2 mm thick. Seven disks were weighed and incubated in 3 ml 50 mM Mes buffer (pH 6.1) in a 25-ml Erlenmeyer flask. Where indicated, PCIB, IAA, 1-aminocyclopropane-1-carboxylic acid (ACC), or aminoethoxyvinylglycine (AVG) was added to the medium.

Determination of Ethylene

For monitoring ethylene production, the vessels containing the plant samples were sealed with a rubber serum cap and shaken in the dark at 27°C. At various periods during incubation, a 2-ml air sample was withdrawn from the vessels with a hypodermic syringe. After each sampling, the vessels were flushed with ethylene-free air and resealed for an additional determination of

ethylene. Ethylene was assayed using a gas chromatograph equipped with an alumina column and a flame ionization detector.

Extraction and Determination of ACC

Mung bean hypocotyls were ground with a mortar and pestle in 2 ml 80% ethanol. The mortar and pestle were washed twice with 2 ml 80% ethanol each time. The extract and washings were combined and centrifuged for 10 min at 10,000*g*. The supernatant was concentrated under reduced pressure at 45°C. Residues were brought to a volume of 2 ml with distilled water and clarified by centrifugation as above.

Apple tissues were homogenized in 4 ml 80% ethanol by means of a Polytron homogenizer. The shaft was washed with additional 4 ml 80% ethanol. The extract and washing were combined and filtered through Miracloth. The ensuing procedure was as for mung bean hypocotyls. ACC in the aqueous extract was assayed by the method of Lizada and Yang (1979).

Assay of EFE Activity

EFE activity was determined by measuring conversion of applied ACC to ethylene in vivo. Tissue was placed in a 25-ml Erlenmeyer flask containing different concentrations of ACC in 1 or 2 ml Mes buffer (pH 6.1). In one experiment using apple peel tissues, AVG was also added to ensure measurement of EFE activity only. Ethylene evolution was measured as described above.

Results

Effect of PCIB on IAA-Induced Ethylene

Consistent with previous data (Yu and Yang 1979), application of 50 μM IAA to mung bean hypocotyls increased ethylene evolution and ACC production (Fig. 1). Treatment with 1 mM PCIB inhibited IAA-induced ethylene. This inhibition was evident after 4 h of incubation, at a time when IAA-induced ethylene started to increase, and it increased gradually with time. After 8 h of incubation, PCIB inhibited IAA-induced ethylene production by 65% (Fig. 1A). Surprisingly, the endogenous level of ACC in IAA-treated hypocotyls was not significantly affected by PCIB (Fig. 1B). Data from similar experiments showed a consistency in the effect of PCIB on IAA-induced ethylene. However, the content of ACC in hypocotyls treated with IAA plus PCIB was either higher or slightly lower than in hypocotyls treated with IAA only.

Effect of PCIB on EFE Activity in Mung Bean Hypocotyls

To examine EFE activity in mung bean hypocotyls, ACC was directly utilized as an ethylene precursor. EFE activity, measured by application of 0.05 and

0.2 mM ACC to hypocotyl sections, was significantly inhibited by PCIB (Fig. 2). In both ACC concentrations, the inhibition was apparent after 2 h of incubation, but it was much higher in the lower concentration of ACC. The inhibition of EFE activity by PCIB increased with time, reaching 95 and 80% of control with 0.05 and 0.2 mM ACC, respectively, after 8 h of incubation.

The effect of different concentrations of PCIB on EFE activity was also examined (Fig. 3). Over a range of 0.05–0.5 mM, the inhibition by PCIB increased progressively from 20 to 75%. The concentration of PCIB required for 50% inhibition of ethylene production was estimated to be about 0.2 mM (Fig. 3).

Attempts to analyze the kinetics of inhibition by a Lineweaver-Burk plot failed to give a clear-cut conclusion about the nature of the inhibition.

Effect of PCIB on Ethylene Production in Apple Tissues

The rate of ethylene production in apple pericarp disks was initially high, but it decreased rapidly during an incubation period of 8 h (Fig. 4A). PCIB significantly inhibited ethylene production, the magnitude of the inhibition reaching ~70% of the control after 8 h of incubation (Fig. 4A). PCIB also reduced ACC level in apple tissues after 8 h of incubation (Figs. 4B, 5B). However, ACC level was only about 20% lower than that in the control, while inhibition of ethylene production was 70% (Fig. 4).

Similar results were obtained in apple peel tissue, where ethylene production was inhibited by 70% and ACC level was reduced by ~20% (Fig. 5). Additional experiments showed that, as in mung bean hypocotyls, there was a consistency in the effect of PCIB on ethylene production (~70% inhibition), but its effect on ACC level varied, so that in most experiments there was a slight reduction whereas in others PCIB somewhat enhanced ACC content (data not shown).

Effect of PCIB on EFE Activity in Apple Peel

The initial ethylene production in freshly excised apple tissues was high (Figs. 4, 5). For measuring EFE activity, AVG was used to accelerate the deprivation of endogenous ACC from apple peel tissue. Measurements of EFE activity started 4 h after excision of the tissues. Under these conditions EFE activity in control tissues was almost constant throughout the experimental period. PCIB reduced EFE activity by ~70% (Fig. 6).

Discussion

The properties of PCIB as an antiauxin agent are well documented (Burstrom 1950, Dohrmann et al. 1978, Jacobs and Hertel 1978). Among its various effects, PCIB was found to inhibit ethylene evolution in Bartlett pears (Frenkel and Haard 1973) and IAA-induced ethylene production in mung bean hypo-

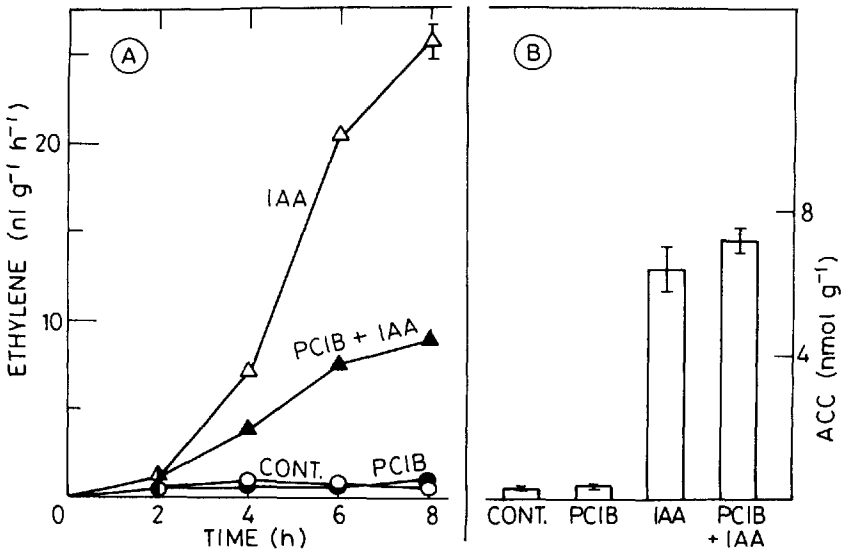

Fig. 1. Effect of PCIB on ethylene production rate (A) and ACC level (B) in mung bean hypocotyls treated with IAA. Ten hypocotyl segments were incubated in Mes buffer (pH 6.1) containing 50 μM IAA with or without 1 mM PCIB. Ethylene production was monitored during an 8-h incubation period. ACC levels were determined at the end of incubation. Bars indicate 1 SE.

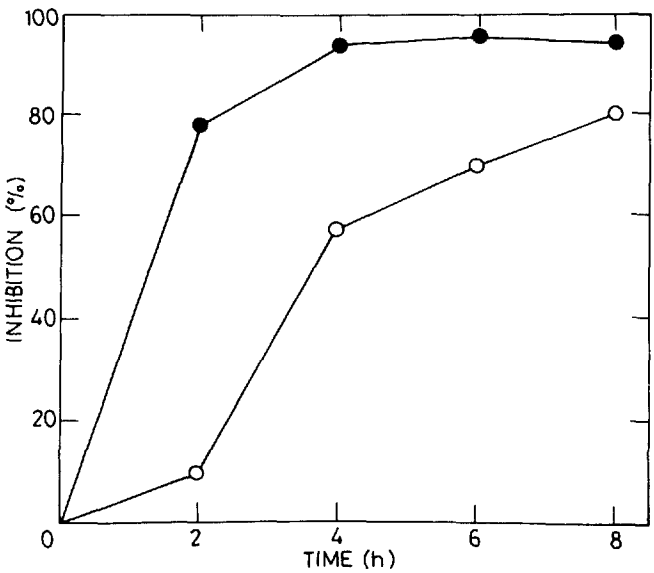

Fig. 2. Time course of the inhibition of EFE activity in mung bean hypocotyl segments by PCIB. Ten hypocotyl segments were incubated in Mes buffer (pH 6.1) containing 1 mM PCIB and 0.05 (●) or 0.2 (○) mM ACC.

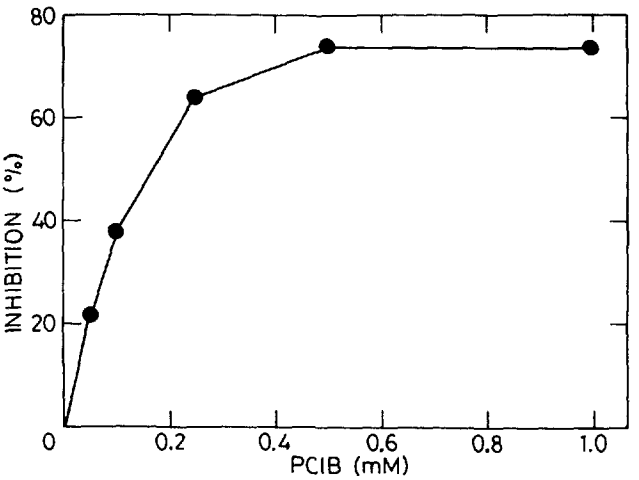

Fig. 3. Dose–response curve for the inhibition of EFE activity by PCIB in mung bean hypocotyls. Ten hypocotyl segments were incubated in Mes buffer (pH 6.1) containing 0.1 mM ACC and various concentrations of PCIB. Ethylene production was measured after an 8-h incubation.

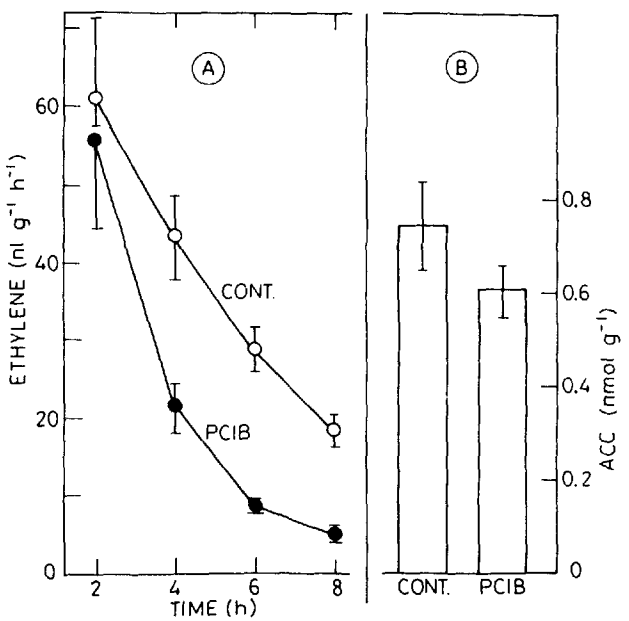

Fig. 4. Effect of PCIB on ethylene production rate (A) and ACC level (B) in apple pericarp discs. Seven disks were incubated in Mes buffer (pH 6.1) containing 1 mM PCIB. Ethylene production was monitored during an 8-h incubation period. ACC level was determined at the end of incubation. Bars indicate 1 SE.

cotyls (Tsai and Arteca 1984). This effect was attributed by the above investigators to the antiauxin properties of PCIB.

It is well accepted that IAA stimulates ethylene production by inducing the synthesis of ACC (Yang and Hoffman 1984). Several studies have shown that

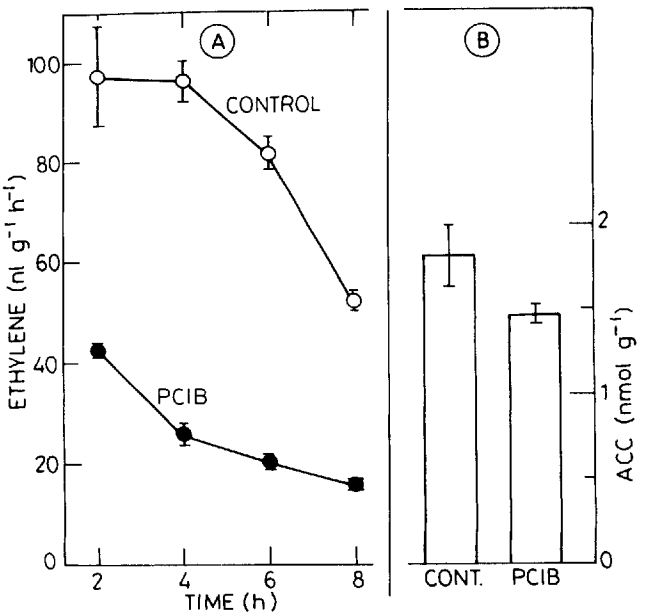

Fig. 5. Effect of PCIB on ethylene production rate (A) and ACC level (B) in apple peel tissue. One 2-cm-long apple peel section was incubated in Mes buffer (pH 6.1) containing 1 mM PCIB. Ethylene production was monitored during an 8-h incubation. ACC content was determined at the end of incubation. Bars indicate 1 SE.

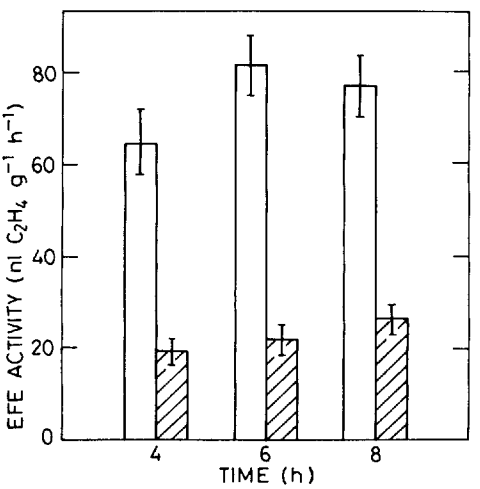

Fig. 6. Effect of PCIB on EFE activity in apple peel tissues. Peel sections were treated with 0.5 mM AVG with or without 1 mM PCIB. EFE activity was measured by application of 0.1 mM ACC. Hatched bars indicate EFE activity when tissue was treated with PCIB. Bars indicate 1 SE.

ethylene production rates induced by IAA closely paralleled the level of endogenous ACC and the activity of ACC synthase (Jones and Kende 1979, Yoshii and Imaseki 1982, Yu and Yang 1979). Accordingly, we expected that inhibition of IAA-induced ethylene production by PCIB (Fig. 1A) would be

accompanied by a parallel reduction in ACC level. Contrary to our expectations, this was not the case. ACC levels in IAA plus PCIB-treated hypocotyls were similar to levels in IAA-treated hypocotyls, and in some experiments even higher (Fig. 1B). Similarly, we observed that PCIB significantly inhibited ethylene production rates in apple tissues, whereas ACC levels were only slightly reduced (Figs. 4, 5). This discrepancy was clarified when the effect of PCIB on EFE activity was studied. PCIB strongly inhibited EFE activity in mung bean hypocotyls (Fig. 2). A significant inhibition was already observed with 0.05 and 0.1 mM PCIB (Fig. 3). Similar concentrations were used for negation of auxin action (Goldschmidt 1968, Rausch et al. 1984, Tsai and Arteca 1984). By using AVG to accelerate the deprivation of endogenous ACC from apple tissues, it could also be shown that PCIB inhibited EFE activity in apple tissues (Fig. 6). Because at present EFE activity can only be measured in vivo, we were unable to obtain reliable data on the nature of the inhibition. In a recent study on the stimulatory effect of thidiazuron on ethylene production, Yip and Yang (1986) also concluded that PCIB does not appear to be a specific inhibitor of IAA-mediated ethylene production.

It seems that the inhibition of ethylene production by PCIB does not result solely from the inhibition of EFE activity. If the conversion of ACC to ethylene were the only step in the ethylene biosynthetic pathway affected by PCIB, ACC levels in PCIB-treated tissues would have been well above those measured in untreated tissues. However, in both mung beans and apples, ACC levels in PCIB-treated tissues did not differ much from the levels of ACC in untreated tissues (Figs. 1B, 4B, 5B). The data suggest that PCIB also inhibits ACC synthase owing to its antiauxin properties. This assumption may be true also for apples, since there is evidence that auxin stimulates ethylene production in fruit tissues (Frenkel and Dyck 1973, Vendrell 1969).

The data obtained in this study may be interpreted to indicate that auxin also regulates EFE activity. It was recently hypothesized that auxins could be involved in the conversion of ACC to ethylene through their effects on membrane polarization (John 1983). Balague and Pech (1985) reported that EFE activity in pear cell cultures was increased by 2,4-D. They also suggested that auxin plays a role in the modulation of EFE activity. We, however, have found that various phenoxy acids, including some of those that possess auxin activity, also inhibited EFE activity in mung bean hypocotyls (data not shown). This indicates that the inhibition of EFE activity by PCIB is probably not related to its antiauxin properties.

Whatever the mechanism of the inhibition of EFE activity by PCIB may be, the use of PCIB to demonstrate the involvement of auxin in various physiological plant processes, and particularly in those in which ethylene is also involved, should be regarded with care.

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