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Inhibition of Growth of Cultured Tobacco Cells at Low Concentrations of Lovastatin Is Reversed by Cytokinin[1]

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ABSTRACT

De novo synthesis of mevalonic acid, which is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase, is the first committed step in the formation of isoprenoid compounds. Various studies have shown that mevalonic acid-derived compounds are required for growth of plant and animal cells, a conclusion supported by the observation that cells treated with lovastatin (a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase) cease growth. We show that *Nicotiana tabacum* BY-2 cells, which require exogenous auxin for growth in culture but do not require exogenous cytokinin, are growth inhibited by 1 μM lovastatin. However, these cells are capable of growing in the presence of 1 μM lovastatin if 8 μM zeatin is supplied in the medium. Furthermore, benzyladenine, kinetin, and thidiazuron effectively reverse the inhibition of growth of these cells at 1 μM lovastatin, whereas adenine and 6-methyladenine have no effect. These results demonstrate that restoration of growth to lovastatin-treated cells is cytokinin specific and is not caused by metabolism of cytokinin into other isoprenoid compounds. Cytokinin does not effectively reverse the effects of higher concentrations of lovastatin, but mevalonic acid does, consistent with the hypothesis that cytokinin biosynthesis is more sensitive to lovastatin than the biosynthesis of other essential isoprenoid compounds in tobacco cells. This observation suggests that lovastatin can be used to induce cytokinin dependence in cytokinin-autonomous tobacco cell cultures.

Cytokinins are growth substances that have profound effects on plant growth and development (naturally occurring cytokinins are N^6-substituted adenine derivatives) (21, 22). For example, cytokinins stimulate chloroplast and lateral bud development (16) and suppress tissue senescence (24, 26). In addition, cytokinins promote cell division in many plant tissue culture systems at concentrations as low as 10^{-10} M (21, 22). These phenomena account, at least in part, for the observation that expression of a cytokinin biosynthetic gene from *Agrobacterium tumefaciens* in plant cells results in the formation of a shooty tumor (1).

In vivo cytokinin studies require the use of cytokinin-depleted plant material. Accordingly, researchers have used primary explants (10), whole plants (12), or cytokinin-starved cell suspensions (7, 11) in their work on the physiology and molecular biology of cytokinin action. Cytokinin-depleted suspension cultures, however, can be difficult to obtain because many plant cell lines undergo cytokinin habituation (i.e. they acquire the ability to produce cytokinin and, therefore, no longer require an exogenous source of cytokinin) (20). Treatment of cytokinin-autonomous cultures with an inhibitor of cytokinin biosynthesis would overcome the problem of habituation, but no specific inhibitor of cytokinin biosynthesis has been found.

Cytokinin is produced by the transfer of an *iso*-pentenyl group from dimethylallyl PPi to AMP and is, therefore, a product of the mevalonate pathway (1, 17). In plants, this pathway participates in the synthesis of ABA, gibberellins, ubiquinone, plastoquinone, dolichols, sterols, carotenoids, Chls, and numerous other isoprenoids (Fig. 1) (1, 3, 5, 9, 13, 17, 25). The first committed step in this pathway, which is catalyzed by HMG CoA[2] reductase (EC 1.1.1.34), is the irreversible reduction of HMG CoA to mevalonate. Plant HMG CoA reductases (3, 4, 6, 8, 27), like mammalian HMG CoA reductases (2, 9, 13, 18), are highly regulated (phytochrome, phytohormones, and other factors have been shown to affect plant HMG CoA reductase activity) and are inhibited by lovastatin, an antibiotic isolated from *Aspergillus terreus* (high concentrations of lovastatin also inhibit a plant sesquiterpene cyclase [28]). Low concentrations of lovastatin (i.e. less than 10 μM) have been shown to decrease phytosterol synthesis in, and inhibit normal growth of, plant seedlings and cell cultures (3-5, 14, 15), suggesting that HMG CoA reductase activity is rate limiting for phytosterol synthesis and normal plant growth (3). However, the primary mechanism of growth inhibition by lovastatin remains to be established.

This work was undertaken to identify the primary cause of growth inhibition by lovastatin and thereby achieve a better understanding of the role of isoprenoids in plant cell growth. Our strategy was to search for an isoprenoid compound (or combination of compounds) that would restore growth to lovastatin-treated tobacco cells. We report that lovastatin inhibits the growth of a cytokinin-autonomous tobacco suspension culture at low concentrations and that this inhibition is reversed by cytokinin. These data suggest that low concentrations of lovastatin specifically inhibit cytokinin biosynthesis.

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[2] Abbreviation: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

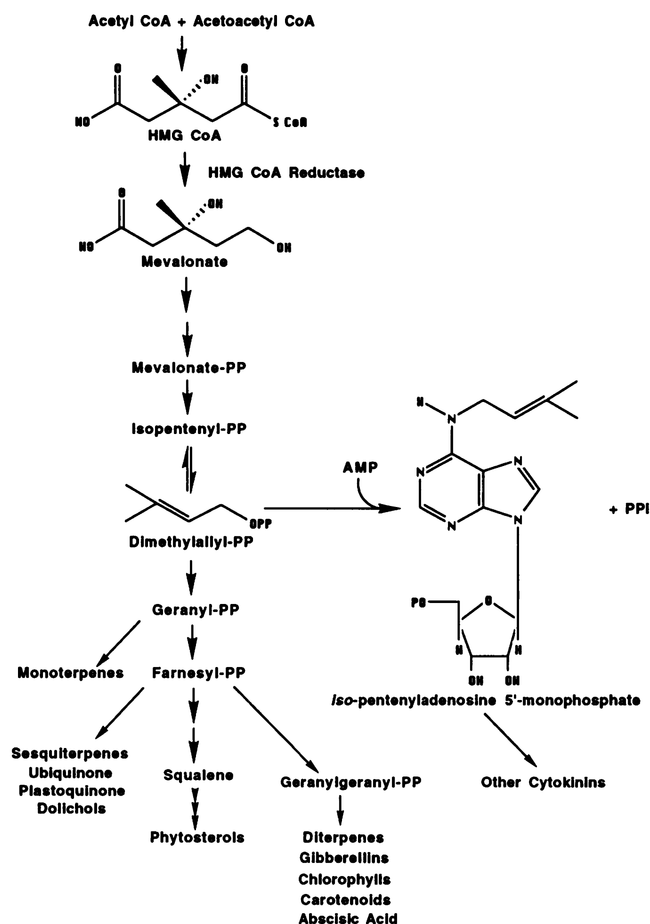

Figure 1. Biosynthesis of isoprenoids in plant cells. The cytokinin synthase shown is a dimethylallyl PPi:AMP-dimethylallyl transferase (Δ^2-isopentenyl PPi:AMP-Δ^2-isopentenyl transferase). However, other acceptor molecules for the dimethylallyl group are possible. Portions were taken from the following published papers: refs. 1, 3, 5, 9, 13, 17, and 25.

MATERIALS AND METHODS

Tissue Culture

All experiments were done on suspension cultures of *Nicotiana tabacum* cell line BY-2 (a cytokinin-independent line derived from *N. tabacum* L. cv Bright Yellow-2 callus), which were grown at 26 ± 1°C in 18 mL of Murashige-Skoog (23) medium containing 0.9 μM (0.2 mg/L) 2,4-D. Cultures were started by adding 3 mL of a 2-fold diluted stationary phase culture (10 d old) to 15 mL of fresh medium and were grown in continuous fluorescent light on a rotary shaker set to 100 rpm. All additions to the cultures (e.g. lovastatin, cytokinin) were made after 24 h to allow the cells to acclimate to fresh medium. (Day 0 is defined as the day that additions were made.) Growth was monitored every 2 d by measuring cell volumes after 10 min of settling (clumping precluded accurate measurement of cell number) and by microscopic examination of the cells (to monitor cell size and cell viability). All experiments were done in duplicate.

All phytohormones were obtained from Sigma (St. Louis, MO) except thidiazuron, which was a generous gift from Dr. Richard Amasino.

Preparation of Chemicals

Lovastatin was kindly provided by A. Alberts of Merck Sharp & Dohme Research Laboratories. A 12.4 mM stock solution of lovastatin was prepared after hydrolyzing the lactone ring in ethanolic NaOH (15% [v/v] ethanol, 0.25% [w/v] NaOH) at 60°C for 1 h. Stock solutions of mevalonic acid (0.34 M) were prepared by the same procedure. A "blank" solution containing only ethanol and NaOH had no effect on the cells used in this study.

All cytokinins were dissolved in 1 M HCl and then diluted such that stock solutions contained 1 mM HCl (pH approximately 3). At the concentrations used in this study, these cytokinins did not affect the pH of the growth medium.

Microscopy

Cells were examined and photographed with a Leitz Dialux 20 microscope at a magnification of ×12.5 after staining with 0.05% Evan's blue for 30 min.

RESULTS

The role of isoprenoids in plant cell growth was studied in a cytokinin-autonomous tobacco culture treated with lovastatin. To minimize the possibility of nonspecific effects, all experiments were done at low concentrations of lovastatin. Accordingly, the growth response of *N. tabacum* cell line BY-2 to various concentrations of lovastatin was determined. As shown in Figure 2, 0.5 μM lovastatin partially inhibited cell growth and 1 μM lovastatin completely inhibited cell growth.

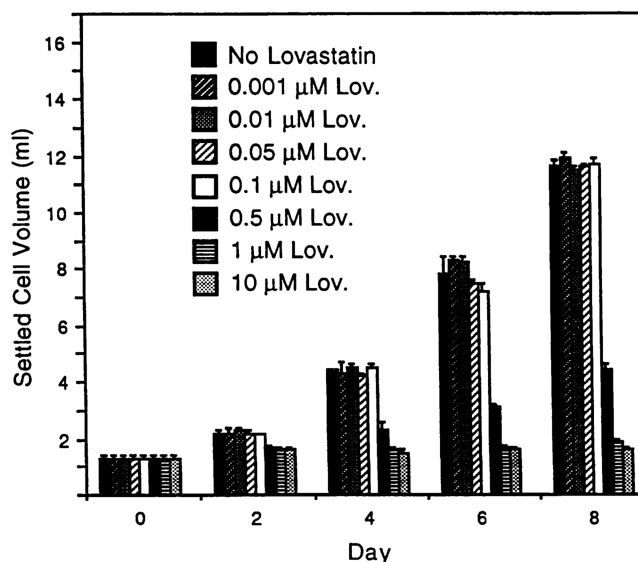

Figure 2. Lovastatin (Lov.) inhibits growth of cultured tobacco cells. All values represent cell volumes after 10 min of settling (measured in mL), which were recorded on days 0, 2, 4, 6, and 8 of the experiment. Error bars represent SD.

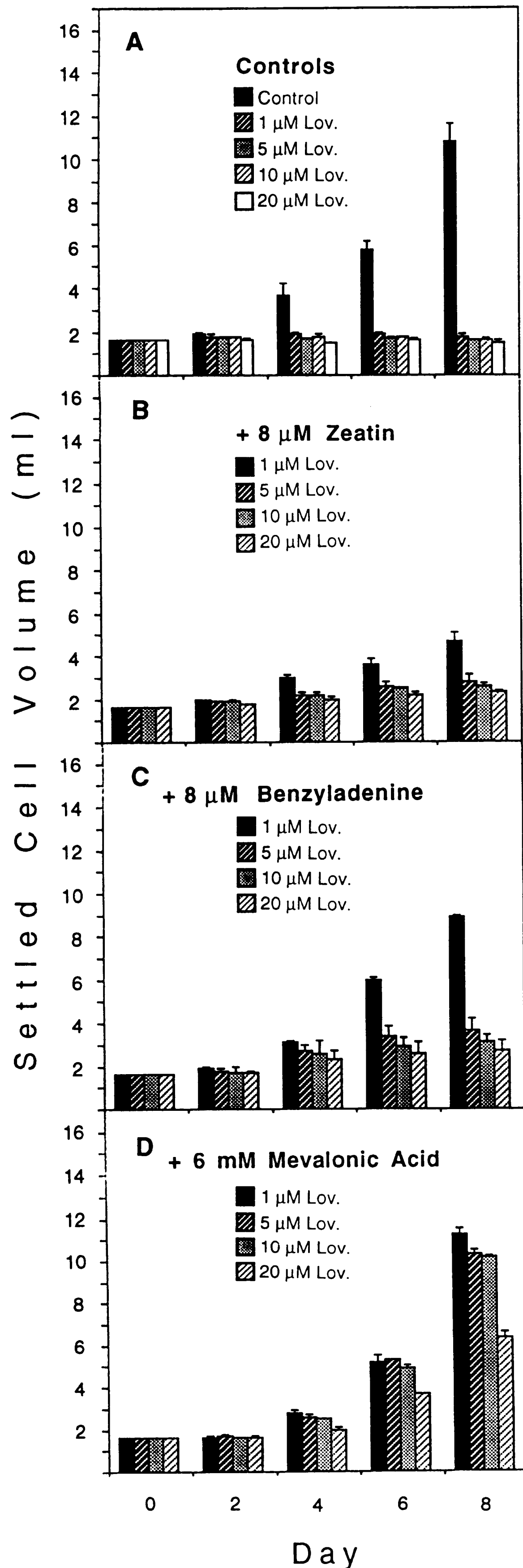

Figure 3. Effects of zeatin, BA, and mevalonic acid on lovastatin-treated cells. A, Growth curves for untreated cells and lovastatin-treated cells. Zeatin (8 μM; B), BA (8 μM; C), and mevalonic acid (6 mM; D) restore growth to lovastatin-treated cells. All values represent cell volumes after 10 min of settling (measured in mL), which were recorded on days 0, 2, 4, 6, and 8 of the experiment. Error bars represent SD. Lov., Lovastatin.

All subsequent experiments were performed at 1 to 20 μM lovastatin.

Zeatin is a mevalonic acid-derived regulator of plant growth and development. Accordingly, 8 μM zeatin was added to tobacco cell cultures containing 1, 5, 10, or 20 μM lovastatin to determine whether this naturally occurring cytokinin would restore growth to lovastatin-treated cells. As shown in Figure 3, zeatin partially restored growth to cultures treated with 1 μM lovastatin, suggesting that growth inhibition at this concentration of lovastatin was caused, at least in part, by reduced endogenous synthesis of cytokinin or other essential isoprenoid(s) that can be synthesized from zeatin. However, 8 μM BA also restored growth to cells treated with 1 μM lovastatin, arguing against the latter possibility (BA has no isoprenoid group to donate). Neither of these cytokinins was effective at lovastatin concentrations greater than 1 μM, indicating that lovastatin inhibits the biosynthesis of other essential isoprenoids or has nonspecific, toxic effects at high concentrations. To test these possibilities, 6 mM mevalonic acid was added to lovastatin-treated cells (Fig. 3) and was found to restore growth at all lovastatin concentrations tested (≤20 μM). Together, these observations suggest that 1 μM lovastatin specifically inhibited cytokinin biosynthesis, whereas higher lovastatin concentrations inhibited the synthesis of other essential isoprenoids as well.

To define the specificity of the cytokinin effect described above, kinetin (a potent cytokinin formed during chemical decomposition of nucleic acids), thidiazuron (a potent cytokinin derived from phenylurea), adenine (a weak cytokinin analog), and 6-methyladenine (another weak cytokinin analog) were tested for their ability to reverse the inhibitory effects of lovastatin on cell growth (19). As shown in Figure 4, 8 μM kinetin restored growth to lovastatin-treated cells, whereas 8 μM adenine had no effect. Furthermore, 8 μM thidiazuron effectively reversed the effects of 1 μM lovastatin but 8 μM 6-methyladenine, like adenine, had no effect (Fig. 5). These results indicate that compounds with cytokinin activity, especially BA, kinetin, and thidiazuron, were able to reverse the inhibitory effects of low concentrations of lovastatin on cell growth. Hence, this phenomenon is unlikely to be due to binding of cytokinin to lovastatin, because cytokinins with dramatically different structures are effective, whereas structurally related analogs with little or no cytokinin activity are not. It is interesting that 8 μM kinetin and 6 mM mevalonic acid had a mild inhibitory effect on growth in the absence of lovastatin (data not shown).

Microscopic analyses indicated that lovastatin, cytokinin, and mevalonic acid have no significant effects on cell size (Fig. 6), arguing that changes in settled cell volume are due to changes in cell number. In addition, treatment with 0.05% Evan's blue, which stains nonviable cells, showed that inhi-

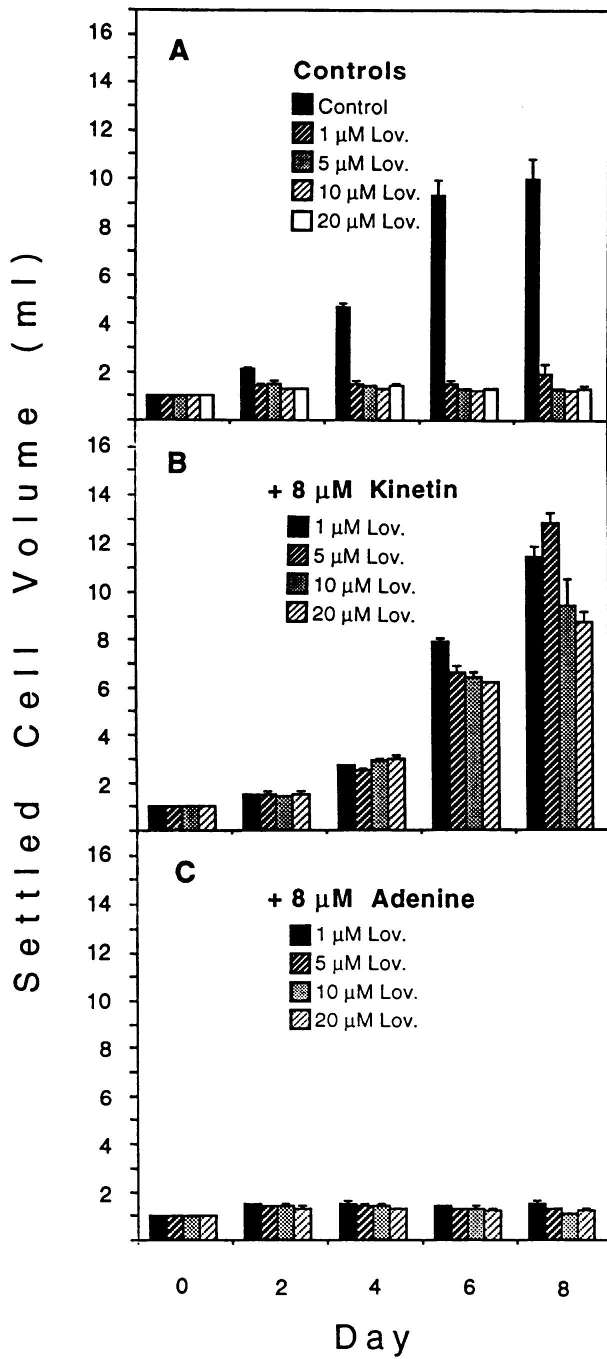

Figure 4. Effects of kinetin and adenine on lovastatin-treated cells. A, Growth curves for untreated cells and lovastatin-treated cells. Kinetin (8 μM; B) restores growth to lovastatin-treated cells, but adenine (8 μM; C) does not. All values represent cell volumes after 10 min of settling (measured in mL), which were recorded on days 0, 2, 4, 6, and 8 of the experiment. Error bars represent SD. Lov., Lovastatin.

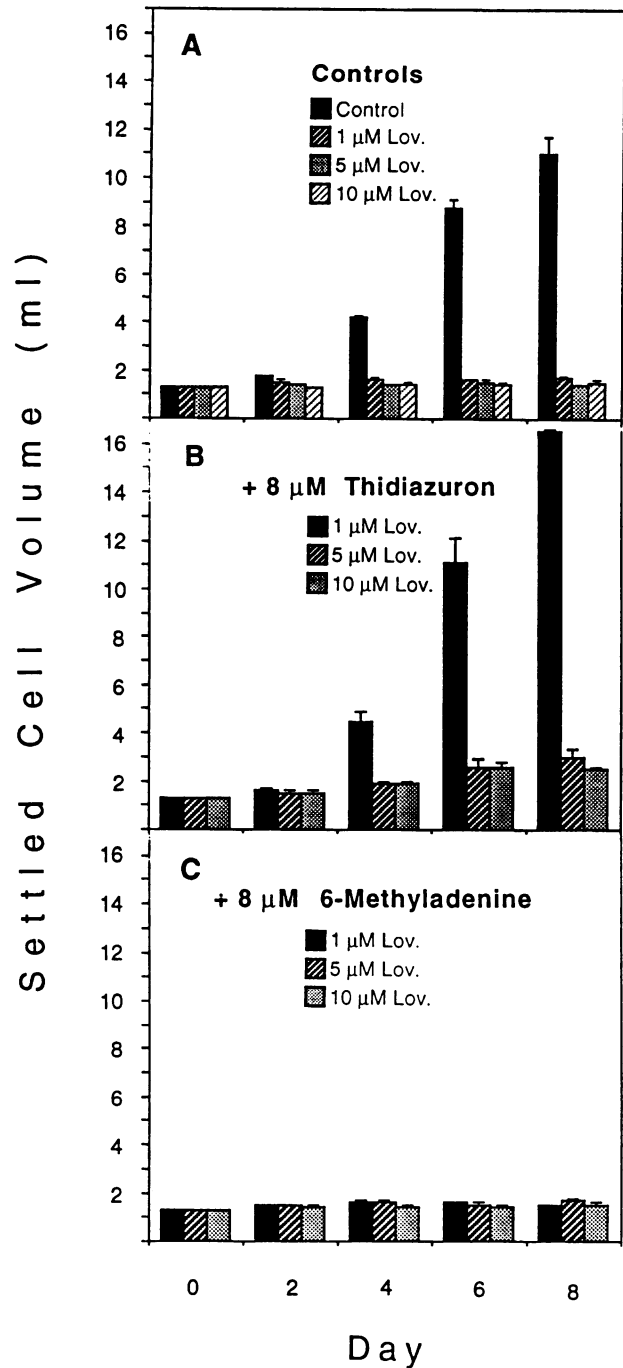

Figure 5. Effects of thidiazuron and 6-methyladenine on lovastatin-treated cells. A, Growth curves for untreated cells and lovastatin-treated cells. Thidiazuron (8 μM; B) restores growth to lovastatin-treated cells, but 6-methyladenine (8 μM; C) does not. All values represent cell volumes after 10 min of settling (measured in mL), which were recorded on days 0, 2, 4, 6, and 8 of the experiment. Error bars represent SD. Lov., Lovastatin.

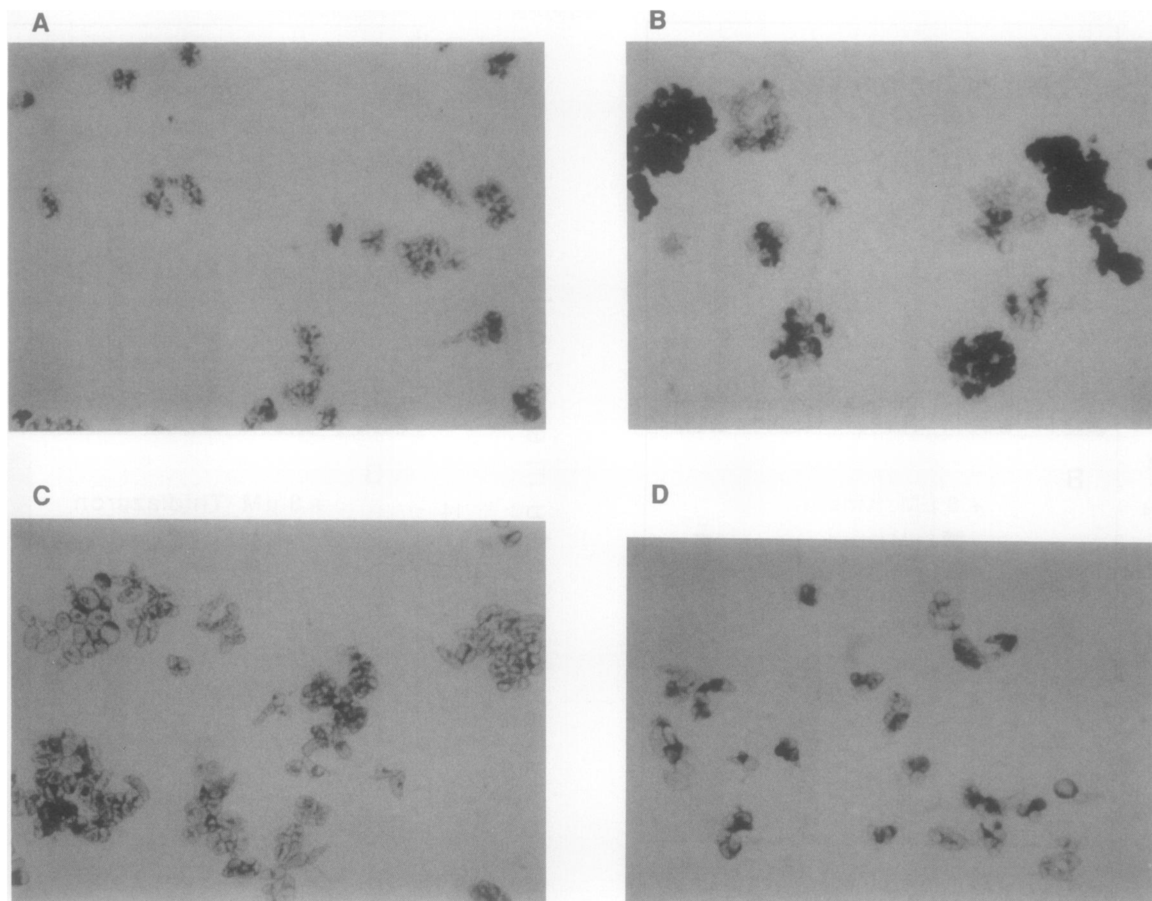

Figure 6. Kinetin (8 μM) and mevalonic acid (6 mM) restore viability to lovastatin-treated cells. Photomicrographs of cells stained with 0.05% Evan's blue for 30 min are shown. A, Untreated cells cultured for 5 d; B, cells cultured for 5 d in the presence of 1 μM lovastatin; C, cells cultured for 5 d in the presence of 1 μM lovastatin and 8 μM kinetin; D, cells cultured for 5 d in the presence of 1 μM lovastatin and 6 mM mevalonic acid. Background staining was observed in all four samples, but cells cultured in the presence of 1 μM lovastatin (B) stain more darkly and uniformly, indicating a loss of viability.

bition of cell growth at 1 μM lovastatin correlated with loss of cell viability and that restoration of growth (i.e. in the presence of kinetin or mevalonic acid) correlated with restoration of cell viability (Fig. 6).

DISCUSSION

The results described in this paper suggest that cytokinin biosynthesis is more sensitive to lovastatin than the biosynthesis of other essential isoprenoids in tobacco cells. Lovastatin is, therefore, a potentially useful tool for manipulating cytokinin levels in vivo.

It is interesting that other researchers have proposed that lovastatin might cause imbalances in phytohormone levels (5). In an earlier study of the effects of compactin (an analog of lovastatin) on growth of cytokinin-dependent tobacco callus, it was found that kinetin lessened the inhibitory effect of 5 μM compactin by 1.4-fold (14). In contrast, we find that, for a cytokinin-independent tobacco culture, kinetin relieves the complete inhibition of growth imposed by 5 μM lovastatin and, thus, has a stimulatory effect on growth in the presence of 5 μM lovastatin greater than 10-fold.

The apparent specificity of lovastatin action at low concentrations can be explained in various ways. For example, it is possible that multiple HMG CoA reductases exist in the plant cell that are dedicated, perhaps by intracellular compartmentation, to different aspects of isoprenoid metabolism. If these HMG CoA reductases differ in their sensitivity to lovastatin, low lovastatin concentrations might thereby exert specific effects on isoprenoid biosynthesis. Alternatively, it is possible that the many enzymes involved in plant isoprenoid metabolism have different affinities for their respective substrates. If the dimethylallyl PPi:AMP-dimethylallyl transferase has a low affinity (i.e. a high K_m) for dimethylallyl PPi, then low concentrations of lovastatin might deplete the pool of isoprenoid precursors such that cytokinin biosynthesis is preferentially inhibited. It is also possible that exogenous cytokinin satisfies a requirement for other essential isoprenoid(s) that become limiting at low concentrations of lovastatin. Or, it is conceivable that cytokinin reverses the effects of low concentrations of lovastatin by inducing HMG CoA reductase synthesis or activity.

We observed that zeatin, BA, and thidiazuron do not restore growth to tobacco cultures treated with higher concentrations of lovastatin (i.e. >1 μM), whereas kinetin and mevalonic acid restore growth at lovastatin concentrations as

high as 20 μM. Recent experiments, however, indicate that mevalonic acid restores growth to cells treated with 40 μM lovastatin, but kinetin does not (data not shown). Several explanations for the effectiveness of kinetin at higher lovastatin concentrations are available. For example, it is possible that lovastatin induces cytokinin turnover at concentrations between 1 and 5 μM and that kinetin is more resistant to degradation than the other cytokinins tested. Alternatively, it can be argued that total cytokinin levels (endogenous and exogenous) fall below a critical threshold between 1 and 5 μM lovastatin, except in the case of exogenously added kinetin (which, again, may be more stable than the other exogenously added cytokinins). Finally, these cytokinins may directly or indirectly compete with lovastatin, either at the level of HMG CoA reductase or at the level of entry into the cell, and kinetin may simply be a more effective competitor than the other cytokinins tested. Resolution of this complicated phenomenon will require a rigorous biochemical analysis of lovastatin-induced changes in cytokinin content, sterol content, and HMG CoA reductase activity in the presence and absence of exogenous cytokinin.

The data presented in this paper provide further evidence of the relationship between the mevalonate pathway and cytokinin biosynthesis in plant cells (1, 17). However, reduction of HMG CoA to mevalonate is probably not the rate-limiting step in cytokinin biosynthesis. Rather, dimethylallyl PPi:AMP-dimethylallyl transferase activity is likely to be rate limiting because overexpression of this activity results in cytokinin overproduction and tumor formation (a potentially misleading result if the enzyme from *Agrobacterium tumefaciens* utilizes stores of dimethylallyl PPi that are unavailable to the native cytokinin synthase) (1). This observation suggests that the isoprenoid precursor to cytokinin, dimethylallyl PPi, is present in an excess in plant cells. The data in this paper also provide evidence that cytokinin-autonomous cell lines require cytokinin for growth, a conclusion based on the observation that inhibition of isoprenoid biosynthesis inhibits growth and that cytokinins restore growth. This conclusion lends support to the hypothesis that cytokinins are universally required for cell division in plants.

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