Effects of Filipin and Cholesterol on $K^+$ Movement in Etiolated Stem Cells of *Pisum sativum* L.\(^1\)

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Received for publication January 5, 1973

ABSTRACT

Filipin, a polyene antibiotic known to induce leakage of materials from various cells, depresses $K^+$ and $NO_3^-$ uptake in etiolated pea epicotyl segments. Filipin concentrations which strongly reduce $K^+$ influx have little effect on efflux; however, high concentrations enhance $K^+$ efflux. Filipin has no effect on respiration rates or cell electropotentials; its action is presumed to be on the cell membranes. Cholesterol, but not a thiol-protecting agent (dithiothreitol), enhances $K^+$ influx and counteracts the inhibition by filipin. Although this effect of cholesterol may be due to an interaction with filipin in the outer solution, there is reason to believe that its major effect is to impart stability to the membrane; filipin is believed to act by interfering with sterol stabilization of phospholipid layers. The predominant native sterols of etiolated pea stem (*Pisum sativum* L. var. Alaska), which cholesterol probably mimics, are $\beta$-sitosterol, campesterol, and stigmasterol.

The polyene antibiotic filipin, isolated from *Streptomyces filipinensis*, is known to change the permeability of artificial (4, 22, 23, 24, 35, 39) and natural membranes (7, 8, 19, 21, 23, 25, 28, 29, 40, 41). The effect of filipin on artificial membranes seems dependent upon prior incorporation of free sterols (20, 23, 24, 30, 39), and free sterols often seem to be able to reverse the actions of filipin on fungi (8). All organisms which are sensitive to filipin contain sterols, but organisms such as blue-green algae and bacteria, which contain no sterols, are resistant to filipin (23, 25, 41).

Filipin induces the loss of cellular materials which absorb at 260 nm from *Neurospora crassa* (19, 21), and potato tuber (29), and of betacyanin from red beet root (29). It inhibits the uptake of glucose in sugarcane while not affecting labeled glucose efflux (28).

In this study, filipin has been shown to reduce the uptake of labeled $K^+$ in etiolated pea stem, and the evidence suggests that the effect is sterol-dependent; the action of filipin is reversed by cholesterol.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* L. cv. Alaska) were grown in darkness for 7 days at 25 C on vermiculite watered with a nutrient solution termed 1X which has the following composition, mm: KCl, 1.0; NaH$_2$PO$_4$, 0.904; Na$_2$HPO$_4$, 0.048; Ca(NO$_3$)$_2$, 1.0; MgSO$_4$, 0.25. The pH of this solution is 5.8. Segments 1 cm long were taken from a point 2 cm from the apical hook. This tissue was pretreated in nutrient solution for 10 to 14 hr prior to use, since it has been found that after such a treatment, uptake of $K^+$ proceeds in a very linear fashion for several days (27, 31). All work was carried out at 20 C.

The chemicals used were reagent grade. Cholesterol was reprecipitated several times from absolute ether and water before use. It has been shown by Berg and Eble (1) that filipin is really a mixture of at least four components, I to IV. Filipin I is in turn made up of other subcomponents. Filipin II, III, and IV are pure pentaenes, accounting for 96% of the total complex. Filipin III constitutes 53% of the total; Sessa and Weissman (35) have shown that the effects of this fraction predominate between 1 nm and 10 $\mu$m. This fraction also appears to have a strong affinity for liposomes prepared with cholesterol (35). The filipin used in this study was the four-component mixture which was kindly supplied by Dr. G. B. Whitfield, Jr., Upjohn Company. The material used had 86% active ingredients according to assay by the Upjohn Company.

Uptake Experiments. Approximately 0.5 g of pea stem tissue was placed in 50 ml of a nutrient solution which contained either 0.5 mm CaSO$_4$ and 0.5 mm KCl or the 1X mixture; both were $^4$K or $^8$Rb labeled to give about 10,000 and 3,000 cpm/0.1 meq $K^+$, respectively. The solutions were placed in 125-ml Erlenmeyer flasks on a reciprocal shaker. It has been demonstrated that $^8$Rb is an effective tracer for $K^+$ in this tissue (D. L. Hendrix, unpublished data). Filipin was added to the uptake solutions by first dissolving it in absolute methanol, then diluting with the nutrient solution to give a final methanol concentration of 0.1%. This concentration of methanol was found to have a negligible effect on $K^+$ uptake, $K^+$ efflux, or tissue respiration. Solutions were prepared just prior to use, since filipin has been shown to be unstable (35). The presence or absence of Ca$^{2+}$ in the uptake solution did not seem to alter the effects of filipin, but it was included in all experiments. After 3 hr of uptake, the tissue was washed with 25 ml of deionized water, then with nonradioactive solution for 15 or 20 min, and finally blotted and weighed. The tissue was extracted with dilute HNO$_3$, and the extract was dried in planchets for counting.

To test the effect of filipin on the $K^+$ influx and content of pea tissue with time, tissue was exposed to filipin as described above and removed at various times for tracer uptake determination. The chemical content of $K^+$ in the same tissue samples was determined from deionized water extracts with a Jarrel-Ash Model 82-700 atomic absorption-flame emission spectrophotometer. Nitrate ion was also measured in these

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\(^1\) This research was supported by National Science Foundation. Grant GB 19201 to N. H. and a fellowship from the National Defense Education Act Title IV to D. L. H.
extracts with an Orion Model 92-07 nitrate-specific ion electrode.

Efflux Experiments. To follow the release of organic material which is detected by absorption at 260 nm (29), approximately 0.5 g of stem segments was placed in plastic test tubes with 10 ml of 1X solution and bubbled with moist air. At half-hour intervals, several milliliters of the incubation medium were removed, and the absorption at 355 and 260 nm was analyzed with a Hitachi Perkin-Elmer Model 124 spectrophotometer, after which the samples were returned to the incubation medium.

To follow the effect of filipin on K+ efflux, approximately 1 g of tissue was cut into 50 ml of 260 nm (29), approximately 0.5 g of stem segments was placed in plastic test tubes with 10 ml of 1X solution and bubbled with moist air. At half-hour intervals, several milliliters of the incubation medium were removed, and the absorption at 355 and 260 nm was analyzed with a Hitachi Perkin-Elmer Model 124 spectrophotometer, after which the samples were returned to the incubation medium.

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the concentration of filipin which strongly inhibits potassium uptake (Fig. 5). It seems strange that "nucleotides" could be released by the lower concentrations of filipin (Fig. 4) which had no effect on the much smaller potassium ion; this appeared to be the case during three repetitions of the experiment shown in Figure 5. We had to use 40 \( \mu M \) filipin to get an increase in K\(^+\) efflux, which is about three times that needed to cause 50\% inhibition of K\(^+\) uptake. This required about 1.5 hr to increase K\(^+\) efflux. Cholesterol, added as a suspension amounting to 25 \( \mu M \) to all solutions 3.5 hr after the start of the efflux, appears to be able to offset the increased rate of potassium loss at higher (40 \( \mu M \)) filament concentrations while not affecting control tissue or tissue at lower concentrations of filament. A possible explanation for the failure of lower concentrations of filament to enhance K\(^+\) efflux is that cytoplasmic potassium is in a bound state and not free to diffuse out; however, we have recently found evidence from nuclear magnetic resonance measurements of cell K\(^+\) activities that this is not the case (J. A. Magnuson, N. S. Magnuson, D. L. Hendrix, and N. Higinbotham, unpublished data).

With the apparent effects on unidirectional K\(^+\) fluxes, it might be expected that filament would change the electro-potential (voltage between the solution and the vacuole) of treated cells. Filament was found not to affect cell electropotentials of tissue when measured as described elsewhere (15, 27, 31, 33). Segments exposed to filament at concentrations up to 40 \( \mu M \) for 3 hr failed to show a significant change in electropotential from that of controls. It might be possible to show a voltage change if one could measure the voltage between the cytoplasm and the solution, something we have not done as yet. However, if anions and cations are similarly affected (Fig. 3), the electropotential could remain the same.

Fig. 2. Effect of filament on the uptake of K\(^+\) per hr, averaged over a 3-hr uptake period and the same solution containing 0.5 mM CaCl \(_2\) plus 0.5 mM KCl (O) and the same solution containing 25 \( \mu M \) suspension of cholesterol (\( \square \)) (four replicates/point, \( \pm \) SD).

Fig. 3. Effect of filament on cell K\(^+\) and NO\(_3^-\) content. Tissue cut into 1X solution at zero time. The increase in K\(^+\) and NO\(_3^-\) content, after the 10-hr lag period, is characteristic of this tissue. Filament was added to 1X nutrient solutions at 0 \( \mu M \) (O), 2 \( \mu M \) (\( \bullet \)) and 20 \( \mu M \) (\( \bigcirc \)) (four replicates/point) to determine the effect of filament on the tissue during the time when its ion content is increasing.

Fig. 4. Time course of absorbance at 260 nm in 1X nutrient solutions containing 0.1% methanol and filament at 0 \( \mu M \) (O), 20 \( \mu M \) (\( \square \)), 40 \( \mu M \) (\( \Delta \)) and 80 \( \mu M \) (hexagon). Approximately 0.5 g of stem tissue was placed in 10 ml of solution. The concentration of filament in the 20 \( \mu M \) system was measured by following the optimal density at 355 nm (\( \square \)). Absorbance changes at 260 nm in a 1X nutrient solution lacking methanol are also shown (\( \bigcirc \)). The absorbance of solutions without tissue did not change during the test period. Points represent the averages of three replicates.
pears to be free sterol in etiolated pea stems which might interact with filipin if the sterol is indeed a membrane component as suggested by Kleinschmidt et al. (24).

It would appear that cholesterol itself has a stimulatory effect on the influx of K⁺ (Table II), since those segments exposed to cholesterol during the 15 hr of the pretreatment and treatment periods showed a 24% faster influx than in the controls; tissue exposed during the pretreatment alone showed a 16% faster rate during the 3-hr uptake period. It also appears that cholesterol is able to offset some of the inhibitory effects of filipin if added with filipin during the uptake period; however, this may be due to the concomitant stimulation by cholesterol shown in Figure 1. Adding cholesterol during the pretreatment alone did not offset the effect of filipin on K⁺ influx. A filipin pretreatment followed by a treatment with cholesterol seemed to stimulate the K⁺ influx rate, even in the continued presence of filipin. Filipin-pretreated tissue, however, failed to gain K⁺ during the pretreatment period; following the 12-hr pretreatment with filipin, there appeared to be no further inhibition on uptake rate although the K⁺ content remained low. It seems difficult to explain why pretreatment with filipin would not lessen the K⁺ uptake rates in 1X, or 1X with filipin solution, after the 12-hr period, unless the K⁺ uptake mechanism involves a protein or some other group which is unaffected by filipin. This “pump” might keep moving K⁺ at the same rate into a cell whose membranes have been made leaky by filipin.

Dithiothreitol at 0.5 mM did not alter the effects of filipin; this is contrary to the counteraction by reducing substances of the action of filipin which has been demonstrated in yeasts (8). This does, however, agree with the lack of effect of glutathione on the filipin-liposome interaction (24).

These results seem to agree with the idea that cholesterol and related free sterols are a stabilizing influence in biological membranes (36-38), and that filipin disrupts this stabilization by interaction with the membrane sterol. Spin-label work has

Table I. Free Sterols in Etiolated Pea Stems

<table>
<thead>
<tr>
<th>Pellet</th>
<th>Cholesterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>β-Sitosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000g</td>
<td>0.322</td>
<td>129</td>
<td>178</td>
<td>765</td>
</tr>
<tr>
<td>500g</td>
<td>0.296</td>
<td>94</td>
<td>120</td>
<td>455</td>
</tr>
<tr>
<td>15,000g</td>
<td>0.540</td>
<td>378</td>
<td>542</td>
<td>2855</td>
</tr>
<tr>
<td>46,000g</td>
<td>0.485</td>
<td>570</td>
<td>932</td>
<td>2430</td>
</tr>
<tr>
<td>Total</td>
<td>1.643</td>
<td>1171</td>
<td>1772</td>
<td>6505</td>
</tr>
</tbody>
</table>

by having the decrease in cation flux being offset by an equal decrease in anion flux in the same direction.

Experiments showed that filipin does not affect respiration in pea stem segments even when they are treated for as long as 12 hr; this is in contrast to the depression of respiration found in yeast (8). Reagents affecting respiration, e.g. CN⁻, azide, and DNP, also may induce leakage, but this leakage seems independent of respiration (13).

As shown in Table I, the most prominent sterol in these pea epicotyl segments is β-sitosterol. Cholesterol is present only in trace amounts. These values are much lower than those found by Grunwald (12) for tobacco tissue and by Bush et al. (2) for etiolated and nonetiolated barley tissue; this was due possibly, to overzealous washing to remove products other than free sterols. Stigmasterol, β-sitosterol, and campesterol have been reported previously in pea tissue (6). The fact that this tissue was etiolated may explain the low value, but Bush et al. (2) demonstrated that the difference in free sterol content between the etiolated and nonetiolated states in barley was less than an order of magnitude. Pea seedlings are known to be low in lipid content (17); nevertheless, there are...
shown that the regularity of molecular orientation in artificial and natural membranes is related to sterols (3, 17, 18). This work correlates well with biological data. *Mycoplasma laidlawii* has a lipoprotein membrane and a nutritional mutant requires sterols for growth (3), but only those sterols which produce the ordering of spin-label will serve as a growth factor.

Cholesterol and filipin form a complex which can be detected by a spectral change in the ultraviolet (24, 30). Norman et al. (30) showed that this change occurred with red blood cell ghosts and in *M. laidlawii* grown with cholesterol. The change did not occur when *M. laidlawii* was grown without cholesterol, indicating that filipin can react with sterol in the cell membranes (30). The lowering of order among phospholipids and alteration of charge density in the lipid regions of the membrane (34) could explain the drop in uptake of potassium and nitrate ions. If this disruption were carried out for a long enough time or were extensive enough, the cells would lose electrolytes and, eventually, larger molecules such as nucleotides. This point to the small amount of free sterols found in pea plants as being important in stabilizing the limiting membranes. Grunwald (10, 11), in fact, has shown that cholesterol, β-sitosterol, and stigmasterol are better stabilizing agents against methanol-induced leakage than is CaCl₂. When the phospholipids are oxidized by H₂O₂, preventing the interaction between sterol and phospholipid postulated above, the sterols can no longer prevent leakage from occurring (11).

Protein portions of the membrane may also be involved. Filipin is not known to inhibit the functioning of enzyme systems in vivo (14, 25) but can modify enzyme systems in intact cells. It seems possible, therefore, that filipin could affect the lipid components of the membrane which could, in turn, alter the conformation of membrane-bound enzymes or even cause the loss of such proteins from the membrane (25, 41). If the movement of K⁺ into the cell is by way of such an enzyme and the outward movement is by passive diffusion, this might explain the differing effects of filipin on the inward and outward movement of K⁺ in pea stems. Filipin concentrations less than 40 μM could alter the conformation of a protein associated with the inward K⁺ movement and yet disturb only the outer half of the lipid bilayer regions. Higher concentrations might affect both sides of the lipid bilayer and increase permeability to K⁺, resulting in an increased K⁺ efflux rate. Alternatively, it seems possible that the difference in effect upon uptake and efflux might result from an effect of filipin at low concentration which affects influx only at the plasmalemma, while efflux, during the time period studied (Fig. 5), might be a nonmastigmatized phenomenon (32).

**LITERATURE CITED**