Cycloheximide Stimulation of Cyanide-Resistant Respiration in Suspension Cultures of Senescent Pear Fruit Cells

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ABSTRACT

Pear fruit cells undergoing a period of senescence in auxin-deprived media develop a substantial cyanide resistant respiration in response to the addition of 0.7 to 3.5 micromolar cycloheximide. The inhibitor does not affect overall cellular respiratory activity and titrations with salicylhydroxamic acid reveal that only a minor portion, about 10%, of the alternate pathway is utilized by the cycloheximide-treated senescent cells. The alternate respiratory pathway appears to be of mitochondrial origin but is not induced by chloramphenicol.

Codron et al. (3) found that auxin deprivation, combined with an increase in medium osmolality, causes cultured pear fruit cells to cease division and to enter a protracted senescent phase. We (12) have since used similar and modified suspension cultures to observe a transient increase in protein synthesis that precedes the onset of cell death. CHi not only affected protein synthesis and the timing of cell death (12), it also appeared to induce the development of CRR. Since CRR has been implicated in the senescence (ripening) of fruit cells (17), we have sought to define the presence and extent of the alternate respiratory pathway in suspension cultures of auxin-deprived pear cells.

MATERIALS AND METHODS

A strain of cells first established from Passe Crassane pear fruit (Pyrus communis L.) in 1972 was employed in these experiments. The growth medium, 'aging' medium (one-quarter concentration of nutrients in growth medium plus 0.4 M mannitol), and conditions of 'batch' culture or culture with continuously renewed media were as previously described (12). The cytokinin autotrophic pear fruit cells were normally subcultured every week. To establish the senescent phase the cells were transferred and held for 9 to 12 days in auxin-deprived growth medium. The cells then were allowed to settle and were washed twice with 'aging' medium before incubation in the latter. As previously described (12), CH was added to the cell suspension after about 6 days of aging. In those experiments where the medium was under continuous renewal CH was also added to the media reservoirs.

Respiration of aliquots of cells withdrawn from culture flasks was measured polarographically with an O2 electrode in a 1-ml respiratory chamber. As cells senesced and declined in respiratory activity it was often necessary to either increase their concentration 1-fold by allowing them to settle and then decanting a portion of the supernatant, or to increase the sensitivity (recorder span) of the O2 measuring system, or both. Percentage of live cells was estimated by their exclusion of Evans blue as observed microscopically.

Mitochondria were isolated by pressing the cells through a fine (250 mm pore size) mesh screen suspended just below the surface of 2 to 3 volumes of isolation medium. Other commonly used forms of cell disruption, e.g. shaking with glass beads or use of a polytron homogenizer, resulted in higher yields but less functional mitochondria. The isolation and wash media, procedures for differential centrifugation and assay medium with succinate as substrate, were similar to those employed in our laboratory for mitochondria from intact fruit (14).

Protein was determined by a modified Lowry procedure (11). SHAM was dissolved in dimethylformamide to avoid a metabolic response to ethanol, the more commonly used solvent. There was no observed cellular response to dimethylformamide.

RESULTS

It has been demonstrated that the respiratory activity of auxin-deprived pear fruit cells undergoes a progressive decline arriving at a reasonably constant basal rate 8 to 10 days after transfer to 'aging' media (12). The same general pattern was observed in the present experiments. Moreover, within the limits of the methods employed, CH at the three concentrations used in these experiments did not affect the magnitude or the time course of cellular respiration (Fig. 1).

The addition of 0.1 mM KCN to aliquots of cells withdrawn from the suspension at various times throughout the senescent phase resulted in a 90 to 100% inhibition of respiratory activity (Fig. 2). However, 1 day after the addition of CH, KCN inhibition was much less. By the 5th day KCN inhibited only 10–15% of cellular respiration signaling the substantial development of an alternate CN-resistant electron transport pathway. As in its inhibition of protein synthesis (12), the CH induction of CRR was transient with a reversion to predominantly KCN sensitive respiration as the cells continued to senesce and approach death. Cellular sensitivity to KCN and SHAM was estimated from O2 electrode traces as shown in the upper portion of Figure 3.

It has been observed (16, 18), and it has been our experience with pear fruit cells as well (12), that cell strains undergo some variability during progressive subcultures and do not always respond in the same way to similar perturbations. We have assessed the CH effect in nine separate experiments with different sequential subcultures of the pear fruit cells over a period of 18 months. Three of the cultures were with continuous media renewal, and six were in batch culture. The amount of CRR present in the control cultures varied from 5 to 45% of the total respiratory

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4Abbreviations: CH, cycloheximide; CRR, cyanide-resistant respiration; SHAM, salicylhydroxamic acid.
activity (Table I). However, in all instances the addition of CH resulted in a marked increase in the potential for CRR so that it equaled from 55 to 92% of the total respiratory activity. In one experiment (No. 4) the addition of KCN actually stimulated cellular respiratory activity, a response also observed with potato tissue slices (8). In all instances there was some, though highly variable, return to a normal respiratory pattern, i.e., a decline in CRR, with continued senescence leading to cell death.

As Theologis and Laties (19) have observed in ripening avocado and banana fruit, the presence of CRR does not necessarily mean that the alternate pathway is being utilized. Using the procedure of Bahr and Bonner (1) which is premised on the use of SHAM as an inhibitor of CRR, we have estimated the fraction, (ρ), of the total alternate respiratory pathway in actual use. Data thus obtained with duplicate CH-treated cell cultures are shown in Figure 4, A and B and replotted in Figure 4A′ and B′ to estimate ρ. Within the limits of accuracy of this method it can be estimated that only about 10% (ρ = 0.1) of the alternate, CRR pathway was being utilized in the CH-treated cells.

It is presumed that mitochondria are the centers of cellular respiratory activity and the sites of the alternate pathway. The data in Table II, derived from cellular and mitochondrial respiratory measurements as shown in Figure 3, demonstrate that enhancement of CRR does occur in the mitochondria of CH-treated cells. In comparable experiments the substitution of 10 mg/l chlorophenicol for the CH did not result in the development of CRR.

Cultured pear cells and mitochondria extracted from them exhibit a residual respiratory activity insensitive to both KCN and

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Table I. Development and Subsequent Decline of the Potential for CRR in Cultured Pear Fruit

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Control</th>
<th>Maximum development</th>
<th>Subsequent decline</th>
</tr>
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<tbody>
<tr>
<td>With media renewal</td>
<td>1</td>
<td>9</td>
<td>86</td>
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<td></td>
<td>2</td>
<td>5</td>
<td>92</td>
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<tr>
<td></td>
<td>3</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>Without media renewal</td>
<td>4</td>
<td>45</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30</td>
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<td></td>
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<td>78</td>
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<td></td>
<td>8</td>
<td>35</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>30</td>
<td>85</td>
</tr>
</tbody>
</table>

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* Respiratory rate in presence of KCN/initial uninhibited respiratory rate less the residual rate in presence of both 0.1 mM KCN and 0.2 mM SHAM. Rates were determined from oxygen electrode traces as shown in Figure 3.

* Value determined at point of maximum observed effect of cycloheximide, generally 4 to 6 days after addition of the inhibitor.

* Subsequent decline in CRR and return to a normal respiration pattern was measured at, and circumscribed by, the onset of cell death.

* In these cycloheximide-treated cells the addition of KCN actually stimulated respiration.
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(5) that

the

development

of

CRR

in

potato

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on

protein

synthesis.

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DISCUSSION

The metabolic effects of CR are ambiguous. McDonald and Ellis (9) noted that

in some plant tissues CH inhibits energy transduction as well as protein synthesis. McMahon (10) observed

additional deleterious effects of CH on Chlamydomonas reinhardii. Cocucci and Marrè (2) discussed these

and other anomalous effects of CH but their observations lead to the suggestion that

CH (18 to 54 μM) depression of respiration in Rhodotorula gracilis

cells resulted from inhibited protein synthesis and the consequent decreased demand for ATP and the increase in energy charge.

It is questionable whether energetics play a dominant role in the CH effect on senescent pear fruit cells whose respiration is already depressed and show no measurable response to CH. It is also questionable whether inhibition of protein synthesis plays a direct role in the development of CRR. Even at the highest concentration (3.5 μM) of CH employed, inhibition of protein synthesis is transient and is followed by a recovery 4 to 6 days after addition of the inhibitor (12). A transient inhibition by CH of protein synthesis in cultured plant cells was also observed by Davies and Exworth (4). If there exists a relationship between protein synthesis and the development of CRR in cultured pear fruit cells, it is likely to be associated with the burst of protein synthesis that follows transient CH inhibition and which precedes the onset of cell death (12). Indeed, the stage of maximum CRR seen in Figure 2 (13th day) corresponds very closely to the stage of maximum recovery in protein synthesis following transient inhibition by CH in these same cells (Fig. 5 in reference 12).

Table II. CRR in Cultured Pear Fruit Cells and Mitochondria Extracted from Them

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRR as % of Normal Respiration*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
</tr>
<tr>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>+CH</td>
<td>74</td>
</tr>
</tbody>
</table>

* Respiratory rate in presence of 0.1 mM KCN/initial uninhibited respiratory rate-less the residual rate in presence of both 0.1 mM KCN and 0.2 mM SHAM. Rates determined from oxygen-electrode traces as shown in Figure 3.

Average of 2 mitochondrial respiratory assays.