Increase by Calcium in Production of Interferon by L929 Cells Induced with Polyriboinosinate-Polyribocytidylate Complex

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SUMMARY

Calcium chloride (5 to 20 mM) potentiated interferon production induced by rI₆:rC₆ in L929 mouse fibroblasts up to a thousand-fold. Higher concentrations of calcium (20 to 65 mM) mixed with rI₆:rC₆ were associated with increased cytotoxicity and a more acidic medium, but were effective in enhancing interferon production if preparations were adjusted to a uniform pH. Although calcium increased cellular binding of ³H-rC₆:rI₆, only a partial correlation between binding and interferon production was observed.

Interferon production, induced by polyriboinosinic acid–polyribocytidylic acid (rI₆:rC₆), is potentiated by polycations such as DEAE-dextran and polyamines (Dianzani et al. 1968; Lampson et al. 1969; Billiau et al. 1970). In addition, these polycationic compounds increase the infectivity of purified virus nucleic acids, probably by enhancing cellular adsorption and perhaps by protecting them from nucleases (Vaheri & Pagano, 1965; McCutchen & Pagano, 1968; Smull & Ludwig, 1962). The infectivity of virus nucleic acids is also increased by calcium (Dubes & Klinger, 1961; Graham & Van der Eb, 1973). These findings led us to examine the influence of calcium on interferon production.

L929 mouse fibroblasts (American Type Culture Collection, CCL 1), grown in a humidified incubator with 5% CO₂ in minimal essential medium (MEM; Earle’s base) supplemented with 10% foetal calf serum (FCS), 2 mM-glutamine, and 50 µg/ml gentamicyn, were used for interferon induction and assay. rI₆:rC₆ (Miles Laboratories, Elkart, Indiana) was suspended in 0.2 M-phosphate buffered saline, pH 7.2, at 1000 µg/ml. This was diluted on the day of use with fresh tris-MEM (3 vol. of MEM, 1 vol. 0.2 M-tris buffer adjusted to pH 7.3 to 7.35) to give a concentration of 200 µg/ml rI₆:rC₆. A 2 M stock solution of CaCl₂ was freshly diluted to twice the desired final concentration in tris-MEM. rI₆:rC₆ and CaCl₂ solutions were mixed 1:1 at room temperature. The inducing mixture without added CaCl₂ contained approx. 1.3 mM-calcium. To make radiolabelled rI₆:rC₆, rI₆ (3.4 µM-P) (Miles Laboratories) was annealed to ³H-rC₆ (1.7 µM-P, 10 µCi) (Schwartz/Mann, Orangeburg, New York) at 70°C and cooled overnight at room temperature. Cells were washed with Earle’s balanced salt solution (EBSS) and overlaid with rI₆:rC₆–CaCl₂. After incubation for 1 h at 37°C, the supernatant was aspirated, the cells were washed three time with EBSS and growth medium was added. After 24 h at 37°C, media were collected for interferon assay. For cell counts, media were aspirated 3 h after induction and cells were scraped from the surface, suspended, and counted in a haemocytometer. Viability was determined by trypan blue exclusion. In isotopic experiments, monolayers, grown on coverslips, were overlaid with ³H-rC₆–CaCl₂. After 1 h at 37°C, monolayers were washed with EBSS and growth medium was added. Following a further 2 h incubation, coverslips were placed in liquid scintillation vials, and disintegrations per minute were determined. Replicate coverslips were incubated for an additional 21 h and the media then collected for interferon assay.
Interferon was assayed and characterized as previously described (Borden & Leonhardt, 1977) by a modification of the dye uptake method (Finter, 1969). Interferon titres were standardized and expressed as reference units by incorporation of a laboratory standard into each assay and repeated comparison to the mouse interferon reference standard preparation G002-902-026 from the National Institutes of Health, Bethesda, Md. All results presented are representative of repeated experiments.

Interferon production in L929 cells, after induction by rI₆·rC₉, has generally been minimal or absent. However, a rI₆·rC₉·CaCl₂ mixture induced interferon production to relatively high levels (Fig. 1). The greatest increases in interferon production were at CaCl₂ concentrations of 4 to 20 mM. CaCl₂ alone (no rI₆·rC₉) at concentrations of 4 to 64 mM did not result in interferon production. Mixing rI₆·rC₉ with varying concentrations of MgSO₄ rather than CaCl₂ did not enhance interferon production. CaCl₂ at concentrations of 2 to 48 mM did not increase interferon production by Newcastle disease virus.

The inhibition of interferon production at high calcium concentrations correlated with a decrease in viable cells (Fig. 1). Both total cell count and viability decreased at higher calcium concentrations. No corresponding decrease in cell count occurred with CaCl₂ alone. The pH of the rI₆·rC₉·calcium mixture also significantly affected interferon production. Mixing of the rI₆·rC₉ with the higher calcium concentrations resulted in a decrease in the pH of the medium. When the pH of the CaCl₂·rI₆·rC₉ inducing mixture was adjusted to maintain a uniform alkaline pH at all calcium concentrations, interferon production was maximally augmented at higher calcium concentrations (30 to 65 mM).

Following the addition of CaCl₂ to rI₆·rC₉, an amorphous precipitate formed in amounts proportional to the concentration of calcium. On the basis of previous work on the effects of CaCl₂ on adenovirus DNA infectivity (Graham & Van der Eb, 1973), it seems likely that the precipitate consisted of rI₆·rC₉ and calcium phosphate. Upon microscopic examination it appeared that this precipitate was cell-associated. To evaluate this further, ³H·rI₆·rC₉ was used with CaCl₂ as an inducer. Enhanced association of ³H·rI₆·rC₉ with cells was observed. However, only a partial correlation existed between increases in cell-associated rI₆·rC₉ and interferon production (Table 1).
Table 1. Effect of added calcium on cellular uptake of $^3$H-$rI_{n:rC_n}$

<table>
<thead>
<tr>
<th>Added CaCl$_2$ (mm)</th>
<th>Cell-associated $^3$H-$rI_{n:rC_n}$ (d/min)$^\dagger$</th>
<th>Interferon produced (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5975</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>5</td>
<td>15600</td>
<td>290</td>
</tr>
<tr>
<td>10</td>
<td>25400</td>
<td>700</td>
</tr>
<tr>
<td>20</td>
<td>50900</td>
<td>380</td>
</tr>
<tr>
<td>50</td>
<td>23300</td>
<td>270</td>
</tr>
</tbody>
</table>

$rI_{n:rC_n}$ at 100 µg/ml in tris-MEM, pH 7.2, used as inducer. Final pH adjusted to 7.2-7.4. Results presented are representative of triplicate experiments.

$^\dagger$ Disintegrations per min/coverslip.

Calcium-mediated enhancement of interferon production by $rI_{n:rC_n}$ is probably a result of a membrane-associated co-precipitate of calcium phosphate and $rI_{n:rC_n}$. Such a precipitate facilitated the infectivity of adenovirus DNA for KB cells and poliovirus RNA for monkey kidney cells (Dubes & Klinger, 1961; Graham & Van der Eb, 1973). However, in contrast to the enhancement by calcium in the infectivity of adenovirus DNA, which occurred at optimal calcium concentrations of 100 mM (Graham & Van der Eb, 1973), the greatest increases in interferon production by $rI_{n:rC_n}$ were in the range, depending on pH, of 5 to 50 mM. Experimental conditions, together with the differences in nucleic acid, cells and biological effects examined, probably account for this discrepancy. If the effects of CaCl$_2$ which we have observed in L929 mouse fibroblasts also occur in other cells, the findings may be of practical significance in induction of interferon.

Other cations such as DEAE-dextran, which enhance interferon production, have also been demonstrated to increase cell adsorption of $rI_{n:rC_n}$ (Bausek & Merigan, 1969; Billiau et al. 1970; Pitha & Carter, 1971). However, no correlation between the amount of $rI_{n:rC_n}$ bound to cells and interferon produced has been observed, and both active and inactive polynucleotides become cell-associated at equivalent rates (Colby & Chamberlin, 1969; De Clercq & De Somer, 1972; Pitha et al. 1972, 1974; Tytell & Field, 1972). Association of $rI_{n:rC_n}$ with cells is a temperature-independent step, but unless a 37 °C incubation follows low temperature treatment, interferon production does not occur (Bausek & Merigan, 1969; Pitha & Carter, 1971). These findings suggest that $rI_{n:rC_n}$ interaction with the cell membrane is a non-specific event.

Both calcium and DEAE-dextran may increase the availability of $rI_{n:rC_n}$ to the specific interferon induction site. Whether this site is on the plasma membrane or inside the cell remains uncertain. Polyene antibiotics such as amphotericin B also increase interferon production by $rI_{n:rC_n}$ up to a thousand-fold (Borden & Leonhardt, 1979). However, enhancement by polyenes does not result from increased adsorption of $rI_{n:rC_n}$ but rather may involve increased intracellular penetration (Borden et al. 1977). DEAE-dextran was most effective in augmenting interferon production when used at concentrations which resulted in maximum reduction of the anionic charge of the polyribonucleotide (Pitha & Carter, 1971). Such a neutral polyanion–polycation complex may well traverse the non-polar lipid membrane more readily. Since the cation, calcium, can result in a similar reduction in negative charge on $rI_{n:rC_n}$ and also can have diverse effects on transport processes and enzymatic function, it may enhance interferon production by a mechanism other than the observed increase in cell membrane adsorption.
Short communications

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REFERENCES


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