Dependence of Guanidine Sensitivity of Poliovirus Replication on the Concentration of Monovalent Cations in the Culture Medium

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SUMMARY

An earlier suggestion that guanidine may inhibit picornavirus replication by interfering with a monovalent cation-mediated event was tested by determining the effect of varying monovalent cation concentration in isotonic medium on the sensitivity of poliovirus replication in HeLa cells to 0.2 mM-guanidine. Lowering [Na+] in the medium to 50 mM had no effect on virus replication. It was found that the degree of inhibition of virus replication by 0.2 mM-guanidine was inversely related to [Na+] in the medium: 99.8%, 99.1%, 38%, and 0% inhibition in the presence of 50, 75, 100 and 145 mM-Na+ respectively. Likewise, guanidine uptake by HeLa cells was also inversely related to [Na+] in the medium. On the other hand, lowering medium [Na+] to 50 or 75 mM resulted in reduced intracellular [Na+] and [K+]. The increased sensitivity of virus replication to guanidine in the presence of low Na+ medium could be abolished with excess K+ in such medium. Excess K+ in low Na+ medium restored intracellular [Na+] and reduced guanidine uptake. Thus, the increased sensitivity of poliovirus replication to guanidine in the presence of low Na+ medium correlated with reduced intracellular [Na+] and [K+] and elevated guanidine uptake.

The protein denaturant guanidinium chloride (guanidine), at millimolar concentrations, inhibits the growth of many picornaviruses (Caliguiri & Tamm, 1973), togaviruses (Friedman, 1970) and plant viruses (Varma, 1968; Dawson, 1976). The primary mechanism of guanidine inhibition of picornavirus replication appears to be inhibition of initiation of viral RNA synthesis (Caliguiri & Tamm, 1973). Synthesis of all three classes of poliovirus RNA, especially single-stranded viral RNA, by a polymerase complex isolated from infected cells was inhibited in vitro by guanidine (Tershak, 1982a). However, it does not appear that poliovirus RNA polymerase itself is the target of guanidine action. Guanidine resistance of poliovirus maps in the gene locus for structural proteins (Cooper et al., 1970) and amino acid changes occur in the structural polypeptides of guanidine-resistant mutants of poliovirus (Korant, 1977). However, guanidine inhibits the synthesis of viral RNA in cells infected with defective interfering poliovirus particles which lack the genes for capsid proteins (Cole & Baltimore, 1973). Therefore, structural protein sequences are not essential for guanidine action. On the other hand, a poliovirus deletion mutant lacking genes for capsid proteins, when mixed with standard virus and passed repeatedly in cells in the presence of guanidine, did not become guanidine-resistant (Tershak, 1982b), suggesting a role for capsid protein genes in guanidine resistance.

More recent evidence points to a picornavirus non-structural protein as the target for guanidine action. This protein, known as 2C according to the unified nomenclature for picornavirus polypeptides (Rueckert & Wimmer, 1984), is altered in guanidine-resistant mutants of poliovirus (Korant, 1977). However, guanidine inhibits the synthesis of viral RNA in cells infected with defective interfering poliovirus particles which lack the genes for capsid proteins (Cole & Baltimore, 1973). Therefore, structural protein sequences are not essential for guanidine action. On the other hand, a poliovirus deletion mutant lacking genes for capsid proteins, when mixed with standard virus and passed repeatedly in cells in the presence of guanidine, did not become guanidine-resistant (Tershak, 1982b), suggesting a role for capsid protein genes in guanidine resistance.
Short communication

Table 1. Dependence of guanidine inhibition of poliovirus replication on monovalent cation concentration in the medium

<table>
<thead>
<tr>
<th>Concentration (mM)* in the medium</th>
<th>Virus yield (p.f.u./culture)</th>
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<tbody>
<tr>
<td>Na⁺ K⁺ Sucrose Guanidine</td>
<td></td>
</tr>
<tr>
<td>50.0 5.0 182.0 0.0</td>
<td>1.8 x 10⁹</td>
</tr>
<tr>
<td>50.0 5.0 182.0 0.2</td>
<td>4.2 x 10⁹</td>
</tr>
<tr>
<td>50.0 96.0 0.0 0.0</td>
<td>1.2 x 10⁹</td>
</tr>
<tr>
<td>50.0 96.0 0.0 0.2</td>
<td>1.2 x 10⁹</td>
</tr>
<tr>
<td>75.0 5.0 132.0 0.0</td>
<td>1.7 x 10⁹</td>
</tr>
<tr>
<td>75.0 5.0 132.0 0.2</td>
<td>1.5 x 10⁹</td>
</tr>
<tr>
<td>100.0 5.0 82.0 0.0</td>
<td>2.1 x 10⁹</td>
</tr>
<tr>
<td>100.0 5.0 82.0 0.2</td>
<td>1.3 x 10⁹</td>
</tr>
<tr>
<td>145.0 5.0 0.0 0.0</td>
<td>1.9 x 10⁹</td>
</tr>
<tr>
<td>145.0 5.0 0.0 0.2</td>
<td>2.2 x 10⁹</td>
</tr>
</tbody>
</table>

* Corrected to the nearest significant figure.

At physiological pH, guanidine is ionized giving rise to the positively charged guanidinium ion which can bind to sites occupied by monovalent cations. Choline chloride, the commonly used substitute for NaCl in physiological experiments, reverses guanidine action (Lwoff, 1965; Mossur et al., 1971). These facts led us to speculate that guanidine may inhibit poliovirus replication by interfering with a monovalent cation-dependent replicative function (Nair et al., 1979). This suggestion anticipates an effect of monovalent cation concentration on guanidine inhibition of virus replication. In this communication it is shown that reducing [Na⁺] in the culture medium enhances inhibition of poliovirus replication by 0.2 mM-guanidine. It is further shown that this increased sensitivity of virus replication to guanidine correlates with reduced intracellular [Na⁺] and [K⁺] ([Na⁺]i and [K⁺]i) and elevated guanidine uptake by HeLa cells.

Confluent monolayer cultures of HeLa cells in 35 mm Falcon Petri plates were infected with poliovirus type 2 at a multiplicity of 200 p.f.u./cell, washed with phosphate-buffered saline (PBS) to remove unattached virus and incubated at 37.5 °C, in the presence or absence of 0.2 mM-guanidine, with minimal essential medium containing various concentrations of Na⁺ and sucrose or K⁺ added to restore tonicity as indicated in Table 1. The medium also contained antibiotics and 2.5% calf serum. After 6 h of incubation, the cultures were frozen and thawed twice and homogenized by forcing the medium and cell debris through a syringe needle. Virus yields in the homogenates were estimated by plaque titration.

The results from a typical experiment shown in Table 1 indicate that lowering [Na⁺] from 145 mM to 50 mM in isotonic medium did not have any effect on viral replication, but had a significant effect on the inhibition of viral replication by 0.2 mM-guanidine. Thus, 0.2 mM-guanidine was not inhibitory in the presence of 145 mM-Na⁺ and was only minimally inhibitory in the presence of 100 mM-Na⁺. Further lowering of medium [Na⁺] to 75 and 50 mM in the presence of 0.2 mM-guanidine resulted in approximately 100- and 400-fold reduction respectively in progeny production. The results also show that the extreme sensitivity of virus replication to guanidine in the presence of isotonic, low Na⁺ medium could be eliminated by replacing sucrose in such medium with Na⁺ or K⁺ to restore osmolarity. It will be noted also that progeny virus production was not significantly lower in the presence of low Na⁺, high K⁺ medium than it was in the presence of medium containing physiological [Na⁺] and [K⁺].

The above results did not indicate whether or not varying [Na⁺] and [K⁺] in the medium affected intracellular concentrations of these ions. To determine this, infected HeLa cells were incubated for 2 h at 37.5 °C with isotonic media containing various concentrations of monovalent cations, washed rapidly with cold (4 °C) isotonic sucrose, and lysed with deionized water. The lysates were lyophilized and the residues were digested with concentrated nitric acid. [Na⁺] and [K⁺] in the digests were estimated by flame photometry. The results (Table 2) show that lowering medium [Na⁺] to 100 mM had no significant effect on [Na⁺], and [K⁺], but further reduction in medium [Na⁺] resulted in lower [Na⁺], and [K⁺]. Furthermore, replacement of
sucre in Na+-deficient medium with K+ restored physiological [Na+]i. Very similar results were obtained when the above experiment was repeated with uninfected cells. These results suggested a correlation between the increased sensitivity of viral replication to 0.2 mM-guanidine in the presence of Na+-deficient medium (Table 1) and reduced [Na+]i and [K+]i.

An explanation for the observed [Na+]i-dependence of guanidine action could be that lowering medium [Na+] resulted in increased guanidine uptake. This was tested by determining steady-state levels of [14C]guanidine uptake by infected cells. Infected dish cultures were incubated with 0.5 μCi/ml of [14C]guanidine carbonate (New England Nuclear; sp. act. 116 mCi/mmol) in media containing different concentrations of [Na+] for 1.5 h. After the removal of radioactive media, washing of the cultures with cold (4°C) PBS and solubilization of the cells, guanidine uptake was determined by scintillation counting. The results revealed an inverse relationship between guanidine uptake by infected cells and medium [Na+] (Table 2). Guanidine uptake from 50 mM-Na+ medium was about 60% higher than that from 145 mM-Na+ medium. Similar results were obtained with uninfected cells.

The results presented in this paper reveal that guanidine inhibition of poliovirus replication in HeLa cells is monovalent cation concentration-dependent and provide an explanation for this observation. Both guanidine inhibition of virus replication and steady-state guanidine uptake by HeLa cells were inversely related to [Na+]i in the medium (Table 2), indicating that [Na+]i affected guanidine action by regulating intracellular guanidine levels. Since K+ was as effective as Na+ in reducing guanidine uptake by cells (Table 2) and reversing guanidine sensitivity of virus replication (Table 1), it may be concluded that monovalent cations influence guanidine action by regulating its uptake. It appears that HeLa cells take up guanidine by more than one mechanism and that the action of at least some anti-guanidine agents such as choline involves the inhibition of guanidine entry into cells (unpublished observations). Like steady-state levels of guanidine uptake (Table 2), the initial rate of guanidine uptake was also dependent on monovalent cation concentration in the medium (unpublished observations). Presumably, guanidinium ions and Na+/K+ compete for binding to a carrier protein involved in guanidine transport. Similarly guanidine may also compete with these ions for binding to a viral precursor polypeptide. The reduction in [Na+]i and [K+]i of cells incubated with low Na+ medium (Table 2) could have facilitated guanidine binding to the putative viral target. The combination of reduced [Na+]i/[K+]i and increased guanidine uptake, rather than increased guanidine uptake alone, probably produced the heightened guanidine sensitivity of virus replication in the presence of low Na+ medium. Thus, these findings may indirectly support the hypothesis that guanidine inhibits picornavirus replication by interfering with a cation-dependent viral function (Nair et al., 1979).

The actual mechanism of guanidine action could be displacement by guanidinium ions of monovalent cations from certain negatively charged sites in viral precursor polypeptides. Unlike cation binding, guanidine binding to these sites may impose conformational constraints on the target molecule(s) and thereby interfere with their processing and/or functions. The
Short communication

observations that the poliovirus capsid precursor VP0 synthesized in the presence of guanidine is not cleaved (Jacobson & Baltimore, 1968) and that capsid proteins fail to associate with the smooth membranes in the presence of guanidine (Yin, 1977) are consistent with this view of guanidine action. Binding to certain sites could be more important than binding to other sites because of their critical involvement in processing and/or functions. Alternatively, the conformation of the target molecule might render certain negatively charged sites more susceptible than others to guanidine binding. A recent report strongly implicated the aspartic acid residue at position 179 in the non-structural protein 2C of poliovirus type 1 in viral sensitivity to relatively high levels of guanidine (Pincus et al., 1986). These authors found that in six of six guanidine-resistant mutants, this particular amino acid residue had been replaced with the neutral amino acids alanine or glycine. It will be noted that guanidinium ions could bind to the negatively charged aspartic acid residues but not to the uncharged residues of glycine or alanine. They also reported that resistance to low levels of guanidine mapped elsewhere, in or out of the gene coding for protein 2C. This observation is consistent with the evidence for alternative sites for guanidine action reported earlier (Cooper et al., 1977; Korant, 1977) and with the view expressed here that guanidine acts by binding to negatively charged amino acid residues. However, it remains to be shown that guanidine binds to a viral precursor polypeptide and that such binding is influenced by the concentration of monovalent cations.

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REFERENCES


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