The Ratio of Plasma Membrane Cholesterol to Phospholipid and the Inhibition of Sindbis Virus Maturation by Low NaCl Medium

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SUMMARY

Sindbis virus maturation is inhibited by low NaCl medium in chick embryo cells and in one strain of BHK cells, but not in another strain of BHK cells which has a different passage history. The plasma membrane of the cells in which Sindbis virus maturation is resistant to low NaCl medium has a higher ratio of cholesterol to phospholipid than the other cells. Cholesterol-containing liposomes, but not cholesterol-free liposomes, can release Sindbis virus from low NaCl-inhibited cells. These results suggest that low NaCl medium may block Sindbis virus maturation by a mechanism which is influenced by the ratio of plasma membrane cholesterol to phospholipid.

Alphaviruses have been employed extensively as models for study of the insertion of proteins into cellular membranes and for the acquisition of membranes by viruses (Brown, 1980; Simons et al., 1982). Sindbis virus, prototype of the group, contains equimolar amounts of two envelope proteins, E1 and E2 (Mr of each is approx. 50000), and a capsid protein, C (Mr approx. 30000) (Schlesinger et al., 1972). In addition to E1 and E2, the virion envelope contains host-derived cholesterol and phospholipid arranged in a bilayer structure (Pfefferkorn & Hunter, 1963). Hydrophobic tails of one or both envelope proteins penetrate this bilayer and interact with the C protein (Harrison et al., 1971; Rice et al., 1982).

Lowering the NaCl concentration of the cellular growth medium from 138 mM to 68 mM inhibits the maturation of Sindbis virus from chick embryo (CE) cells at a stage after the nucleocapsids have bound to the plasma membrane (Waite & Pfefferkorn, 1968, 1970; Waite et al., 1972; Bell et al., 1978; Strauss et al., 1980). Returning low NaCl-inhibited cells to normal medium rapidly reverses the inhibition; the number of virus particles produced within minutes after medium shift is the same or greater than that released by cultures incubated in normal medium throughout (Waite & Pfefferkorn, 1968; Pierce et al., 1974; Garry et al., 1979).

Experiments using antiserum specific for E1 and E2 or lactoperoxidase radioiodination indicate that low NaCl medium inhibits virus release at a step after the cleavage of PE2, a polyprotein precursor to E2 and E3 (a non-structural protein) (Bell et al., 1978). E3, which is normally shed into the medium of Sindbis virus-infected cells after its cleavage from PE2, fails to accumulate in cells exposed to low NaCl medium (Bell et al., 1978; Mayne et al., 1984).

Waite & Pfefferkorn (1968) demonstrated that Sindbis virus maturation was similarly inhibited in both CE cells and in baby hamster kidney (BHK) cells incubated in low NaCl medium (Waite & Pfefferkorn, 1968). In contrast, Pierce et al. (1974) reported that, although virus maturation was blocked by low NaCl medium in CE cells, the inhibition did not occur in BHK cells. To determine whether the differences in the effects of low NaCl medium might be accounted for by differences between BHK cell strains, the effects of low NaCl medium on Sindbis virus maturation were examined in several strains of BHK cells with different passage histories. As reported previously (Waite & Pfefferkorn, 1968; Pierce et al., 1974), virus

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maturation in CE cell cultures was inhibited more than 95% when the NaCl concentration of the medium was reduced below 68 mM (Fig. 1). This block in maturation was rapidly reversed by incubation in medium containing normal amounts of NaCl. Total virus yields (low plus normal NaCl harvests) from CE cells incubated in low NaCl medium then exposed to normal medium may exceed those from CE cells grown under isotonic conditions throughout infection (Pierce et al., 1974; Garry et al., 1979). A similar inhibitory effect of low NaCl medium on Sindbis virus maturation was observed in a strain of BHK cells (BHK-sensitive; BHK-S) originally obtained through E. Pfefferkorn in which Sindbis virus morphogenesis had previously been shown to be sensitive to low NaCl medium (Waite & Pfefferkorn, 1968). The effects of low NaCl media on Sindbis virus maturation in CE cells and BHK-S cells contrasted sharply with the effect of these media on Sindbis virus maturation in certain other independently-passaged strains of BHK cells. One of these strains (BHK-resistant; BHK-R) from the American Type Culture Collection (ATCC-CCL10) was selected for further study. Although total virus production by BHK-R cultures incubated in low NaCl medium was reduced, there was no selective inhibition of virus maturation (Fig. 1).

Both BHK-S and BHK-R were obtained as BHK-21 cells and proved to be hamster cells by a karyotype analysis (data not shown). Both showed slight variation in the number of hamster chromosomes per nucleus as is typical for BHK-21 cells. BHK-R cultures contained significantly more ‘giant’ cells, showed more karyotypic variation, and displayed more orderly orientation in cell culture than BHK-S. The significance of the differences between the two strains of BHK cells with respect to their sensitivity to low NaCl medium is not known.

Since low NaCl medium blocks Sindbis virus maturation at a step following the binding of nucleocapsids to the plasma membrane (Waite et al., 1972), we compared the composition of the plasma membranes of infected and uninfected CE, BHK-S and BHK-R cells and the compositions of the membranes of virus released from these cell types (Table 1). The ratio of cholesterol to phospholipid was significantly higher in the plasma membranes of infected BHK-R cells compared to either infected CE cells or BHK-S cells. BHK-R cells had more protein, while BHK-S cells had somewhat lower levels of protein, than the CE cells. Measurements of the cholesterol and phospholipid contents of CE cell plasma membranes obtained by previous
Table 1. Composition of plasma membranes from CE, BHK-R and BHK-S and of Sindbis virus membranes prepared from each cell type*

<table>
<thead>
<tr>
<th></th>
<th>Plasma membrane (uninfected)</th>
<th>Plasma membrane (infected)</th>
<th>Sindbis virus membrane</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CE</td>
<td>BHK-S</td>
<td>BHK-R</td>
</tr>
<tr>
<td>Protein</td>
<td>38.2 ± 2.7</td>
<td>30.8 ± 2.6†</td>
<td>42.6 ± 3.1†</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>12.3 ± 0.3</td>
<td>12.7 ± 0.3</td>
<td>17.2 ± 1.5†</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>50.1 ± 1.8</td>
<td>56.4 ± 3.9</td>
<td>40.0 ± 1.6†</td>
</tr>
</tbody>
</table>

* Plasma membranes were prepared from infected cells by two cycles of gradient centrifugation by the method of Bosmann et al. (1968) as previously described (Bose & Brundige, 1972). Between 3 and 5% of total cellular glucose 6-phosphatase activity, a standard marker enzyme of the endoplasmic reticulum (Bosmann et al., 1968; Bose & Brundige, 1972), was present in the plasma membrane preparations indicating that maximum contamination with endoplasmic reticulum accounted for no more than 10% by weight. Sindbis virus was purified by three cycles of velocity gradient centrifugation (Waite & Pfefferkorn, 1968). Virus preparations contained only Sindbis virus proteins and no host proteins detectable by SDS–polyacrylamide gel electrophoresis. Protein was determined by the method of Lowry et al. (1951). This value for Sindbis virus membranes was obtained by assuming that E1, E2 and C were present in the virion in a 1:1:1 ratio and had a total Mr of 138000 while the capsid protein has an Mr of 30000. Lipids were extracted by the method of Folch et al. (1956). Neutral lipids were chromatographed on silica gel TLC plates in a solvent system of hexane:ether:formic acid (180:150:3). Spots corresponding to cholesterol were located by co-chromatography with an authentic standard (Sigma), scraped from the plates, extracted with chloroform, evaporated to dryness, resuspended in chloroform and quantified as described by Zlatkis et al. (1953). Total phosphorus and the amount of phospholipid were determined by the method of Bartlett (1959). All values are expressed as the mean ± the standard error of the mean (mg/100 mg total weight). Total weights were the sum of the weights of the protein, phospholipid and cholesterol. Statistical significance was determined by comparison with the corresponding value obtained in uninfected CE cell plasma membranes by Student's t-test (n = 8 or 9 for plasma membrane preparations; n = 6 for virus preparations) (Scheller, 1969).

† P < 0.01 when compared to value obtained in uninfected CE cell plasma membranes. Values which did not differ significantly from the corresponding value obtained in uninfected CE cells have no symbol.
workers (Renkonen et al., 1971; Quigley et al., 1972; Pessin & Glaser, 1980) were very similar to the values reported here. Differences in the composition of plasma membranes from CE cells and certain strains of BHK cells have been observed in previous studies (David, 1971) and different strains of BHK cells have been shown to differ in the relative content of plasma membrane cholesterol and phospholipid (Renkonen et al., 1971). The plasma membranes of infected CE, BHK-S and BHK-R cells contained somewhat lower ratios of cholesterol to phospholipid than the plasma membranes of uninfected cells. Lower ratios of cholesterol to phospholipid were also observed after infection of BHK cells by Semliki Forest virus, another alphavirus (Renkonen et al., 1971). The levels of protein and the ratio of cholesterol to phospholipid were higher in the Sindbis virus envelopes than in the plasma membranes of any of the infected cell types examined. These values for the protein and lipid compositions of alphavirus membranes were comparable to previously reported values (Renkonen et al., 1971; David, 1971). Despite differences in the cholesterol to phospholipid ratios in the plasma membranes of the different cell types, the ratios in virions grown in the different cells were similar. Because Sindbis virus infection does not significantly increase the relative overall levels of plasma membrane cholesterol, the process of virus budding must selectively occur in or generate regions of plasma membrane with higher ratios of cholesterol to phospholipid.

Since the ratios of cholesterol to phospholipid were lower in the plasma membranes of low NaCl-sensitive cells than in the resistant cells or virus derived from all three cell types, we reasoned that the relative cholesterol content of the membrane might influence the effect of low NaCl medium on Sindbis virus budding. If so, changing the relative cholesterol content of the plasma membrane may affect virus release from the low NaCl-sensitive cells. One way to alter the composition of the plasma membrane rapidly and selectively is to incubate cells with liposomes of various compositions (Inbar & Shintzky, 1974). In preliminary experiments we found that the fusion of liposomes occurred eight to ten times more effectively with Sindbis virus-infected cells than with uninfected cells. The enhanced fusion of liposomes to infected cells may be a consequence of the fusogenic properties of the virus glycoproteins inserted in the plasma membrane. Previous studies have shown that incorporation of myxovirus or herpesvirus glycoproteins in liposomal membranes dramatically increases fusion with plasma membranes (Huang et al., 1980; Johnson et al., 1984). Using liposomes containing labelled cholesterol or oleic acid, we found that treatment of infected, sensitive cells (CE or BHK-S) with liposomes containing a high content of cholesterol resulted in sufficient incorporation of cholesterol to increase the ratio of cholesterol to phospholipid in the plasma membrane transiently to values comparable to those in BHK-R cells. To determine whether this modification could alter the response of Sindbis virus-infected cells to low NaCl medium, Sindbis virus-infected sensitive cells (both BHK-S and CE cells) were exposed in low NaCl solution to either cholesterol-free liposomes or to liposomes containing a high content of cholesterol. As shown in Table 2, cholesterol-enriched liposomes, but not cholesterol-free liposomes, caused a rapid release of Sindbis virus from the low NaCl-inhibited cells. Liposomes containing lower ratios of cholesterol to phospholipid did not release Sindbis virus from low NaCl medium-inhibited cells. It has been reported that cholesterol is required for the binding and fusion of alphaviruses with liposomes (Mooney et al., 1975; White & Helenius, 1980). Fusion of host plasma membranes containing Sindbis virus envelope proteins and nucleocapsids with the liposomes could directly release virus from the intact cells. However, this is difficult to visualize topologically because of the orientation of the envelope proteins. Increasing the ratio of cholesterol to phospholipid in the region of the plasma membrane of the cell containing virion proteins could alter the response to low NaCl medium. Thus, Sindbis virus release from cells in which virus release had been inhibited due to the low NaCl medium could be a secondary consequence of the change in the ratio of cholesterol to phospholipid in the plasma membrane.

Strauss et al. (1980) have reported that changing the medium supplement from bovine calf serum to foetal bovine serum altered the response of their line of BHK cells to low NaCl medium such that Sindbis virus maturation was inhibited by low NaCl medium. Preliminary evidence suggests that this medium modification may decrease the ratio of plasma membrane cholesterol to phospholipid in certain subclones of BHK cells. Differences in the medium supplementation
Table 2. Release of Sindbis virus from low NaCl-inhibited cells by cholesterol-enriched liposomes*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time post-infection (h)</th>
<th>Virus release (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low NaCl medium</td>
<td>0-6</td>
<td>CE 110</td>
</tr>
<tr>
<td>LBSS</td>
<td>6-6.5</td>
<td>BHK-S 65</td>
</tr>
<tr>
<td>Cholesterol-free liposomes in LBSS</td>
<td>6-6.5</td>
<td>Cholesterol-enriched liposomes in LBSS 1850</td>
</tr>
<tr>
<td>Cholesterol-enriched liposomes in LBSS</td>
<td>6-6.5</td>
<td>HBSS 2040</td>
</tr>
<tr>
<td>HBSS</td>
<td>6-6.5</td>
<td></td>
</tr>
</tbody>
</table>

* CE, BHK-S and BHK-R cells were infected with Sindbis virus (m.o.i. 5) in normal medium. After 1 h for virus adsorption cells were incubated in low NaCl medium for 6 h. This medium was removed and the cells were incubated for an additional 0-5 h in either Hanks' balanced salt solution (HBSS), cholesterol-free liposomes in LBSS (HBSS containing 68 mM-NaCl), or cholesterol-enriched liposomes in LBSS. Liposomes were prepared by rotary vacuum evaporation as previously described (Straub et al., 1974). Cholesterol-free liposomes contained phosphatidylethanolamine:sphingomyelin:stearylamine:diacetylphosphate (30:5:6:3 by wt.) and cholesterol-enriched liposomes contained phosphatidylethanolamine:cholesterol:sphingomyelin:stearylamine:diacetylphosphate (10:30:5:6:3 by wt.). Liposomes were dialysed against LBSS prior to use. Approximately 10 mg of liposome suspension in 1 ml was added to each 100 mm dish (5 x 10^6 cells). Virus release was assayed on CE cell monolayers as previously described (Pfefferkorn & Hunter, 1963).

Based on present information regarding Sindbis virus morphogenesis it is possible to envisage several models of how a higher ratio of plasma membrane cholesterol to phospholipid could overcome the inhibition of Sindbis virus maturation imposed by low NaCl medium. Conformational or locational changes in E2 which follow its cleavage from PE2 are known to be affected by low NaCl medium (Bell et al., 1978). Several cellular plasma membrane modifications must occur either prior to or concomitant with this change in E2 to permit virus budding. Host-derived proteins must be excluded from the membrane. However, if this were a step limited by low NaCl medium, then specific host proteins would have to be involved since BHK-R cells contain somewhat more protein in their plasma membranes than BHK-S cells. Alternatively, low NaCl medium may inhibit the sequestration of cholesterol in the region of the budding virion. Membranes with a higher initial concentration of cholesterol might be less susceptible to such effects. Based on differences in the response of plaque morphology variants to low NaCl medium, Strauss et al. (1980) suggested that low NaCl medium may inhibit Sindbis virus maturation because of charge effects on the budding viral envelope proteins. The results presented here are not at all inconsistent with that hypothesis. Our results suggest that charge effects on the membrane lipid components may also be involved in the inhibition of Sindbis virus maturation by low NaCl medium. BHK-S and CE cells are relatively low in membrane cholesterol which has a neutral charge, and high in phospholipid which has a negative charge. Insufficient cations to neutralize the negative charges of the phospholipid may be contained in low NaCl medium. A higher ratio of neutral cholesterol to negatively charged phospholipid in the plasma membrane, as in BHK-R cells, could make the membrane less subject to such an effect of lowered NaCl in the medium.

The present studies suggest that the incorporation of a relatively high ratio of envelope cholesterol to phospholipid is important in the maturation of Sindbis virus in vertebrate cells. It should be noted, however, that a relatively high ratio of cholesterol to phospholipid is not an absolute requirement for maturation or infectivity of Sindbis virus, since virus grown in *Aedes albopictus* (mosquito) cells is deficient in membrane cholesterol compared to virus grown in vertebrate cells (Luukkonen et al., 1977).

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REFERENCES


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