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# Cerulenin, an inhibitor of lipid synthesis, blocks vesicular stomatitis virus RNA replication

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The replication of genomes of animal viruses in the cytoplasm of susceptible cells is usually coupled to specialized membrane structures. The inhibitor of lipid synthesis cerulenin blocks the formation of vesicular stomatitis virus polypeptides when added to cells soon after virus entry, but has much less effect on viral translation, or the acylation of the glycoprotein G, when cerulenin is added later during infection. By contrast, cerulenin powerfully blocks viral RNA synthesis or the incorporation of glycerol into lipids when present at any time after VSV-infection. These findings suggest that the synthesis of VSV RNA is dependent on continuous synthesis of lipids.

Cerulenin; Viral RNA replication; Phospholipid synthesis; Vesicular stomatitis virus

## 1. INTRODUCTION

Vesicular stomatitis virus (VSV) is a member of the *Rhabdoviridae* family which has been well characterized at the molecular level [1]. It contains a single molecule of negative-strand RNA as genome [1,2]. This molecule of 11 162 nucleotides [3] codes for at least five polypeptides [4,5]. The bullet-shaped virus particle is enveloped by a lipid bilayer [6]. After virus entry and partial decapsidation, the minus-strand RNA is first transcribed by the virion polymerases that forms part of the nucleocapsid giving rise to five distinct mRNA molecules, each encoding for a single viral polypeptide [2,7]. Translation of the VSV mRNAs produces five proteins known as N, NS, M, G and L, encoded in this order in the RNA genome (3′ to 5′) [1,2,7]. The progeny genomes are produced as in all RNA viruses in two stages. First, the genome is copied to produce a full-length positive strand RNA molecule, which represents an exact copy of the genome (−)-RNA [2,7]. These (+)-RNAs then act as templates for the synthesis of more genomic copies of (−)-RNA [2,7]. Both types of RNA molecules are encapsidated in nucleocapsids but only the (−)-RNA are assembled, after budding, in whole virions [1]. The regulation of replication and transcription of the viral genome may be governed by the amount of free N protein available [9]. Although detailed studies have been carried out on the requirements of VSV transcription in cell-free systems, much less is known about the cellular processes that govern VSV RNA synthesis in intact cells [2,7].

We described recently that the replication of poliovirus RNA requires continuous synthesis of lipids, most probably to provide new membranes where viral replication complexes attach [10]. Preliminary evidence indicated that the requirement of lipid synthesis for virus genome replication could be a widespread phenomenon [10]. Therefore, we have now analyzed the implications that the inhibition of phospholipid synthesis has for the replication of VSV RNA and the results are presented here.

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## 2. MATERIALS AND METHODS

### 2.1. *Cell cultures and virus*

Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% calf serum was used for growth and maintenance of HeLa cell cultures. BHK-21 cells were grown in DMEM containing 10% foetal bovine serum. Vesicular stomatitis virus, Indiana strain, was propagated and titered in monolayers of BHK-21 cells.

### 2.2. *Analysis of proteins by polyacrylamide gel electrophoresis and autoradiography*

HeLa cell monolayers were infected at a multiplicity of 50 pfu per cell in DMEM containing 2% calf serum. Proteins were labeled by the addition of 20 μCi/ml [$^{35}$S]methionine (1450 Ci/mmol; Amersham) contained in methionine-free DMEM, or 50 μCi/ml [$^{3}$H]palmitic acid (40–60 Ci/mmol; Amersham). After 1 h of incubation with the radioactive medium, the label was removed, the cells were harvested and the proteins analyzed as described [10].

### 2.3. *Incorporation of uridine into RNA*

Monolayers of HeLa cells were infected with VSV at a m.o.i. of 50 in the presence of 5 μg/ml actinomycin D. 10 μCi/ml of [$^{3}$H]uridine (25–30 Ci/mmol; Amersham) were added at the indicated intervals. After a 60 min labeling period the medium was discarded, and the cells were treated with 0.5 ml of 5% trichloroacetic acid, washed twice with ethanol, dried under an infrared lamp and dissolved in 200 μl of 0.1 N NaOH/1% SDS. Samples of 150 μl were counted in a liquid scintillation spectrometer.

### 2.4. *Northern blot analysis of RNA*

HeLa cell monolayers grown in 60 mm dishes were infected with VSV at a m.o.i. of 50. Cerulenin was added after adsorption of the

virus. At the times indicated the cells were lysed in a buffer containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl and 0.65% NP-40. After removing nuclei by low speed centrifugation, supernatants were mixed with an equal volume of a buffer containing 20 mM Tris-HCl, pH 7.8, 350 mM NaCl, 20 mM EDTA and 1% SDS. Samples were extracted with a mixture of phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v) and RNA was precipitated with 2 vols of ethanol. 5 $\mu$g of denatured RNA were electrophoresed in a 1.2% agarose/formaldehyde gel and transferred to nitrocellulose filters. RNA samples immobilized on nitrocellulose were hybridized under standard conditions to plus or minus strand-specific M gene RNA probes generated by in vitro transcription of a cDNA of the M gene of VSV (kindly provided by Dr R.R. Wagner; University of Virginia). Bands obtained in the films were quantitated in a computing densitometer (model 300A, Molecular Dynamics).

### 2.5. *Estimation of glycerol incorporation into lipids*

HeLa cells grown in 24-well plates were infected with VSV at a m.o.i. of 50 p.f.u. per cell. Every hour after infection cells were incubated in medium containing 10 $\mu$Ci/ml of [$^3$H]glycerol (1-3 Ci/mmol; Amersham) at 37°C for 1 h. Afterwards, the labeling medium was removed, and the cells were harvested in 200 $\mu$l of distilled water. The lipid fraction was obtained by extraction with chloroform/methanol 2:1 and washed with 0.9% NaCl/methanol/chloroform (47:48:3). The chloroform fraction was dried under vacuum and counted in Bray's scintillation fluid.

### 2.6. *Phospholipid analysis by thin-layer chromatography*

HeLa cells grown in 60 mm dishes were infected with VSV at a m.o.i. of 50 p.f.u. per cell. Lipids were labeled by adding a medium containing 10 $\mu$Ci/ml [$^3$H]glycerol and incubating the cells for 2 h at 37°C. When indicated, cerulenin was present in the medium at a concentration of 0.08 mM. Lipid fractions were extracted as described above. Samples were redissolved in 100 $\mu$l of chloroform and applied to activated silicagel thin layer plates. The plates were developed in chloroform/methanol/acetic acid/water (25:15:4:2) dried, and visualized with iodine prior to autoradiography. Densitometric scanning was performed in a computing densitometer.

## 3. RESULTS

### 3.1. *Action of cerulenin on VSV macromolecular synthesis*

The infection of HeLa cells by VSV causes a profound inhibition of host translation and the synthesis of viral proteins soon after infection (Fig. 1A). Cerulenin, an inhibitor of lipid synthesis, both in prokaryotic and eukaryotic cells [11] profoundly inhibits the appearance of viral proteins, if added from the beginning of infection, but after the absorption and entry of VSV

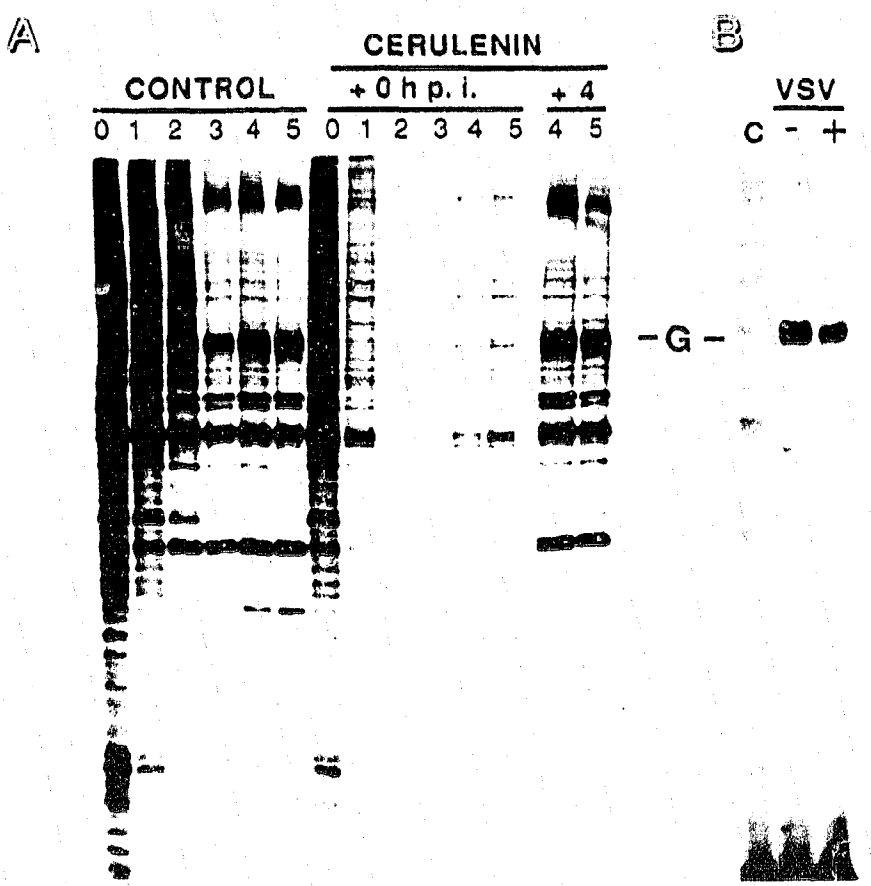


Fig. 1. Effect of cerulenin on viral protein synthesis. (A) HeLa cells grown in 24-well plates were infected with vesicular stomatitis virus (VSV) at a multiplicity of infection of 50 pfu per cell. After a 60 min adsorption period, cells were labelled every hour with 20 $\mu$Ci/ml [$^{35}$S]methionine for 1 h. Proteins were analyzed on a 15% polyacrylamide SDS gel. 0.08 mM cerulenin was added at the times indicated. (B) VSV-infected HeLa cells were labeled from 5 to 6 h post-infection with [$^3$H]palmitic acid (50 $\mu$Ci/ml). C = mock-infected cells; + = 0.1 mM cerulenin present from 3 h post-infection.

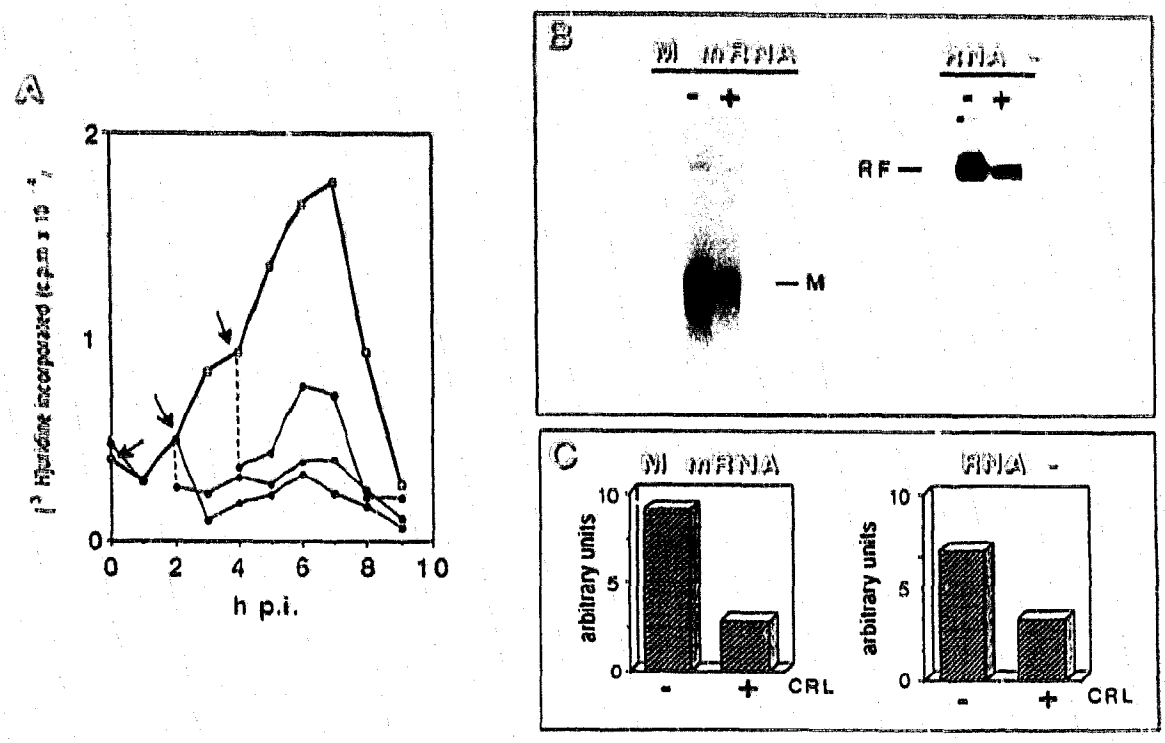


Fig. 2. Inhibition of VSV RNA synthesis by cerulenin. Monolayers of HeLa cells infected with VSV at a m.o.i. of 50 pfu/cell were incubated in the presence of 5 μg/ml actinomycin D. (A) Time-course of [$^3$H]uridine labeling. (□) Control VSV-infected cells; (●) 0.08 mM cerulenin was added as indicated by the arrows. (B) Northern blot analysis of RNA extracted at 6 h p.i. from VSV-infected HeLa cells. Selective plus or minus riboprobes that hybridize with the VSV M gene are used. + = 0.08 mM cerulenin was added at 0 h of infection. (C) Quantitation by densitometric scan of the bands shown in (B).

(herewith designated as 0 hour p.i.). If the inhibitor is added later (4 h p.i.) there is no effect on viral translation up to, at least two hours after addition of cerulenin. Although cerulenin blocks the appearance of viral proteins when present early in infection, it does not prevent the inhibition of host translation induced by VSV infection. On the other hand, those concentrations of cerulenin had no effect on cellular translation [10].

The VSV glycoprotein G is known to be post-translationally modified by the covalent binding of fatty acids [3,12]. Since cerulenin is an inhibitor of fatty acid synthesis it was of interest to test to what extent the acylation of the G glycoprotein was affected by cerulenin. Fig. 1B shows that the G glycoprotein is labeled with palmitic acid and cerulenin only partially blocked this acylation [13].

To test the effects of cerulenin on VSV nucleic acid synthesis, we measured the incorporation of [$^3$H]uridine into RNA in VSV-infected cells. Actinomycin D was added from the beginning of infection in order to block cellular RNA synthesis. The results shown in Fig. 2A clearly indicate that cerulenin is a powerful inhibitor of viral RNA synthesis when added at any time after infection. The presence of this antibiotic for a period of 9 h had no inhibitory effect on cellular RNA synthesis [10] (results not shown).

In order to measure the influence of cerulenin on the formation of the viral replicative complex and the synthesis of M protein mRNA, we took advantage of a plasmid containing the cDNA that codes for the VSV M protein [13] and made selective plus- or minus-stranded riboprobes. Fig. 2B shows that both the formation of the replicative form that contains plus- and minus-stranded RNA, and the synthesis of the mRNA coding for the M protein are inhibited by cerulenin. These results agree well with our previous findings on picornavirus-infected cells, which indicated that cerulenin is also a selective inhibitor of poliovirus RNA synthesis and has no effect on viral translation itself [10].

### 3.2. *Effects of viral infection and cerulenin on the synthesis of lipids*

Little is known about the effects of VSV infection on the metabolism of phospholipids. This subject has been analyzed in more detail in picornavirus- and togavirus-infected cells [14–19] indicating that the synthesis of phospholipids is stimulated after virus infection. Fig. 3A shows that the incorporation of [$^3$H]glycerol into chloroform-soluble material decreases as infection with VSV progresses, suggesting that VSV, in contrast to other RNA-containing viruses, reduces the synthesis of lipids after infection. Notably, the addition of

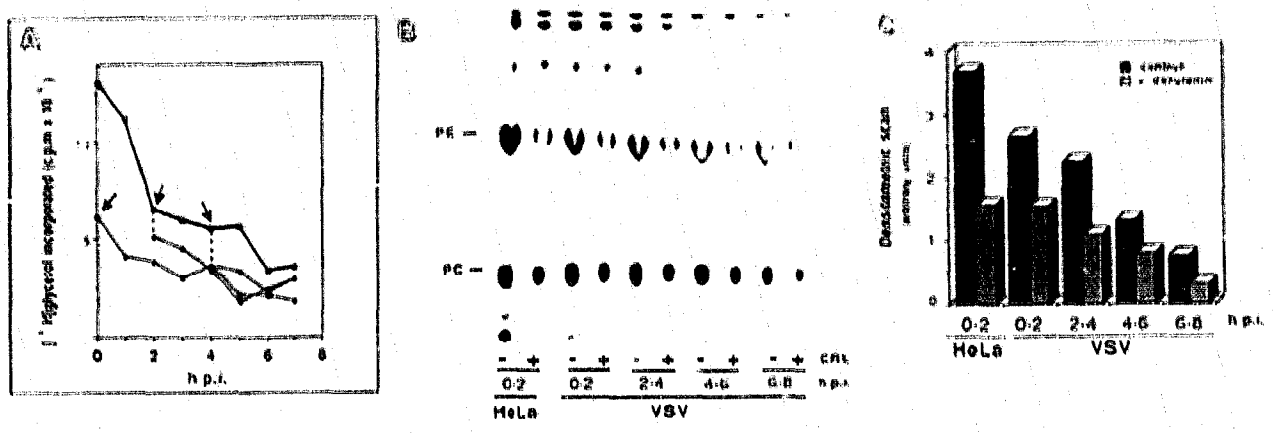


Fig. 3. Lipid synthesis in VSV-infected cell. Effect of cerulenin. (A) HeLa cells grown in 60 mm dishes were infected with VSV (50 pfu/cell). At different times post-infection, cells were incubated in medium containing 10 μCi/ml [$^3$H]glycerol. Total lipids were extracted as described in Materials and Methods. (□) Control VSV-infected cells; (●) plus cerulenin added to the medium at the times indicated by the arrows. (B) Analysis by thin-layer chromatography (TLC) of phospholipids extracted from VSV-infected HeLa cells, incubated in the presence (+), or absence (−) of 0.08 mM cerulenin. 10 μCi/ml [$^3$H]glycerol was present for 2 h. (C) Quantitation by densitometric scanning of the thin-layer autoradiography shown in (B).

cerulenin significantly inhibited the synthesis of lipids both in mock-infected and VSV-infected HeLa cells. In order to analyse specifically the synthesis of phospholipids, they were separated by thin-layer chromatography. Fig. 2B and C indicate that certainly the synthesis of phospholipids decreases throughout infection and that the presence of cerulenin further reduces this synthesis. Therefore, these results indicate that this inhibitor blocks the synthesis of both cellular phospholipids and viral RNA, soon after its addition.

## 4. DISCUSSION

The infection of cells by viruses that replicate in the cytoplasm induces the formation of special structures where viral genomes replicate. These structures, known as factories [20], viroplasm [21], or cytopathic vacuoles [22,23] are formed by special membrane structures. Little is known about the mechanism by which membrane traffic during virus infection is diverted from the rough endoplasmic reticulum to form those special membrane complexes. For picornaviruses a great number of membrane vesicles are induced during infection, filling most of the cytoplasm of infected cells, where viral RNA is replicated and encapsidated to form new virions [21,24,25]. Inhibition of lipid synthesis has consequences for new membrane formation and membrane traffic, in such a way that blockade of lipid synthesis leads to impairment of new membrane formation, as revealed by sucrose density gradient [10]. In turn, the interference with these membranes leads to a reduction of poliovirus genome replication [10]. Thus, it was of interest to test if a negative-stranded RNA virus, such as VSV, would also be sensitive to the inhibition of lipid synthesis. Our present results suggest that this is so, because the addition of cerulenin to VSV-infected cells has a clear inhibitory action on glycerol incorporation into lipids and uridine incorporation into RNA. It appears that not only is the synthesis of viral RNA physically connected with membrane structures, but also that the synthesis of new lipids regulates the formation of viral genomes. A number of questions arise that need further research: for instance, why is viral RNA synthesis coupled to membrane structures? What viral components are involved in the coupling between membranes and viral replicative complexes? Can selective inhibitors of viral RNA synthesis be designed that interfere with the coupling of membranes and viral genomes? We are presently attempting to answer some of these questions.

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