

Coxsackievirus protein 2B modifies endoplasmic reticulum membrane and plasma membrane permeability and facilitates virus release

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Digital-imaging microscopy was performed to study the effect of Coxsackie B3 virus infection on the cytosolic free Ca²⁺ concentration and the Ca²⁺ content of the endoplasmic reticulum (ER). During the course of infection a gradual increase in the cytosolic free Ca²⁺ concentration was observed, due to the influx of extracellular Ca²⁺. The Ca²⁺ content of the ER decreased in time with kinetics inversely proportional to those of viral protein synthesis. Individual expression of protein 2B was sufficient to induce the influx of extracellular Ca²⁺ and to release Ca²⁺ from ER stores. Analysis of mutant 2B proteins showed that both a cationic amphipathic α -helix and a second hydrophobic domain in 2B were required for these activities. Consistent with a presumed ability of protein 2B to increase membrane permeability, viruses carrying a mutant 2B protein exhibited a defect in virus release. We propose that 2B gradually enhances membrane permeability, thereby disrupting the intracellular Ca²⁺ homeostasis and ultimately causing the membrane lesions that allow release of virus progeny.

Keywords: endoplasmic reticulum/enterovirus/
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interactions

Introduction

The genus *Enterovirus* of the family of Picornaviridae comprises polioviruses, Coxsackie group A and B viruses, ECHO viruses and several unnamed enteroviruses. The molecular biology of these cytolitic animal viruses is relatively well known, mainly from the extensive studies on poliovirus. Enteroviruses are non-enveloped viruses that contain a single-stranded RNA genome of positive polarity. After cell entry and virion uncoating, the RNA molecule acts as an mRNA directing the synthesis of a single polyprotein. This polyprotein is subsequently processed by virus-encoded proteases to produce the structural capsid proteins and the non-structural proteins that have been implicated in viral RNA (vRNA) replication (for review see Porter, 1993; Wimmer *et al.*, 1993). For replication, the genomic RNA is used as a template to

synthesize a complementary minus-strand, which, in turn, is transcribed into new molecules of RNA. Plus-strand RNA synthesis occurs at the outer surface of virus-induced membranous vesicles that proliferate and accumulate in the cytoplasm of infected cells (Bienz *et al.*, 1994). During infection, enteroviruses induce a number of alterations in metabolic functions and morphological structures of the cell, most of which serve to facilitate viral replication. These include inhibition of host cell protein and RNA synthesis (Holland, 1962; Ehrenfeld, 1982), stimulation of lipid synthesis (Mosser *et al.*, 1972) and inhibition of vesicular protein transport (Doedens and Kirkegaard, 1995).

The molecular mechanism employed by enteroviruses to induce cell lysis and release virion progeny is largely unknown. Cell lysis is presumably the ultimate result of the increase in plasma membrane permeability that occurs from the third hour post-infection (Carrasco, 1995). This modification is such that gradients of monovalent ions are gradually destroyed and compounds that normally do not pass the membrane leak out of the cell or flow into the cytoplasm (Carrasco, 1995). It has long been thought that either the bulk of viral gene expression or the formation and accumulation of virus particles is responsible for enhancing membrane permeability and lysis of the cell. However, recent data suggest that a single viral protein may be responsible for the enhancement of plasma membrane permeability: individual expression of the 2B proteins of both poliovirus and Coxsackievirus in mammalian cells led to an increased permeability of the plasma membrane to the non-permeative translation inhibitor hygromycin B (Doedens and Kirkegaard, 1995; van Kuppeveld *et al.*, 1997). Increased membrane permeability was also observed in *Escherichia coli* cells that expressed protein 2B (Lama and Carrasco, 1992). The mechanism of this activity and its relevance to the viral life cycle remain to be established. No experiments have yet directly addressed the role of 2B, or any other viral protein, in virus release or cell lysis.

Enterovirus 2B is a small hydrophobic protein that has been localized at the rough ER membrane and the outer surface of the ER-derived membranous vesicles at which plus-strand RNA replication takes place (Bienz *et al.*, 1987, 1994). Biochemical analysis of the isolated vesicles confirmed that the membranes are derived at least in part from the ER (Schlegel *et al.*, 1996). The ER is the major intracellular store of Ca²⁺ ions (Carafoli, 1987). Based on its localization and proposed potential to modify membrane permeability, we hypothesized that protein 2B might release Ca²⁺ from internal stores. To examine this, we measured the intracellular Ca²⁺ concentration ([Ca²⁺]_i) and the Ca²⁺ content of the ER in Coxsackie B3 virus (CBV3)-infected cells. Here, we demonstrate that the Ca²⁺ gradients maintained by the plasma membrane and ER

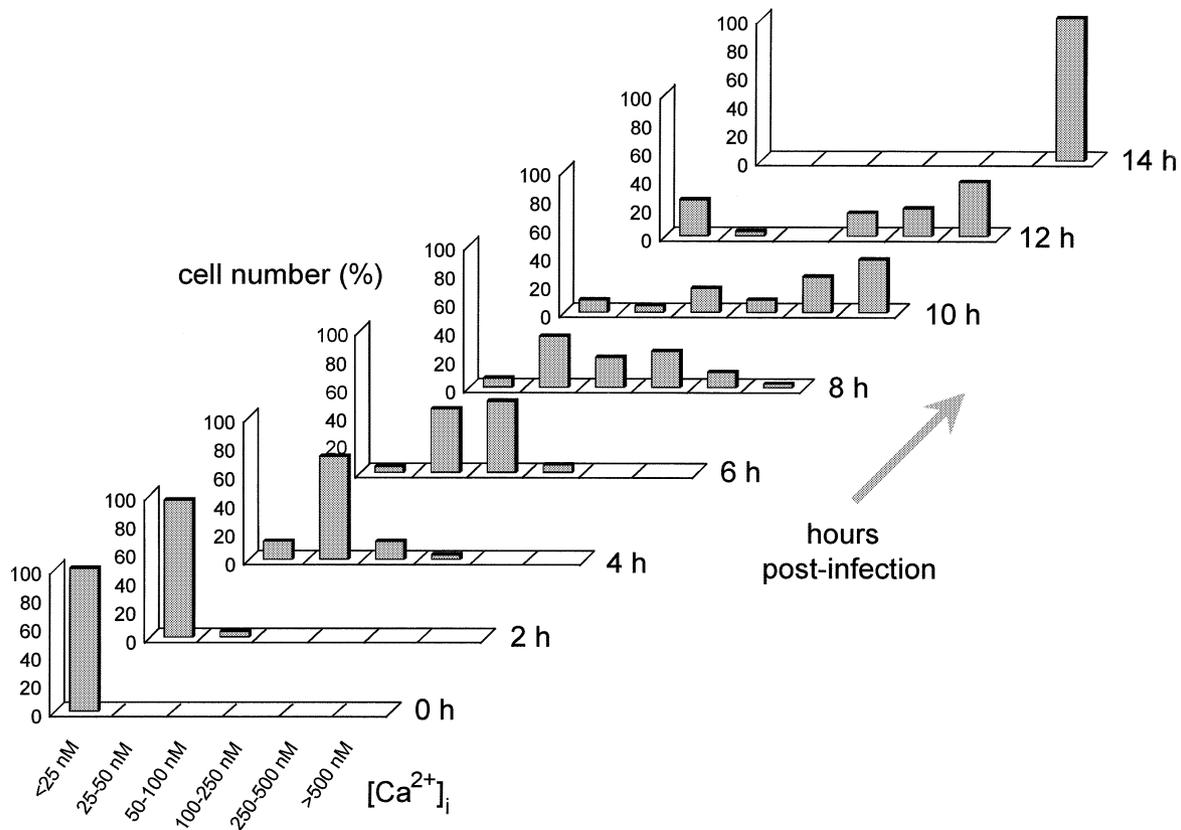


Fig. 1. $[\text{Ca}^{2+}]_i$ during CBV3 infection. HeLa cells grown on coverslips were infected with CBV3 at an m.o.i. of 25 TCID₅₀ per cell for 30 min at room temperature and grown at 37°C. The cells were loaded with the fluorescent Ca^{2+} indicator fura-2 (2.5 μM) for 30 min at 37°C, starting at 30 min before the indicated times. At each time, the $[\text{Ca}^{2+}]_i$ in 250 individual cells was determined using digital-imaging microscopy, focusing on independently chosen cells lying in different areas of the coverslip. The $[\text{Ca}^{2+}]_i$ was calculated from the fluorescence ratio of the emission intensities measured at 340 and 380 nm as described in Materials and methods.

membrane are disrupted during infection. Individual expression of protein 2B appeared to be sufficient to induce these effects. Analysis of mutant 2B proteins showed that both a cationic amphipathic α -helix and a second hydrophobic domain were required for these activities. The ability of protein 2B to enhance membrane permeability served to facilitate virus release. The increase in $[\text{Ca}^{2+}]_i$ further potentiated virus release. We propose that protein 2B by forming membrane-embedded pores accounts for the disturbance of the intracellular Ca^{2+} homeostasis and, ultimately, the formation of the membrane lesions that allow virus release. A putative function of the release of ER-stored Ca^{2+} in the assembly of the viral replication complex is proposed.

Results

Enhanced intracellular Ca^{2+} concentration during Coxsackie B3 virus infection

To monitor the $[\text{Ca}^{2+}]_i$ during CBV3 infection, we carried out digital-imaging microscopy using the fluorescent Ca^{2+} indicator fura-2. HeLa cells grown on coverslips were infected with wild-type CBV3 at a multiplicity of infection (m.o.i.) of 25 TCID₅₀ (50% tissue culture infective doses) per cell. At various times post-infection (p.i.), the $[\text{Ca}^{2+}]_i$ in 250 individual cells was determined. The basal $[\text{Ca}^{2+}]_i$ in uninfected HeLa cells was ~20 nM. Figure 1 shows that the $[\text{Ca}^{2+}]_i$ in infected cells gradually increased from

2 h p.i. Cells that showed the highest $[\text{Ca}^{2+}]_i$, which were also those that exhibited the most severe cytopathic effects (e.g. rounding and granulation), started to detach from the coverslip from 10 h p.i. The few cells that were still attached to the coverslip at 14 h p.i. all exhibited an enormous increase in $[\text{Ca}^{2+}]_i$.

Two possible mechanisms may explain the rise in $[\text{Ca}^{2+}]_i$. One possibility is that the virus induces the influx of extracellular Ca^{2+} . Alternatively, the virus may release Ca^{2+} from intracellular stores. To examine this, the $[\text{Ca}^{2+}]_i$ in CBV3-infected HeLa cells that were supplied with medium without Ca^{2+} was measured. In the absence of extracellular Ca^{2+} , no increases in $[\text{Ca}^{2+}]_i$ were observed (data not shown). Moreover, lowering of the external Ca^{2+} concentration of cells exhibiting an increase in $[\text{Ca}^{2+}]_i$ resulted in an immediate decrease in $[\text{Ca}^{2+}]_i$ (see Figure 2B, lower panel). From this it follows that the Ca^{2+} responsible for increasing the $[\text{Ca}^{2+}]_i$ comes mainly from the external medium.

Leakage of Ca^{2+} from the endoplasmic reticulum during infection

We have measured the inducible Ca^{2+} release of ER stores at various times during infection using thapsigargin, a specific inhibitor of the ER Ca^{2+} -ATPase (Lytton *et al.*, 1991). This Ca^{2+} -ATPase transports Ca^{2+} from the cytoplasm into the ER, an activity that is required to compensate for the continuous leakage of stored Ca^{2+} through

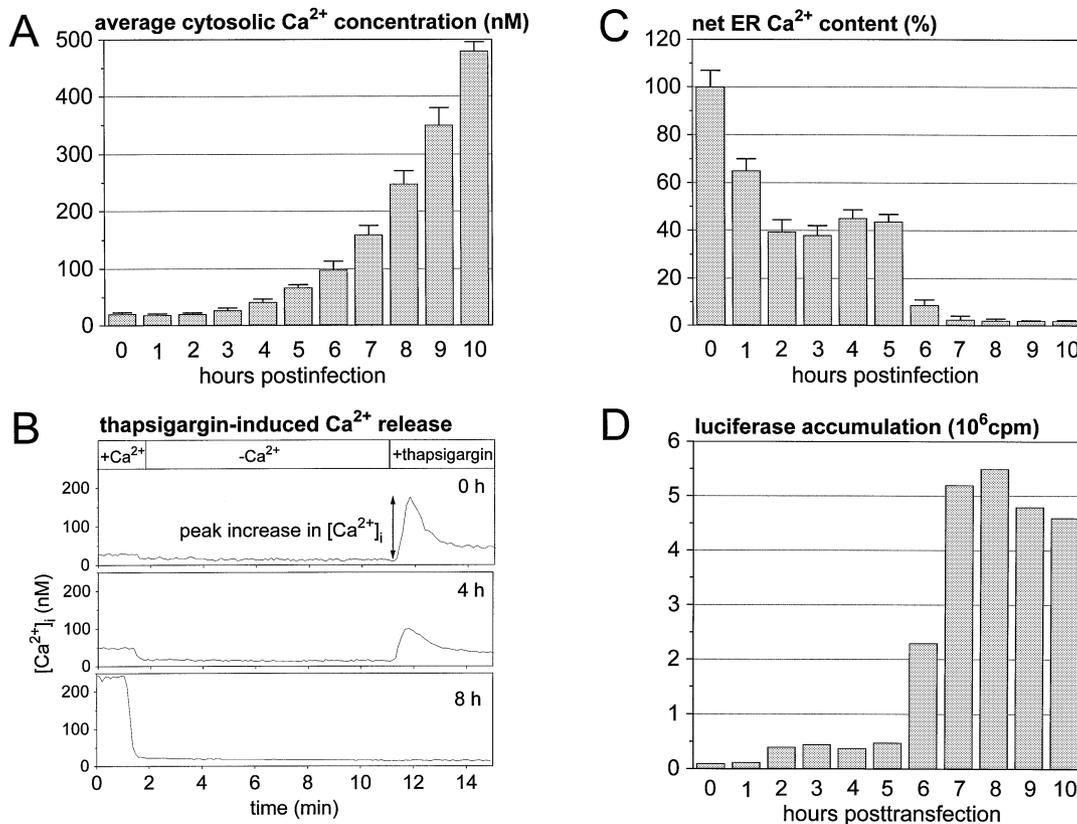


Fig. 2. (A–C) $[Ca^{2+}]_i$ and the Ca^{2+} content of the ER in CBV3-infected cells at various times post-infection. HeLa cells grown on coverslips were infected with CBV3 at an m.o.i. of 25 TCID₅₀ per cell for 30 min at room temperature and grown at 37°C. At the indicated times, first the average $[Ca^{2+}]_i$ in groups of fura-2-loaded cells was determined (A). The cells were then stimulated with 1 μ M thapsigargin following a 10 min incubation in Ca^{2+} -free medium and the average thapsigargin-induced peak increase in $[Ca^{2+}]_i$, a reflection of the ER Ca^{2+} content, was determined. Representative traces measured at 0, 4 and 8 h p.i. are shown in (B). Peak increases in response to thapsigargin measured hourly between 1 and 10 h p.i. relative to that of non-infected cells are shown in (C). Data in (A) and (C) are means \pm SEM of measurements of two independent experiments. Five groups of cells (5–10 cells per group, all groups lying in the same area of the coverslip) were analysed in each experiment. (D) Kinetics of viral protein synthesis. HeLa cell monolayers were transfected with 1 μ g of copy RNA transcripts of pCB3/T7-LUC, a subgenomic CBV3 replicon that contains the luciferase gene in place of the capsid coding region. At various times post-transfection, cells were lysed and the luciferase activity was determined.

channels in the ER membrane (Carafoli, 1987). When this ATPase is inhibited, Ca^{2+} that leaks from the ER is not resealed and accumulates in the cytosol. In Ca^{2+} -free medium, this accumulation is transient (minutes) because the Ca^{2+} ions that leak out of the ER are rapidly pumped out of the cell by Ca^{2+} -ATPases present in the plasma membrane (Carafoli, 1987). Under the latter condition (Ca^{2+} -free medium), the size of the thapsigargin-induced peak increase in $[Ca^{2+}]_i$ may be considered as a reflection of the Ca^{2+} content of the ER.

HeLa cells grown on coverslips were infected with wild-type CBV3 at an m.o.i. of 25 TCID₅₀ per cell. At various times post-infection, first the average $[Ca^{2+}]_i$ of groups of fura-2-loaded cells was determined. The cells were then incubated in Ca^{2+} -free medium and challenged with thapsigargin after 10 min. Figure 2A shows that the average $[Ca^{2+}]_i$ gradually increased and that there is an ~2-fold increase at 4 h, a 5-fold increase at 6 h, and a 10-fold increase at 8 h p.i. The average Ca^{2+} content of ER was calculated relative to that of mock-infected cells (0 h p.i.). In these cells, the thapsigargin-induced Ca^{2+} release caused a peak increase in $[Ca^{2+}]_i$ of 130 ± 10 nM (mean \pm SEM) (Figure 2B, upper panel). Representative traces measured at 4 and 8 h p.i. are shown in Figure

2B (middle and lower panel, respectively). The relative responses to thapsigargin measured hourly between 1 and 10 h p.i. are shown in Figure 2C. From this figure it can be seen that the virus-induced decrease in the ER Ca^{2+} content followed triphasic kinetics. Infected cells showed a decrease in the amount of releasable Ca^{2+} already at 1 h p.i. (phase I). At this time, the thapsigargin-induced peak increase in $[Ca^{2+}]_i$ was only 65% of that of control cells. From 2 to 5 h p.i., a relatively constant response to thapsigargin (~40% of that of control cells) was observed (phase II). Thereafter, the inducible Ca^{2+} release rapidly declined and from 7 h p.i. onwards virtually no response to thapsigargin was observed (phase III).

The kinetics of Ca^{2+} depletion from the ER were compared with those of viral protein synthesis. Viral translation was visualized by transfection of HeLa cells with copy RNA transcripts of pCB3/T7-LUC, a chimeric CBV3 replicon that contains the luciferase gene in place of the capsid coding region (van Kuppeveld *et al.*, 1995). In transfected cells, the replicon RNA drives translation of a chimeric polyprotein that is processed to yield luciferase and all non-structural proteins involved in RNA replication. The monocistronic nature of the RNA guarantees that luciferase accumulation faithfully repre-

sents levels of viral proteins produced. Figure 2D shows that, in transfected cells, a triphasic pattern of luciferase accumulation was observed; luciferase activity initially increased as a result of translation of the input RNA (phase I), remained constant between the second and fifth hours (phase II), and showed a second increase as a result of translation of newly synthesized chimeric RNA strands (phase III). The triphasic kinetics of Ca^{2+} depletion from the ER and those of viral protein synthesis are inversely proportional. This suggests that a viral protein is directly responsible for the leakage of ER-stored Ca^{2+} .

Expression of protein 2B is sufficient to increase $[\text{Ca}^{2+}]_i$

Transient expression of each non-structural protein of poliovirus indicated that protein 2B, and also its precursor 2BC, strongly enhanced membrane permeability to the hydrophilic antibiotic hygromycin B (Doedens and Kirkegaard, 1995; Aldabe *et al.*, 1996). This ability was found to be conserved in CBV3 protein 2B (van Kuppeveld *et al.*, 1997). To examine whether expression of CBV3 protein 2B was also sufficient to increase $[\text{Ca}^{2+}]_i$, COS cells were transfected with plasmids that allowed efficient expression of this protein or that encoded no protein. At 2 days post-transfection, the $[\text{Ca}^{2+}]_i$ in 250 individual cells was determined and expression of 2B was tested by immunofluorescence microscopy.

Figure 3A shows that in the cell cultures transfected with the 2B-encoding plasmid, ~20% of the cells exhibited an increased $[\text{Ca}^{2+}]_i$ (>500 nM). A similar percentage of cells showed reactivity with a polyclonal antiserum against 2B (Figure 3B). No immunoreactivity was observed in cell cultures that were transfected with the plasmid that encoded no protein. These latter cells displayed a $[\text{Ca}^{2+}]_i$ of ~40 nM, which is similar to that of non-transfected COS cells. The small portion (~3%) of the cells that displayed an elevated $[\text{Ca}^{2+}]_i$ (>500 nM) probably represented necrotic, membrane-permeable cells, the occurrence of which is most likely due to the electroporation procedure. That protein 2B is expressed in ~20% of the cells and that a similar percentage of cells exhibited an increase in $[\text{Ca}^{2+}]_i$ strongly suggests that 2B is sufficient to increase $[\text{Ca}^{2+}]_i$. Figure 3C shows the $[\text{Ca}^{2+}]_i$ in COS cells transfected with the 2B-encoding plasmid as a function of time post-transfection.

Protein 2B causes leakage of Ca^{2+} from the endoplasmic reticulum

To examine whether protein 2B could also induce leakage of ER-stored Ca^{2+} , we compared the ER Ca^{2+} content of COS cells exhibiting an increased $[\text{Ca}^{2+}]_i$ (>500 nM), which were considered as 2B-expressing cells, with that of cells present on the same coverslip and displaying a normal $[\text{Ca}^{2+}]_i$. The latter cells most likely represented non-transfected and non-2B-expressing cells and were further used as control cells. Fura-2-loaded cells were stimulated with thapsigargin following a 10 min incubation in Ca^{2+} -free medium. At the end of each experiment, the thapsigargin-induced peak increase in $[\text{Ca}^{2+}]_i$ in individual 2B-expressing cells and control cells was determined and the average peak increase was calculated. Because Ca^{2+} releases at differing $[\text{Ca}^{2+}]_i$ are not comparable, 2B-expressing cells that had not been able to lower their

$[\text{Ca}^{2+}]_i$ to control levels during the 10 min incubation in Ca^{2+} -free medium were not considered. A total of six experiments was performed (between 32 and 48 h post-transfection). In control cells, the basal $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free medium was ~20 nM and the thapsigargin-induced net change in $[\text{Ca}^{2+}]_i$ was 50 ± 15 nM (mean \pm SEM). The net change in $[\text{Ca}^{2+}]_i$ in 2B-expressing cells relative to that of control cells, which was adjusted to 100% in each experiment, was on average $41 \pm 13\%$ (Figure 4). These results provide evidence that protein 2B indeed induces leakage of Ca^{2+} from ER stores ($P < 0.02$).

A cationic amphipathic α -helix in protein 2B is required to elevate $[\text{Ca}^{2+}]_i$

CBV3 protein 2B contains two hydrophobic domains. One of these domains (aa 37–54) has the potential to form a cationic amphipathic α -helix with a structural arrangement typical of lytic polypeptides (van Kuppeveld *et al.*, 1996a). The second domain (aa 63–80) displays characteristics of multimeric transmembrane domains (van Kuppeveld *et al.*, 1995). To examine the importance of these structural motifs for the ability of protein 2B to increase $[\text{Ca}^{2+}]_i$, five 2B proteins carrying mutations in these domains were tested. Three proteins contained mutations in the amphipathic α -helix (Figure 5A). In protein 2B–K[41,44,48]E, the cationic character of the helix is disturbed by the replacement of the three lysines with glutamic acid residues. In protein 2B–ins[44]L, the amphipathic character of the helix is disturbed by the insertion of a leucine. In protein 2B–K[41,44]L, the amphipathy is diminished, but not abolished, due to the substitution of two of the lysines with leucines. Two proteins contained mutations in the second hydrophobic domain. These mutations either severely increased (2B–S[77]M/C[75]M) or decreased (2B–I[64]S/V[66]S) the hydrophobicity of this domain.

COS cells were transfected with plasmids that expressed either wild-type or mutant 2B. It seemed possible that the mutant 2B proteins might increase $[\text{Ca}^{2+}]_i$ in a delayed manner. Therefore, the $[\text{Ca}^{2+}]_i$ was measured (in 250 individual cells) after both 30 h (Figure 5B) and 50 h (Figure 5C), the 30 h time point being the time at which the number of cells displaying a marked increase in $[\text{Ca}^{2+}]_i$ (>500 nM) first reached its maximum (Figure 3C). The 2B proteins carrying mutations K[41,44,48]E and ins[44]L were unable to elevate $[\text{Ca}^{2+}]_i$. Protein 2B carrying mutation K[41,44]L was still able to increase $[\text{Ca}^{2+}]_i$, but its activity was considerably delayed. The 2B proteins that carried mutations in the second hydrophobic domain exhibited different abilities in increasing $[\text{Ca}^{2+}]_i$; protein 2B–I[64]S/V[66]S was unable to elevate $[\text{Ca}^{2+}]_i$, whereas protein 2B–S[77]M/C[75]M showed a wild-type 2B activity in elevating $[\text{Ca}^{2+}]_i$. Using immunofluorescence, it was confirmed that all mutant 2B proteins were expressed in ~20% of the cells, similar to wild-type 2B (data not shown). These data demonstrate that both the cationic amphipathic α -helix and the second hydrophobic domain are required to increase $[\text{Ca}^{2+}]_i$.

Intracellular distribution of protein 2B

To gain more insight into the mechanism by which CBV3 protein 2B affects Ca^{2+} homeostasis, we examined its subcellular localization in both transfected COS cells and

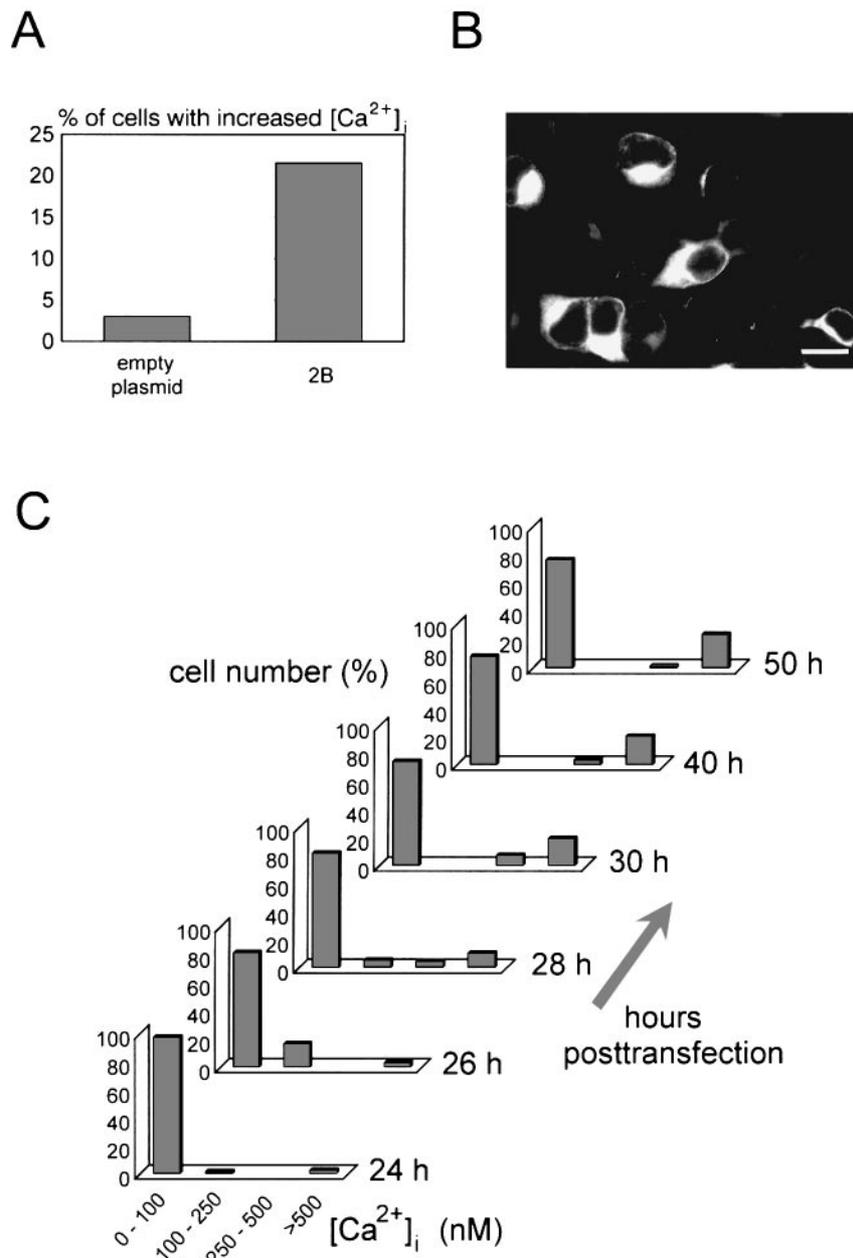


Fig. 3. (A) Effect of CBV3 protein 2B on $[Ca^{2+}]_i$. COS cells were transfected with plasmids containing the coding sequence of 2B (plasmid pC2 α 6) or no viral protein (plasmid pLINK α 6) and grown at 37°C. At 2 days post-transfection, the $[Ca^{2+}]_i$ in individual cells was determined. (B) Immunofluorescence micrograph of 2B-expressing COS cells. Protein 2B was detected using rabbit polyclonal serum raised against 2B and fluorescein isothiocyanate-conjugated goat-anti-rabbit IgG. Bar, 10 μm (magnification $\times 1000$). (C) Determination of $[Ca^{2+}]_i$ in individual cells following transfection of COS cells with the CBV3 2B protein-encoding plasmid. The $[Ca^{2+}]_i$ in 250 individual fura-2-loaded cells was determined at each time.

infected HeLa cells. Immunoelectron microscopic analysis of 2B-expressing COS cells showed intense immunolabelling of the plasma membrane and, to a lesser extent, of ER membranes (Figure 6A–C). Protein 2B did not have an indiscriminate affinity for all membranes, since the nuclear and mitochondrial membranes were not labelled. In CBV3-infected HeLa cells (Figure 6D–F), protein 2B occurred predominantly in the cytoplasm, where it has been localized at the outer surface of the virus-induced, ER-derived membranous vesicles that surround the viral replication complex (Bienz *et al.*, 1994). In addition to its cytoplasmic localization (Figure 6F), a small fraction of

the 2B proteins seemed to occur at the plasma membrane level (Figure 6E). No immunolabelling was found in mock-infected HeLa cells or COS cells that were transfected with a plasmid that encoded no protein (data not shown).

Protein 2B promotes release of virus progeny from infected cells

Based on its observed potential to enhance membrane permeability, we reasoned that a function of protein 2B might be to release virion progeny from infected cells. To establish such a role, virus release of vCB3-2B-K[41,44]L-infected cells was examined. This virus produces 2B–

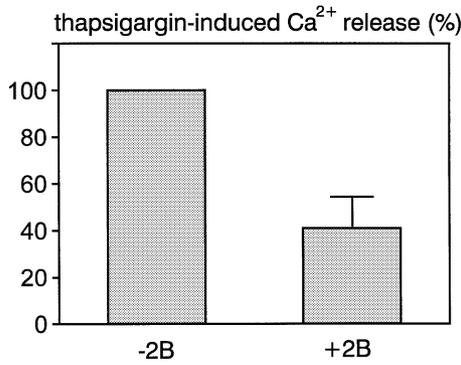
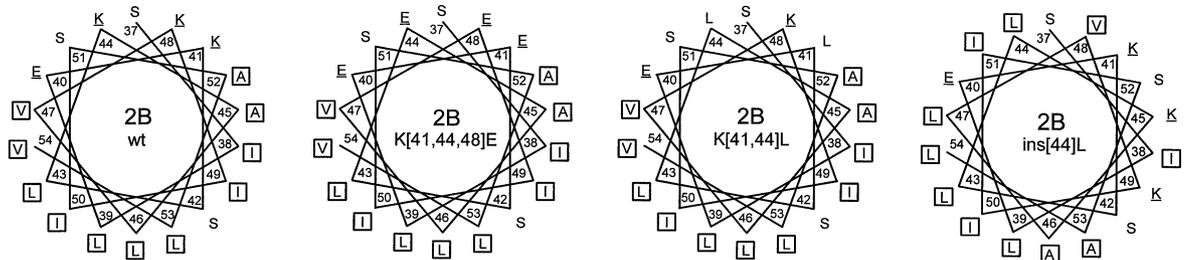


Fig. 4. Protein 2B causes release of Ca²⁺ from ER stores. COS cells were transfected with a 2B-encoding plasmid and grown at 37°C. Fura-2-loaded cells were stimulated with 1 μM thapsigargin following a 10 min incubation in Ca²⁺-free medium; 25–50 cells were monitored. At the end of the experiment, the average thapsigargin-induced peak increase in [Ca²⁺]_i, a reflection of the Ca²⁺ content of the ER, was calculated from the 2B-expressing cells that had been able to discharge their Ca²⁺ content during the 10 min incubation in Ca²⁺-free medium (for explanation, see text) and an equal amount of control cells. The average net change in [Ca²⁺]_i in control cells was adjusted to 100% in each experiment. A total of six experiments was performed. The relative response of the 2B-expressing cells represents the mean ± SEM. Experiments were performed between 32 and 48 h post-transfection.

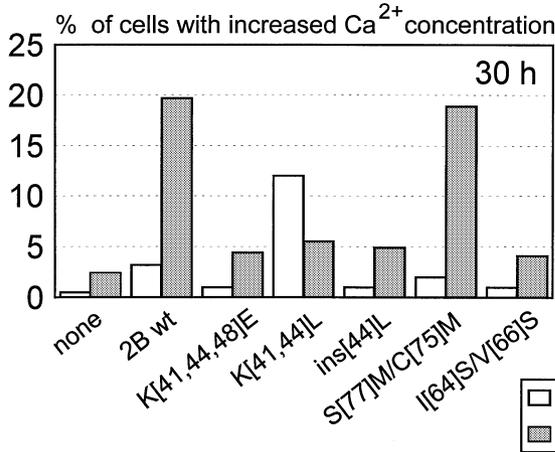
K[41,44]L, i.e. the mutant 2B protein that showed a delay in increasing [Ca²⁺]_i, most likely as a result of an impaired ability to increase membrane permeability.

HeLa cells were infected with either wild-type virus or vCB3-2B-K[41,44]L and the production and release of viruses was compared. In the same experiment, we also tested vCB3-3'UTR-A[7335]C, a mutant virus that exhibits a reduction in virus growth and yield due to a nucleotide substitution in its 3' untranslated region (Melchers *et al.*, 1997). This virus was included to allow a comparison of the kinetics of virus release of vCB3-2B-K[41,44]L with that of a mutant virus that produces a wild-type 2B protein and which accumulates virion progeny to considerably lower levels. Figure 7 shows the amounts of viruses released at various times post-infection. In cells infected with wild-type virus or vCB3-3'UTR-A[7335]C, nearly all virion progeny was released at 16 h p.i. At this time, only 1% of the progeny of vCB3-2B-K[41,44]L was released. With the latter mutant, virus release was not complete before 32 h p.i. These results support the idea that virus release is dependent on the membrane-disturbing action of protein 2B, rather than on the accumulation of virion particles (which in vCB3-2B-K[41,44]L-infected cells is more than 100-fold higher than in vCB3-3'UTR-A[7335]C-infected cells).

A



B



C

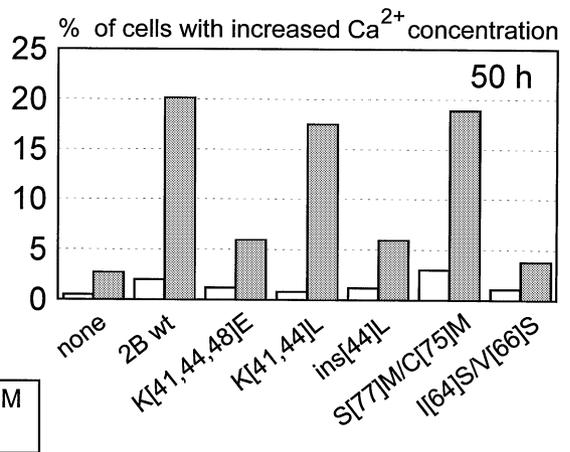


Fig. 5. Ability of mutant 2B proteins to increase [Ca²⁺]_i. (A) Helical wheel diagrams of mutants that carry alterations in the putative cationic amphipathic α-helix formed by 2B amino acids 37–54. Charged residues are underlined. Hydrophobic amino acids are boxed. (B and C) [Ca²⁺]_i measured at 30 h (B) and 50 h (C) after transfection of COS cells with plasmids that direct the synthesis of wild-type 2B or the indicated mutant 2B proteins. The [Ca²⁺]_i in 250 individual fura-2-loaded cells was determined at each time.

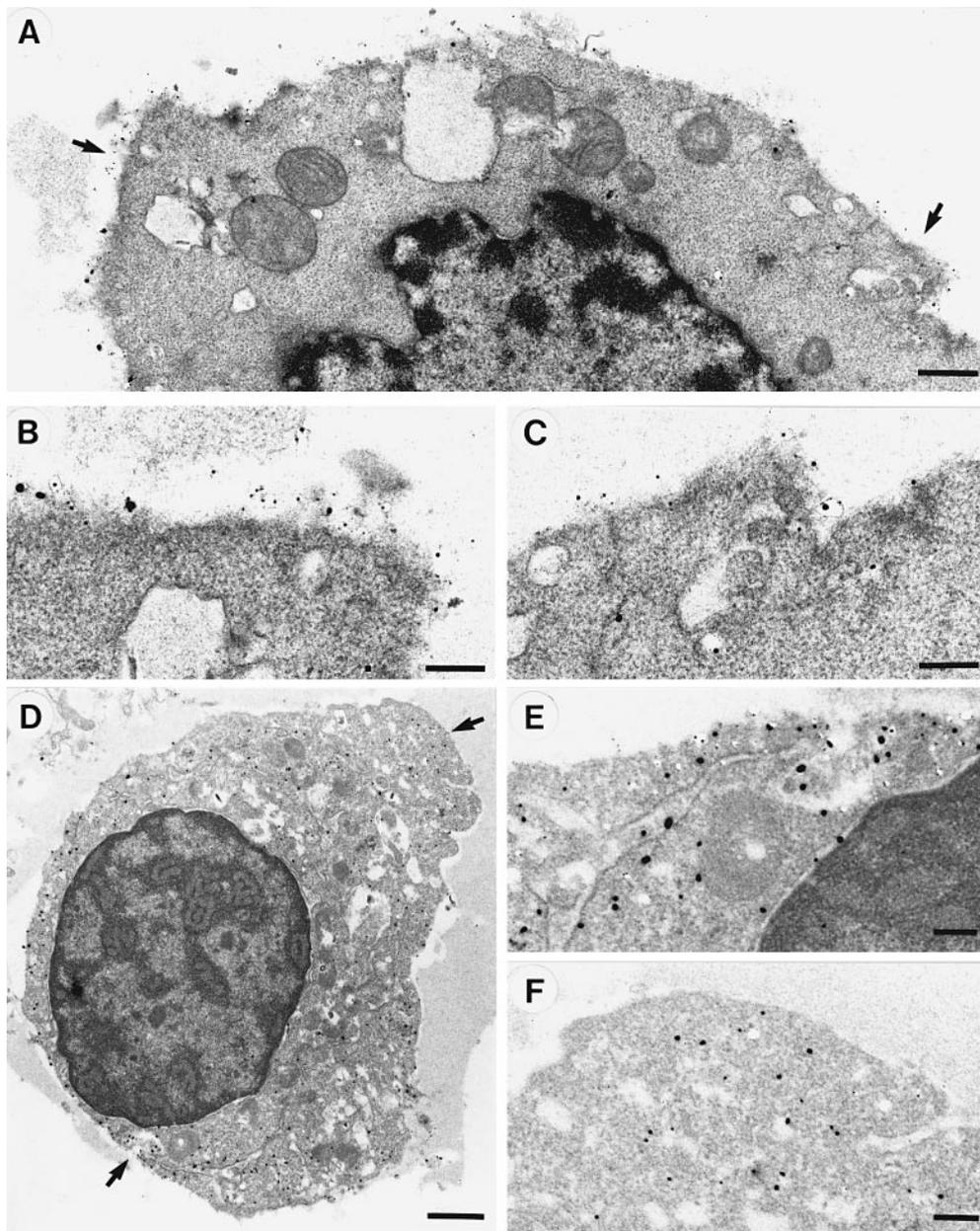


Fig. 6. Immunoelectron microscopic localization of protein 2B in 2B-expressing COS cells (A–C) and CBV3-infected HeLa cells (D–F). COS cells were transfected with a CBV3 2B-encoding plasmid and grown at 37°C for 40 h. HeLa cells were infected with CBV3 at an m.o.i. of 25 TCID₅₀ per cell and grown at 37°C for 5 h. Cryostat sections (25 µm) were generated and processed as described in Materials and methods. Protein 2B was visualized with 1 nm colloidal gold beads. The gold particles have a size distribution due to enhancement of the signal using HQ silver. The arrows in (A) and (D) point to the places that are shown enlarged in sections (B) and (C), and in sections (E) and (F), respectively. Bar in section (A), 0.5 µm (magnification ×19 000). Bar in sections (B) and (C), 0.25 µm (magnification ×40 000). Bar in section (D), 1 µm (magnification ×9100). Bar in sections (E) and (F), 0.25 µm (magnification ×30 000).

Do increased [Ca²⁺]_i levels contribute to certain stages of the viral replicative cycle?

The question of whether the virus-induced increase in [Ca²⁺]_i serves to modulate a viral process or function, or is just a side effect of the enhanced permeability of the plasma membrane, was addressed. For this, we compared the efficiency and course of several stages of the viral replicative cycle taking place in CBV3-infected cells supplied with medium with or without Ca²⁺.

The importance of Ca²⁺ for the shutoff of cellular protein synthesis and the efficiency of viral translation was examined by pulse-labelling of infected cells at

various times post-infection. Figure 8A shows that extracellular Ca²⁺ is dispensable for the inhibition of host cell protein synthesis (which is evident at 3 h p.i.). In the absence of extracellular Ca²⁺, a small delay in the onset of viral protein synthesis was observed. However, once initiated, the rate of viral translation was not affected.

The role of Ca²⁺ in virus amplification was analysed by measuring the virus titre at several times post-infection. Figure 8B shows that virus growth is largely independent of [Ca²⁺]_i. In the absence of extracellular Ca²⁺, a small reduction in virus titre was observed at 6 h p.i., but viruses grew to similar titres at 8 h p.i. That viruses replicated

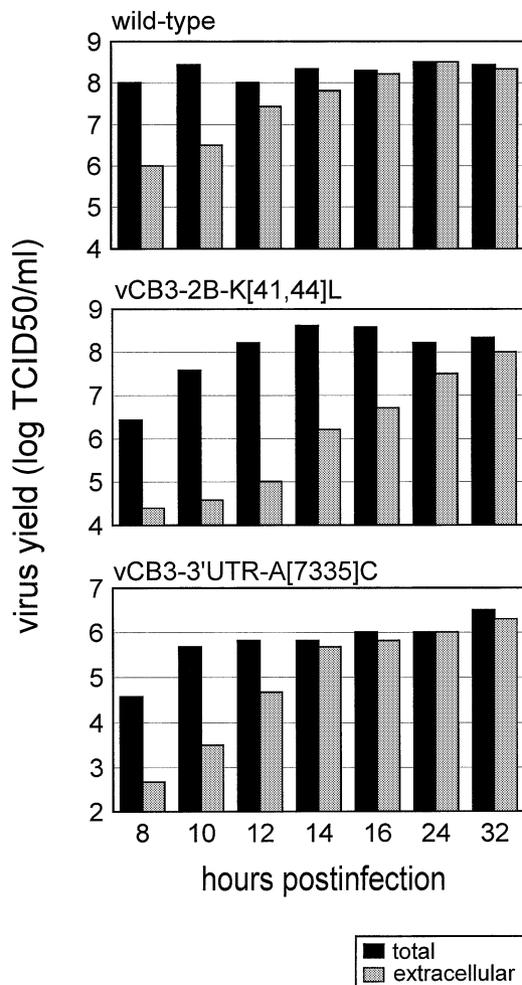


Fig. 7. Release of virion progeny from cells infected with wild-type CBV3, vCB3-2B-K[41,44]L, or vCB3-3'UTR-A[7335]C. HeLa cell monolayers were infected at an m.o.i. of 5 TCID₅₀ per cell and grown at 37°C. At the indicated times post-infection, a small portion of the medium was removed from the flasks. Cells and the remainder of the medium were subjected to three cycles of freezing and thawing to release intracellular viruses. The medium sample that was kept apart was microcentrifuged for 5 min at 1500 *g* to remove intact cells. The number of viruses present in the medium (containing extracellular viruses only) and the flasks (containing total virus, i.e. extracellular and intracellular viruses) was determined by endpoint titration. TCID₅₀, 50% tissue culture infective dose. One of two representative experiments is shown.

efficiently in the absence of extracellular Ca²⁺ implies that increases in [Ca²⁺]_i are dispensable for processes like viral minus and plus-strand RNA synthesis and virion assembly.

The possibility was investigated that the elevated [Ca²⁺]_i might aid in increasing plasma membrane permeability. First, the sensitivity of translation to hygromycin B in infected HeLa cells was assayed. The effect of hygromycin B was tested at 6 h p.i., a time at which host cell translation is shut off and only viral translation takes place. Figure 8C shows that the inhibition of translation by hygromycin B occurred irrespective of the [Ca²⁺]_i. We also tested the permeability of the plasma membrane at increasing times post-infection. Also, permeability was assessed by the uptake of propidium iodide, a membrane-impermeable, fluorescent DNA stain. The percentage of permeable cells

was then determined by flow cytometry. Figure 8D shows that during infection the number of permeable cells rapidly increased independently of the presence of extracellular Ca²⁺. These findings suggest that it is unlikely that the 2B-induced increase in plasma membrane permeability is facilitated by the increase in [Ca²⁺]_i.

To examine whether the increase in [Ca²⁺]_i contributes to the release of virus progeny, the amount of total virus and extracellular virus produced and released in the presence or absence of extracellular Ca²⁺ was compared (Figure 8E). The amount of total virus produced in the absence of Ca²⁺ was found to be similar to that produced in the presence of Ca²⁺ and is therefore not shown. In the presence of Ca²⁺, it took ~16 h before all viruses were released from the cells, i.e. when the amount of total virus and extracellular virus were the same. In the absence of Ca²⁺, however, virus release was not complete before 24 h p.i. Between 8 and 14 h p.i., the amount of virus that was released in the absence of Ca²⁺ was 5- to 10-fold lower than that released in the presence of Ca²⁺. Thus, increases in [Ca²⁺]_i facilitate the release of virus progeny.

Discussion

Protein 2B facilitates virus release by modifying membrane permeability

Cytolytic animal viruses need to modify membrane permeability in order to release their progeny. This may be particularly important for non-enveloped viruses that do not leave the cells by budding but that need to lyse the membrane. The molecular mechanisms employed to disturb the membrane are as yet poorly understood. It has been suggested that membrane permeability is due to the expression of a single or a limited number of virus genes rather than to the bulk of viral gene expression or the formation and accumulation of virus particles (Carrasco, 1995). Several results presented here provide ample evidence for this suggestion. First, we have shown that CBV3 infection leads to disruption of the Ca²⁺ gradients maintained by the plasma membrane and ER membrane, and that protein 2B is sufficient to induce these effects. Second, viruses carrying a mutant 2B protein exhibited an impairment in the release of virion progeny. Third, mutant viruses that showed a 100-fold reduction in accumulation of virion particles, but which produced a wild-type 2B protein, showed a wild-type efficiency of virus release. These findings strongly suggest that 2B is a protein targeted to enhance plasma membrane permeability, and that a function of this activity is to facilitate the release of virion progeny.

The recognition of virus proteins capable of enhancing membrane permeability is currently leading to the description of a new family of virus proteins, the vioporins (Carrasco, 1995). Until now, only two such proteins have been identified; influenza virus protein M2 and togavirus 6K protein, small proteins that contain a predicted amphipathic α -helix. Both proteins are able to modify membrane permeability in the absence of other viral proteins (Pinto *et al.*, 1992; Guinea and Carrasco, 1994; Sanz *et al.*, 1994), and deletion or blockage of the function of these proteins caused a decrease in the release of virions due to a defect in budding from the cell surface (Liljeström *et al.*, 1991; Ruigrok *et al.*, 1991). CBV3 protein 2B

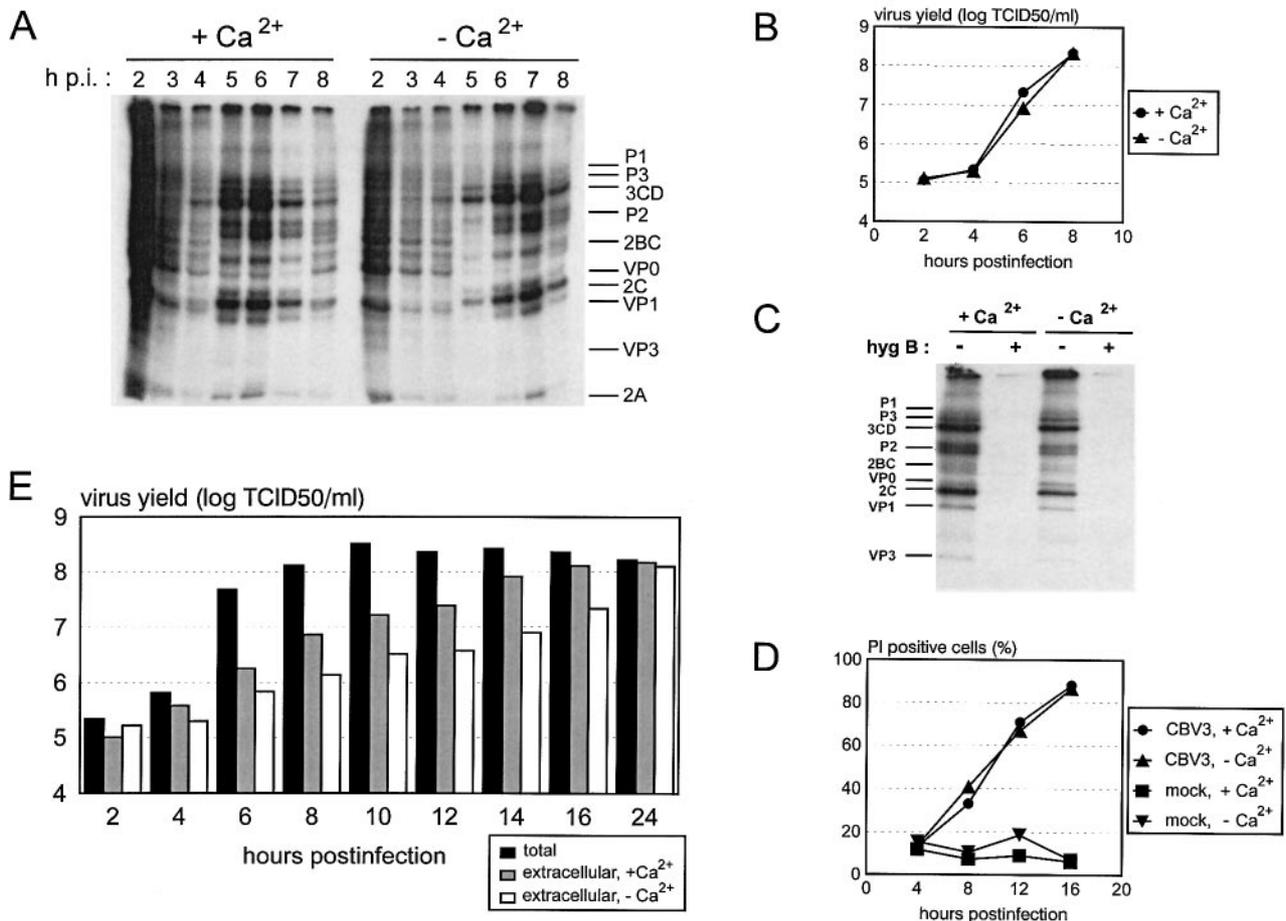


Fig. 8. Importance of Ca²⁺ for several stages of the viral replicative cycle. In all panels, HeLa cell monolayers were infected with CBV3 at the indicated m.o.i. (given in TCID₅₀ per cell), supplied with medium with or without Ca²⁺, and grown at 37°C until further analysis. (A) Pulse-labelling of infected cells (m.o.i. 25) at various times post-infection (indicated above each lane). At the indicated times, cells were incubated for 30 min in methionine-free MEM, with or without Ca²⁺, in the presence of 10 μCi of Tran³⁵S-label (a mixture of [³⁵S]methionine and [³⁵S]cysteine). Labelled proteins were analysed on an SDS–12.5% polyacrylamide gel. Viral proteins are indicated. (B) Single-cycle growth curves of infected cells (m.o.i. 5). At the indicated times, cells were subjected to three cycles of freezing and thawing. Virus titres were determined by endpoint titration. (C) Permeability of the plasma membrane of infected cells (m.o.i. 25) to hygromycin B. At 6 h p.i., cells were pulse-labelled with 10 μCi of Tran³⁵S-label for 30 min in methionine-free MEM, with or without Ca²⁺, and in the presence or absence of 500 μg hygromycin B (hyg B) per ml. (D) Permeability of infected cells (m.o.i. 5) and mock-infected cells to propidium iodide (PI). At the indicated times, cells were processed for propidium iodide uptake and flow cytometric analysis as described in Materials and methods. (E) Release of virion progeny from infected cells (m.o.i. 5). Titres of total and extracellular virus were determined as described in the legend of Figure 7. One of two representative experiments is shown.

represents the first example of a viroporin of a naked virus. An understanding of the mechanism by which 2B affects membrane permeability will be of great interest in understanding how cytolitic viruses release their progeny and will contribute to our knowledge on the structure of proteins designed to lyse cellular membranes.

Destabilization of the lipid bilayer by membrane-disturbing proteins is a widespread strategy used in nature to lyse cells. Among these proteins are the holins—proteins from bacteriophages that lyse bacteria by forming holes in their membranes (Witte *et al.*, 1990; Young, 1992); haemolysins—cytolitic toxins of bacterial origin (Bernheimer and Rudy, 1986); cecropines—peptides that occur in the venom of several insect species (Segrest *et al.*, 1990); magainins—antibiotic peptides that occur in the skin of amphibia (Zasloff, 1987); and defensins—vertebrate antimicrobial peptides (Cociancich *et al.*, 1993). A general feature of these membrane-disturbing proteins is the occurrence of amphipathic α-helices, mostly con-

taining cationic amino acids in their hydrophilic faces. Two molecular models of action have emerged from structural and functional studies on these proteins (Bernheimer and Rudy, 1986; Shai, 1995). According to the first type of model, the helices form aqueous pores by spanning the membrane and forming oligomers, exposing their hydrophobic sides to the lipid bilayer and their hydrophilic faces forming the aqueous interior. The second model suggests that the amphipathic helix is lying parallel to the membrane plane and penetrates a few Angstroms into it, thereby making the phospholipids more susceptible to degradation by phospholipases. The distinction between these models may not be so clear because membrane-embedded pores may also activate phospholipase activity (Young, 1992).

Analysis of mutations in individually expressed 2B proteins confirmed that the predicted cationic amphipathic α-helix in 2B is a major determinant for its ability to increase [Ca²⁺]_i. In addition to the amphipathic helix,

however, the second hydrophobic domain also appeared to be involved in this ability. This argues against an independent role of the amphipathic α -helix (i.e. the second model) in increasing membrane permeability. It should therefore be considered that multimers of 2B form membrane-embedded pores and that the formation of these pores depends on a correct positioning of the second hydrophobic domain in the membrane.

When considering the mechanism by which protein 2B increases plasma membrane permeability, the subcellular localization of the protein should be taken into account. Studies examining the localization of poliovirus 2B showed that the protein is contained exclusively centrally in the cytoplasm, where it is associated with the ER-derived, membranous vesicles that surround the viral replication complex (Bienz *et al.*, 1994). The possibility that the 2B-induced release of ER-stored Ca^{2+} is indirectly responsible for the increase in plasma membrane permeability seems unlikely, because the enhancement of plasma membrane permeability did not rely on the presence of Ca^{2+} ions. In addition, the thapsigargin-induced evacuation of ER-stored Ca^{2+} in non-infected cells had no effect on plasma membrane permeability to hygromycin B (unpublished data). Another possibility is that a small fraction of the 2B proteins associate directly with the plasma membrane. The immunocytochemical studies reported here provide a first indication for this possibility. In CBV3-infected cells, protein 2B was not only observed centrally in the cytoplasm, but also at the plasma membrane level. Whether these 2B proteins actually represent membrane-associated proteins or, alternatively, proteins that are associated with viral replication complexes that lie in the close vicinity of the plasma membrane remains to be established. That protein 2B is able to associate with the plasma membrane, however, is strongly suggested by the plasma membrane labelling observed in 2B-expressing cells.

A second activity identified for protein 2B is the ability to interfere with protein trafficking through the vesicular system (Doedens and Kirkegaard, 1995). It could be postulated that this activity is indirectly responsible for the increase in plasma membrane permeability by altering the protein and lipid composition of the membrane. However, this possibility seems unlikely for two reasons. First, expression of enterovirus protein 3A, which is an even more potent inhibitor of vesicular protein transport than 2B, does not lead to enhanced plasma membrane permeability (Doedens and Kirkegaard, 1995). Second, analysis of the abilities of mutant CBV3 2B proteins to increase membrane permeability and inhibit protein transport suggested that these two activities are separate functions of protein 2B rather than that one of these effects is the consequence of the other (van Kuppeveld *et al.*, 1997).

It is tempting to speculate that, by forming pores in both the ER membrane and the plasma membrane, 2B is responsible for the disturbance of ionic gradients and the activation of phospholipases. Increased phospholipase C activities have indeed been reported in poliovirus-infected cells (Guinea *et al.*, 1989) and it is likely that these contribute to a further disruption of the membrane, resulting in increased pore sizes. A progressive recruitment of 2B proteins may also cause widening of the pore. The

idea that 2B forms pores that gradually increase in size is consistent with the phenomenology observed in poliovirus-infected cells: initially, ionic gradients are disrupted in both directions; then low-molecular weight compounds can pass the membrane; and finally enzymes leak out of the infected cells (Carrasco, 1995). It is likely that this membrane damage will ultimately allow release of newly formed viruses. The observation that polioviruses are released exclusively from the apical surface of polarized human intestinal epithelial cells (Tucker *et al.*, 1993) may be due to a focal distribution of 2B proteins at the apical plasma membrane.

Disruption of intracellular Ca^{2+} homeostasis by protein 2B

CBV3 infection gradually enhances $[\text{Ca}^{2+}]_i$, similarly to that recently reported in poliovirus-infected cells (Irurzun *et al.*, 1995). The increase in $[\text{Ca}^{2+}]_i$ in both CBV3-infected HeLa cells and 2B-expressing COS cells is mainly due to the influx of extracellular Ca^{2+} . This influx may be explained by the increased plasma membrane permeability. However, the influx of Ca^{2+} may also be triggered by the partial emptying of the ER. Reductions in the state of filling of intracellular Ca^{2+} stores stimulate the influx of Ca^{2+} by opening specific Ca^{2+} channels in the plasma membrane, a phenomenon known as capacitative Ca^{2+} entry (reviewed by Berridge, 1995). The capacitative Ca^{2+} entry mechanism is present in many cells and can be switched on by a great variety of stimuli, all of which share a common property of releasing stored Ca^{2+} (Putney and Bird, 1994). Capacitative Ca^{2+} entry is therefore likely to occur in both CBV3-infected HeLa cells and 2B-expressing COS cells. The relative contribution and importance of the capacitative Ca^{2+} entry in increasing the $[\text{Ca}^{2+}]_i$ is difficult to define because the time at which the $[\text{Ca}^{2+}]_i$ starts to increase in infected cells (i.e. from 4 h p.i.) is the same as that at which the permeability of the plasma membrane starts to increase (Carrasco, 1995). Besides, the capacitative Ca^{2+} entry channel has not been identified and specific inhibitors are as yet unknown.

It seems plausible that protein 2B releases Ca^{2+} from the ER by increasing the permeability of the ER membrane. However, alternative mechanisms should be considered. The release of ER-stored Ca^{2+} could be caused by: (i) the enhanced activity of phospholipases, generating increased levels of inositol-1,4,5-trisphosphate (IP_3); (ii) a direct inhibition of the ER Ca^{2+} -ATPase; or (iii) a direct stimulation of the ER IP_3 receptor. The first possibility seems unlikely because increased levels of IP_3 in poliovirus-infected cells were detected only at 3–4 h p.i. (Guinea *et al.*, 1989), whereas the Ca^{2+} content of the ER is already decreased at 1 h p.i. Direct interactions of protein 2B with the ER Ca^{2+} -ATPase or the ER IP_3 receptor also seem unlikely, since we have demonstrated that mutations that abrogate the membrane-disturbing potential of 2B also abolish its ability to increase $[\text{Ca}^{2+}]_i$. However, the possibility that membrane-embedded 2B proteins sterically influence the functioning of these proteins cannot be excluded.

Until now, only one other viral protein capable of mobilizing Ca^{2+} from the ER has been identified; rotavirus NSP4. This non-structural ER transmembrane glycoprotein contains a predicted amphipathic α -helix and this helix

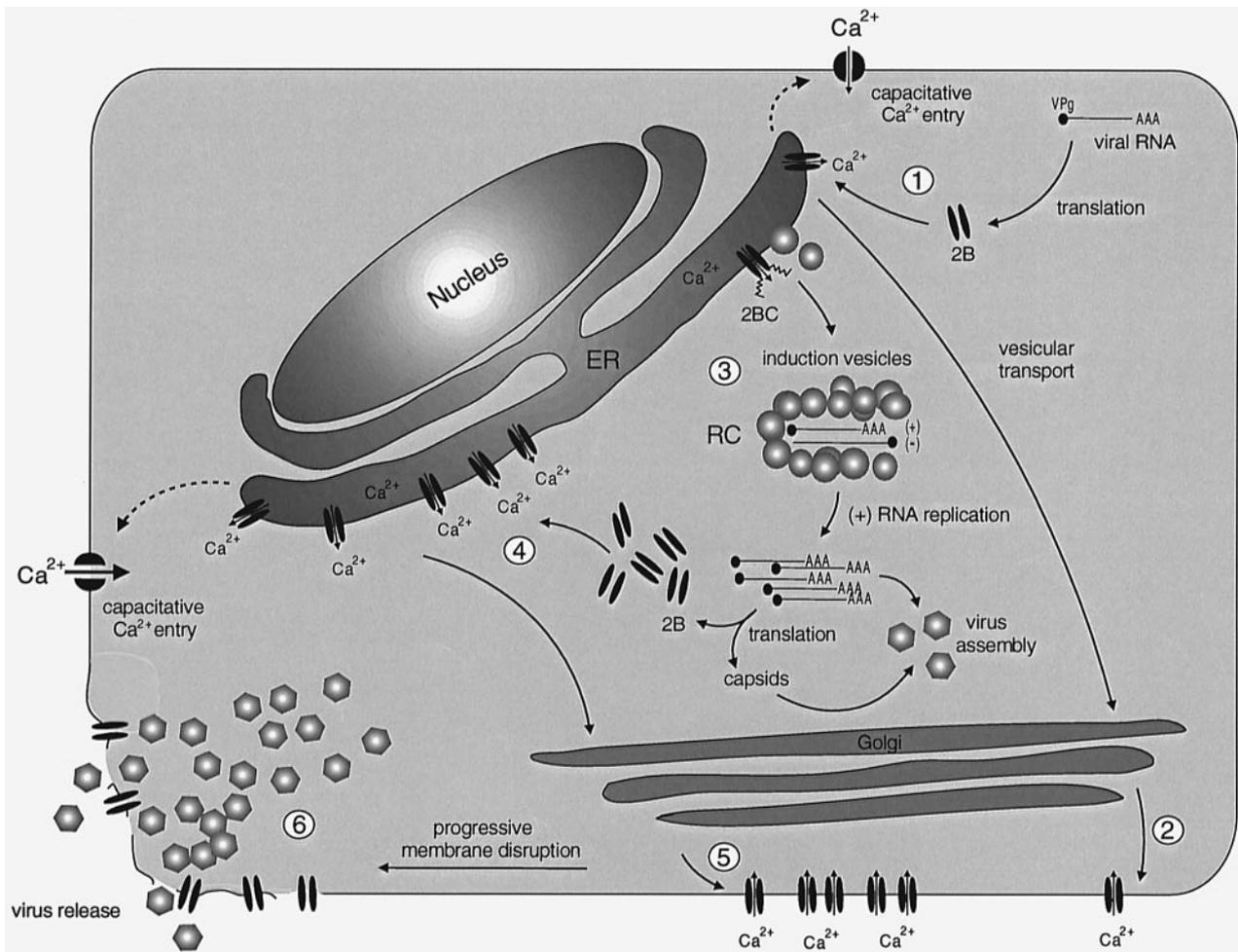


Fig. 9. Model for the mobilization of ER-stored Ca^{2+} , the influx of extracellular Ca^{2+} and the release of virus progeny by protein 2B through the formation of membrane-embedded pores. Early events (up to 4 h p.i.) in the viral replicative cycle involve: (1) production of protein 2B and insertion in the ER membrane, leading to a release of Ca^{2+} and, as a consequence, opening of capacitative Ca^{2+} entry channels; (2) transport of 2B to the plasma membrane where it is inserted and causes influx of extracellular Ca^{2+} ; (3) induction of the membranous vesicles by protein 2BC that are required for viral plus-strand RNA replication (possibly through the release of ER-stored Ca^{2+}). Late events (from 5 h p.i.) in the viral replicative cycle involve: (4) translation of newly formed plus-strand RNAs yielding large numbers of protein 2B; insertion of these proteins in the ER membrane causes a rapid evacuation of Ca^{2+} and gives rise to an increased capacitative Ca^{2+} entry; (5) collapse of Ca^{2+} gradient maintained by the plasma membrane by massive insertion of 2B proteins; (6) progressive increase in the size of the pores formed by 2B causes disruption of the membrane and results in the membrane lesions that allow virus release. For details, see text. RC, replication complex.

has been shown to be involved in the mobilization of Ca^{2+} from the ER (Tian *et al.*, 1995). The formation of cation channels has been proposed as a possible mechanism by which NSP4 alters the ER permeability. Thus, rotavirus NSP4 and 2B may release ER-stored Ca^{2+} by a common mechanism.

A model that may account for the alterations in Ca^{2+} homeostasis that take place with time during CBV3 infection, and for the release of virus progeny from infected cells is illustrated in Figure 9. We propose that early in infection (during the first 2 h) the 2B protein is produced and inserted into the ER membrane, where it promotes a partial release (of ~50%) of stored Ca^{2+} by forming membrane-embedded pores. As a consequence, capacitative Ca^{2+} entry will be induced. However, the $[\text{Ca}^{2+}]_i$ remains constant during the first 3–4 h p.i., as a result of the action of the plasma membrane Ca^{2+} -ATPase. A small fraction of the 2B proteins is transported to the plasma membrane, where it causes the influx of Ca^{2+} (evident from 4 h p.i.). Between 2 and 5 h p.i., the Ca^{2+}

content of the ER remains constant. This steady-state phase probably reflects the time needed to replicate the plus-strand genomic RNA via a complementary minus-strand RNA intermediate. Translation of these newly synthesized plus-strand RNAs (between 5 and 6 h p.i.) gives rise to the production of large numbers of 2B protein, which cause a rapid evacuation of ER-stored Ca^{2+} by further disturbing the ER membrane. Progressive insertion of 2B proteins in the plasma membrane results in a further collapse of ionic gradients. An increased influx of Ca^{2+} ions through both capacitative Ca^{2+} entry channels and pores formed by 2B will be the result. A rapid increase in $[\text{Ca}^{2+}]_i$ occurs from 6 h p.i., most likely because from this time the Ca^{2+} influx exceeds the pumping capacity of the plasma membrane Ca^{2+} -ATPase. Progressive membrane disruption ultimately allows the release of virus progeny.

Studies of the potential contribution of the enhanced $[\text{Ca}^{2+}]_i$ to different stages of the viral life cycle showed that increases in $[\text{Ca}^{2+}]_i$ were dispensable for the shutoff

of cellular translation, the rate of viral translation, viral minus-strand and plus-strand RNA replication, virion assembly and virus release. This latter process, however, appeared to be potentiated by increases in $[Ca^{2+}]_i$. The reason for this is unknown. A possible explanation is that the elevated $[Ca^{2+}]_i$ is cytotoxic and enhances cell lysis. Increases in $[Ca^{2+}]_i$ have been shown to be involved in animal virus-induced cytopathic effects and cell killing (Nokta *et al.*, 1987; Michelangeli *et al.*, 1991; Tian *et al.*, 1994; Sanderson *et al.*, 1996). Whether the increase in $[Ca^{2+}]_i$ in CBV3-infected cells also contributes to cytopathology, and whether this contributes to virus release, remains to be established.

Putative function of the mobilization of Ca^{2+} from the ER in viral genome replication

We have shown that CBV3 protein 2B enhances membrane permeability and that this activity serves to release virus progeny. However, it seems unlikely that this is the only function of 2B. Mutations in the 2B gene that disrupt essential structural domains abolish vRNA replication and virus growth (van Kuppeveld *et al.*, 1995, 1996a,b). If the only function of 2B were to modify plasma membrane permeability in order to release virus progeny, then genomic RNAs carrying mutations in their 2B gene would be expected to be replicated and encapsidated to produce viruses that accumulate in the cell. Additional functions of 2B connected with its ability to disturb membrane integrity should therefore be considered. A possible explanation is that the release of ER-stored Ca^{2+} is required for vRNA replication. Viral plus-strand RNA synthesis takes place at the outer surface of virus-induced membrane vesicles that originate from the ER (Bienz *et al.*, 1994). Protein 2BC has been identified as the viral protein responsible for the induction of these vesicles (Bienz *et al.*, 1983; Cho *et al.*, 1994; Barco and Carrasco, 1995). The function of 2B in precursor 2BC may involve its ability to release ER-stored Ca^{2+} . The ER contains a gel of Ca^{2+} -binding proteins that is responsible for the sequestration of Ca^{2+} ions. These Ca^{2+} -binding proteins form coordination complexes with Ca^{2+} ions that are bound to the negatively charged phospholipid head groups on the luminal face of the ER membrane, thereby stabilizing the underlying membrane and preventing its vesiculation (Sambrook, 1990). It has been predicted that transport vesicles derived from the ER normally bud from regions of membrane that are not stabilized by the Ca^{2+} -protein gel. The artificial evacuation of Ca^{2+} ions from the lumen of the ER that occurs when cells are treated with Ca^{2+} ionophores has been proposed to destabilize the entire Ca^{2+} -protein gel and its associated membrane, leading to a mass vesiculation (Sambrook, 1990). By analogy with the effect of the ionophores, the release of ER-stored Ca^{2+} by the 2B polypeptide in protein 2BC may be involved in the massive induction of ER-derived membrane vesicles by 2BC (Figure 9). According to this model, mutations that disrupt the ability of 2B to enhance membrane permeability will interfere with the ability of protein 2BC to induce these membrane vesicles and thereby account for the defects in vRNA replication and virus growth.

The exact mechanism by which protein 2BC induces membrane proliferation and vesicle accumulation is still puzzling. The release of ER-stored Ca^{2+} may be involved,

but is clearly not sufficient for these activities, as protein 2B alone is unable to account for these structural alterations. Therefore, a function of protein 2C in precursor 2BC seems also to be required. Protein 2C has been identified as an NTPase that shares structural similarities with the group of small GTPases involved in the regulation of vesicle transport (Rodriguez and Carrasco, 1993; Mirzayan and Wimmer, 1994), but its exact function is unknown. Further investigations are required to define the contribution of proteins 2B and 2C to the ability of protein 2BC to rearrange membranes and to induce membranous vesicles.

Materials and methods

Cells, media, virus infections and titrations

COS-1 cells were grown in Dulbecco's modified Eagle's medium (Gibco). HeLa Ohio cells were grown in minimal essential medium (MEM) (Gibco). Media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were grown at 37°C in a 5% CO₂ incubator.

Ca^{2+} -free MEM was the same as regular MEM except that it lacked CaCl₂. In experiments in which the effects of extracellular Ca^{2+} were assayed, as Ca^{2+} -containing medium we used Ca^{2+} -free medium to which CaCl₂ was added to a final concentration of 1.8 mM. Both media were adjusted to pH 7.3. To Ca^{2+} -free MEM, 0.5 mM ethylene glycol-bis(β-amino ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 7.3, was added immediately before use.

All viruses used in this study are recombinant CBV3 viruses derived from plasmid pCB3/T7 (Klump *et al.*, 1990), which contains a full-length cDNA of CBV3 strain Nancy behind a T7 RNA polymerase promoter. HeLa Ohio cell monolayers were infected with virus at the indicated m.o.i. for 30 min at room temperature. After this, cells were washed three times with phosphate-buffered saline (PBS), supplied with MEM (with or without Ca^{2+}) containing 3% FBS, and grown at 37°C.

Virus yields were determined by endpoint titration. Serial 10-fold dilutions were tested in eight replicative wells of 96-well plates as described (van Kuppeveld *et al.*, 1995). Virus titres were calculated and expressed as TCID₅₀ values (Reed and Muench, 1938).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by using the fluorescent Ca^{2+} indicator fura-2 essentially as described previously (Willems *et al.*, 1993). Briefly, at 30 min before measurement, the cells were incubated in new medium containing 2.5 μM fura-2 in its membrane-permeant acetoxymethyl (AM) ester form (Molecular Probe, Inc., Eugene, OR). Cells were incubated for 30 min at 37°C and then washed three times with medium to remove extracellular probe. Cells were supplied with preheated medium and the coverslip was introduced to a thermostatted (37°C) incubation chamber on the stage of an inverted fluorescence microscope (Nikon Diaphot). Routinely, an epifluorescent ×40 magnification oil immersion objective was used, allowing simultaneous monitoring of ~25–50 single cells. Dynamic video imaging was carried out using the MagiCal hardware and TARDIS software (Joyce Loebel, UK). Fluorescence was measured every 2 s with the excitation wavelength being altered between 340 and 380 nm and the emission fluorescence being recorded at 492 nm (binding of Ca^{2+} shifts the absorbance spectra from 380 to 340 nm). At the end of each experiment, a region free of cells was selected and one averaged background frame was collected at each excitation wavelength. The averaged background frames were subtracted from the corresponding experimental frames. After this, the fluorescence ratio R , which equals F_{340}/F_{380} , where F_{340} and F_{380} are the emission intensities at 340 and 380 nm excitation, respectively, was calculated. $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times (F_{min} / F_{max})$, where R_{min} and R_{max} are the minimum and maximum fluorescence ratios, obtained in the presence of 10 mM EGTA and 1 μM ionomycin (Sigma), respectively, and F_{min} and F_{max} are the emission fluorescence values at 380 nm at these two extreme conditions. The equilibrium dissociation constant (K_d) for the Ca^{2+} -fura-2 complex at 37°C was taken to be 224 nM (Grynkiewicz *et al.*, 1985).

Measurement of Ca^{2+} release from endoplasmic reticulum stores

Cells on coverslips were loaded with fura-2 as described above and placed on the stage of the microscope. After recording cells in Ca^{2+} -

containing medium, the medium was replaced with 200 μ l of Ca^{2+} -free medium. After 10 min, 800 μ l of Ca^{2+} -free medium containing 1 μ M (final concentration) thapsigargin (LC Services, Woburn, MA) was added. The $[\text{Ca}^{2+}]_i$ was measured as described above.

Plasmids

For the expression of both the wild-type CBV3 2B protein and the mutant 2B proteins, we made use of previously described pC2B α 6 plasmids (van Kuppeveld *et al.*, 1997). These dicistronic plasmids were constructed by cloning of the coding region of 2B, with an additional methionine at its 5' end and a stop codon at its 3' end, as the first cistron in plasmid pLINK α 6. The plasmids contain an SV40 origin of replication to allow their amplification in COS-1 cells and an SV40 late promoter to drive transcription of RNA.

Transfections

COS-1 cell monolayers grown to confluency of 70% were harvested by trypsinization, collected by centrifugation for 5 min at 1500 g, and resuspended in PBS to a density of 1×10^7 cells per ml. 2×10^6 COS-1 cells were transfected with 15 μ g of plasmid DNA by electroporation at 300 V and 125 μ F using the Gene Pulser (Biorad). After electroporation, cells were resuspended in fresh medium and seeded onto coverslips (diameter, 2.4 cm) placed inside the wells of six-well plates. Cells were seeded at a density of 2×10^5 cells per well and grown at 37°C until further analysis.

Transfection of HeLa cell monolayers with *in vitro*-transcribed copy RNA of chimeric replicon pCB3/T7-LUC and measurement of the luciferase activity were performed as described (van Kuppeveld *et al.*, 1995).

Indirect immunofluorescence

Transfected COS-1 cells grown on coverslips were fixed by treatment with cold methanol, followed by treatment with cold acetone, each for 2 min. The coverslips were incubated for 1 h at 37°C with rabbit polyclonal serum raised against CBV3 protein 2B (kind gift from the laboratory of R.Kandolf, University of Tübingen, Germany) that was diluted 1:40 in PBS. After two washes with PBS, the coverslips were incubated for 1 h at 37°C with fluorescein isothiocyanate-conjugated goat-anti-rabbit IgG (Cappel Laboratories, Cochranville, PA), which was diluted 1:40 in 0.2% Evans blue/PBS. The coverslips were washed with PBS, dried, overlaid with a 1:1 mixture of glycerol and PBS, and viewed with a Leitz dialux 20 EB microscope. The percentage of protein-expressing cells was determined *de visu* of 500 cells.

Immunoelectron microscopy

CBV3-infected HeLa cells and transfected COS-1 cells were harvested by scraping in MEM without FBS and collected by centrifugation for 5 min at 1500 g. After fixation in periodate-lysine-2% paraformaldehyde for 2 h at room temperature, cells were pelleted in 15% gelatin. Gelatin pellets were cut into small blocks, cryoprotected in 2.3 M sucrose for 45 min, and snap-frozen in liquid nitrogen. Cryostat sections (25 μ m) were washed with PBS and incubated in 50 mM glycine, pH 7.4, for 10 min. After two washes in PBS, the sections were incubated overnight at 4°C with anti-2B serum (see above) that was diluted 1:40 in 1% bovine serum albumin (BSA)/PBS. Sections were washed in PBS and incubated for 1 h with goat-anti-rabbit antibodies, coupled to colloidal gold of 1 nm (Nanoprobes Immunosome, Stony Brook, NY), that were diluted 1:50 in 1% BSA/PBS. This was followed by a 1 h incubation in PBS and post-fixation with 1% glutaraldehyde/PBS. After three washes in distilled water, the gold signal was enhanced using HQ silver (Nanoprobes Immunosome) for 7 min. Sections were incubated overnight in distilled water and treated with 1% OsO_4 for 30 min. After this, they were dehydrated in alcohol, embedded in Epon 812, and viewed with a JEOL 1200 EX II electron microscope.

Analysis of viral protein synthesis *in vivo*

HeLa cell monolayers were infected at an m.o.i. of 25 TCID₅₀ per cell. After infection, cells were supplied with MEM, with or without Ca^{2+} , and incubated at 37°C. At various times post-infection, protein synthesis was monitored by pulse labelling with 10 μ Ci of Tran³⁵S-label (a mixture of [³⁵S]methionine and [³⁵S]cysteine, ICN) in methionine- and serum-free MEM or methionine- and serum-free MEM without CaCl_2 for 30 min. Lysis of cells and analysis of labelled proteins by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed as described previously (van Kuppeveld *et al.*, 1995).

In experiments in which the permeability of cells to hygromycin B was assayed, cells were incubated in methionine- and serum-free MEM,

with or without Ca^{2+} , and in the presence or absence of 500 μ g hygromycin B (Sigma) per ml, for 15 min before the addition of 10 μ Ci of Tran³⁵S-label to the medium.

Viral growth curves

Confluent HeLa cell monolayers in T25 flasks were infected with virus at an m.o.i. of 5 TCID₅₀ per cell. After infection, cells were supplied with 5 ml of MEM, with or without Ca^{2+} , and grown at 37°C. At the indicated times post-infection, flasks were subjected to three cycles of freezing and thawing to release intracellular viruses. Viruses contained in these flasks represented the total virus population (i.e. extracellular plus intracellular virus). Virus titres were determined by endpoint titration.

In experiments in which the release of viruses from the cells was studied, 1 ml of medium was removed before freezing of the flasks. This sample was microcentrifuged for 5 min at 1500 g to remove intact cells. The titre of extracellular viruses maintained in the supernatant was determined as described above.

Propidium iodide uptake

HeLa cell monolayers were infected with CBV3 at an m.o.i. of 5 TCID₅₀ per cell. After infection, cells were supplied with MEM, with or without Ca^{2+} , and grown at 37°C. At the desired time post-infection, the medium was collected. Cells remaining at the surface of the flask were harvested by incubating in 1 ml of PBS containing 50 mM EDTA and combined with the medium. Cells were collected by centrifugation for 5 min at 1500 g and resuspended in 300 μ l of medium. Propidium iodide (Sigma) was added to a final concentration of 5 μ g. Cells were incubated for 10 min at room temperature. The number of permeable cells was determined using a Coulter XL flow cytometer (Coulter Corp., Hialeah, FL).

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