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The Rotavirus Nonstructural Glycoprotein NSP4 Mobilizes Ca^{2+} from the Endoplasmic Reticulum

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We previously reported that expression of rotavirus nonstructural glycoprotein NSP4 is responsible for an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in *Spodoptera frugiperda* (Sf9) insect cells (P. Tian, Y. Hu, W. P. Schilling, D. A. Lindsay, J. Eiden, and M. K. Estes, J. Virol. 68:251–257, 1994). The purpose of the present study was to determine the mechanism by which NSP4 causes an increase in $[Ca^{2+}]_i$ by measuring the permeability of the cytoplasmic and endoplasmic reticulum (ER) membranes in recombinant-baculovirus-infected Sf9 cells. No obvious change in plasmalemma permeability to divalent cations was observed in cells expressing NSP4 compared with that in cells expressing another rotaviral glycoprotein (VP7) when the influx of Ba^{2+}, a Ca^{2+} surrogate, was monitored. The basal Ca^{2+} permeability of the internal Ca^{2+} store was evaluated by measuring the release of Ca^{2+} induced by ionomycin, a Ca^{2+} ionophore, or thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase pump, following suspension of the cells in Ca^{2+}-free extracellular buffer. Releasable Ca^{2+} decreased with time to a greater extent in cells expressing NSP4 compared with that in cells expressing VP7, suggesting that NSP4 increases the basal Ca^{2+} permeability of the ER membrane. To determine the possible mechanism by which NSP4 increases ER permeability, purified NSP4 protein or a 22-amino-acid synthetic peptide consisting of residues 114 to 135 ($NSP4_{114–135}$) was added exogenously to noninfected Sf9 cells during measurement of $[Ca^{2+}]_i$. Both NSP4 and the $NSP4_{114–135}$ peptide produced a time-dependent increase in $[Ca^{2+}]_i$ that was attenuated by prior inhibition of phospholipase C with U-73122. Pretreatment of the cells with thapsigargin completely blocked the increase in $[Ca^{2+}]_i$ produced by $NSP4_{114–135}$, but the peptide only partially reduced the change in $[Ca^{2+}]_i$ produced by thapsigargin. No changes in $[Ca^{2+}]_i$ were seen in cells treated with control peptides. These results suggest that (i) exogenous NSP4 increases $[Ca^{2+}]_i$ through the activation of phospholipase C, (ii) Ca^{2+} release by exogenous NSP4 is from a store that is a subset of the thapsigargin-sensitive compartment, and (iii) amino acid residues 114 to 135 of NSP4 are sufficient for this activity. In contrast to exogenous NSP4, the mechanism by which endogenously expressed NSP4 increases $[Ca^{2+}]_i$ appears to be unrelated to phospholipase C, since no effect of U-73122 was seen on the elevated $[Ca^{2+}]_i$ in cells expressing NSP4 and exogenously applied $NSP4_{114–135}$ caused a further increase in $[Ca^{2+}]_i$ in cells expressing NSP4 protein. We propose that alteration in the Ca^{2+} permeability of the ER membrane during viral expression of NSP4 plays an important role in virus maturation and that release of NSP4 following cell lysis and the concomitant stimulation of Ca^{2+} signal transduction in neighboring cells may contribute to altered ion transport in the intestinal epithelium. The actual mechanism by which NSP4 alters ER permeability is unknown. NSP4 may (i) form a cation channel in the ER membrane, (ii) increase Ca^{2+} leakage through the inositol-1,4,5-trisphosphate receptor, and/or (iii) have action on the phospholipids leading to membrane disruption.

Rotaviruses are nonenveloped, triple-layer viruses with a genome of 11 segments of double-stranded RNA and are recognized as the most important cause of severe viral gastroenteritis in humans and animals. Rotaviruses have a unique morphogenesis in which particles obtain a transient membrane envelope that is formed by the budding of newly made subviral particles into the endoplasmic reticulum (ER) (15). This process is mediated by the rotavirus nonstructural transmembrane ER-specific glycoprotein NSP4 (formerly called NS28 [1, 2, 28, 31]).

Ca^{2+} is important for rotavirus replication and cytopathogenesis. The physical integrity of rotavirus particles requires Ca^{2+}. Treatment of infectious triple-layer particles with Ca^{2+} chelating agents results in the loss of infectivity and the removal of the two outer capsid proteins, VP4 and VP7 (11, 18, 41). Increasing the intracellular Ca^{2+} concentration during the early stage of replication blocks virus uncoating (26). Ca^{2+} also plays a major role in the acquisition of specific conformations necessary for the correct association of proteins during virus maturation in the ER. Ca^{2+} is required for the oligomerization of the virus-encoded proteins VP4, VP7, and NSP4 and the stability of VP7 and of neutralizing epitopes on VP7 (13, 14, 32, 40).

A significant increase in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) has been reported in rotavirus-infected MA104 cells. This increased $[Ca^{2+}]_i$ has been correlated with rotavirus-specific protein synthesis (29) and has been suggested to be

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related to the cytotoxicity observed in infected cells (29, 40, 45). We previously reported that expression of NSP4 causes an increase in basal $[Ca^{2+}]_i$ in Sf9 insect cells infected with recombinant baculovirus containing the NSP4 cDNA (45), but the mechanism by which NSP4 causes $[Ca^{2+}]_i$ to increase remains unknown. The increased $[Ca^{2+}]_i$ could result from a change in the net permeability of either the plasmalemma or the ER membrane. Michelangeli et al. (29) reported that changes in cytoplasmic membrane permeability were responsible for Ca^{2+} increases (Ca^{2+} influx) in rotavirus-infected MA104 cells. However, this increase of Ca^{2+} influx may have resulted from a depletion of the internal Ca^{2+} pool. It has been documented in both mammalian cells and *Spodoptera frugiperda* Sf9 cells that the depletion of Ca^{2+} from the internal pool results in a significant increase in Ca^{2+} influx (23, 33–35). NSP4 has been recognized as a transmembrane protein of the ER, an organelle which functions as an internal Ca^{2+} pool (3, 6, 9, 16). We hypothesized that the expression of NSP4 might increase $[Ca^{2+}]_i$ by increasing the release of Ca^{2+} from the ER. Alternatively, NSP4 could affect the normal Ca^{2+} signal transduction mechanisms of the cell. Stimulation of specific surface membrane receptors (e.g., muscarinic acetylcholine receptors) in a variety of nonexcitable cells, including epithelial cells, causes activation of phospholipase C (PLC), an increase in inositol-1,4,5-trisphosphate ($InsP_3$), and the release of Ca^{2+} from internal storage sites. Direct stimulation of PLC or interaction of NSP4 with ER IP_3 receptors or Ca^{2+} pumps could result in altered Ca^{2+} homeostasis.

In the present study we have used the fluorescent Ca^{2+} indicator fura-2 to measure plasmalemma and ER membrane permeability in Sf9 cells expressing or exposed to NSP4. The Sf9 insect cell is useful for the functional expression of heterologous proteins following infection with recombinant baculovirus containing the cDNA for the protein of interest. This system has been used for the functional expression of the M5 muscarinic receptor (23), the human B2 bradykinin receptor (24, 36), and the human thrombin receptor (10). Stimulation of Sf9 cells expressing these receptors with their respective agonists causes a change in $[Ca^{2+}]_i$ essentially identical to that observed in mammalian cells. Thus, the Sf9 cell serves as a model system to investigate the mechanisms of action of proteins thought to affect Ca^{2+} homeostasis. The results of the present study suggest that NSP4 can increase Ca^{2+} permeability of the ER membrane through two distinct mechanisms, both of which could play an important role in the physiological consequences of rotavirus infection of intestinal epithelial cells.

MATERIALS AND METHODS

Cells and viruses. Recombinant baculoviruses expressing the rotavirus nonstructural glycoprotein NSP4 (1), the full-length rotavirus capsid protein VP7 (12), and a rat brain M5 muscarinic acetylcholine receptor (23) were used to infect Sf9 insect cells at a multiplicity of infection of 10 PFU per cell. A recombinant baculovirus expressing a *Drosophila* protein, the *trpl* (transient receptor potential-like [25]) protein, was used to infect Sf9 cells at a multiplicity of infection of 3 PFU per cell. The infected cells were harvested for analysis at 36 h postinfection. This time postinfection was chosen on the basis of our earlier work in which increases in intracellular calcium in cells expressing NSP4 were found to be maximal at 36 h postinfection and there was no effect in cells infected with wild-type baculoviruses (45). Sf9 cells were grown and maintained in TNM-FH media containing 10% fetal bovine serum as previously described (17).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by using the fluorescent Ca^{2+} indicator fura-2 as previously described (45). Briefly, 10^8 cells were suspended in 20 ml of extracellular buffer (ECB; 10 mM $CaCl_2$, 60 mM KCl, 17 mM $MgCl_2$, 10 mM NaCl, 10 mM morpholineethanesulfonic acid [MES], 4 mM glucose, 110 mM sucrose, and 0.1% bovine serum albumin, pH 6.2) containing 2 μM fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes, Eugene, Oreg.). Following a 30-min incubation at room temperature, the cell suspension was subjected to centrifugation, resuspended in 20 ml of ECB, and incubated for an additional 30 min. The cell suspension was again subjected to centrifugation, and the cells were suspended in fresh ECB. Cells (5×10^6) from this final suspension were washed twice in ECB or Ca^{2+}-free ECB ($CaCl_2$ was replaced by $MgCl_2$) immediately prior to the measurement of fluorescence. Aliquots of cells were placed in a quartz cuvette with a magnetic stirrer, and fluorescence was measured in an SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, Ill.) with the excitation wavelength being altered between 340 and 380 nm and emission fluorescence being recorded at 510 nm. All measurements were performed at room temperature and corrected for autofluorescence by using unloaded cells. Calibration of the fura-2 associated with the cells was accomplished by lysing the cells in the presence of saturated divalent cation and then adding ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA; pH 8.5). $[Ca^{2+}]_i$ was calculated with the equations of Grynkiewicz et al. (22) with an equilibrium dissociation constant for Ca^{2+} binding to fura-2 of 278 nM at 22°C (42).

Measurement of plasmalemma permeability. Ba^{2+} influx was used to measure plasmalemma permeability in recombinant-baculovirus infected Sf9 cells. Ba^{2+} is a Ca^{2+} surrogate which is transported across the cytoplasmic membrane through Ca^{2+} channels but is not a substrate for the carriers and pumps that normally transport Ca^{2+} across cell membranes. Ba^{2+} influx in Sf9 cells was measured as described previously (25, 37). Briefly, the cells were suspended in Ca^{2+}-free ECB immediately before measurements were made. Ba^{2+} (10 mM) was added to the cells 2 min later. The fluorescence was measured at excitation wavelengths of 350 and 390 nm. An increased Ba^{2+} influx was indicated by an increase in the 350-nm/390-nm fluorescence ratio (23, 25).

Measurement of Ca^{2+} release from internal Ca^{2+} stores. Internal Ca^{2+} stores include the ER, the mitochondria, and other intracellular organelles (6). Two chemicals, ionomycin (a Ca^{2+} ionophore) and thapsigargin (a specific inhibitor of the ER Ca^{2+}-ATPase pump), were used to release Ca^{2+} from internal stores. Ionomycin transports Ca^{2+} across biological membranes, including the cytoplasmic membrane, the ER membrane, the mitochondrial membrane, and other intracellular organelle membranes. In the absence of extracellular Ca^{2+}, the increase in $[Ca^{2+}]_i$ induced by ionomycin was used as an index of Ca^{2+} released from the total internal Ca^{2+} store. The ER membrane has channels that release Ca^{2+} into the cytoplasm. A Ca^{2+}-ATPase pump in the ER membrane transports Ca^{2+} from the cytoplasm back into the ER. When the ATPase pump in the ER is inhibited by thapsigargin, Ca^{2+} that leaks from the ER is not resequestered by the pumps and Ca^{2+} accumulates in the cytosol. The thapsigargin-induced increase in $[Ca^{2+}]_i$ is thought to represent Ca^{2+} released from the ER store. Fura-2/AM-loaded Sf9 cells were washed twice in Ca^{2+}-free ECB and suspended in Ca^{2+}-free ECB containing 1 mM EGTA immediately before fluorescence measurement. At various times after suspension of the cells in Ca^{2+}-free buffer, 200 nM (final concentration) thapsigargin (Calbiochem, San Diego, Calif.) or 2 μM ionomycin (Calbiochem) was added to the ECB and the $[Ca^{2+}]_i$ was measured as described above.

Purification of NSP4 and VP6. NSP4 was purified from Sf9 cells infected with a recombinant baculovirus expressing the rotavirus gene 10 by fast-performance liquid column chromatography on an anion-exchange quaternary methylamine anion exchange (QMA) column (44). The purity of NSP4 was examined by silver staining of the protein analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, by densitometry scanning of these gels, and by Western blot (immunoblot) analysis using a polyclonal antipeptide antiserum against a synthetic peptide consisting of residues 114 to 135 ($NSP4_{114–135}$). These analyses showed three major bands of NSP4 (28K, 26K, and 20K) which represented ~70% of the total protein. The total protein concentration was determined by the Bio-Rad protein assay, and the NSP4 concentration was estimated to be 70% of this total concentration.

VP6 was purified from the medium of Sf9 cells infected with baculovirus recombinant pAC461/SA11-6 (17) by pelleting oligomers through a 35% sucrose cushion by centrifugation in a Beckman SW28 rotor for 90 min at 25,000 rpm. The pelleted VP6 was then banded by equilibrium gradient centrifugation in CsCl (starting refractive index of 1.3610) in phosphate-buffered saline (PBS), pH 7.4, in a Beckman SW50.1 rotor for 18 h at 35,000 rpm. A visible band was collected by side puncture and dialyzed against 10 mM Tris-buffered saline, pH 7.4. SDS-polyacrylamide gel electrophoresis and silver staining of 20 μg of protein showed a single protein band of VP6, indicating that VP6 was >95% pure.

Synthetic peptides. All peptides were synthesized by fluorenylmethoxycarbonyl chemistry (8) by the Peptide Synthesis Facility at the University of Pittsburgh (Pittsburgh, Pa.). Peptides were cleaved from their solid resin support and purified by a series of cold ether extractions, conventional gel filtration chromatography (Sephadex G-25; Sigma), and reverse-phase high-pressure liquid chromatography (Delta Pak C_4; Waters, Milford, Mass.) or multiple gel filtration runs. The predominant peptide fraction was characterized by mass spectroscopy. Only those peptides with the correct theoretical mass corresponding to 90% or greater of the final peptide product were employed in this study. The following peptides were used in this study: rotavirus $NSP4_{114–135}$ (DKLTTREIEQVELL KRIYDKLT); $NSP4_{2–22}$ (EKLTDLNYTLSVITLMNNTLH); $NSP4_{90–123}$ (TT KDEIEQMDRVVKEMRRQLEMIDKLTTREIEQ); a Norwalk virus-specific peptide, NVSP (SHVAKIRGTSNGTVINLTELD); and a C-terminal Norwalk virus peptide, NVCP (DTGRNLGEFKAYPDGFLTCV). Each peptide was purified again with a Sephadex G-25 column (Pharmacia) equilibrated in 10% acetic acid. After lyophilization, the peptides were dissolved in sterile PBS.

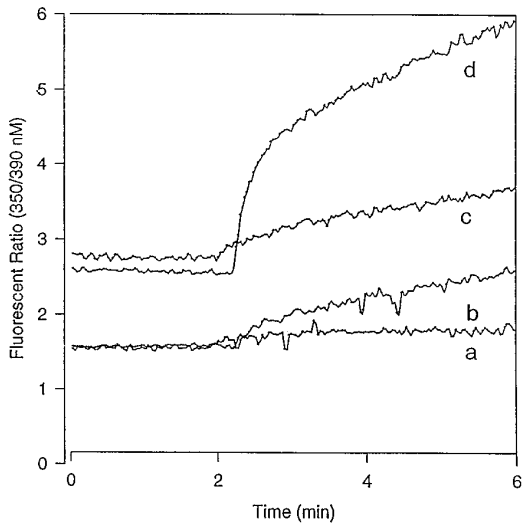

FIG. 1. Ba^{2+} influx in noninfected Sf9 insect cells and in cells expressing NSP4, VP7, and the *trpl* protein. Sf9 cells were harvested at 36 h postinfection. Fura-2-loaded Sf9 cells were incubated in Ca^{2+}-free ECB for 2 min. Ba^{2+} was added at a final concentration of 10 mM to uninfected Sf9 cells (trace a) or cells expressing VP7 (trace b), NSP4 (trace c), or *trpl* (trace d). A representative trace from three experiments is shown for each protein. Duplicate or triplicate traces were obtained in each experiment.

RESULTS

Plasmalemma permeability changes are not responsible for the increase in $[Ca^{2+}]_i$ observed in Sf9 cells expressing NSP4. Expression of NSP4 is associated with an increased basal $[Ca^{2+}]_i$ (45). This may reflect an increase in Ca^{2+} influx or a decrease in Ca^{2+} efflux from the cytosol. To distinguish between these two possibilities, we examined basal Ba^{2+} influx in Sf9 cells expressing the NSP4 protein. Ba^{2+} is known to be permeable through Ca^{2+} channels but is not a substrate for the intracellular pumps that normally function to remove Ca^{2+} from the cytosol. As a positive control for measuring changes in the plasmalemma permeability, the *Drosophila trpl* protein was used. An increased Ba^{2+} influx in Sf9 cells expressing *trpl* has been shown to be related to a permeability change in the cytoplasmic membrane and increased basal $[Ca^{2+}]_i$ (25). The rotavirus ER-associated glycoprotein VP7 was used as a negative control since expression of this protein is not associated with an increase in $[Ca^{2+}]_i$ (45). Addition of Ba^{2+} to uninfected Sf9 cells incubated in Ca^{2+}-free buffer produced little change in the fluorescence ratio, suggesting that noninfected Sf9 cells are relatively impermeable to Ba^{2+} (Fig. 1, trace a). A slight increase in Ba^{2+} influx was observed in cells expressing VP7 and NSP4 (traces b and c) examined at 36 h postinfection. In contrast, a dramatic increase in the fluorescence ratio was observed upon addition of Ba^{2+} to cells expressing *trpl* (trace d). It is striking that although basal levels of $[Ca^{2+}]_i$ in the cells expressing the *trpl* protein and NSP4 were significantly elevated compared to the basal $[Ca^{2+}]_i$ in uninfected cells or VP7-expressing Sf9 cells, the Ba^{2+} influx was much higher in the cells expressing the *trpl* protein compared to the influx in cells expressing NSP4. These results suggest that the small increase in Ba^{2+} permeability of the cells expressing NSP4, which presumably reflects the basal permeability of the plasmalemma for Ca^{2+}, did not contribute significantly to the increased $[Ca^{2+}]_i$ observed in these cells.

Ca^{2+} level in the internal stores in insect cells expressing NSP4 or VP7. An alternative source for the elevated $[Ca^{2+}]_i$ in cells expressing NSP4 is the internal Ca^{2+} store. The amount of total releasable Ca^{2+} and the amount of Ca^{2+} in the ER were determined by monitoring the changes in $[Ca^{2+}]_i$ induced by ionomycin and thapsigargin, respectively, in cells expressing NSP4 and VP7 and incubated in Ca^{2+}-free buffer containing 1 mM EGTA (Fig. 2). Ca^{2+} released from the ionomycin-sensitive pool 2 min after suspension of the cells in Ca^{2+}-free buffer (as estimated by the net change in $[Ca^{2+}]_i$) was 448 ± 88 and 464 ± 78 nM (mean ± standard deviation) in cells expressing NSP4 and VP7, respectively. Likewise, Ca^{2+} release from the thapsigargin-sensitive pool 2 min after suspension of the cells in a Ca^{2+}-free buffer (as estimated by the net change in $[Ca^{2+}]_i$) was 129 ± 22 and 112 ± 19 nM in cells expressing NSP4 and VP7, respectively. These results suggest that the initial load of Ca^{2+} in the internal store is not significantly different in cells expressing NSP4 and VP7 ($P > 0.05$). However, since the amount of Ca^{2+} within the ER reflects a balance between the leakage of Ca^{2+} out of the ER and the reuptake of Ca^{2+} into the ER by the Ca^{2+} pumps, it is possible that the ER permeability to Ca^{2+} is increased in cells expressing NSP4 but that Ca^{2+} in the ER is maintained at a near normal level by an increase in pump activity.

The ER membrane permeability changes in cells expressing NSP4. To determine the basal Ca^{2+} permeability of the ER membrane, cells were challenged with either ionomycin or thapsigargin, 6 and 10 min after suspension of the cells in Ca^{2+}-free buffer. Representative traces are shown in Fig. 2. The rate of Ca^{2+} loss from the internal store was quantified as the net change in $[Ca^{2+}]_i$ relative to the 2-min value (Fig. 3). Ca^{2+} release induced by ionomycin and thapsigargin decreased significantly faster in cells expressing NSP4 compared with that in cells expressing VP7 ($P < 0.05$), suggesting that the basal Ca^{2+} efflux of the internal store was increased by the expression of NSP4.

Partially purified NSP4 causes Ca^{2+} release from the internal Ca^{2+} stores. To determine the mechanism by which NSP4 increased ER Ca^{2+} permeability, partially purified (~70% pure) NSP4 protein was purified and added to fura-2-loaded noninfected Sf9 cells. Addition of NSP4 caused an increase in $[Ca^{2+}]_i$ that peaked within 1 min and subsequently declined with time toward the basal level (Fig. 4A, trace a). To determine if Ca^{2+} release from internal stores contributed to this response, NSP4 was added to cells in the absence of extracellular Ca^{2+} (Fig. 4A, trace b). Under this condition basal $[Ca^{2+}]_i$ was reduced, but addition of NSP4 increased $[Ca^{2+}]_i$ with a time course similar to that obtained with cells bathed in normal Ca^{2+} buffer. This result suggests that exogenous application of NSP4 releases Ca^{2+} from intracellular stores in Sf9 cells. The response to the NSP4 protein was dose dependent. At the higher concentrations, the transient nature of this response can clearly be seen; $[Ca^{2+}]_i$ peaks within 1 min and returns close to basal level within 3 min of addition of the protein (Fig. 4B, trace c). As a control, purified VP6 (3 μM) from recombinant-baculovirus-infected Sf9 cells was added to cells; no effect on the intracellular Ca^{2+} levels was observed (data not shown).

A region corresponding to amino acid (aa) residues 114 to 135 of NSP4 appears to correspond to the functional domain of NSP4 responsible for the $[Ca^{2+}]_i$ change. To determine if a specific domain in NSP4 is responsible for increasing $[Ca^{2+}]_i$ in Sf9 cells, several synthetic peptides were tested in fura-2-loaded, noninfected Sf9 cells. One of the peptides tested, $NSP4_{114–135}$, has a predicted amphipathic alpha-helix structure. Amphipathic helices have frequently been associated with biologically active peptides and proteins and were originally

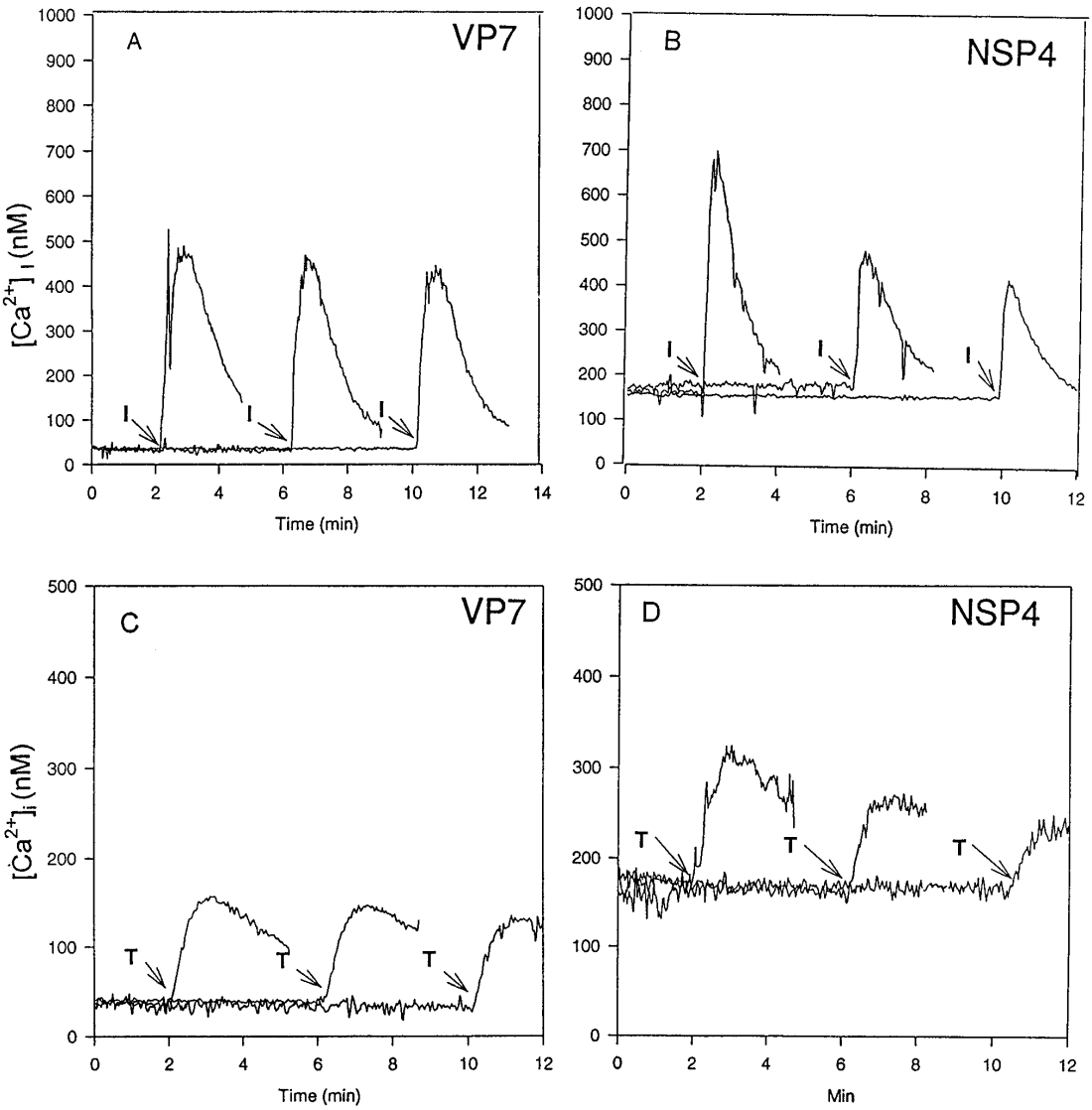

FIG. 2. The release of Ca^{2+} from the ionomycin-sensitive Ca^{2+} pool (A and B) or thapsigargin-sensitive pool (C and D) in Sf9 cells expressing VP7 (A and C) and NSP4 (B and D) was examined in fura-2-loaded Sf9 cells suspended in Ca^{2+}-free ECB containing 1 mM EGTA. The Sf9 cells were harvested and tested 36 h postinfection. Three traces are superimposed in each panel. At 2, 6, or 10 min, 2 μM ionomycin (I) or 200 nM thapsigargin (T; final concentrations) was added, and the fluorescence was recorded. Representative traces from three independent experiments are shown. Duplicate or triplicate traces were obtained in each independent experiment.

recognized as a protein motif that interacts with lipids (38, 39). A significant increase in $[Ca^{2+}]_i$ was observed immediately after the addition of $NSP4_{114–135}$ to Sf9 cells. The increased $[Ca^{2+}]_i$ induced by $NSP4_{114–135}$ was dose dependent (Fig. 5A and B), but the response was relatively sustained in comparison to that induced by the NSP4 protein. Increases in $NSP4_{114–135}$ concentration greater than 100 μM did not further increase the $[Ca^{2+}]_i$ levels. The increase in $[Ca^{2+}]_i$ induced by $NSP4_{114–135}$ was specific. Other synthetic peptides, including an N-terminal NSP4 peptide ($NSP4_{2–22}$), a peptide of NSP4 ($NSP4_{90–123}$) which had a higher predicted amphipathic score (AS; AS = 70) than did the $NSP4_{114–135}$ peptide, a Norwalk virus-specific peptide (NVSP), and a Norwalk virus C-terminal peptide (NVCP), did not cause an increase in $[Ca^{2+}]_i$ (Fig. 5C).

The $NSP4_{114–135}$ peptide also causes Ca^{2+} release from the ER. To determine whether $NSP4_{114–135}$ causes Ca^{2+} release from the internal Ca^{2+} pool, the $[Ca^{2+}]_i$ increase induced by $NSP4_{114–135}$ in Sf9 cells in the presence or absence of extracellular Ca^{2+} was measured. A 3.1-fold increase and a 2.66-fold increase in $[Ca^{2+}]_i$ were induced by $NSP4_{114–135}$ (100 μM) in cells incubated in the presence (Fig. 6A) and absence (Fig. 6B), respectively, of extracellular Ca^{2+}, suggesting that $NSP4_{114–135}$ caused Ca^{2+} release from the internal Ca^{2+} store. The observed increase of calcium release induced by $NSP4_{114–135}$ in the presence of extracellular Ca^{2+} was statistically significant ($P < 0.05$) compared with the increase in $[Ca^{2+}]_i$ observed in the absence of extracellular Ca^{2+}, suggesting that exogenously added peptide could mobilize Ca^{2+} from the extracellular medium as well as from the ER store.

To further confirm that $NSP4_{114–135}$ releases Ca^{2+} from the ER, a competitive depletion assay was performed. Ca^{2+} in the ER was depleted by thapsigargin or $NSP4_{114–135}$, and then $NSP4_{114–135}$ or thapsigargin was added 6 min later. If the

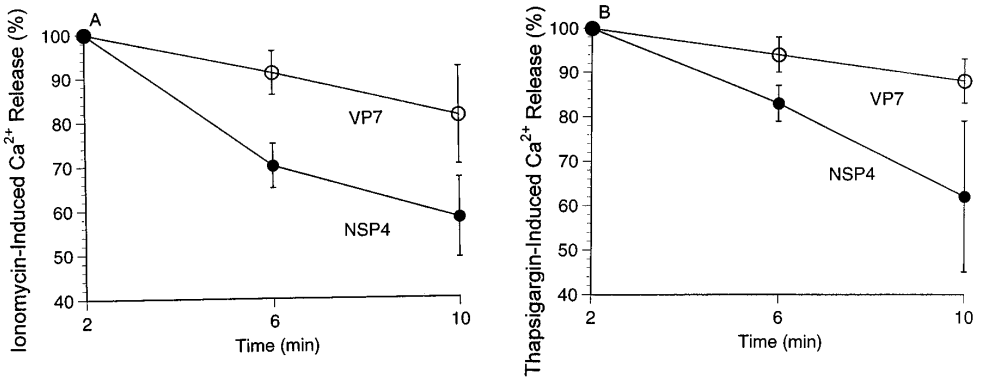

FIG. 3. Ca^{2+} efflux from ionomycin- and thapsigargin-sensitive internal stores. Basal $[Ca^{2+}]_i$ was subtracted from the peak change in $[Ca^{2+}]_i$ following addition of ionomycin or thapsigargin at 2, 6, and 10 min as described in the legend to Fig. 2. Each point represents the mean ± standard deviation net change in $[Ca^{2+}]_i$ obtained from three independent experiments for cells expressing NSP4 or VP7.

$NSP4_{114–135}$ peptide released Ca^{2+} from the same pool as thapsigargin, a significant decrease in the Ca^{2+} release induced by $NSP4_{114–135}$ would be observed when Ca^{2+} was depleted by thapsigargin. As seen in Fig. 7, the increase in $[Ca^{2+}]_i$ induced by $NSP4_{114–135}$ was significantly lower when Ca^{2+} in the ER was depleted with thapsigargin (a 1.2-fold increase in $[Ca^{2+}]_i$ in Fig. 7B, peak d) than in cells without depletion (a 2.9-fold increase in $[Ca^{2+}]_i$ in Fig. 7A, peak a). Furthermore, the increase in $[Ca^{2+}]_i$ induced by thapsigargin was significantly lower when the cells were first exposed to $NSP4_{114–135}$ (a 2.9-fold increase in $[Ca^{2+}]_i$ in Fig. 7A, peak b, versus a 3.3-fold increase in $[Ca^{2+}]_i$ in Fig. 7B, peak c). These results suggest that the $NSP4_{114–135}$-sensitive pool is a subset of the thapsigargin-sensitive compartment.

The Ca^{2+} release from the ER induced by exogenously added NSP4 and $NSP4_{114–135}$ can be blocked by U-73122, a PLC inhibitor. To determine if the Ca^{2+} released from the ER induced by exogenously expressed NSP4 and $NSP4_{114–135}$ was mediated by activation of a PLC pathway, the effect of NSP4 or $NSP4_{114–135}$ was examined in the presence of a PLC inhibitor, U-73122. As a positive control, the effect of U-73122 was examined in cells expressing the M5 muscarinic receptor. Stimulation of this receptor by specific agonists such as carbachol causes an increase in $[Ca^{2+}]_i$ in Sf9 cells and mammalian cells. A threefold increase in $[Ca^{2+}]_i$ was observed in cells expressing the M5 receptor following addition of the calcium-elevating agonist carbachol (Fig. 8A, trace a). However, when cells expressing M5 receptors were pretreated with U-73122 (10 μM), the carbachol-induced increase in $[Ca^{2+}]_i$ was attenuated (Fig. 8A, trace b). Furthermore, addition of U-73122 at the peak of the carbachol response resulted in the rapid return of $[Ca^{2+}]_i$ to the basal level (Fig. 8A, trace c). Similar results were observed for the effect of U-73122 on the increase in $[Ca^{2+}]_i$ produced by exogenous addition of NSP4 (not shown) or the $NSP4_{114–135}$ peptide (Fig. 8B). When $NSP4_{114–135}$ (40 μM) was added to noninfected Sf9 cells, $[Ca^{2+}]_i$ was increased twofold (Fig. 8B, trace a). Pretreatment with U-73122 attenuated the increase in $[Ca^{2+}]_i$ induced by $NSP4_{114–135}$ (Fig. 8B, trace b), and addition of U-73122 at the peak of the response to $NSP4_{114–135}$ returned $[Ca^{2+}]_i$ toward basal levels (Fig. 8B,

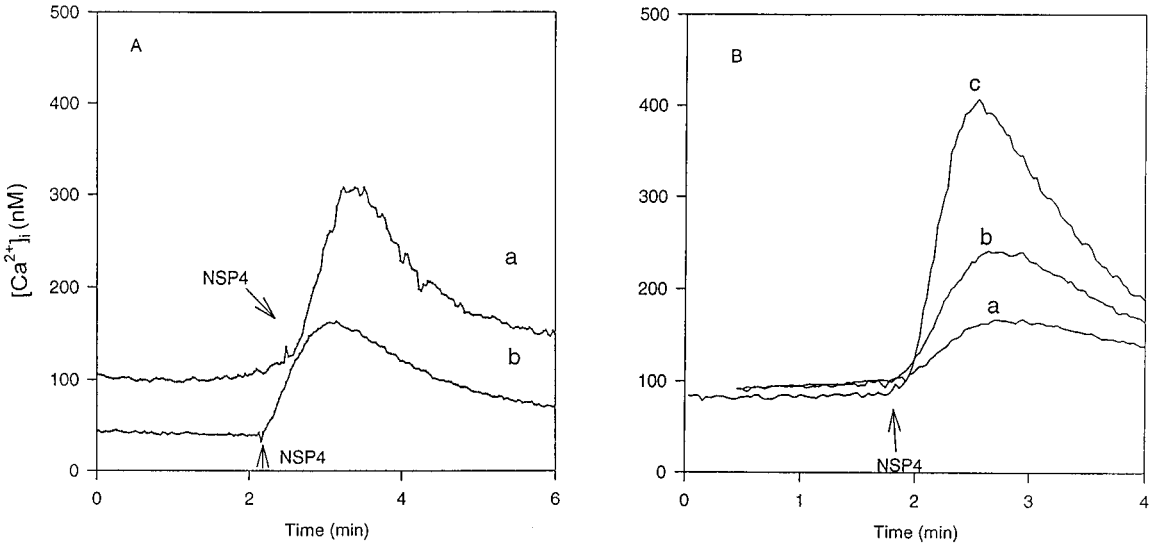

FIG. 4. Effect of NSP4 on $[Ca^{2+}]_i$ in Sf9 cells in the presence or absence of extracellular Ca^{2+}. (A) Fura-2-loaded noninfected Sf9 cells were suspended in ECB containing 10 mM Ca^{2+} (trace a) or Ca^{2+}-free ECB containing 1 mM EGTA (trace b). NSP4 (final concentration, 3 μM) was added at the time indicated in each trace. (B) Three traces are superimposed. NSP4 protein was added to fura-2-loaded Sf9 cells at a concentration of 1.5 μM (trace a), 3 μM (trace b), or 6 μM (trace c) at the time indicated. The cells were in ECB containing 10 mM Ca^{2+}.

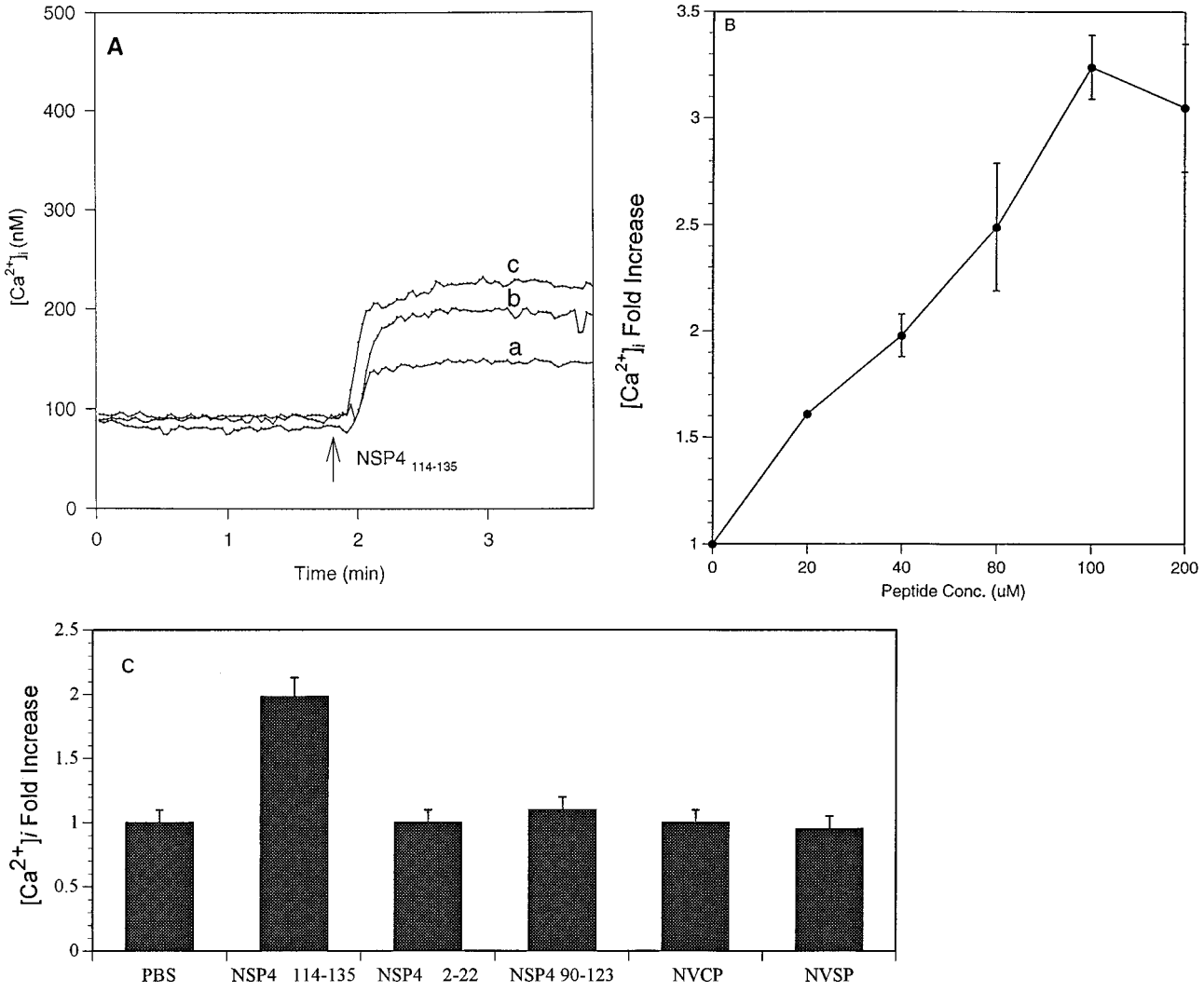

FIG. 5. Effect of a synthetic peptide, $NSP4_{114–135}$ on $[Ca^{2+}]_i$ in noninfected Sf9 cells suspended in ECB containing 10 mM Ca^{2+}. (A) Three traces are superimposed. $NSP4_{114–135}$ was added to fura-2-loaded noninfected Sf9 cells at the time indicated in each trace at a concentration of 20 μM (trace a), 40 μM (trace b), or 80 μM (trace c). (B) The peak increase in $[Ca^{2+}]_i$ from recordings as in panel A was expressed as the increase relative to the basal $[Ca^{2+}]_i$ before the addition of the peptide. The means ± standard deviations were calculated from triplicate traces obtained in a representative experiment. (C) The indicated synthetic peptides were added to fura-2-loaded Sf9 cells at a final concentration of 40 μM. The increase in $[Ca^{2+}]_i$ is shown relative to the value observed before the addition of the peptides (PBS).

trace c). These results suggest that exogenously added NSP4 protein and $NSP4_{114–135}$ peptide increase $[Ca^{2+}]_i$ in Sf9 cells by a PLC-dependent mechanism.

$NSP4_{114–135}$ increases $[Ca^{2+}]_i$ in cells expressing NSP4. If endogenously expressed NSP4 protein increases $[Ca^{2+}]_i$ through a mechanism similar to that of the $NSP4_{113–135}$ peptide, addition of the peptide to cells expressing NSP4 should have little effect on $[Ca^{2+}]_i$. As seen in Fig. 9 (trace a), the peptide clearly increased $[Ca^{2+}]_i$ in cells expressing NSP4. Furthermore, addition of U-73122 had no significant effect on $[Ca^{2+}]_i$ in cells expressing NSP4, suggesting that the elevated $[Ca^{2+}]_i$ is unrelated to PLC activity (Fig. 9, trace b).

DISCUSSION

Ca^{2+} plays a significant role in the rotavirus replication cycle. Shahrabadi and Lee (41) and Shahrabadi et al. (40) reported that reduction of extracellular Ca^{2+} levels decreased bovine rotavirus maturation and cytopathic effect. Michelan-

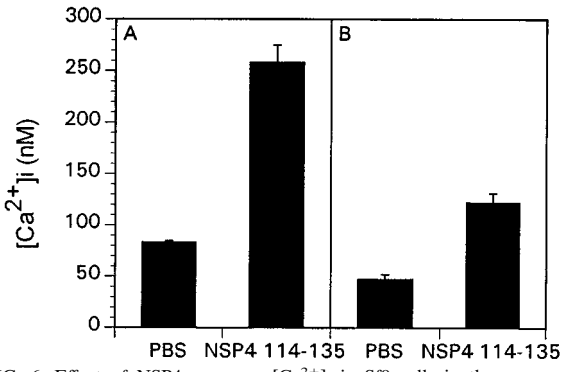

FIG. 6. Effect of $NSP4_{114–135}$ on $[Ca^{2+}]_i$ in Sf9 cells in the presence or absence of extracellular Ca^{2+}. Sf9 cells were suspended in ECB containing 10 mM Ca^{2+} (A) or Ca^{2+}-free ECB containing 1 mM EGTA (B). After 2 min, $NSP4_{114–135}$ (100 μM) was added to fura-2-loaded noninfected Sf9 cells at the time indicated, and the peak response was recorded. Values represent the means ± standard deviations from three independent experiments each performed in triplicate. The *t* test was used for statistical analysis.

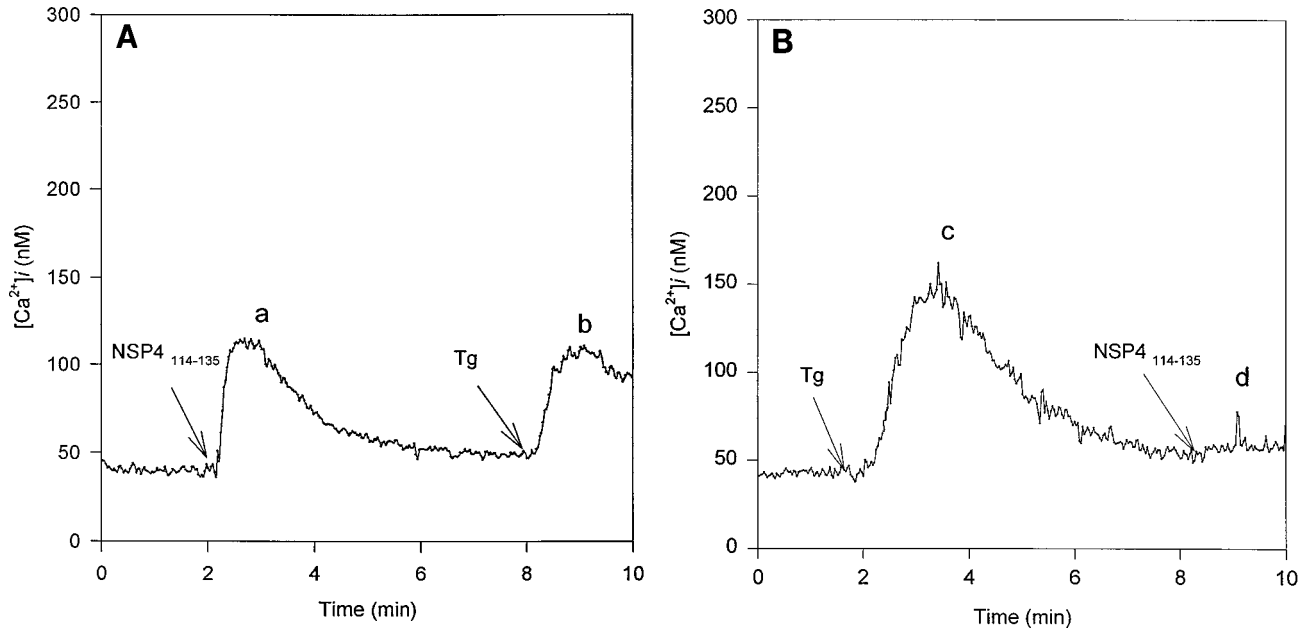

FIG. 7. $NSP4_{114–135}$ releases Ca^{2+} from the thapsigargin-sensitive pool. Sf9 cells were washed twice with Ca^{2+}-free ECB and suspended in Ca^{2+}-free ECB containing 1 mM EGTA. $NSP4_{114–135}$ (100 μM) and thapsigargin (Tg; 200 nM) were added at the times indicated in each panel. Traces shown are representative of three independent experiments. Duplicate or triplicate traces were obtained in each experiment.

geli et al. (29) reported that rotavirus protein synthesis was required to induce increased $[Ca^{2+}]_i$ and that this appeared to be related to cell death caused by rotavirus in MA104 cells. We have shown that the rotavirus nonstructural ER transmembrane glycoprotein, NSP4, is the only rotavirus protein that increases $[Ca^{2+}]_i$ when expressed in Sf9 insect cells (45).

The mechanism responsible for the increase in $[Ca^{2+}]_i$ by NSP4 is unknown. Eukaryotic cells have specific mechanisms for regulating intracellular Ca^{2+} levels. Intracellular Ca^{2+} homeostasis is controlled by the concerted operation of plasmalemmal Ca^{2+} translocation and intracellular compartmentalization (6). Ca^{2+}-ATPase pumps are responsible for seques-

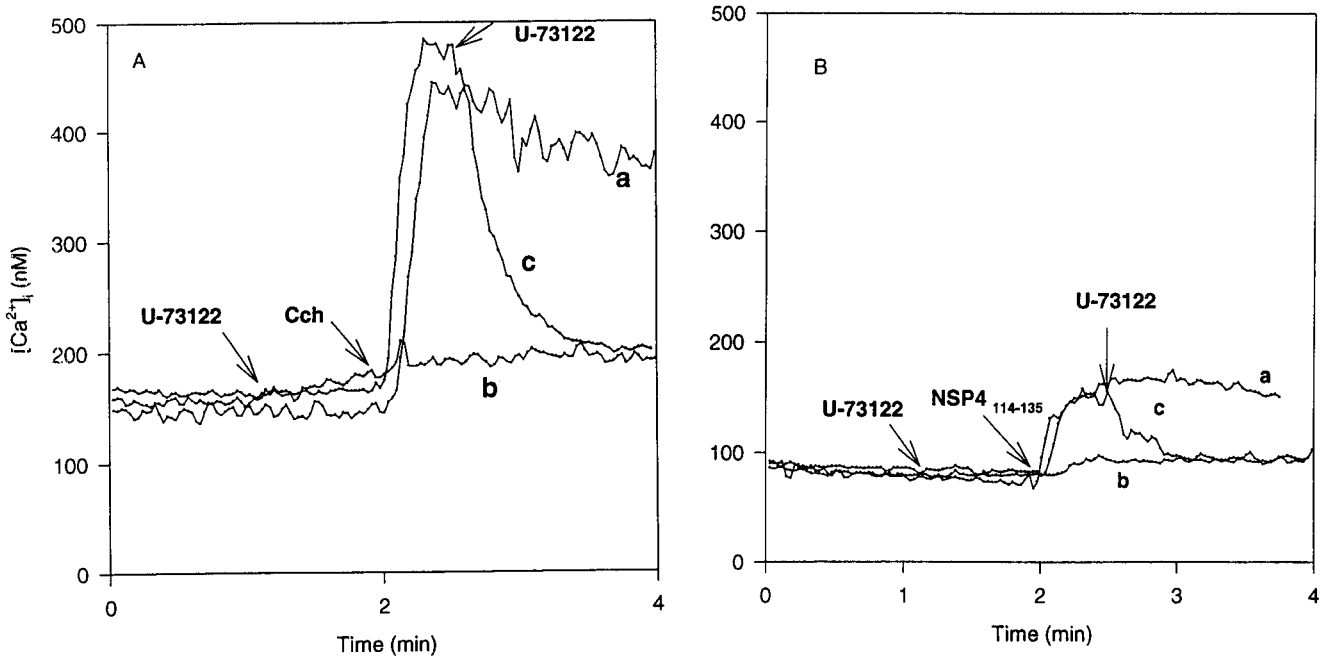

FIG. 8. Effect of U-73122, an inhibitor of PLC, on the change in $[Ca^{2+}]_i$ produced by $NSP4_{114–135}$. Three traces are superimposed in each panel. (A) Sf9 cells expressing the M5 muscarinic acetylcholine receptor were loaded with fura-2 and suspended in ECB. Carbachol (Cch; 100 μM) was added at the time indicated in the absence (trace a) or presence (trace b) of U-73122 (10 μM), or U-73122 was added at the peak of the response to Cch (trace c). (B) Same as panel A with $NSP4_{114–135}$ (40 μM) added instead of Cch. Traces are representative of three experiments each performed in triplicate.

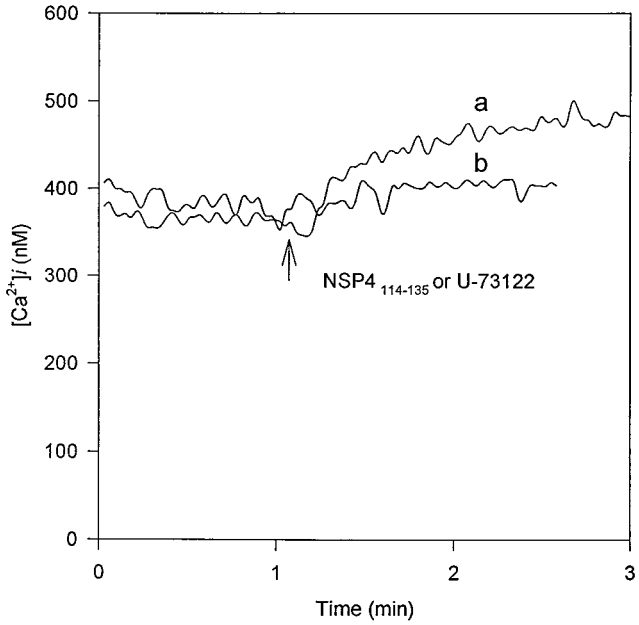

FIG. 9. Effect of $NSP4_{114–135}$ and U-73122 on $[Ca^{2+}]_i$ in Sf9 cells expressing NSP4. Fura-2-loaded Sf9 cells expressing NSP4 protein 36 h postinfection were suspended in normal ECB. Two traces are superimposed. At the time indicated, $NSP4_{114–135}$ (40 μM; trace a) or U-73122 (10 μM; trace b) was added and the fluorescence was recorded. The traces shown are representative of two experiments.

tering Ca^{2+} from the cytoplasm by transport either to the extracellular space or into intracellular Ca^{2+} pools, including the ER (7). The opening of Ca^{2+} channels on the cytoplasmic membrane or the ER membrane allows $[Ca^{2+}]_i$ to increase (30). The channels on the ER are regulated by $InsP_3$ produced by PLC which is activated by a surface receptor via a GTP-binding protein (4). Release of Ca^{2+} from internal stores by $InsP_3$ or by inhibition of the ER Ca^{2+}-ATPase pump by thapsigargin stimulates Ca^{2+} entry across the plasmalemma through an unknown mechanism and pathway. This Ca^{2+} entry is important for refilling the internal Ca^{2+} store following termination of receptor stimulation. The increased $[Ca^{2+}]_i$ seen in NSP4-expressing Sf9 cells could result from altered Ca^{2+} fluxes across the plasmalemma or the ER membrane either directly (e.g., a channel formed by the NSP4 protein) or via interaction with one or more steps in the normal Ca^{2+} signal transduction cascade.

To understand the mechanism of NSP4 action on cellular Ca^{2+} homeostasis and to determine the source of increased $[Ca^{2+}]_i$ in cells infected with recombinant baculovirus expressing NSP4, we first measured the influx of Ba^{2+}. To avoid any potential nonspecific effects of baculovirus infection on cytoplasmic membrane permeability, the Ba^{2+} influx in infected cells was measured at a relatively early stage (36 h postinfection). Previously, we had shown that increases in $[Ca^{2+}]_i$ were maximal at this time postinfection and there were no effects in cells infected with wild-type baculovirus (45). In addition, the Ca^{2+} in the ER pool at this time postinfection was the same in cells expressing NSP4 or VP7. In uninfected cells, Ba^{2+} influx was small. In all Sf9 cells infected with recombinant baculoviruses which expressed NSP4, VP7, VP6, and the M5 receptor (data not shown) and a recombinant baculovirus lacking an insert, a slight increase in basal Ba^{2+} influx was observed. This small increase in divalent cation influx probably reflects a nonspecific effect of the baculovirus infection. Although Ba^{2+} influxes in cells expressing NSP4 and cells expressing VP7 were quite similar, the basal levels of Ca^{2+} in cells expressing NSP4 were significantly higher than those in cells expressing VP7. In addition, although the basal levels of $[Ca^{2+}]_i$ in cells expressing NSP4 and in cells expressing *trpl* were similar, the Ba^{2+} influx was significantly higher in cells expressing *trpl* than in cells expressing NSP4. These results suggest that the increase in $[Ca^{2+}]_i$ in Sf9 insect cells expressing NSP4 is not due to an increased influx of Ca^{2+} from the extracellular space. These experiments do not eliminate the possibility that expression of NSP4 activates or forms a channel in the plasmalemma that is permeable to Ca^{2+} but not Ba^{2+}. However, preliminary patch clamp experiments comparing the plasma membrane permeability of Sf9 cells expressing NSP4 with that of cells expressing VP7 exclude this possibility (data not shown).

Michelangeli et al. (29) reported that an increase in Ca^{2+} influx in porcine rotavirus (OSU strain)-infected MA104 cells reflects an increase in the cytoplasmic membrane permeability to Ca^{2+} caused by rotavirus infection. The basal $[Ca^{2+}]_i$ was increased as early as 5 h postinfection and reached a plateau (fourfold increase) at 8 h. Although Ca^{2+} influx was not measured, $^{45}Ca^{2+}$ uptake 10 min after addition of isotope to OSU-infected MA104 cells was greater than that observed in noninfected cells. These authors concluded that the increased $^{45}Ca^{2+}$ uptake represents an increased Ca^{2+} influx and enhanced accumulation of Ca^{2+} in the ER in OSU-infected MA104 cells (29). However, it has been shown that depletion of Ca^{2+} from the ER can stimulate Ca^{2+} influx in mammalian cells and Sf9 cells (19, 23, 25, 37). Therefore, the increased Ca^{2+} uptake observed in rotavirus-infected MA104 cells might be a secondary, rather than a primary, event, due to alterations in Ca^{2+} homeostasis at the ER membrane. Interpretation is further complicated by the infection time course. Changes in $[Ca^{2+}]_i$ at early times postinfection, which could play a role in viral maturation, may occur through mechanisms entirely different from those responsible for the changes in $[Ca^{2+}]_i$ seen later in the infection cycle. The later changes may be associated with virus-induced cell death. Importantly, the early changes in $[Ca^{2+}]_i$ could contribute to alterations in transepithelial ion movement which can be triggered by elevations in $[Ca^{2+}]_i$. Our initial finding that expression of NSP4 gives rise to an increase in basal $[Ca^{2+}]_i$ suggests that it plays an important role in these early changes in Ca^{2+} homeostasis.

Although endogenous expression of NSP4 does not seem to increase the plasmalemma permeability for Ca^{2+}, the expression of NSP4 in the Sf9 cell is associated with a change in the ER membrane. When cells were incubated in the absence of Ca^{2+} and then challenged with either ionomycin or thapsigargin, the total amount of releasable Ca^{2+} and the amount of Ca^{2+} in the ER decreased over time in cells expressing NSP4 but not in cells expressing VP7. This was not due to a decreased pumping activity in the NSP4 cells, since sequestered Ca^{2+} was the same in both cell types before (or up to 2 min after) incubation in Ca^{2+}-free buffer. Thus, it appears that expressed NSP4 increases basal ER permeability to Ca^{2+}. The observed decrease in the total amount of releasable Ca^{2+} in the ER amount over time in cells expressing NSP4 and in media lacking extracellular Ca^{2+} can be explained in the context of a pump-leak model. In this model, the elevated $[Ca^{2+}]_i$ in cells expressing NSP4 at 36 h postinfection without a significant decrease in the ER Ca^{2+} store is only seen in cells incubated in medium containing Ca^{2+} or at early times (2 min) after placement of the cells in medium lacking Ca^{2+}. The increase in Ca^{2+} efflux from the ER causes an increase in $[Ca^{2+}]_i$ which activates the Ca^{2+} pump of the ER. A new steady state is achieved when uptake equals efflux. The conse-

quence is an increase in $[Ca^{2+}]_i$ with little change in stored Ca^{2+}. However, as the permeability of the ER continues to increase, $[Ca^{2+}]_i$ will increase until the pump is saturated. Further increases in ER Ca^{2+} permeability and $[Ca^{2+}]_i$ cannot be balanced by the pump, efflux from the ER will exceed influx, and stored Ca^{2+} will decline with time. This presumably occurs late in the infection cycle and is probably associated with a loss of cell viability and death. Although the effect of NSP4 on ER permeability can explain the change in $[Ca^{2+}]_i$, the actual mechanism by which NSP4 alters ER permeability is unknown. NSP4 may (i) form a cation channel in the ER membrane, (ii) increase Ca^{2+} leak through the $InsP_3$ receptor, and/or (iii) have action on the phospholipids leading to membrane disruption.

In contrast to the effects of endogenously expressed NSP4, several lines of evidence suggest that exogenous application of purified NSP4 protein mobilizes Ca^{2+} from internal stores in Sf9 cells. First, exogenous addition of partially purified NSP4 protein to Sf9 cells resulted in an increase in $[Ca^{2+}]_i$ in both the presence and absence of extracellular Ca^{2+}. This effect of exogenous NSP4 was blocked by the PLC inhibitor U-73122. This compound is a novel organic compound that specifically blocks receptor-stimulated PLC activity and the associated increases in $[Ca^{2+}]_i$ in a variety of cell types (5, 46–48). Similar results were obtained in Sf9 cells expressing the M5 muscarinic receptor. The change in $[Ca^{2+}]_i$ produced by carbachol was blocked by U-73122 at a concentration shown in many other cells to block PLC activity. These results suggest that exogenous NSP4 increases $[Ca^{2+}]_i$ by a PLC-dependent mechanism. It seems likely that exogenously applied NSP4 protein is acting at the extracellular membrane surface. Whether this is a receptor and G-protein-mediated event remains to be determined.

In order to identify the structural features of NSP4 responsible for the increase in $[Ca^{2+}]_i$ observed with exogenous application of the protein, we examined several peptides. A predicted amphipathic alpha-helix structure is present between aa 90 and 135 of NSP4. Amphipathic alpha-helical structures have been reported to function in lipid association, membrane perturbation, hormone receptor catalysis, and transmembrane signal transduction (38). Several pieces of evidence suggest that this domain is part of the functional domain responsible for the Ca^{2+} release. First, when this domain ($NSP4_{114–135}$) with an AS of 24 (27) was added to cells, a significant increase in $[Ca^{2+}]_i$ levels was observed. Other peptides, including an N-terminal peptide of NSP4 with an AS of 6.5, another NSP4 peptide with an AS of 70, a Norwalk virus-specific peptide (NVSP), and a Norwalk virus C-terminal peptide (NVCP) which also has an amphipathic alpha-helix structure with an AS of 25, did not cause a significant increase in $[Ca^{2+}]_i$ at the same concentration (40 μM) or at higher concentrations (100 μM) (data not shown). Second, the effect of $NSP4_{113–135}$ was attenuated by inhibition of PLC by U-73122, suggesting that the peptide acts in a fashion similar to that of the native NSP4 protein. Third, when $NSP4_{114–135}$-specific antiserum was incubated with the partially purified NSP4, the Ca^{2+}-releasing effect of NSP4 was totally blocked (data not shown). These results suggest that the amino acid residues 114 to 135 of NSP4 correspond to a region in the functional domain responsible for the $[Ca^{2+}]_i$ change.

$NSP4_{114–135}$ partially depleted the thapsigargin-sensitive calcium pool (ER) whereas, thapsigargin completely eliminated the response of cells to $NSP4_{114–135}$. This result demonstrates that the peptide mobilizes intracellular Ca^{2+} and suggests that $NSP4_{114–135}$ releases calcium from a subcompartment of the thapsigargin-sensitive pool. However, in the presence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ response of the cells to exogenous $NSP4_{114–135}$ is sustained for a longer period of time (Fig. 5A) and exhibits a greater increase (Fig. 6) compared with the values obtained in the absence of extracellular Ca^{2+} (Fig. 6 and 7A). Thus, at least part of the response is dependent on extracellular Ca^{2+}. This is not unexpected, since most agonists that mobilize intracellular Ca^{2+} through a PLC-dependent mechanism (see above) also stimulate Ca^{2+} influx from the extracellular medium, i.e., through the so-called capacitative Ca^{2+} entry mechanism (33, 34).

The partially purified NSP4 protein induced Ca^{2+} release in Sf9 cells more efficiently than did the synthetic peptide. The maximal Ca^{2+} change induced by the partially purified NSP4 was 4.5-fold at a final concentration of 6 μM, and the maximal Ca^{2+} change induced by $NSP4_{114–135}$ was 3.3-fold at a much higher concentration (100 μM). Possible explanations for these results include the following: (i) the peptide does not represent the entire functional domain of NSP4, (ii) the peptide does not adopt the correct conformation, (iii) other remote domains in NSP4 may also play a role in $[Ca^{2+}]_i$ change, and (iv) tiny amounts of cellular proteins in the partially purified NSP4 enhance the ability of NSP4 to induce Ca^{2+} release. Most likely, the peptide represents only part of the complete functional domain. This idea is supported by the fact that the calculated AS is 70 for aa 95 through 119 of NSP4. Therefore, we propose that the entire functional domain responsible for the observed Ca^{2+} change begins at or immediately after aa 95 and ends around aa 135 of NSP4. The fact that a peptide from aa 90 through 123 did not alter intracellular Ca^{2+} levels suggests that the functional domain begins after aa 123 or essential functional residues are present between aa 123 and 135.

Although the PLC inhibitor U-73122 inhibited the Ca^{2+} release induced by exogenously added NSP4 and $NSP4_{114–135}$, the administration of U-73122 to cells expressing NSP4 endogenously had no effect on the increased $[Ca^{2+}]_i$ levels. In addition, the treatment of cells expressing NSP4 with the $NSP4_{114–135}$ peptide caused a further increase in $[Ca^{2+}]_i$ levels. Taken together, our results clearly demonstrate that expressed NSP4 and exogenously applied NSP4 protein or peptide act to increase $[Ca^{2+}]_i$ via different mechanisms.

Although the results reported here describe responses in insect cells, we propose that similar responses occur in mammalian cells. However, this will need to be validated. In particular, it will be necessary to confirm that the conclusions made here with exogenously added NSP4 or peptide reflect what happens in mammalian cells, in which NSP4 is expressed endogenously. We propose that expression of NSP4 during rotavirus infection alters Ca^{2+} homeostasis at the ER level and that this plays an important role in the viral maturation process. However, at later times postinfection, NSP4 protein or peptide fragments of NSP4 gain access to the extracellular solution and stimulate neighboring cells. This results in activation of the Ca^{2+} signal transduction cascade which may alter Ca^{2+}-dependent ion transport across the intestinal epithelium and affect normal cellular function. Although NSP4 is an ER membrane protein, small amounts of NSP4 can be detected in the supernatant of recombinant-baculovirus-infected Sf9 cells late in infection (49), and specific cleavage products of NSP4 have been reported in cells expressing NSP4 (45). Topographic models of NSP4 suggest that the C terminus of NSP4 is in the cytoplasm (3, 9), and a specific cleavage of NSP4 might result in the release of the C terminus of NSP4 from the ER membrane. This released C-terminal segment, which would contain aa 114 to 135, may allow the proposed functional domain to activate the PLC-mediated pathway. It is also possible that NSP4 activates the PLC-mediated pathway

inside of the cells. PLC has been localized in cytosolic fractions (20, 21, 43) and the ER membrane (21). The role of PLC-mediated and other pathways in the rotaviral replication cycle and in $[Ca^{2+}]_i$ homeostasis in rotavirus-infected cells, and perhaps in neighboring epithelial cells, requires further investigation.

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