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Mouse Hepatitis Virus Infection Induces an Early, Transient Calcium Influx in Mouse Astrocytoma Cells

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Mouse hepatitis virus (MHV), a murine coronavirus, utilizes murine carcinoembryonic antigens as receptors. The events that follow virus–receptor binding and eventually lead to virus entry are poorly understood. We studied the possible effects of MHV infection on intracellular calcium in a mouse astrocytoma cell line. Using the calcium-sensitive dye fluo-3 and confocal laser scanning microscopy, we found that MHV strain JHM induced an immediate (within 20 s) and transient (lasting no longer than 2 min) calcium increase in about 5% of the infected cells. The calcium increase was blocked by antibodies against the viral spike protein, suggesting that it was specifically triggered by the interaction of the viral spikes with cells. It was also inhibited by L-type calcium channel blockers and was not detected in calcium-free medium, suggesting that the calcium increase was caused by calcium influx from the extracellular medium. Studies of the kinetics of viral replication by immunofluorescence staining of the viral nucleocapsid protein revealed that at 3 h postinfection there was roughly the same percentage of cells (5%) that produced the viral protein as the percentage of cells that had responded with a calcium signal. This finding and the virus dilution studies together suggest that calcium responders may represent cells that had been infected with multiple viruses and undergone rapid viral replication. Furthermore, calcium channel blockers, including verapamil and cadmium chloride, and the calcium chelator EGTA inhibited virus infection. Therefore, the transient intracellular calcium increase reported here may be an early signaling event associated with virus infection.

Key Words: **mouse hepatitis virus; mouse astrocytoma cells; intracellular calcium; fluo-3; confocal laser scanning microscopy; immunofluorescence.**

INTRODUCTION

Murine coronavirus mouse hepatitis virus (MHV) is an enveloped virus with a large, 31-kb, positive-stranded RNA genome that is known to cause hepatitis, vasculitis, encephalitis, and demyelination in mice [1]. MHV utilizes members (principally biliary glycoproteins) of the murine carcinoembryonic antigen (CEA) family as receptors, with the prototypic receptor being $MHVR_1$ [2]. Other identified murine receptors include $MHVR_2$, BGP2, and bCEA [3–5]. The virus binding to the receptors is mediated by the viral envelope spike (S) protein. It is not known how S protein–receptor interaction leads to virus entry by either endocytosis or membrane fusion [6]. Conceivably, virus binding triggers a series of signal transduction events that culminate in virus entry; however, the precise molecular mechanism involved has not yet been characterized.

Free calcium functions as an intracellular second messenger in various pathways. As a prerequisite for this function, the cell must precisely regulate its calcium level. A sustained increase in intracellular free calcium is often a sign of increased membrane permeability, loss of energy, and finally cell death. Viruses have been shown to influence intracellular free calcium in various ways. An immediate calcium response after virus or envelope protein binding has been reported for human immunodeficiency virus (HIV) and influenza virus [7–11]. Whether these calcium responses are a prerequisite of viral infection or a mechanism of viral pathogenesis is not clear. Elevation in intracellular free calcium during the later stage of the viral life cycle also has been shown for rotaviruses or polioviruses, which result from the interaction of the newly synthesized viral proteins with the cell membrane, leading to calcium influx from the extracellular medium [12, 13].

To understand the mechanism of MHV entry, we studied the possible effects of MHV on intracellular free calcium in a mouse astrocytoma cell line using the calcium-sensitive dye fluo-3 and confocal laser scanning microscopy. We found that MHV induced an immediate (within 20 s) and transient increase in intracellular free calcium upon the addition of the virus to

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the cells. This increase was caused by calcium influx through the cell membrane and was sensitive to the L-type calcium channel blockers nifedipine and verapamil. Furthermore, calcium channel blockers inhibited virus infection. Therefore, calcium influx may represent the earliest detectable molecular event following virus infection.

MATERIALS AND METHODS

Cell culture, virus, and antibodies. DBT cells, a murine astrocytoma cell line [14], were grown in Eagle's minimal essential medium (MEM) supplemented with heat-inactivated 7.5% newborn calf serum, 1% glutamine, 1% penicillin/streptomycin, and tryptose phosphate broth. In this study, MHV strain JHM-DL, propagated in DBT cells under serum-free conditions, was used [15]. MEM and Joklik-modified MEM were obtained from GIBCO Life Technologies (Gaithersburg, MD). Monoclonal antibodies specific for the S and nucleocapsid (N) protein of JHM have been described earlier [16].

Calcium measurements and imaging. To determine intracellular calcium, 10^4 cells/well were plated in eight-well coverglass tissue culture chambers (Nunc Inc., Naperville, IL) 24 h before measurements. Cells were loaded with 2 μM fluo-3/AM (Molecular Probes, Eugene, OR) in serum-free MEM at room temperature for 20 min, and fluorescence measurements of intracellular calcium ($[Ca^{2+}]_i$) were performed within 2 h after dye loading. Chambers were mounted on the microscope stage of an inverted confocal laser scanning microscope (LSM410; Zeiss, Germany) equipped with an external argon–krypton laser. Time series of images of whole microscopic fields were taken and stored for further analysis. In each experiment, two images of nonstimulated cells (baseline calcium) were recorded before addition of virus, antibodies, or control liquids. MHV prepared in serum-free MEM medium was added to the cells with multiplicity of infection (m.o.i.) ranging from 1 to 10 pfu/cell. Images were printed with a Fujix Pictrography 3000 color printer (Fujifilm, Japan) using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Calculation of calcium concentration. Calculation of the intracellular calcium concentration was performed *in situ* as described [17]. After recording of the MHV-induced calcium response, Ca^{2+} ionophore ionomycin (Sigma, St. Louis, MO) was added to the cells in the presence of extracellular Ca^{2+} to saturate the intracellular dye and thereby obtain the maximal fluorescence (F_{max}). Subsequently, the minimal fluorescence (F_{min}) was measured after addition of an excess of EGTA (5 mM). Fluorescent intensities (F) were then translated into $[Ca^{2+}]$ using the equation $[Ca^{2+}] = K_d(F\text{-}F_{min}/F_{max}\text{-}F)$, where K_d is the dissociation constant [18]. K_d is 320 nM for fluo-3 as indicated by Molecular Probes. Calculations were performed separately for selected equal-size areas (pixels/pixels) located in the cytoplasm and in the nucleus because of the heterogeneity of the fluorescence within the image.

Statistical analysis. The percentage of cells showing an increase in intracellular free calcium was calculated from intensity-based area measurements of microscopic fields using the image analysis program KS 300 (Kontron, Germany). Three thresholded images (pixels × pixels) were calculated per time series and used for estimation: (I) The *total cellular area* was calculated from an image of nonstimulated cells before the measurement (baseline calcium), using a low threshold [10 arbitrary units (AU) over background intensity]. (II) From the same image of nonstimulated cells, an "area of high intensity" using a high threshold (twofold higher than the mean intensity of nonstimulated cells) was calculated. This area was used for correction of the "*responding area*" (III) for high-intensity values occasionally observed in nonstimulated cells due to dye compartmentalization. (III) The "*responding area*" was calculated from the sum of all images of a time series using a high threshold (twofold higher than the mean intensity of nonstimulated cells). The calculation of the "*responding area*" was done based on the sum of all images rather than using a single time point because the cells responded to the addition of the virus at different time points. Finally, the "*responding cellular area*" was determined by subtraction of area (II) from the responding area (III), and expressed as percentage of the total cellular area (I).

Immunofluorescence. DBT cells in eight-well coverglass tissue culture chambers (10^4 cells/well) were infected with MHV (at a m.o.i. of 5) in serum-free MEM at 37°C for 1 h. After washing, the cells were incubated at 37°C for an additional 3 h in MEM supplemented as described above. Immunofluorescence staining of the cells was performed after fixation in 2% paraformaldehyde in PBS, followed by permeabilization with 0.5% Triton X-100 in PBS, using a monoclonal antibody against the N protein [19] and a rhodamine-conjugated anti-mouse secondary antibody (Jackson Immuno Research, West Grove, PA). Images of 512 × 512 pixels were recorded with a confocal laser scanning microscope and printed as described above. The percentage of stained cells was calculated essentially as described under *statistical analysis.*

Virus inhibition studies. DBT cells were grown in 60-mm plates to 80% confluence, washed with MEM, and incubated with various inhibitors at the desired concentration for 2 h at 37°C. Subsequently, cells were washed with MEM, and virus mixed with inhibitors (in 500 μl total volume) was added to the cells at a m.o.i. of 0.05 to 1 and incubated at 37°C for 2 h. The cells were washed again with MEM and incubated with MEM containing 1% newborn calf serum and the inhibitors for an additional 7 h at 37°C. The supernatant was harvested at 9 h postinfection and used for plaque assay on DBT cells to determine virus titers. The concentrations of the inhibitors used were predetermined to be free of cytotoxicity.

RESULTS

Calcium measurements in DBT cells after MHV infection. We investigated whether MHV infection induced changes in $[Ca^{2+}]_i$ in mouse astrocytoma cells, using the calcium indicator fluo-3 and confocal laser scanning microscopy. Under resting conditions, fluo-3-loaded DBT cells showed a homogeneous fluorescence in the cytoplasm and a 1.2- to 1.3-fold higher fluorescence intensity in the nucleus (Fig. 1A, 0 s). Addition of MHV (at a m.o.i. of 5) to DBT cells evoked in some cells an immediate increase in fluo-3 fluorescence in the cytoplasm as well as in the nucleus that ranged between 1.5- and 4.7-fold (mean, 2.9; SD, 0.8; $n = 26$ cells) of the basal intensity level (Figs. 1A and 2A). An increase in fluorescence could be detected as early as 12 s after the addition of the virus and was most intense at the 24-s time point. The fluorescence intensity gradually decreased within the next minute and returned to the resting level, in general, within 120 s after the addition of the virus (Fig. 1A). This result shows that the addition of MHV to DBT cells invoked an immediate and transient increase of the intracellular calcium level in some cells. Since the fluorescence was not homogeneously distributed within individual cells, changes in fluorescence intensity and hence in $[Ca^{2+}]_i$ were separately analyzed in equal-sized regions (pixel by pixel) of the cytoplasm and of the nucleus. Analysis of single cells showed that the increase of fluorescence intensity after the addition of virus occurred in a similar manner in both compartments, with higher inten-

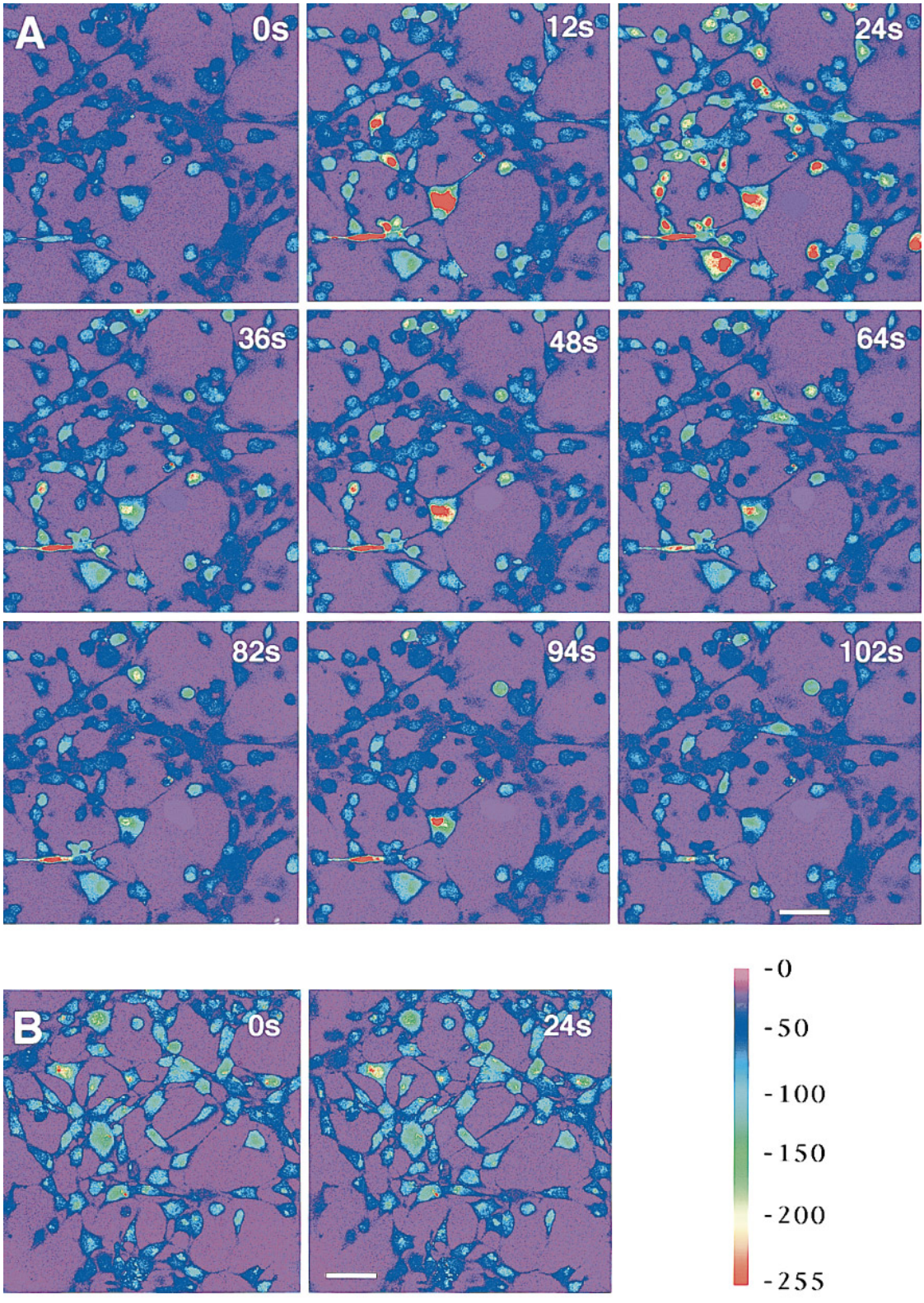

FIG. 1. Microscopic analysis of the intracellular calcium increase in mouse astrocytoma cells after MHV infection. (A) Fluo-3-loaded DBT cells were recorded every 4 s over 120 s. MHV was added at time point 4 s. The gallery shows selected images from the time series at the indicated time points. (B) MHV was preincubated with α-S protein mab at 37°C for 1 h before addition to DBT cells and the recording of the intracellular calcium as under A. Color bar displays gray levels (relative fluorescence intensities) that correspond to colors. Bars, 50 μm.

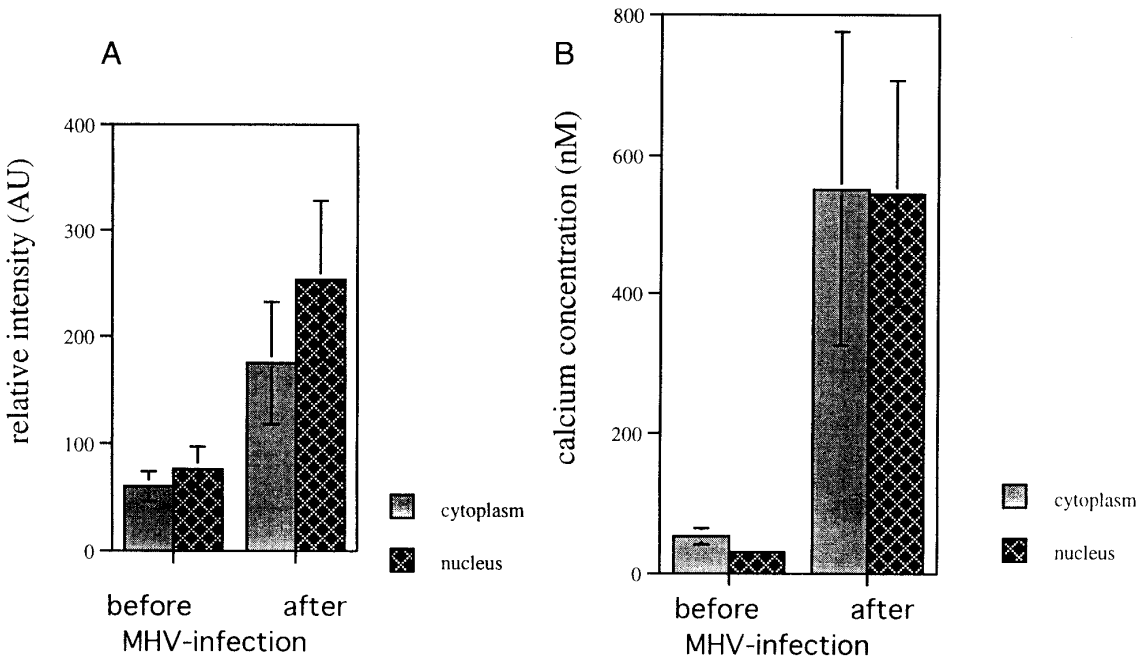

FIG. 2. Mean calcium concentration in cytoplasmic and nuclear areas of DBT cells (n = 24) before and after addition of MHV (5 pfu/cell). The measurements were done on the cells at the resting level (as, for example, 0 s in Fig. 1A) and peak fluorescence intensity (as, for example, 24 s in Fig. 1A). Mean fluo-3 intensities in cytoplasm and nucleus were measured in equal-sized areas (pixel/pixel) (A) and transformed into calcium concentrations after calibration (B).

sity values for the nuclear compartment (Fig. 2A). However, transformation of the fluorescence intensities into $[Ca^{2+}]_i$ after calibration of the respective areas (see Materials and Methods) demonstrated that baseline $[Ca^{2+}]_i$ is slightly lower in the nucleus than that in the cytoplasm (Fig. 2B). After the addition of MHV, the $[Ca^{2+}]_i$ is 10-fold higher than that in control cells. A similar difference was observed in both the nucleus and the cytoplasm (Fig. 2B).

There were some variations in the pattern of $[Ca^{2+}]_i$ increases in individual cells in response to the addition of MHV. In most of the cells, $[Ca^{2+}]_i$ rose sharply and transiently as a single peak, which subsided to the resting level after approximately 20 s and remained at that level thereafter (Fig. 3A). In some cells, multiple oscillations of $[Ca^{2+}]_i$ were observed (Fig. 3B). In either case, $[Ca^{2+}]_i$ spikes usually occurred within 20 s after the addition of the virus. Occasionally, however, late responders were observed, in which the sharp increase of $[Ca^{2+}]_i$ did not occur until 1 or 2 min after the addition of virus. Nevertheless, in all cases, cells returned to the resting calcium level within 4 min; no longer lasting changes in $[Ca^{2+}]_i$ could be observed (data not shown).

The percentage of DBT cells that responded, expressed as "responding cellular area" (see Materials and Methods), ranged from 3 to 15%, depending on the experiment, with a mean of 5.04% (SD, 5.7; n = 14). Figure 1A shows a time series of a selected field of view with approximately 15% responding cells. A higher virus titer (m.o.i. of 10) only marginally increased the percentage of responding cells (data not shown), but decreasing virus titers resulted in an exponential reduction of the number of responding cells. For example, at a m.o.i. of 1, the percentage of responding cells was reduced to 0.29% (SD, 0.63; n = 11), and a further dilution to 0.5 pfu/cell practically failed to evoke any calcium response in DBT cells. This result suggests that multiple virus particles per cell may be necessary for inducing $[Ca^{2+}]_i$ changes. In all subsequent experiments, MHV infection was performed at a m.o.i. of 5, and every experiment was repeated at least five times.

Specificity of the MHV-induced calcium increase. To assess the specificity of the $[Ca^{2+}]_i$ increases observed following the addition of MHV to DBT cells, we first attempted to rule out the possibility that calcium increases were caused by components of media. Cells were incubated with the conditioned medium from DBT cell culture; no change in intracellular calcium was observed (Table 1). Furthermore, a related coronavirus, bovine coronavirus, which belongs to the same serogroup as MHV but cannot infect DBT cells [6], did not induce any changes in the intracellular calcium level, suggesting that this calcium change was specifically associated with MHV. To further demonstrate this specificity, MHV was preincubated with a monoclonal antibody against the JHM spike protein (α-S protein mab) that is known to neutralize MHV infec-

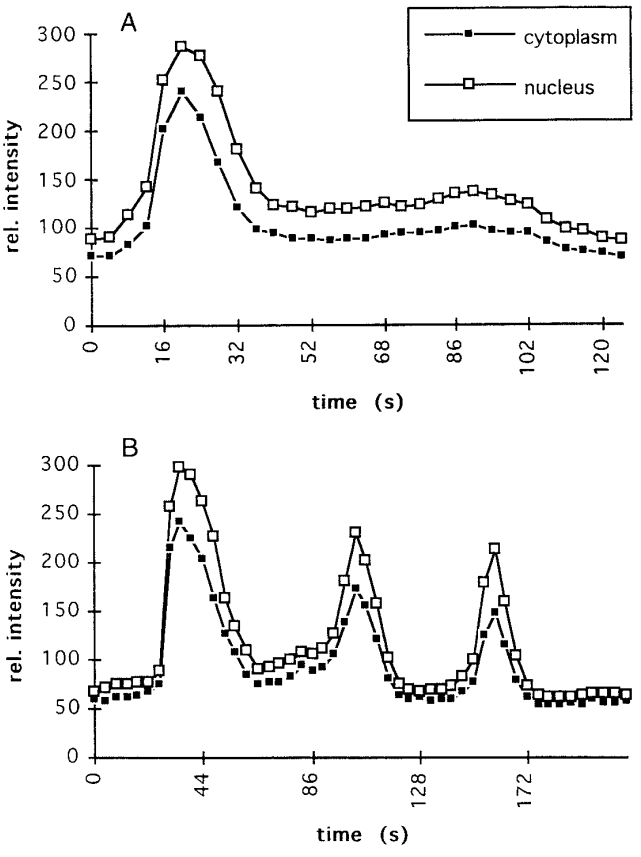

FIG. 3. Time course of intracellular calcium changes in individual MHV-infected cells: Single peak pattern (A) and oscillatory pattern (B). Images were taken every 4 s, and MHV was added to DBT cells at time point 4 s. Shown are relative intensities in equal-sized areas in the cytoplasm and in the nucleus of two individual cells.

tion of DBT cells [16]; the calcium increase was abolished (Fig. 1B and Table 1). In contrast, preincubation of the virus with a monoclonal antibody directed against an internal viral protein (α-N protein mab) or an unrelated antibody (α-MHC class II mab) did not inhibit the MHV-induced intracellular calcium response. These data suggest that the observed rise in $[Ca^{2+}]_i$ following the addition of MHV to DBT cell culture was most likely due to a specific interaction of the viral spike protein with the cells.

Source of MHV-induced calcium increase. A rise in intracellular calcium can be caused by Ca^{2+} mobilization from intracellular stores and/or Ca^{2+} influx through calcium channels in the cell membrane. To distinguish between these two possibilities, we examined whether MHV could induce an intracellular calcium increase if the cells were bathed in medium lacking Ca^{2+} (Joklik-modified MEM) prior to infection. Incubation of DBT cells in this calcium-free MEM for 10 min did not alter the resting $[Ca^{2+}]_i$ levels (in comparison to those of control cells bathed in regular MEM). When MHV (virus preparation in serum-free MEM

TABLE 1

MHV Induces a Specific calcium Increase in DBT Cells

Cells challenged with	Cellular area responding (%)[a]
Conditioned medium	0
MHV (mouse hepatitis virus)	3.8 (2.2)
BCV (bovine coronavirus)	0.04 (0.07), s, $P < 0.005$
MHV+ α-S protein mab	0.07 (0.18), s, $P < 0.005$
MHV+ α-N protein mab	4.6 (2.5), ns, $P = 0.4$
MHV+ α-MHC II mab	3.7 (1.8), ns, $P = 0.9$

Note. Fluo-3-loaded cells were incubated with conditioned medium or infected with MHV or BCV at a m.o.i. of 5. Blocking experiments were performed by preincubation of MHV with the indicated antibodies at 37°C for 1 h before addition to fluo-3-loaded cells. From a series of 16 images recorded over 160 s, the percentage of responding cells (expressed as % cellular area responding) was calculated.

[a] Data are expressed as an arithmetic mean (and standard deviation) of 5 to 10 independent experiments. Calculation of significance compared to MHV-infected cells by *t* test. s, significant; ns, not significant; *P*, probability.

supplemented with 1.8 m*M* EGTA) was added to DBT cells preincubated in Joklik medium, no $[Ca^{2+}]_i$ response was observed (Table 2), indicating that the MHV-mediated calcium rise in DBT cells was caused by Ca^{2+} influx from the extracellular medium. To further assess the involvement of Ca^{2+} channels in this process, cells were preincubated with the L-type channel blockers nifedipine (1 μM) or verapamil (1 μM) for 10 min prior to the addition of virus. Both reagents significantly reduced the MHV-induced calcium response (Table 2). Similar results were obtained in the presence of cadmium chloride (20 μM), a divalent cation that blocks almost all types of calcium channels (Table 2). In contrast, a sodium channel inhibitor, tetrodotoxin (1 μM),

TABLE 2

The Effects of Media and Calcium Channel Blockers on MHV-Induced Calcium Response

DBT cells preincubated in	Cellular area responding, (%)[a]
MEM	4.04 (2.4)
Calcium-free medium (Joklik medium)	0.02 (0.042), s, $P < 0.005$
MEM + 1 μm nifedipine	0, s, $P < 0.005$
MEM + 1 μm verapamil	0.06 (0.1), s, $P < 0.005$
MEM + 20 μm cadmium chloride	0.76 (0.5), s, $P < 0.005$
MEM + 1 μm tetrodotoxin	4.57 (1.9), ns, $P = 0.6$

Note. Fluo-3-loaded cells were preincubated for 10 min with the indicated reagents before addition of MHV (m.o.i. of 5). From a series of 16 images recorded over 160 s, the percentage of responding cells (expressed as % cellular area responding) was calculated.

[a] Data are expressed as an arithmetic mean (and standard deviation) of 5 to 10 independent experiments. Calculation of significance compared to MHV-infected cells, preincubated in regular MEM by *t* test. s, significant; ns, not significant; *P*, probability.

TABLE 3

The Effects of Calcium Chelator and Calcium Channel Blockers on MHV Infection

Treatment	Inhibition (%)
MEM	—
MEM + EGTA (1.8 m*M*)	99.8 ± 0.05
MEM + verapamil (10 μM)	84.5 ± 9.5
MEM + verapamil (40 μM)	97.7 ± 2.4
MEM + cadmium chloride (20 μM)	99.4 ± 0.4
MEM + tetrodotoxin (1 μM)	0

Note. DBT cells were incubated with MEM medium containing the indicated inhibitors for 2 h before and throughout the MHV infection. The supernatant was collected 9 h postinfection and plaque assayed for virus titer. Percentages of inhibition (±standard deviation) were calculated by comparing with the virus titers in MEM only.

did not inhibit the MHV-induced calcium increase (Table 2). Taken together, our results suggest that the MHV-induced $[Ca^{2+}]_i$ increase in DBT cells is caused by Ca^{2+} influx through the cell membrane involving L-type or related calcium channels.

Effects of calcium channel blockers and EGTA on MHV infection. To investigate whether this transient calcium increase is required for virus infection, we studied the effects of calcium channel blockers and the calcium chelator EGTA on MHV infection in DBT cells. Cells were incubated in medium containing verapamil, cadmium chloride, or EGTA for 2 h before and throughout MHV infection. At 9 h postinfection, the media were harvested, and the titers of virus released from the infected cells were determined by plaque assay. The results showed that verapamil (at 10 μM) caused an 84.5% reduction of MHV titer produced by the virus-infected cells; at 40 μM, the inhibition reached 97.7% (Table 3). However, at a lower concentration (1 μM) of verapamil, no significant inhibition of MHV titer was noted (data not shown). Cadmium chloride, at the concentration (20 μM) that is required to block calcium channels [20], also showed a significant inhibitory effect on virus infectivity. In contrast, the sodium channel inhibitor tetrodotoxin (1 μM) did not have any effect on the MHV titer. Similar to the effect of calcium channel blockers, chelating of the extracellular calcium with EGTA (1.8 m*M*) caused a 99% inhibition of the MHV titer (Table 3). These results suggest that calcium influx may play an important role in virus infection.

Calcium measurements at later stages of MHV infection. Virus-infected DBT cells were examined for changes of intracellular calcium at various time points for up to 15 h postinfection. No changes in fluo-3 fluorescence intensity could be found when compared to that in noninfected cells, suggesting that MHV does not disturb calcium homeostasis over a longer period of time (data not shown).

Immunofluorescence of MHV infection in DBT cells. Since only an average of 5% of cells exhibited a measurable transient increase of $[Ca^{2+}]_i$ upon addition of virus (at a m.o.i. of 5), we attempted to determine whether there was a correlation between the $[Ca^{2+}]_i$ increases and the establishment of viral infection. DBT cells infected with MHV (at a m.o.i. of 5) were examined by immunofluorescence using an antibody against the N protein of MHV at 3 and 6 h after infection. Interestingly, at 3 h postinfection, approximately 4.33% (SD, 1.7; $n = 10$) of cells are expressing the viral N protein. All of the cells remained as individual cells (Fig. 4A). This percentage of the N-protein-positive cells was roughly equivalent to the percentage of cells that had exhibited MHV-induced $[Ca^{2+}]_i$ increases. At 6 h postinfection, most of the cells formed syncytia, which expressed the viral N protein (Fig. 4B). Thus, the cells expressing viral N protein at 3 h postinfection may represent only a subset of virus-infected cells, probably only those in which virus replicated early. These cells may have been infected with multiple virus particles. Alternatively, the N-protein-producing cells may represent all of the virus-infected cells early in infection, and the syncytia seen at 6 h p.i. may be the result of fusion between the virus-infected and the uninfected cells.

DISCUSSION

Virus binding to host cell receptors is an essential first step of viral infection that triggers the internalization of virus particles into cells. However, virus–receptor binding is necessary but not sufficient for virus entry, as many cell types have receptors for MHV, and yet, the virus cannot enter the cells [3, 21]. MHV receptors are expressed in many nontarget tissues, e.g., kidney, which are resistant to virus infection [22, 23]. Therefore, additional molecular events must occur to trigger virus internalization following virus binding. In this paper, we have demonstrated that MHV infection induces an immediate and transient calcium increase in DBT cells. This response is specific for MHV and triggered by an interaction between the MHV spike protein and DBT cell surface molecules. Furthermore, our results suggest that the calcium increase was the result of the influx of calcium from the extracellular medium through L-type or related calcium channels. A similar calcium increase through influx from the extracellular medium has been reported to be induced by the HIV envelope protein gp120 in primary cultures of neurons [7, 8]. Similarly for MHV, the transient calcium increase was also mediated by the viral spike protein and not by other components of cell products or media, since this response was blocked by the neutralizing antibody and not induced by the conditioned media.

The immediate and transient nature of the MHV-

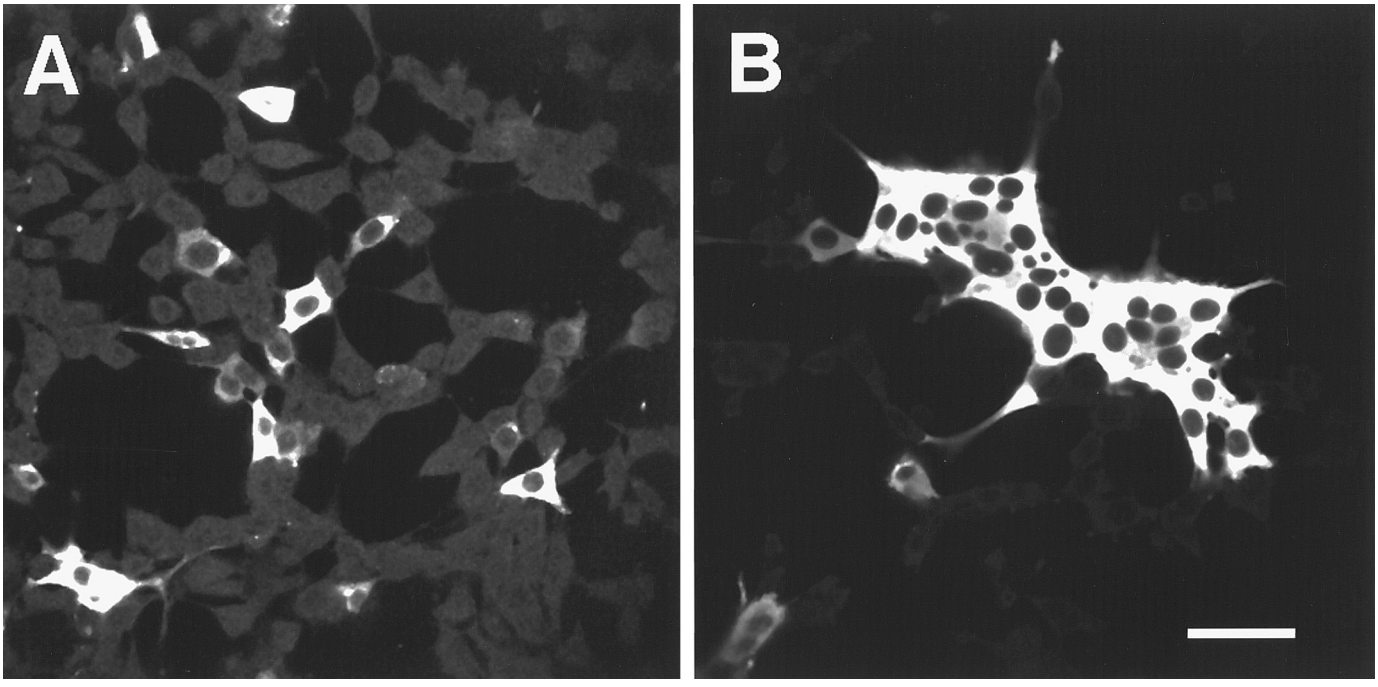

FIG. 4. Detection of MHV N protein during infection. MHV-infected DBT cells were labeled by indirect immunofluorescence with the α-N protein mab at 3 (A) and 6 h (B) postinfection. Bar, 50 μm.

induced calcium response in DBT cells suggests its role in signal transduction. The influx of calcium following virus binding may trigger subsequent signal transduction events that activate the cellular machinery for endocytosis or cell fusion and eventually lead to viral entry. Earlier work has suggested that an increase in intracellular free calcium is necessary to produce Sendai-virus-induced cell fusion at the maximum rate [24]. However, this result was obtained by an indirect method using the Ca^{2+}-activated photoprotein obelin, and calcium changes were monitored for the entire cell population and not for single cells as demonstrated in this study. Recently, the requirement of an intracellular calcium increase for the cellular entry of the bacterium *Salmonella typhimurium* was demonstrated [25]. Alternatively, the MHV-induced calcium increase could be a contributing factor to MHV pathogenesis. For example, an HIV-induced calcium increase has been suggested to play a role in AIDS-related symptoms, such as lymphocyte activation and neuro- and enteropathy [7, 8, 10, 11]. In the case of influenza A virus, a virus-induced calcium increase appears to be important for the impairment of neutrophil functions in influenza [9].

We attempted to establish the fact that the MHV-induced calcium response is required for virus infection. Such a connection was suggested from the result of the immunofluorescence analysis of the MHV-infected cells, which showed that, at 3 h postinfection, the percentage of individual cells that had expressed MHV N protein was similar to the percentage of cells that responded with an intracellular calcium increase. These cells probably represent those in which the virus had undergone very rapid internalization and proliferation, so that viral proteins could be detected at such an early time point. Further evidence for the involvement of calcium in MHV infection came from the studies which showed that calcium channel blockers or EGTA decreased virus yield (Table 3). Preliminary studies have further shown that these inhibitors did not affect the virus particle itself or the virus–cell binding, but acted on an early step of virus infection, probably a virus entry step (D. S. Chen, H.-P. Li, and M. M. C. Lai, unpublished observation). It should be noted that the cadmium concentration required for inhibition of virus production was the same as that for inhibition of calcium influx; however, the concentration of verapamil required for inhibition of virus production (10 μM) was higher than that for inhibition of calcium influx (1 μM). Thus, other factors associated with calcium influx may contribute to virus infection.

It is curious that only about 5% of cells responded with a detectable intracellular calcium increase. One would have expected a higher percentage of responders at an m.o.i. of 5, if every infected cell had responded with a calcium increase. One likely possibility is that these responders represent cells which were infected with multiple virus particles. Indeed, virus dilution studies showed that there was a precipitous drop in the percentage of responders as the m.o.i. was decreased

(0.29% at a m.o.i. of 1 and nearly 0% at a m.o.i. of 0.5). This is consistent with the multiple-hit hypothesis. Perhaps the cells infected with only one virus particle produced only minimal calcium changes that could not be detected by the procedure used here. It is enlightening that roughly the same percentage of cells produces abundant viral proteins at a very early time point of infection (3 h p.i.), although most of the cells appeared to have been infected, as detected by immunofluorescence staining of infected cells at 6 h p.i. Thus, the calcium responders may represent virus-infected cells in which virus entry occurs rapidly, so that viral proteins could be detected as early as 3 h postinfection. Still, another possibility is that the virus could infect only approximately 5% of the cells under the culture conditions used; the N-protein-positive syncytia may be the result of fusion between the virus-infected and the uninfected cells. In either scenario, there is an apparent correlation between the number of calcium responders and the number of virus-infected cells.

Thus far, MHV and HIV are the only two viruses that are capable of activating calcium channels immediately following the binding of virus or viral surface molecules to the cells. The early and transient nature of the MHV-induced calcium increase, the apparent correlation between the percentage of calcium responders and early producers of viral protein, and the inhibition of virus infection by calcium channel blockers and EGTA suggest that this event is involved in virus entry. The investigation of further downstream signals following calcium increase will be of great interest to clarify its significance and understand the mechanism of virus entry.

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