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Influence of Calcium on the Early Steps of Rotavirus Infection

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The structure of rotaviruses and many steps of their replication cycle depend on the concentration of calcium in the microenvironment. In this work, to learn about the role of calcium during the early steps of the infection, we characterized the effect of increasing the calcium concentration in the medium on the infectivity of rotaviruses. We found that a fivefold increase in the calcium concentration of the cell culture medium results in an increased viral titer in all rotavirus strains tested. The effect of this divalent ion seems to be mainly on the viral particle and not on the surface of the cell. Analysis of the intrinsic fluorescence spectra of purified triple-layered particles revealed that changes in the environment of tryptophan residues occurred as calcium concentration increased, suggesting that conformational changes in the viral particle might be responsible for the effect of this ion on the viral infectivity.

Key Words: rotavirus; rotavirus infectivity; viral entry; calcium; intrinsic fluorescence; conformational changes.

INTRODUCTION

Rotaviruses are the major cause of severe acute diarrhea of infants and young children under 2 years of age and are important pathogens in the young of many avian and mammalian species. These agents, members of the family *Reoviridae,* are nonenveloped icosahedric viruses consisting of three concentric layers of protein that surround the genome composed of 11 segments of double-stranded RNA. The outermost layer is composed of two proteins, VP4 and VP7, which are responsible for the initial interactions of the virus with the host cell. The smooth external surface of the virus is made up of 780 copies of glycoprotein VP7 arranged in trimers and of 120 copies of protein VP4, which form 60 dimeric spike-like structures that extend from the surface of the particle (Estes, 1996).

VP4 has essential functions in the early virus–cell interactions, including receptor binding and cell penetration (Crawford *et al.,* 1994; Ludert *et al.,* 1996; Mendez *et al.,* 1996; Zarate *et al.,* 2000). This protein is cleaved by trypsin into subunits VP5 and VP8, and it has been shown that this proteolytic treatment results in an enhancement of rotavirus infectivity (Arias *et al.,* 1996; Estes *et al.,* 1981; Lopez *et al.,* 1985). The role of VP7 during the early interactions of the virus with the cell has not been defined, but it has been proposed that it may modulate some functions of VP4 (Beisner *et al.,* 1998; Mendez *et al.,* 1996; Xu and Woode, 1994) and interact with cell surface molecules after the initial attachment of the virus through the VP4 protein (Coulson *et al.,* 1997; Mendez *et al.,* 1999).

Recently, it has been established that the interaction of rotavirus with its host cell is a multistep process in which sequential contacts of the viral capsid with the cell surface take place (Mendez *et al.,* 1999). Accordingly, different cellular molecules have been described as possible rotavirus receptors and coreceptors; among them gangliosides GM1 and GM3 (Delorme *et al.,* 2001; Guo *et al.,* 1999; Rolsma *et al.,* 1998) and integrins $\alpha 2\beta 1$, $\alpha 4\beta 1$, and $\alpha v\beta 3$ (Coulson *et al.,* 1997; Guerrero *et al.,* 2000a; Hewish *et al.,* 2000) have been found to play an important role during virus binding to, and penetration into, the cell.

The replication cycle and the structure of rotaviruses are strongly dependent on the concentration of the ion calcium in the environment (Gajardo *et al.,* 1997; Ruiz *et al.,* 2000). During the replication cycle, the viral particles travel through different cellular compartments, each characterized by a different calcium concentration that is determinant for each step of the virus cycle. During cell entry, the triple-layered particle (TLP) loses the outer layer proteins, VP4 and VP7, and the double-layered particle (DLP), active in transcription, reaches the cytoplasm. Although still controversial, the low-calcium environment of the cell has been proposed to be needed for this uncoating process (Cuadras *et al.,* 1997; Ludert *et al.,* 1987). During virus assembly, the double-layered particle buds from the cytoplasm into the lumen of the endoplasmic reticulum (ER), which has a high-calcium environment, acquiring during the process a transient lipid en-

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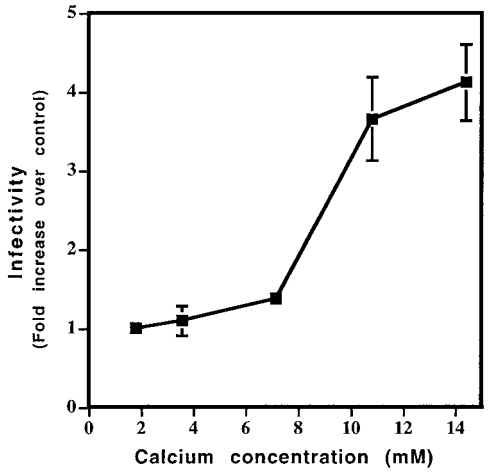

FIG. 1. Effect of calcium concentration on the infectivity of rotavirus RRV. MA104 cells in 96-well plates were infected with trypsin-activated virus in MEM containing the indicated calcium concentrations. After 1 h of adsorption at 37°C, the virus inoculum was removed and fresh MEM (1.8 mM $CaCl_2$) was added. The infected cells were incubated for 14 h at 37°C; after this time, the cells were fixed and stained by an immunoperoxidase assay as described under Materials and Methods. Data are expressed as fold increases over control (virus titer in 1.8 mM $CaCl_2 = 2 \times 10^7$ ffu/ml). The arithmetic mean ± standard deviation of three independent experiments performed in duplicate is shown.

velope. The formation of mature virus, in which the transient envelope is replaced by the outer layer proteins VP4 and VP7, is strictly dependent on the concentration of calcium present in the ER lumen (Sharabari and Lee, 1987). Compounds that alter the concentration of this ion in the ER, such as tapsigargin, which depletes the calcium from the ER lumen, block the maturation of rotaviruses at the enveloped stage, and the mature triple-layered particle is not formed (Poruchynsky *et al.,* 1991). Also, the synthesis of the viral proteins alters the calcium homeostasis of the cell, which favors the morphogenesis of the virus, and induces cell death (Dong *et al.,* 1997).

The structure of the viral particle is also dependent on the calcium concentration. The outermost layer of rotavirus contains calcium (Sharabari and Lee, 1987). *In vitro,* when the viral particles are treated with calcium chelators, the virus uncoats, releasing the transcriptionally active double-layered particle (Cohen *et al.,* 1979). The sensitivity of the viral particle to low concentrations of calcium segregates with VP7 (Gajardo *et al.,* 1997), and more recently it has been shown that calcium binds within VP7 trimers and that these calcium-stabilized trimers are the basic building block of the outer layer of the rotavirus particle. Also, treatment of double-layered particles with high concentrations of calcium (>1 M) causes the intermediate layer of the particle, formed by VP6, to disassemble (Lepault *et al.,* 2001).

In this work we characterized the effect of increasing the calcium concentration in the cell culture medium on the infectivity of rotaviruses. We found that a fivefold increase over the basal calcium concentration of the cell culture medium resulted in an increased viral titer in all rotavirus strains tested. The effect of this divalent ion was found to be mainly on the viral particle, and not on the molecules present on the surface of the cell. Analysis of the intrinsic fluorescence spectra of purified TLPs suggests that conformational changes in the viral particle might explain the effect of this ion on the enhancement of viral infectivity.

RESULTS

The infectivity of rhesus rotavirus RRV is increased by high concentrations of calcium

Since many steps of the viral cycle of rotaviruses are modulated by the concentration of calcium in the microenvironment, the effect of this ion on the infectivity of rhesus rotavirus RRV was tested. In this assay, MA104 cells were infected with RRV rotavirus suspended in Eagle's minimal medium (MEM) containing increasing concentrations of $CaCl_2$, starting from 1.8 mM, which is the regular concentration of $CaCl_2$ in the MEM. The increase in calcium concentration in the medium during the incubation of the virus with the cells resulted in an enhancement of viral infectivity, reaching a maximum titer of virus at 14.4 mM $CaCl_2$, which represented a fourfold increase in the infectivity compared to the virus titer obtained in 1.8 mM $CaCl_2$ (Fig. 1). It was not possible to test higher concentrations of calcium in the medium since the viability of the MA104 cells was affected (data not shown). Based on these data, to characterize the effect of Ca^{2+} on the infectivity of rotaviruses, we used in the following assays 10 mM $CaCl_2$ as the highest concentration and 2 mM $CaCl_2$ as the standard condition.

To analyze whether the effect of calcium on the viral infectivity was specific to this ion, the effect of other divalent cations (Ba^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, and Zn^{2+}) on rotavirus infectivity was tested. We found that in addition to Ca^{2+}, the only other divalent ion that caused an increase in the infectivity of RRV was Sr^{2+} (Table 1). This was not surprising, since in addition to having an ionic radius very similar to that of calcium, Sr^{2+} has been reported to share some functional features with calcium, such as binding to calmodulin (Missiaen *et al.,* 1999;

TABLE 1

Effect of Different Divalent Ions on the Infectivity of RRV Rotavirus

Divalent ion	Infectivity[a]
$BaCl_2$	0.95 ± 0.04
$CaCl_2$	5.65 ± 0.21
$SrCl_2$	3.70 ± 0.56
$MgCl_2$	0.85 ± 0.07

[a] Expressed as fold increase over control (viral infectivity in 2 mM $CaCl_2$). The arithmetic mean ± standard deviation of three independent experiments performed in duplicate is shown.

Yamamoto *et al.,* 1987). Strontium has also been reported to be the only other divalent cation important for the stability of rotaviral particles (Shirley *et al.,* 1981). Magnesium and barium did not have an effect on the viral titer at a concentration of 10 mM; at this same concentration, manganese and zinc were toxic to the cells; at 4 mM, which was the highest concentration tolerated by the cells, these two ions did not affect the infectivity of the virus (not shown).

A high calcium concentration enhances the infectivity of several rotavirus strains

To study whether the effect of the calcium ions was general for rotavirus strains isolated from different animal species, we infected MA104 cells with different rotavirus strains in medium containing either 2 or 10 mM $CaCl_2$, and the titer of each virus was determined. Table 2 shows that the infectivity of all rotavirus strains tested increased in 10 mM Ca^{2+}, although the magnitude of the increment was different depending on the rotavirus strain; for example, the titer of the human strain DS1 increased about 50-fold over its control in 2 mM calcium, while the infectivity of the bovine strain NCDV increased by only a factor of 2. It is noteworthy that in general the viruses that had the lowest titers in the control medium had the largest increments when their infectivity was assayed in medium containing 10 mM calcium.

To determine the specificity of this effect, the infectivity of two other nonenveloped viruses, poliovirus and reovirus, in 2 and 10 mM $CaCl_2$ was assayed. We found that the increase in the calcium concentration did not alter the infectious titer of either of these two viruses, indicating that the effect of calcium on the infectivity was specific for rotaviruses.

TABLE 2

Effect of Calcium on the Infectivity of Different Rotavirus Strains

Virus	Origin	Virus titer[a] in 2 mM $CaCl_2$	Virus titer[a] in 10 mM $CaCl_2$	Fold increase[b]
DS-1[c]	Hu	6.2×10^4	3.5×10^6	56.5 ± 19.0
ST3[d]	Hu	2.1×10^4	8.7×10^5	41.3 ± 16.0
TY-1[c]	Av	3.5×10^5	6.6×10^6	18.9 ± 5.0
CH-2[c]	Av	5.2×10^5	7.0×10^6	13.5 ± 0.2
nar3[e]	Si	6.7×10^5	7.0×10^6	10.4 ± 1.1
Wa[f]	Hu	3.9×10^6	2.9×10^7	7.4 ± 0.3
UK[d]	Bo	1.2×10^6	8.5×10^6	7.1 ± 0.2
L338[d]	Eq	9.4×10^6	5.8×10^7	6.2 ± 3.1
B223[c]	Bo	5.7×10^5	2.7×10^6	4.8 ± 0.1
RF[g]	Bo	4.0×10^6	1.8×10^7	4.7 ± 0.4
YM[e]	Po	3.9×10^6	1.7×10^7	4.3 ± 0.5
H2[d]	Eq	1.4×10^6	5.6×10^6	4.0 ± 1.7
69M[h]	Hu	5.8×10^6	2.0×10^7	3.4 ± 0.3
RRV[f]	Si	1.1×10^7	5.0×10^7	4.8 ± 1.3
SA11 4S[i]	Si	4.3×10^7	1.1×10^8	2.5 ± 0.2
NCDV[d]	Bo	6.9×10^7	1.6×10^8	2.3 ± 0.6
Poliovirus[j]	Hu	4.0×10^4	4.0×10^4	1.0 ± 0.2
Reovirus[k]	Hu	2.7×10^5	3.0×10^5	1.1 ± 0.2

[a] The arithmetic mean of at least three independent experiments performed in duplicate is shown.

[b] Fold increase over control (infectivity in 2 mM $CaCl_2$). The arithmetic mean ± standard deviation of at least three independent experiments is shown.

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Calcium affects the early interactions of rotavirus with the cell surface

To determine whether the calcium increase in the culture medium favored the binding or the entry of rotavirus to the cell, we took advantage of the fact that rotaviruses attach to the cell at 4°C, but penetrate only at 37°C. In these assays, twofold dilutions of trypsin-activated RRV virus, in MEM containing either 2 or 10 mM $CaCl_2$, were adsorbed to monolayers of MA104 cells for 30 min at 4°C; the unbound virus was removed, and warm medium containing 10 or 2 mM $CaCl_2$ was added to the cells for 30 min at 37°C. After this incubation period the cells were washed, MEM containing 2 mM $CaCl_2$ was added, and the infection was left to proceed for 14 h at 37°C, at which time the cells were fixed and immunostained. Figure 2 shows that the viral infectivity increased only when 10 mM $CaCl_2$ was present during the adsorption period, but not when the medium containing 10 mM calcium was added after the virus was bound in 2 mM calcium, suggesting that the effect of this ion could be during the binding step.

To discover whether the increase in viral titer at 10 mM calcium was due to an improved attachment of the viral particles to the cell surface, the amount of virus bound to the cells was determined. For this, purified TLPs in medium containing 2 or 10 mM $CaCl_2$ were incubated on monolayers of MA104 cells in 48 well-plates, for 1 h at 4°C. After the unbound virus was removed, the cells were washed extensively with MEM containing 2 mM calcium and then lysed by two rounds of freeze–thaw. The cell-bound virus present in the lysate was determined by an enzyme-linked immunosorbent assay (ELISA), as previously described (Zarate *et al.,* 2000), and its infectious titer was determined. We found no difference in the amount of viral particles bound to the cells in medium containing 2 or 10 mM $CaCl_2$, as determined by the ELISA (Fig. 3A). However, when the titer of these same preparations was analyzed, we found that the virus

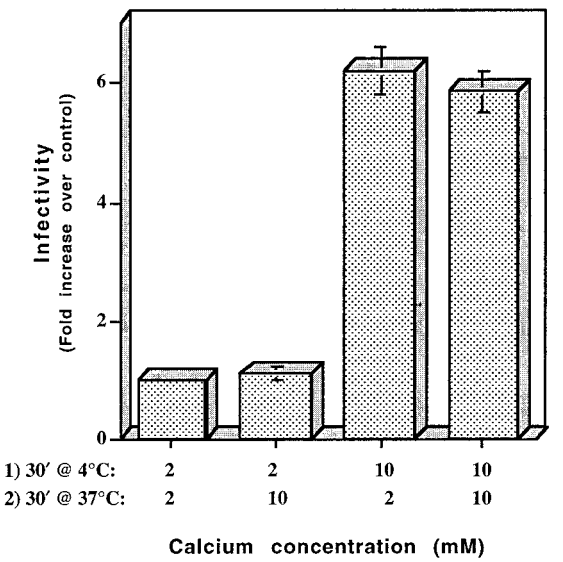

FIG. 2. Effect of calcium concentration on the early interactions of rotavirus with the host cells. Trypsin-activated virus in MEM containing either 2 or 10 mM $CaCl_2$ was adsorbed to a confluent monolayer of MA104 cells for 30 min at 4°C. After this time the unbound virus was removed, and warm MEM containing 10 or 2 mM $CaCl_2$ was added to the cells for 30 min at 37°C. The cells were washed twice and the infection was left to proceed in MEM containing 2 mM calcium for 14 h at 37°C, the cells were then fixed and stained by an immunoperoxidase assay as described under Materials and Methods. Data are expressed as fold increases over control (virus titer in 2 mM $CaCl_2$ = 1.8×10^6 ffu/ml). The arithmetic mean ± standard deviation of three independent experiments performed in duplicate is shown.

that was bound to the cells in 10 mM calcium had a titer two- to threefold higher than the virus bound in 2 mM calcium (Fig. 3B). These results suggest that even though the same amount of physical particles bound to the cell surface in 2 or 10 mM calcium, the ratio of infectious to physical particles changed with the increased concentration of $CaCl_2$ used, probably because more viral particles were stabilized by the calcium to be able to penetrate and/or initiate the replication of the virus. These results suggest that the effect of the calcium is mainly on the viral particle, although the possibility cannot be eliminated that the calcium might be favoring or stabilizing a specific virus–cell interaction, which finally leads to infection.

The effect of calcium on the infectivity of RRV is irreversible once the virus is bound to the cell surface

To determine whether the effect of calcium on the viral particle was reversible, we performed the following experiments. CsCl-purified RRV TLPs, adjusted to either 2 or 10 mM $CaCl_2$ for 30 min at 4°C, were shifted for different periods of time to 10 or 2 mM $CaCl_2$, respectively, and the titer of the virus at these different time points after the change in calcium concentration was determined (Fig. 4A). We found that when the virus in 2 mM calcium was taken up to 10 mM, the viral infectivity increased almost immediately, since at the first time point measured (1 min) the titer of the virus was already sevenfold higher than the control virus that was kept at 2 mM during the experiment. However, in the reverse experiment in which the virus resuspended in 10 mM calcium was diluted to 2 mM, we found that it took 90 min after the calcium concentration was reduced for the titer of the virus to return to the level observed for the control virus that was kept at 2 mM calcium, suggesting that the effect of the increase in calcium concentration was primarily on the viral particle and that this effect was reversible. In a second set of experiments the shift in calcium concentration was performed on virus that was already bound to the cells. In this case, twofold dilutions of CsCl-purified TLPs in medium containing either 2 or 10 mM $CaCl_2$ were initially bound to a monolayer of MA104 cells for 30 min at 4°C, and then the medium was replaced with medium containing either 10 or 2 mM $CaCl_2$ for different periods of time at 4°C. The time points were stopped by replacing the MEM with warm MEM containing 2 mM $CaCl_2$. The infection was left to proceed for 14 h at 37°C, and the titer of these viruses was determined (Fig. 4B). We found that, in contrast to the previous results, the titer of the virus that was initially

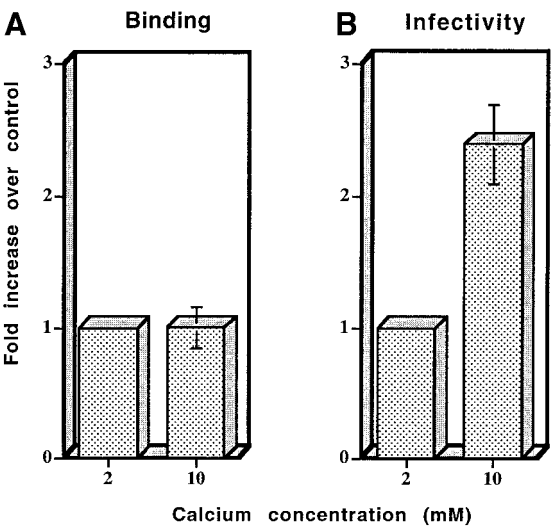

FIG. 3. Effect of calcium concentration on the binding of RRV TLPs to the surface of MA104 cells. Confluent monolayers of MA104 cells in 48-well plates were preincubated with MEM–1% BSA for 1 h at room temperature. Ice-cold purified TLPs (500 ng/well) suspended in MEM–1% BSA containing 2 or 10 mM $CaCl_2$ were added to the cells for 1 h at 4°C, with gentle shaking. Cells were washed, MEM with 2 mM calcium was added, and the cells were lysed by two rounds of freeze–thaw. (A) The cell-bound virus present in these lysates was quantified by an ELISA as described under Materials and Methods. (B) The viral titer in these lysates was determined by an immunoperoxidase focus assay, as described under Materials and Methods. Data are expressed as fold increases over control (A, OD at 405 nm of the virus bound in 2 mM $CaCl_2$; or B, titer of the virus bound in 2 mM $CaCl_2$ = 0.9×10^6 ffu/ml). The arithmetic mean ± standard deviation of three independent experiments performed in duplicate is shown.

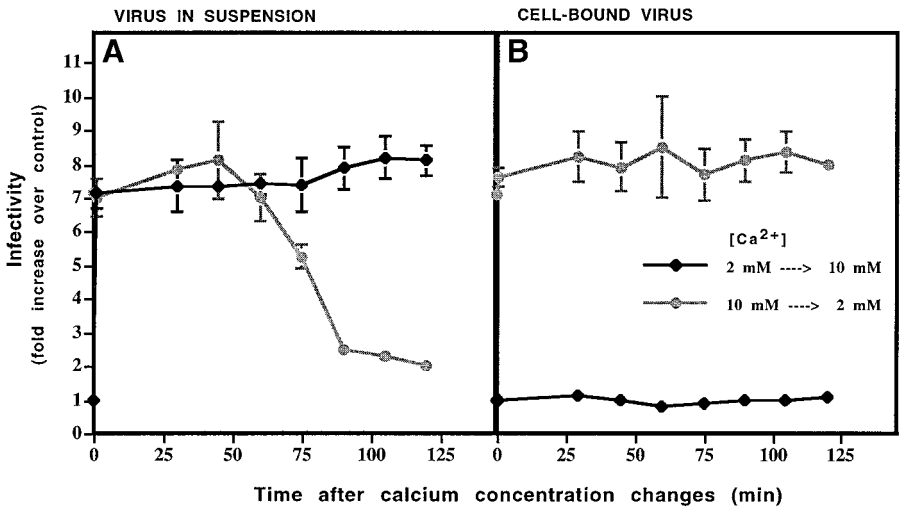

FIG. 4. Effect of calcium concentration changes on the viral particles in suspension, or bound to the cells. (A) Twofold dilutions of trypsin-activated purified TLPs of RRV in suspension were diluted in MEM with 2 or 10 mM $CaCl_2$ and kept for 30 min at 4°C. The virus was then taken up to a concentration of 10 mM calcium or diluted to 2 mM $CaCl_2$ respectively, and at the indicated time points, the virus was adsorbed to the cells for 30 min at 4°C, and then warm MEM containing 2 mM calcium was added and the infection was left to proceed for 14 h and processed as described under Materials and Methods. (B) Twofold dilutions of trypsin-activated purified TLPs of RRV were adsorbed to confluent monolayers of MA104 cells in MEM containing 2 or 10 mM $CaCl_2$ for 30 min at 4°C. After this time, the unbound virus was removed and the calcium concentration in the medium was shifted to 10 or 2 mM $CaCl_2$ respectively, and at the indicated time points at 4°C the cells were washed, warm MEM containing 2 mM calcium was added, and the infection was left to proceed for 14 h. The cells were fixed and stained by an immunoperoxidase assay as indicated under Materials and Methods. Data are expressed as fold increases over control (viral infectivity in 2 mM $CaCl_2$ = 2.5×10^7 ffu/ml or viral infectivity in 10 mM $CaCl_2$ = 1.8×10^8 ffu/ml). The arithmetic mean ± standard deviation of three independent experiments performed in duplicate is shown.

bound at either concentration of calcium remained unchanged for the period of time tested, irrespective of the shift in calcium concentration. These results indicate that once the virus was bound to the cell surface, a decrease or an increase in calcium concentration no longer affected its interaction with the cell surface.

Calcium reduces the sialic acid dependence of RRV

RRV requires the presence of sialic acid on the cell surface to bind and to infect MA104 cells, and treatment of cells with neuraminidase (NA) diminishes its infectivity drastically (Méndez *et al.,* 1993). To study whether the change in calcium concentration altered the initial interaction of RRV with sialic acid, MA104 cells treated with different concentrations of NA were infected with RRV and its NA-resistant variant nar3 (Méndez *et al.,* 1993), in medium containing 2 or 10 mM $CaCl_2$. Figure 5 shows that, as previously observed, the infectivity of RRV in cells treated with 20 mU/ml of NA decreased about 75%, while the infectivity of nar3 was not affected even at higher concentrations of NA. However, when this same assay was performed in the presence of 10 mM calcium the infectivity of RRV was less sensitive to the treatment of the cells with NA, so that 20 mU of enzyme, the infectivity of this virus decreased only 15%. As expected, the infectivity of nar3 did not change in the NA-treated cells, even though the infection titer of this virus in MEM containing 10 mM $CaCl_2$ increased 10-fold with respect to the titer in 2 mM $CaCl_2$ (see Table 2). These results indicate that RRV in 10 mM calcium became less dependent on the presence of sialic acids on the cell surface, suggesting that the viral particles might have suffered a conformational change, such that the initial contact with the neuraminidase-sensitive receptor could be surpassed.

Fluorescence emission spectra of rotavirus RRV

Since we suspected that the increase in calcium concentration induced a conformational change in the viral particle that would allow the virus to better interact with the cell surface, we used fluorescence spectroscopy to detect possible conformational changes in the emission spectra of purified TLPs and DLPs of RRV, in either 2 or 10 mM calcium. The TLPs of RRV show a characteristic tryptophan fluorescence emission spectrum, with a peak at 328 nm. Addition of 10 mM $CaCl_2$ to this preparation showed a reproducible increase in the intrinsic fluorescence of the particles, but did not cause a shift in the emission spectrum (Fig. 6A). The inset in Fig. 6A shows the calcium concentration dependence of fluorescence of the TLPs, which is suggestive of a conformational change in the viral particle (Lakowicz, 1986). The increase in fluorescence was specific for the triple-layered particle, since the emission spectra of the DLPs did not change with the addition of 10 mM calcium (Fig. 6B), and the fluorescence intensity of the DLPs did not change with the addition of calcium (Fig. 6B, inset). The increase in fluorescence was specific for the ion calcium since when the fluorescence spectra of the TLPs were carried

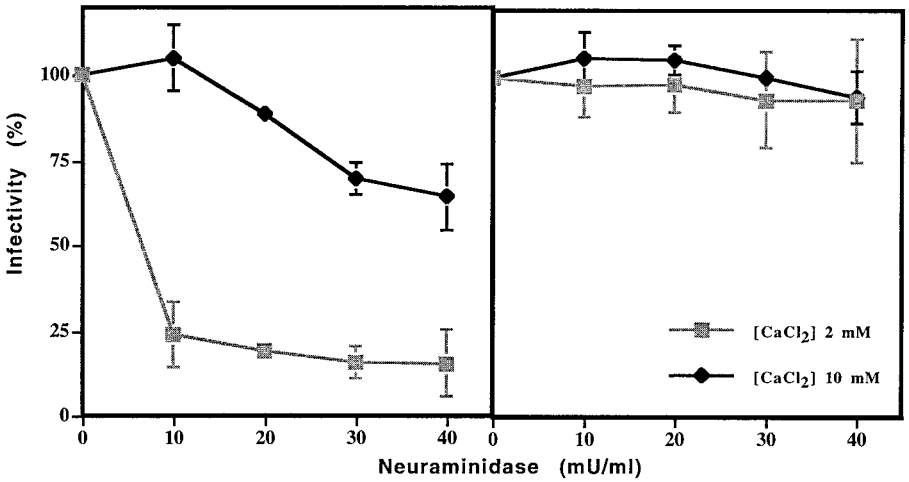

FIG. 5. Effect of calcium concentration on the infectivity of rotavirus in neuraminidase-treated cells. MA104 cells in 96-well plates were treated with the indicated concentrations of NA from *A. ureafaciens* for 1 h at 37°C, washed, and subsequently infected with 1000 ffu/well of trypsin-activated rotaviruses RRV or nar3, in MEM containing 2 or 10 mM $CaCl_2$ for 1 h at 37°C. The virus inoculum was removed, fresh MEM with 2 mM calcium was added, and the infection was left to proceed for 14 h at 37°C. The cells were fixed and stained by an immunoperoxidase assay as described under Materials and Methods. Data are expressed as the percentage of infectivity in control, untreated cells in 2 $CaCl_2$ or 10 mM $CaCl_2$. The arithmetic mean ± standard deviation of three independent experiments performed in duplicate is shown.

out using 10 mM $MgCl_2$, a shift in the fluorescence intensity of the viral particle was not detected (Fig. 6C), in agreement with the fact that magnesium did not have an effect on the viral titer. A solution of tryptophan, used as a control, did not change its fluorescence when 10 mM calcium was added (Fig. 6D). Addition of 10 mM calcium to purified TLPs of nar3 and Wa rotaviruses had the same effect on their emission spectra (data not shown). Taken together, these results suggest that the increase in the intrinsic fluorescence of the rotavirus TLPs might reflect a change in the exposure and/or environment of the tryptophan residues of the viral particle upon addition of calcium, which in turn might indicate a conformational change in the rotavirus TLPs. Since the fluorescence spectra of the DLPs did not vary under the different concentrations of calcium tested, the conformational changes induced by Ca^{2+} most likely occur in the proteins VP4 and VP7, which compose the outer layer of the virion.

Determination of the tryptophan residues exposed in rotavirus particles

Aqueous fluorescence quenchers such as KI and acrylamide measure the exposure of tryptophan residues to the aqueous environment. To determine whether the change in the fluorescence spectra observed when the TLPs were incubated with 10 mM $CaCl_2$ was due to an increase in the tryptophan residues exposed to the solvent, fluorescence quenching studies using KI were carried out, and the degree of quenching was calculated by the modified Stern–Volmer equation (see Materials and Methods). Table 3 shows that there was a higher degree of exposure of tryptophan residues when the TLPs of RRV and nar3 viruses were in 10 mM $CaCl_2$, compared to the TLPs in 2 mM calcium, indicating that the increase in calcium concentration induced a conformational change in the viral particles that resulted in a higher level of exposure of tryptophan residues to the aqueous environment.

DISCUSSION

A growing number of examples show that for a virus to enter its host cell, it must establish several sequential interactions with cell receptors and coreceptors, and it has been shown that as a consequence of these interactions the viral particle may undergo conformational changes. These changes can be influenced by the presence of divalent ions, such as calcium. For example, this ion plays an important role in the stability of the viral capsids of SV40 (Liddington *et al.,* 1991), black beetle

TABLE 3

Determination of Tryptophan Residues Exposed in Rotavirus Particles[a]

Virus	2 mM $CaCl_2$	10 mM $CaCl_2$
RRV	35.5% ± 2.18	45.77% ± 4.21
nar3	22.43% ± 0.96	39.88% ± 2.31

[a] Data are expressed as the percentage of tryptophan residues exposed in the viral particles at the indicated calcium concentration. These results represent the arithmetic mean ± standard deviation of three independent experiments.

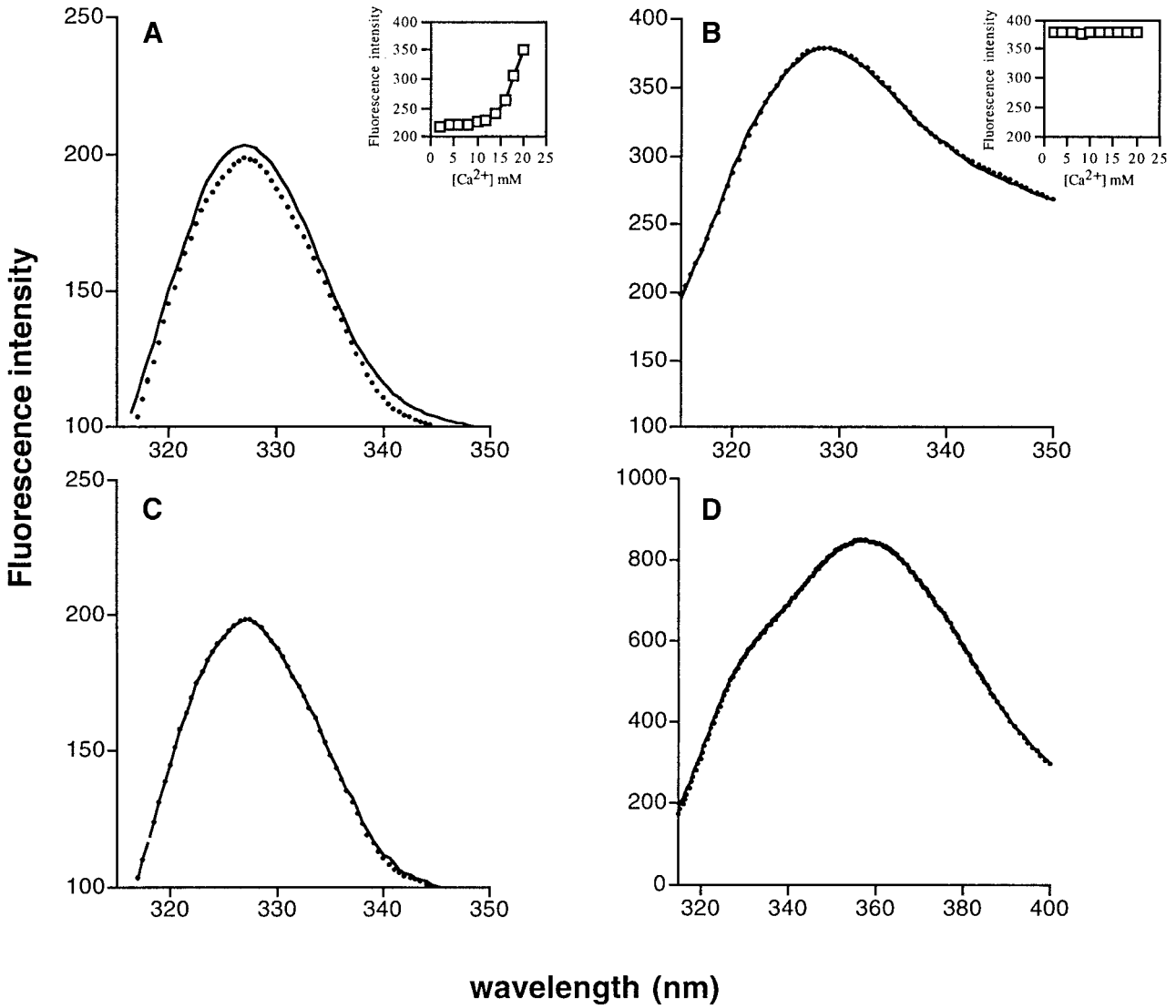

FIG. 6. Fluorescence emission spectra from rotavirus particles. (A) Samples of purified TLPs of rotavirus RRV (10 μg/ml) in physiological solution containing 2 mM $CaCl_2$ (dotted line) or after addition of calcium to a final concentration of 10 mM (continuous line). (B) Fluorescence spectra of RRV-DLPs (10 μg/ml) in physiological solution containing 2 mM (dotted line) or 10 mM $CaCl_2$ (continuous line). The insets in A and B show the fluorescence intensity of the TLPs or DLPs at 328 nm at increasing calcium concentrations. (C) TLPs of rotavirus RRV (10 μg/ml) in physiological solution containing 2 mM $CaCl_2$ (dotted line) or after addition of $MgCl_2$ to a final concentration of 10 mM (continuous line). (D) Tryptophan (10 ng/ml) in physiological solution with 2 mM (dotted line) or 10 mM $CaCl_2$ (continuous line). The excitation wavelength was 295 nm.

nodavirus (Wery *et al.,* 1994), polyoma virus (Haynes *et al.,* 1993), rhinovirus (Zhao *et al.,* 1997), rotavirus (Cohen *et al.,* 1979), and several plant viruses (Durham and Haidar, 1977; Durham and Hendry, 1977; Hull, 1978; Robinson and Harrison, 1982). It has also been observed that the binding of some viruses to their cellular receptors depends on the presence of calcium, as in hepatitis A virus (Bishop and Anderson, 1997) and adenovirus type 37 (Wu *et al.,* 2001). Calcium ions are also required for the cell fusion mediated by the interactions between CD4 and the HIV-1 envelope glycoproteins (Dimitrov *et al.,* 1993; Doranz *et al.,* 1999; Jernigan *et al.,* 2000).

The role of calcium on the stability of the rotaviral capsid was recognized shortly after the discovery of the virus (Cohen *et al.,* 1979), and thereafter it has been observed that many of the steps involved in the replication cycle of the virus depend on the calcium concentration of the microenvironment [reviewed in Ruiz *et al.* (2000)]. In this work, we characterized the effect of calcium on the infectivity of rotaviruses. We found that increases of this ion in the cell culture medium resulted in an enhanced infectivity of all rotavirus strains tested. This effect was found to be specific for rotaviruses since the infectivity of reovirus and poliovirus was not modified. We also found that the effect is specific for calcium (and strontium), since other divalent ions did not en-

hance the viral infectivity, suggesting that the increase in infectivity is due to the calcium ion per se, and not only due to the positive charges of this cation.

The results obtained in this work suggest that the effect of calcium is on the viral particle, since the infectivity was increased when the virus was either bound to cells or preincubated in suspension in 10 mM calcium; this enhancement of infectivity was maintained even if the calcium concentration was reduced to 2 mM after the virus was bound to the cells or after the preincubation of the virus in suspension.

We found that even though the same amount of viral particles bound to the cell surface in both 2 and 10 mM calcium, as determined by an ELISA, the infectious titer of the particles bound to cells in 10 mM calcium was higher than that of the viral particles bound in 2 mM calcium. This observation suggests that when adsorbed to the cell surface in 10 mM calcium, more viral particles were competent to proceed with the infection. It is known that of the total amount of virus produced as a result of an infection, there is a high proportion of viral particles that are noninfectious. In rotavirus the infectious to physical particle ratio is low, varying between different strains of rotavirus. For example, for rotavirus RRV it has been estimated that there is one infectious particle (IP) for every 100–300 physical, noninfectious particles (PP); and in the case of the human strain Wa this ratio is even lower, with one IP per $1–4 \times 10^4$ PP (Mendez *et al.,* 1999). The reason why the physical particles are noninfectious is not known, but the fact that the infectious titer of a viral stock is enhanced in the presence of 10 mM calcium suggests that this cation induces a change in the conformation of the viral capsid that makes the particles probably more stable and able to initiate a productive infection. Recently, Dormitzer *et al.* (2000) reported that the trimerization of VP7 depends on the presence of calcium, and they suggested that dissociation of these trimers might be the biochemical basis for the EDTA-induced uncoating of rotavirus particles.

We have proposed that the entry of rotavirus to its host cell is a multistep process, in which the viral proteins interact with at least three different cellular molecules in a sequential manner (Mendez *et al.,* 1999). We hypothesized that the virus could interact with these different molecules by experimenting conformational changes that allow the virion to expose the protein domains responsible for each of these interactions, although at present there has not been a direct observation of any of these putative conformational changes in the viral particle. In this work, we found that rotavirus RRV, which requires sialic acid to bind to and to infect cells, became less dependent at 10 mM calcium on the presence of sialic acid on the surface of the cells. This observation suggests that incubation of the virus in this calcium concentration favors a conformational change in the RRV particle that allows the virus to interact with the cell surface, in a neuraminidase-resistant manner, surpassing the initial interaction of this virus with a sialic acid-containing receptor. The fact that other rotavirus strains, like nar3, which is a neuraminidase-resistant variant of RRV, and human strains Wa and DS1, which are naturally resistant to the neuraminidase treatment of the cells, also increased their titer upon addition of calcium suggests that the favored virus–cell interaction is shared among these strains. The precise virus–cell contact improved by the high calcium concentration needs to be defined.

In this work we used the intrinsic tryptophan fluorescence of purified TLPs to monitor the conformational changes in the viral particles induced by calcium. Tryptophan fluorescence is strongly influenced by the environment of its indole side chain and has thus proved to be a useful tool for studying conformational changes in proteins, protein–protein interactions, and protein–membrane interactions (Carneiro *et al.,* 2001; Lakowicz, 1986).

The intrinsic fluorescence studies on the TLPs of RRV, nar3, and Wa (shown only for RRV) revealed that changes in the solvent exposure of the tryptophan residues occurred as calcium increased, which is an indication of a conformational change in the viral particle. The quenching assays with KI further confirmed this observation, since it was determined that the percentage of tryptophan residues exposed in the nar3 and RRV TLPs in the presence of 10 mM calcium was higher than when the TLPs were suspended in 2 mM calcium.

Recently, it was suggested that the formation of the outer layer during viral assembly and the loss of the outer layer during the entry of the virus into the cell are mediated by a calcium-dependent conformational change in VP7 (Dormitzer *et al.,* 2000). In this work we found that the calcium concentration induced conformational changes in the complete triple-layered viral particle, which might be contributed in part by the changes that occur in VP7, although the contribution of VP4 cannot be neglected.

Rotaviruses have a very specific cell tropism, infecting only the enterocytes on the tip of intestinal villi (Kapikian and Chanock, 1996). The digestive tract is exposed to a wide variation in the concentrations of calcium; in this regard, it is interesting to note that the young of the rotavirus-susceptible mammalian species are prone to develop the rotavirus disease, at an age where the principal component of the diet is milk, which contains a mean calcium content of 10 mM (Mataloun and Leone, 2000; Meschy, 2000), thus providing an excellent microenvironment for the virus infection. From the practical point of view, the observations made in this work might prove to be useful for the growth of those rotavirus strains that typically are fastidious to work with, given their low infectious titer in tissue culture.

MATERIALS AND METHODS

Cells and viruses

MA 104 cells, L929 (L) cells, and HeLa cells were grown in Eagle's MEM supplemented with 10% fetal bovine serum (FBS). Throughout this work, the MEM employed for the calcium assays was initially prepared without calcium and was supplemented with the indicated concentrations of $CaCl_2$.

Rotavirus strains DS-1, 69M, Wa, ST3, B223, NCDV, RF, UK, MDR-13, YM, H2, L338, SA114S, RRV, nar3, TY-1, and CH-2, reovirus type 1, and poliovirus type 3 (Leon strain) were obtained from different laboratories as indicated in Table 2. All rotavirus strains were propagated in MA104 cells, in MEM containing 1.8 mM $CaCl_2$ (Espejo *et al.,* 1980); poliovirus and reovirus were grown in HeLa, and L cells, respectively.

To prepare purified virus, virus-infected cells were harvested after complete cytopathic effect was attained, the cell lysate was frozen and thawed twice, and the virus was pelleted by centrifugation for 60 min at 25,000 rpm at 4°C in an SW28 rotor (Beckman). The virus pellet was resuspended in TNC buffer [10 mM Tris–HCl (pH 7.5), 140 mM NaCl, 10 mM $CaCl_2$], extracted with Freon, and subjected to isopycnic centrifugation in cesium chloride gradients as previously described (Espejo *et al.,* 1981). DLPs were prepared by treatment of purified TLPs with 50 mM EDTA for 30 min at 37°C and then purified by equilibrium centrifugation in CsCl gradients as described previously. The opalescent band corresponding to DLPs was collected and rebanded in a second CsCl gradient. The protein composition of purified DLPs and TLPs was verified by SDS–PAGE, and their protein content was determined by the Bradford protein assay (Bio-Rad).

Infectivity assays

Confluent monolayers of MA104 cells in 96-well tissue culture plates were infected for 1 h at 37°C with twofold dilutions of trypsin-activated rotavirus (10 μg of trypsin per milliliter for 30 min at 37°C). After this time, the excess virus was removed; the cells were washed twice and then incubated for 14 h at 37°C in MEM. The virus-infected cells were determined by an immunoperoxidase focus assay, using a rabbit hyperimmune serum to porcine rotavirus YM, as previously described (Arias *et al.,* 1987). This assay measures the expression of viral antigens during the first round of infection. The virus titer was expressed as focus-forming units (ffu) per milliliter. The focus-forming units were counted with the help of a Visiolab semiautomatic system as described previously (Guerrero *et al.,* 2000b).

For the infectivity assays in the presence of different divalent ions, the MEM was supplemented with $CaCl_2$, $MgCl_2$, $SrCl_2$, $BaCl_2$, $ZnCl_2$, or $MnCl_2$ (Sigma Chemical Co.) to achieve a final concentration of 10 mM for each cation.

Neuraminidase treatment

MA 104 cells in 96-well plates were treated with different concentrations of NA from *Arthrobacter ureafaciens* (Sigma Chemical Co.) for 1 h at 37°C, as previously described (Méndez *et al.,* 1993). After two washes with MEM, the cells were infected with 1000 ffu/well of RRV or nar3 lysates, which were previously titrated in MEM containing either 2 or 10 mM $CaCl_2$, and processed as described above.

Binding assays

Confluent monolayers of MA104 cells in 48-well plates were incubated with MEM containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Purified TLPs (500 ng/well) in ice-cold MEM–1% BSA with either 2 or 10 mM $CaCl_2$ in a final volume of 500 μl were added to the cells for 1 h on ice with gentle shaking, and then the unbound virus was removed and the cells were washed three times with cold MEM. Finally, 120 μl of MEM was added to each well, the cells were subjected to two rounds of freeze–thaw, and the lysed cells were stored at −70°C. The cell-bound virus present in these lysates was detected by an ELISA as described previously (Zarate *et al.,* 2000), and its viral titer was determined by an immunoperoxidase focus assay as described above.

Fluorescence measurements

Fluorescence spectra were obtained using a Perkin–Elmer luminescence spectrometer (Model LS-50B), at 25°C in 1-cm quartz cells (Perkin–Elmer) with a magnetic stirrer. Intrinsic fluorescence was measured by exciting the samples at 295 nm and collecting the emission between 300 and 400 nm, at 1500 nm/min. The intrinsic tryptophan fluorescence of purified viral particles (10 μg/ml) was measured in physiological solution [130 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.5 mM $NaHPO_4$, 5 mM HEPES–Na, 5 mM glucose (pH 7.4)] (Santi *et al.,* 1998), containing 2 or 10 mM $CaCl_2$. Fluorescence quenching studies were carried out by adding aliquots of freshly prepared 5 mM KI (Yang and Teng, 1998) to virus suspensions in either 2 or 10 mM calcium. The degree of quenching was calculated by the modified Stern–Volmer equation (Lakowicz, 1986; Lehrer, 1971)

$$F_0/(F_0 - F) = ((1/F_a) + ((1/F_a K[Q])),$$

where F_0 and F are fluorescence intensities of the protein at an appropriate wavelength in the absence and in the presence of the quencher, respectively; F_a is the fraction of fluorescence groups accessible to the quencher; K is the Stern–Volmer quenching constant; and Q is the concentration of quencher. A plot of F_0/

($F_0 - F$) versus 1/[Q] will yield a straight line with a slope of 1/($F_a K$) and an intercept of 1/F_a.

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