

Received 6 October 2003

Accepted 8 January 2004

CAVEOLAE AS AN ADDITIONAL ROUTE FOR INFLUENZA VIRUS ENDOCYTOSIS IN MDCK CELLS

ISABEL NUNES-CORREIA^{1,2}, ANA EULÁLIO^{1,2}, SHLOMO NIR³
and MARIA C. PEDROSO DE LIMA^{1,2,*}

¹Department of Biochemistry, University of Coimbra, Apartado 3126, 3000 Coimbra, Portugal, ²Center for Neuroscience and Cell Biology, University of Coimbra, 3000 Coimbra, Portugal, ³Seagram Center for Soil and Water Sciences, Faculty of Agricultural, Food and Environmental Quality Sciences, Hebrew University of Jerusalem, Rehovot 76100, Israel

Abstract: Clathrin-mediated endocytosis has been described as the primary internalization pathway for many viruses, including the influenza virus. However, caveolae, an alternative clathrin-independent endocytotic pathway, has also been described as mediating the entry of some molecules, including viruses. To address the question of pathway selection by the influenza virus, we have investigated whether the virus is internalized via clathrin-coated pits and/or caveolae in Madin Darby canine kidney (MDCK) cells. By applying pharmacological manipulations to selectively disrupt the cell internalization pathways, we found that, in MDCK cells, the influenza virus may be internalized via caveolae in addition to entry by clathrin-mediated endocytosis. However, a small contribution by another mode of entry, as recently proposed [Sieczkarski, S.B. and Whittaker, G.R., *J. Virol.* **76** (2002) 10455-10464], cannot be excluded.

Key Words: Influenza Virus, MDCK Cells, Endocytosis, Clathrin, Caveolae

INTRODUCTION

Influenza virus is a lipid-enveloped virus that belongs to the family of orthomyxoviruses. This virus is a common pathogen of humans and of a wide variety of animal species [1], causing significant morbidity and mortality. One of the major surface viral proteins, which projects radially from the outer lipid

* Corresponding author: Maria C. Pedroso de Lima; E-mail: mdelima@ci.uc.pt

Abbreviations used: HA – hemagglutinin; M β CD – methyl- β -cyclodextrin; MDCK – Madin Darby canine kidney; NEM – *N*-ethylmaleimide; R18 – octadecylrhodamine B chloride.

bilayer, is hemagglutinin (HA) [2-4], the most studied fusion protein. HA mediates the binding of the virus to sialic acid-containing receptors on the target cell surface [5-7]. After virus-cell binding, influenza virus is internalized by receptor-mediated endocytosis [8]. Virions are, therefore, delivered into endosomes, compartments involved in intracellular sorting [8]. The acid pH generated in these organelles by the vacuolar proton ATPase induces a conformational change in the viral HA, which triggers fusion between the viral envelope and endosomal membranes [2, 9, 10].

Several viruses, including influenza virus, were identified in clathrin-coated vesicles during early stages of internalization [11-13]. However, non-coated vesicles containing the virus were also observed in these studies [12]. Caveolae, an alternative clathrin-independent endocytotic pathway, has recently been described as mediating the entry of some molecules and viruses, such as SV-40 [14-18]. Caveolae are small flask-shaped invaginations of the plasma membrane, characterized by high levels of cholesterol and glycosphingolipids and also by the presence of caveolin, an integral membrane protein [19, 20]. These structures are found most abundantly in certain endothelia of the continuous type but also to various degrees in many, if not all, cell types [20].

Cholesterol, a lipid that regulates both the flexibility and mechanical stability of the membrane bilayer, plays a critical role in assembling membrane microdomains, such as rafts and caveolae, in a separate phase from the rest of the bilayer [21-23]. Thus, the most effective way of disrupting caveolae function involves the use of sterol-binding drugs that sequester cholesterol. The depletion, redistribution, or removal of this lipid results in the flattening and disassembly of caveolae, and unclustering of receptors, leading to the loss of caveolae-mediated endocytosis [20, 24, 25]. Such sterol-binding drugs include nystatin, filipin and methyl- β -cyclodextrin (M β CD) [16, 20, 25, 26], none of which typically affect clathrin-mediated endocytosis [19, 25, 26].

To address the question of pathway selection by influenza virus, we investigated whether the virus is internalized via clathrin-coated pits and/or caveolae in Madin Darby canine kidney (MDCK) cells. MDCK cells were used because they exhibit endocytotic activity through both pathways [12, 27, 28] and also because of our previous studies on the characterization of influenza virus interactions with this cell line [29, 30]. To quantify influenza virus fusion activity with the endosomal membrane of MDCK cells, we used the fluorescent probe octadecylrhodamine B chloride (R18). As we previously described [31], this fluorescent probe seems to be the probe of choice to monitor membrane fusion when it is necessary to label pre-existing bilayers. By applying pharmacological manipulations to selectively disrupt the cell internalization pathways, we found that influenza virus may be internalized via caveolae in addition to entry by clathrin-mediated endocytosis. Recently, it was demonstrated that influenza virus might infect HeLa cells by an endocytotic pathway, which is not clathrin or caveolae-dependent [32]. The current study does not exclude the possibility of such a mode of entry in the case of MDCK cells, but by focusing on the issue of binding and fusion, we find that both clathrin

and caveolae-mediated endocytosis play an important role in the pH-dependent fusion of influenza virus with endosomes.

MATERIALS AND METHODS

Materials

The A/PR8/34 (H1N1) strain of the influenza virus, was obtained from SPAFAS (Preston, CT). The virus was grown for 48 h at 37°C in the allantoic cavity of 11-day-old specific pathogen-free embryonated eggs, purified by discontinuous sucrose density gradient centrifugation and stored at -70°C in phosphate saline buffer. Cells were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Antimycin A, NaN₃, Sephadex G-25, filipin, chlorpromazine, M β CD and *N*-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (St. Louis, MO), nystatin from Calbiochem (San Diego, CA), trypsin from Gibco (Grand Island, NY) and octadecylrhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR).

Viral labeling

Viral preparations were labeled with the fluorescent probe octadecylrhodamine B chloride as previously described [7, 29, 31, 33-34]. The final self-quenching concentration of added probe corresponded to approximately 5 mol % of total viral lipid and that of ethanol was less than 1% (v/v). The mixture was incubated in the dark for 30-45 min at room temperature. R18-labeled virus was separated from noninserted fluorophore by passage through a column (Biorad bio-spin) of Sephadex G-25, and was collected under centrifugation at 850 \times g for 4 min. The protein concentration of the labeled virus was determined using the Sedmak assay [35].

Cells

MDCK cells were maintained in DME medium containing 10 mM Hepes and 1 g/L sodium bicarbonate, supplemented with 10% fetal bovine serum, pH 7.4. The cells were grown in T-75 flasks under a 5% CO₂/95% air atmosphere at 37°C up to cell confluence. As this cell line grows adherent to the bottom of the flasks, just before experiments, cells were placed in suspension after a brief treatment with trypsin (0,05%). The cells were harvested and washed by centrifugation at 180 \times g for 5 min at room temperature in DME medium, and then once in medium A containing 110 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM Hepes, 10 mM Mes and 10 mM sodium acetate, pH 7.4. The cells were resuspended in the latter buffer and kept on ice in polypropylene tubes until use. Cell viability was determined using Trypan blue exclusion and was routinely above 95%.

Cell treatments

MDCK cells (5.0×10^6 cells) were preincubated for 30 min at 37°C in 2 ml of medium A containing chlorpromazine (10 μ g/ml) or nystatin (25 μ g/ml).

Alternatively, MDCK cells (5.0×10^6 cells) were preincubated for 15 min at 37°C in 2 ml of medium A containing filipin (1 µg/ml or 5 µg/ml) or NEM (1 mM). Every agent was included in the medium throughout the subsequent incubations. Stock solutions of filipin and NEM were made in ethanol and the final added ethanol concentration was 1% (v/v) or less.

Potassium depletion of MDCK cells

MDCK cells were rinsed with K^+ -free buffer (140 mM NaCl, 20 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml D-glucose, pH 7.4) and subsequently rinsed for 5 min with hypotonic buffer (K^+ -free buffer diluted 1:1 with water). Cells were then washed twice with K^+ -free buffer before fusion experiments. As a control, cells were incubated in a similar buffer containing 5 mM KCl.

Fusion of R18 labeled influenza virus with MDCK cells

Fusion was monitored continuously using the R18 fluorescence assay [7, 29, 31, 33-34] at 37°C. Fusion of influenza virus with the endosomal membrane was monitored following rapid injection of R18-labeled virus (2 µg viral protein) into a cuvette containing untreated (control) or treated MDCK cells (5×10^6 cells) in a final volume of 2 ml of Medium A, at pH 7.4. Parallel experiments were performed in the presence of the following metabolic inhibitors of endocytosis: 1 µg/ml antimycin A, 10 mM NaF and 1 mg/ml sodium azide [36]. Fusion of influenza virus with the plasma membrane was triggered by lowering the pH of the external medium to 5 following a 5 min virus preincubation with cells at pH 7.4. The fluorescence scale was calibrated such that the initial fluorescence of the R18-labeled virus and cell suspension was set at 0% fluorescence. The value obtained by lysing the virus and cellular membranes after each experiment with $C_{12}E_8$ (at a final concentration of 2 mM), was set at 100% fluorescence. Fluorescence measurements were performed in a Spex Fluorolog 2 fluorometer using the front-face configuration in the emission channel, with excitation at 560 nm and emission at 590 nm. A high-pass filter (50% transmission at 590 nm: Schott Glass OG590, Melles-Griot) was placed between the cuvette and the emission monochromator. The sample chamber was equipped with a magnetic stirring device and the temperature was controlled with a thermostatted circulating water bath.

Cell binding and cell association

Fluorescently labeled influenza virus was incubated with control or treated MDCK cells (5.0×10^6 cells), in a final volume of 2 ml of medium A for various times at 4°C or 37°C. The cells were sedimented by centrifugation at 4°C for 8 min at $180 \times g$, and the fluorescence was measured at 37°C in the supernatant and in the pellet following detergent lysis of the virus and the membranes after each experiment with $C_{12}E_8$ (at a final concentration of 2 mM). The percentages of cell binding and cell association were calculated according to the following equation:

$$\% \text{ binding or cell association} = \frac{F_{\text{pellet}}}{F_{\text{supernatant}} + F_{\text{pellet}}} \times 100$$

where F is the value of fluorescence.

RESULTS AND DISCUSSION

Involvement of clathrin-mediated endocytosis in influenza virus entry

The entry of influenza virus into MDCK cells by clathrin-mediated endocytosis has already been described [12]. However, we addressed the question whether additional entry pathways could exist. To distinguish between the relative involvement of a clathrin-mediated *versus* non-clathrin mediated internalization pathway for influenza virus endocytosis in MDCK cells, we investigated the effect of pretreating the cells with chlorpromazine on virus-cell interaction. This cationic amphiphilic drug causes clathrin to localize and accumulate in late endosomes, thereby inhibiting coated pit endocytosis [16, 37]. Preincubation of MDCK cells with chlorpromazine and keeping the drug present during the incubation of influenza virus with the cells at 37°C and pH 7.4 caused an inhibition on virus fusion with the endosomal membrane of MDCK cells of approximately 60% at 30 min incubation (Fig. 1).

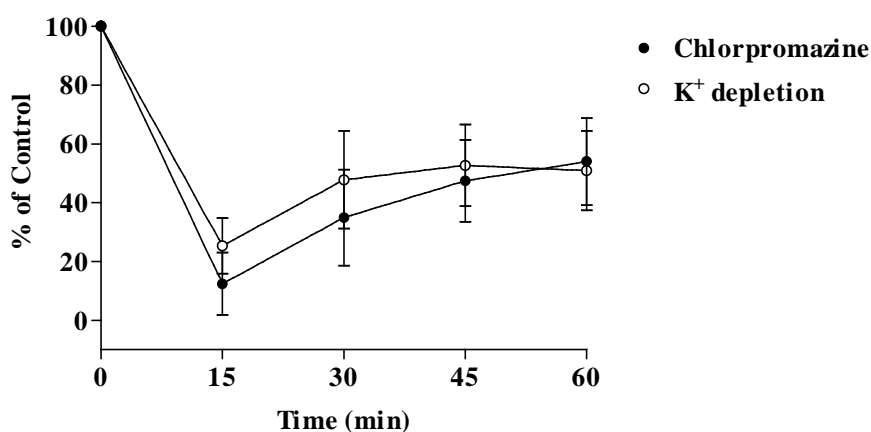


Fig. 1. Effect of chlorpromazine and potassium depletion on the lipid mixing kinetics of influenza virus with the endosomal membrane of MDCK cells. MDCK cells (5×10^6) were preincubated with chlorpromazine (10 $\mu\text{g}/\text{ml}$) (●) for 30 min at 37°C or subjected to K⁺ depletion (○). Thereafter, R18 labeled influenza virus (2 μg viral protein) was added to the cells and the kinetics of lipid mixing was followed for 60 min at 37°C in a final volume of 2 ml at pH 7.4. The results are expressed as the percentage of the untreated control cells (100%). Each point represents the mean \pm standard deviation of at least four experiments.

Since cell treatment with chlorpromazine has been reported to be non-specific [32], we used the more specific and less toxic technique of potassium depletion combined with hypotonic shock to obtain further insight into the role of clathrin-

mediated internalization pathway for influenza virus endocytosis in MDCK cells. Such treatment has been well established to reversibly arrest clathrin-coated-pit formation [38]. As illustrated in Fig. 1, a 50% inhibition of influenza virus fusion with the endosomal membrane of potassium-depleted MDCK cells at the 30 min incubation point was detected, similar to what was observed with chlorpromazine. However, the inhibition was not complete, indicating that clathrin-mediated endocytosis is not the only internalization pathway for influenza virus in MDCK cells. In potassium-depleted and chlorpromazine-treated cells, the decrease in the extent of fusion is more pronounced at early times of virus-cell incubation (15 min) than later. However, due to the high variability of the experimental results, we cannot conclude whether this apparent recovery of virus internalization is significant.

In order to account for some unspecific exchange of the R18 probe, the values obtained for the fluorescence increase from parallel experiments performed in the presence of metabolic inhibitors of endocytosis were subtracted from those in the absence of the inhibitors, both in chlorpromazine treated and potassium-depleted cells.

Involvement of caveolae-mediated endocytosis in influenza virus entry

To investigate the potential contribution of caveolae-mediated endocytosis in influenza virus internalization, we examined the effects of treating MDCK cells with nystatin and filipin, on virus fusion with the endosomal membrane. Nystatin and filipin are sterol-binding agents that sequester cholesterol, a prominent component of lipid rafts involved in caveolae formation, thus disrupting caveolae structure and function [16, 20, 24, 25]. Both drugs are believed to selectively inhibit caveolae invaginations, leaving coated-pit organization unaffected and without perturbing the uptake of ligands through this pathway [19, 24]. Preincubating MDCK cells with nystatin and maintaining nystatin presence during the subsequent virus-cell incubation did not significantly reduce the fusion activity of influenza virus towards the endosomal membrane of MDCK cells (Fig. 2). These results seem to indicate that influenza virus does not require the caveolae uptake pathway in MDCK cells. However, the exposure of MDCK cells to filipin greatly inhibited fusion of influenza virus with the endosomal membrane, 1 $\mu\text{g/ml}$ of filipin being sufficient to decrease the extent of lipid mixing by nearly 50%, thus indicating that influenza virus entry in MDCK cells may occur to some extent by caveolae-mediated endocytosis. It should be noted that viability of filipin-treated cells (at both 1 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ of filipin), as assessed by Trypan blue exclusion, was always $\geq 90\%$ of the untreated control cells (data not shown).

In order to account for some unspecific exchange of the R18 probe, the values obtained for the fluorescence increase from parallel experiments performed in the presence of metabolic inhibitors of endocytosis were subtracted from those in the absence of the inhibitors, both in the case of nystatin and filipin treatment.

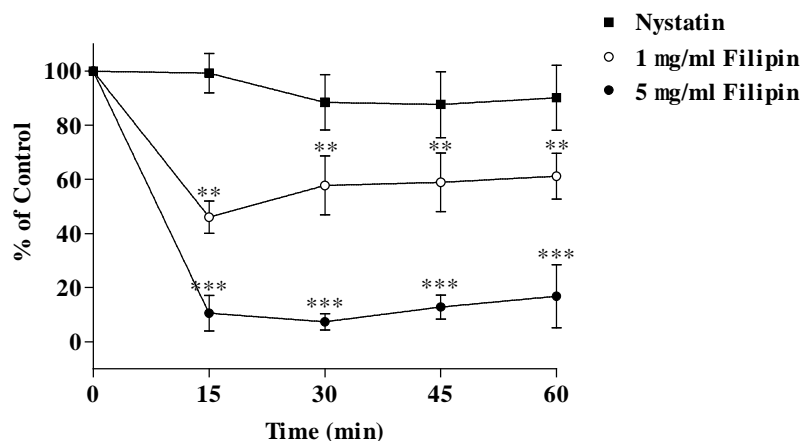


Fig. 2. Effect of nystatin and filipin on the lipid mixing kinetics of influenza virus with the endosomal membrane of MDCK cells. MDCK cells (5×10^6) were preincubated with nystatin (25 $\mu\text{g}/\text{ml}$) (■) for 30 min at 37°C or with 1 $\mu\text{g}/\text{ml}$ (○) or 5 $\mu\text{g}/\text{ml}$ (●) of filipin for 15 min at 37°C. Thereafter, R18 labeled influenza virus (2 μg viral protein) was added to the cells and the kinetics of lipid mixing was followed for 60 min at 37°C in a final volume of 2 ml at pH 7.4. The results are expressed as the percentage of the untreated control cells (100%). Each point represents the mean \pm standard deviation of at least four experiments. Symbols indicate significance relative to control by unpaired *t*-test, ** $P \leq 0.01$, *** $P \leq 0.001$.

Usually, when nystatin and filipin are used to block caveolae-mediated endocytosis, they are described as having similar effects [20, 39]. Therefore, and since the inhibitory effect of filipin on virus fusion with the endosomal membrane could potentially result from its direct interaction with the receptor and/or ligand, preventing cell surface binding, we tested the effect of filipin on influenza virus binding to the plasma membrane of MDCK cells. For this purpose, experiments were carried out by preincubating the cells with filipin for 15 min at 37°C before performing the binding assay at 4°C for 60 min. We found no evidence for interference with the binding (Fig. 3). Next, we considered the possibility that the decrease in the extent of virus fusion with the endosomal membrane, upon cell treatment with filipin, would be due to an inhibition of membrane lipid mixing. To test this possibility, we investigated the effect of filipin on the low pH-induced fusion of influenza virus with the plasma membrane of MDCK cells. As shown in Fig. 3, cell treatment with filipin did not result in any inhibitory effect. From these results, we can conclude that the inhibitory effect of filipin on virus fusion with the endosomal membrane is attributed to a decrease in the extent of virus internalization. Since cell association encompasses virus binding, internalization and fusion with the endosomal membrane, such a decrease might be reflected in a reduction of the percentage of virus that associated with filipin-treated cells. In fact, we observed a slight but statistically significant decrease in cell association following virus-cell incubation for 30 min at 37°C upon cell treatment with filipin (Fig. 3). It

should be recalled that a significant fraction of virus association with these cells following 30 min incubation arises from its binding. These results confirm our hypothesis that the inhibitory effect of filipin is exerted at the level of virus internalization.

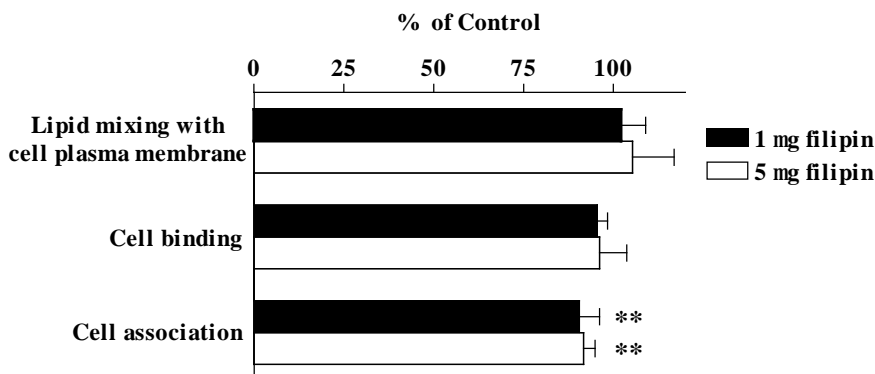


Fig. 3. Effect of filipin on influenza virus interaction with MDCK cells: cell binding, cell association and fusion with the cell plasma membrane. MDCK cells (5×10^6) were incubated with 1 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$ of filipin for 15 min at 37°C in a final volume of 2 ml at pH 7.4. Thereafter, R18 labeled influenza virus (2 μg viral protein) was added to the cells. In cell binding experiments, the virus was incubated with the cells for 60 min at 4°C, while in cell association experiments, virus-cell incubation was performed for 30 min at 37°C. The experimental values for virus cell binding/cell association were determined from fluorescence values in the pellet and supernatant, after the addition of C_{12}E_8 to dequench the R18. Lipid mixing of influenza virus with the plasma membrane of MDCK cells was monitored for 10 min at 37°C at pH 5, following virus-cell preincubation for 5 min at 37°C at pH 7.4. The results are expressed as a percentage of untreated control cells (100%). Each point represents the mean \pm standard deviation of at least four experiments. Symbols indicate significance relative to the control by unpaired *t*-test, $^{***}P \leq 0.01$.

While this study was being conducted, it was reported that influenza virus might infect HeLa cells by an additional non-clathrin-dependent, non-caveolae-dependent endocytotic pathway [32]. These authors used nystatin and M β CD to block caveolae-mediated endocytosis. M β CD has been described to bind cholesterol with high specificity [40] and has therefore been extensively used as a cholesterol-depleting reagent [26, 32, 41-43]. However, other studies reported that M β CD induces the inhibition of clathrin-mediated endocytosis, whereas non-clathrin-mediated endocytosis is much less affected [44]. In our studies, attempts made to use M β CD failed, because this cyclodextrin interfered with R18 fluorescence (data not shown). Nystatin had no effect on the lipid mixing kinetics of influenza virus with the endosomal membrane using 25 $\mu\text{g/ml}$, indicating that caveolae may not be involved in influenza virus entry in MDCK cells, as had been observed with HeLa cells [32]. However, as we observed an inhibitory effect at the level of virus internalization upon cell treatment with filipin, we believe that caveolae may be involved at some extent in the internalization of

influenza virus in MDCK cells. It is possible that the use of 25 $\mu\text{g/ml}$ of nystatin is not sufficient to induce a similar inhibition to that observed with filipin on influenza virus entry in MDCK and in HeLa cells.

Effect of NEM on influenza virus endocytosis

It is well established in a variety of cell types and in reconstituted cell-free assays *in vitro* that transport mediated by membrane vesicular carriers between specific intracellular compartments can be inhibited by NEM. It was demonstrated that this thioalkylating agent inhibits transferrin uptake, which is mediated by clathrin-coated vesicles, and caveolae-mediated intracellular and transcellular transport [14, 27, 45]. These vesicular pathways may require key intracellular component(s) that are sensitive to alkylation with NEM [45]. Accordingly, we decided to investigate the effect of this thioalkylating agent in order to obtain additional experimental support for the involvement of caveolae in influenza virus cell uptake. Fig. 4A shows that fusion of influenza virus with the endosomal membrane of MDCK cells is essentially abolished upon cell treatment with 1 mM of NEM. It should be noted that the results shown in Fig. 4 represent the difference between the values observed for the fluorescence increase in the absence of metabolic inhibitors of endocytosis and those obtained from parallel experiments performed in the presence of the same inhibitors. Similarly to what was observed with filipin, NEM had no effect on binding and on low pH-induced fusion of influenza virus with the plasma membrane of MDCK cells (Fig. 4B). However, we observed a significant reduction of the percentage of virus that associated with NEM-treated cells, indicating that this thioalkylating agent exerts its inhibitory effect at the level of virus internalization by MDCK cells, similarly to what was observed with filipin. Cell viability after NEM treatment was $\geq 85\%$ of the untreated control cells (data not shown).

The observation that the inhibition of influenza virus fusion with the endosomal membrane of MDCK cells following cell treatment with NEM is more extensive than that obtained with chlorpromazine and potassium depletion gives support to the notion that influenza virus may be internalized via caveolae-mediated endocytosis and/or by an additional non-clathrin-dependent, non-caveolae-dependent endocytotic pathway, in addition to clathrin-mediated endocytosis in MDCK cells. The interaction between influenza virus and MDCK cells at neutral pH resulted in similar values of fluorescence increase using NEM or the metabolic inhibitors of endocytosis (antimycin A, Sodium azide and NaF). These results provide evidence that under the conditions used to quantify R18 unspecific probe transfer, the endocytotic pathways are completely inhibited, and therefore the fluorescence increase observed with time is exclusively due to unspecific R18 probe transfer.

The results obtained in this study show that caveolae-mediated endocytosis may play an important role in influenza virus entry into MDCK cells in addition to clathrin-mediated endocytosis. However, an additional non-clathrin-dependent,

non-caveolae-dependent endocytotic pathway cannot be excluded. It should be stressed that the caveolae pathway and that involving clathrin-coated pits are not mutually exclusive and thus each one can yield varying contributions to the entry of influenza virus into cells depending on the cell type.

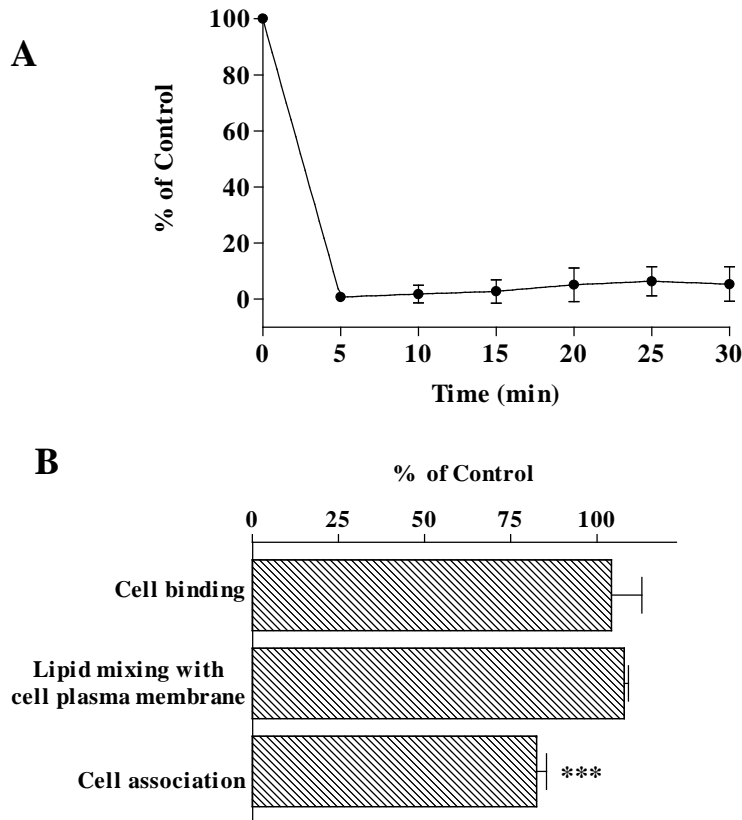


Fig. 4. Effect of *N*-ethylmaleimide on influenza virus interaction with MDCK cells. A: lipid mixing kinetics with the endosomal membrane. B: cell binding, cell association and fusion with the cell plasma membrane. MDCK cells (5×10^6) were incubated with NEM (1 mM) for 15 min at 37°C in a final volume of 2 ml at pH 7.4. Thereafter, R18 labeled influenza virus (2 μ g viral protein) was added to the cells. Lipid mixing kinetics between influenza virus and the endosomal membrane of MDCK cells was followed for 30 min at 37°C. In cell binding experiments, the virus was incubated with the cells for 60 min at 4°C, while in cell association experiments, virus-cell incubation was performed for 30 min at 37°C. The experimental values for virus cell binding/cell association were determined from fluorescence values in the pellet and supernatant, after the addition of $C_{12}E_8$ to dequench the R18. Lipid mixing of influenza virus with the plasma membrane of MDCK cells was monitored for 10 min at 37°C at pH 5, following virus-cell preincubation for 5 min at 37°C at pH 7.4. The results are expressed as a percentage of untreated control cells (100%). Each point represents the mean \pm standard deviation of at least four experiments. Symbols indicate significance relative to control by unpaired *t*-test, *** $P \leq 0.001$.

Acknowledgement. This study was supported by Grant PRAXIS XXI/BD/21347/99 from FCT, Portugal (I. N-C.) and by Grant POCTI/CVT/42700/2001 from FCT, Portugal (M. C. P. L.).

REFERENCES

1. Cox, N.J. and Fukuda, K. Influenza. **Infect. Dis. Clin. North Am.** 12 (1998) 27-38.
2. Hernandez, L.D., Hoffman, L.R., Wolfsberg, T.G. and White, J.M. Virus-cell and cell-cell fusion. **Annu. Rev. Cell Dev. Biol.** 12 (1996) 627-661.
3. Hoekstra, D. and Pedroso de Lima, M.C. Membrane interactions of HIV: Implications for pathogenesis and therapy in AIDS. in: **Advances in membrane fluidity** (Aloia, R.C. and Curtain, C.C., Eds.), Wiley-Liss, New York, 1992, 71-97.
4. Wiley, D.C. and Skehel, J.J. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. **Annu. Rev. Biochem.** 56 (1987) 365-394.
5. Bergelson, L.D., Bukrinskaya, A.G., Prokazova, N.V., Shaposhnikova, G.I., Kocharov, S.L., Shevchenko, V.P., Kornilaeva, G.V. and Fomina-Ageeva, E.V. Role of gangliosides in reception of influenza virus. **Eur. J. Biochem.** 128 (1982) 467-474.
6. Paulson, J.C., Rogers, G.N., Murayama, J.I., Sze, G. and Martin, E. Biological implications of influenza virus receptor specificity. in: **Virus attachment and entry into cells.** (Crowell, R.L. and Lonberg-Holm, K., Eds.) American Society for Microbiology, Washington, DC, 1986, 144-151.
7. Pedroso de Lima, M.C., Ramalho-Santos, J., Flasher, D., Slepishkin, V.A., Nir, S. and Düzgünes, N. Target cell membrane sialic acid modulates both binding and fusion activity of influenza virus. **Biochim. Biophys. Acta** 1236 (1995) 323-330.
8. Mellman, I. Endocytosis and molecular sorting. **Annu. Rev. Cell Dev. Biol.** 12 (1996) 575-625.
9. Gaudin, Y., Ruigrok, R.W.H. and Brunner, J. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. **J. Gen. Virol.** 76 (1995) 1541-1556.
10. Ramalho-Santos, J. and Pedroso de Lima, M.C. The influenza virus hemagglutinin: a model protein in the study of membrane fusion. **Biochim. Biophys. Acta** 1376 (1998) 147-154.
11. Marsh, M. and Helenius, A. Adsorptive endocytosis of Semliki Forest virus. **J. Mol. Biol.** 142 (1980) 439-454.
12. Matlin, K.S., Reggio, H., Helenius, A. and Simons, K. Infectious entry pathway of influenza virus in a canine kidney cell line. **J. Cell Biol.** 91 (1981) 601-613.

13. Matlin, K.S., Reggio, H., Helenius, A. and Simons, K. Pathway of vesicular stomatitis virus entry leading to infection. **J. Mol. Biol.** 156 (1982) 609-631.
14. Anderson, R.G. The caveolae membrane system. **Annu. Rev. Biochem.** 67 (1998) 199-225.
15. Kartenbeck, J., Stukenbrok, H. and Helenius, A. Endocytosis of simian virus 40 into the endoplasmic reticulum. **J. Cell Biol.** 109 (1989) 2721-2729.
16. Orlandi, P.A. and Fishman, P.H. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. **J. Cell Biol.** 141 (1998) 905-915.
17. Pelkmans, L., Kartenbeck, J. and Helenius, A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. **Nat. Cell Biol.** 3 (2001) 473-483.
18. Ros-Baró, A., López-Iglesias, C., Peiró, S., Bellido, D., Palacín, M., Zorzano, A. and Camps, M. Lipid rafts are required for GLUT4 internalization in adipose cells. **Proc. Natl. Acad. Sci. USA** 98 (2001) 12050-12055.
19. Lefkowitz, R.J. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. **J. Biol. Chem.** 273 (1998) 18677-18680.
20. Schnitzer, J.E., Oh, P., Pinney, E. and Allard, J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of selected macromolecules. **J. Cell Biol.** 127 (1994) 1217-1232.
21. Brown, D.A. and London, E. Functions of lipid rafts in biological membranes. **Annu. Rev. Cell Dev. Biol.** 14 (1998) 111-136.
22. Brown, D.A. and London, E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. **J. Biol. Chem.** 275 (2000) 17221-17224.
23. Simons, K. and Ikonen, E. Functional rafts in cell membranes. **Nature** 387 (1997) 569-572.
24. Rothberg, K.G., Ying, Y.S., Kamen, B.A. and Anderson, R.G. Cholesterol controls the clustering of the glycopospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. **J. Cell Biol.** 111 (1990) 2931-2938.
25. Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.S., Glenney, J.R. and Anderson, R.G. Caveolin, a protein component of caveolae membrane coats. **Cell** 68 (1992) 673-682.
26. Neufeld, E.B., Cooney, A.M., Pitha, J., Dawidowicz, E.A., Dwyer, N.K., Pentchev, P.G. and Blanchette-Mackie, E.J. Intracellular trafficking of cholesterol monitored with a cyclodextrin. **J. Biol. Chem.** 271 (1996) 21604-21613.
27. Hoekstra, D. and Kok, J.W. Entry mechanisms of enveloped viruses. Implications for fusion of intracellular membranes. **Biosci. Rep.** 9 (1989) 273-305.

28. Thomsen, P., Roepstorff, K., Stahlhut, M. and van Deurs, B. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. **Mol. Biol. Cell** 13 (2002) 238-250.
29. Nunes-Correia, I., Ramalho-Santos, J., Nir, S. and Pedroso de Lima, M.C. Interactions of influenza virus with cultured cells: detailed kinetic modeling of binding and endocytosis. **Biochemistry** 38 (1999) 1095-1101.
30. Nunes-Correia, I., Nir, S. and Pedroso de Lima, M.C. Kinetics of influenza virus fusion with the endosomal and plasma membranes of cultured cells: effect of temperature. **J. Memb. Biol.** 195 (2003) 21-26.
31. Nunes-Correia, I., Eulálio, A., Nir, S., Düzgünes, N., Ramalho-Santos, J. and Pedroso de Lima, M.C. Fluorescent probes for monitoring virus fusion kinetics: comparative evaluation of reliability. **Biochim. Biophys. Acta** 1561 (2002) 65-75.
32. Sieczkarski S.B. and Whittaker, G.R. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. **J. Virol.** 76 (2002) 10455-10464.
33. Hoekstra, D., de Boer, T., Klappe, K. and Wilschut, J. Fluorescent method for measuring the kinetics of fusion between biological membranes. **Biochemistry** 23 (1984) 5675-5681.
34. Ramalho-Santos, J., Pedroso de Lima, M.C. and Nir, S. Partial fusion activity of influenza virus towards liposomes and erythrocyte ghosts is distinct from viral inactivation. **J. Biol. Chem.** 271 (1996) 23902-23906.
35. Sedmak, J.J. and Grossero, F.E. A rapid sensitive and versatile assay for protein using coomassie brilliant Blue G250. **Anal. Biochem.** 79 (1977) 544-552.
36. Lee, K.-D., Nir, S. and Papahadjopoulos, D. Quantitative analysis of liposome-cell interactions in vitro: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. **Biochemistry** 32 (1993) 889-899.
37. Wang, L.H., Rothberg, K.G. and Anderson, R.G. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. **J. Cell Biol.** 123 (1993) 1107-1117.
38. Larkin, J.M., Brown M.S., Goldstein J.L. and Anderson R.G. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. **Cell** 33 (1983) 273-285.
39. Okamoto, Y., Ninomiya H., Miwa, S. and Masaki, T. Cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits in Chinese hamster ovary cells. **J. Biol. Chem.** 275 (2000) 6439-6446.
40. Pitha, J., Irie, T., Sklar, P.B. and Nye, J.S. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. **Life Sci.** 43 (1988) 493-502.

41. Chen, X. and Resh, M D. Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor. **J. Biol. Chem.** 277 (2002) 49631-49637.
42. Hooper, N.M. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). **Mol. Membr. Biol.** 16 (1999) 145-156.
43. Ostermeyer, A.G., Beckrich, B.T., Ivarson, K.A., Grove, K.E. and Brown, D.A. Glycosphingolipids are not essential for formation of detergent-resistant membrane rafts in melanoma cells. **J. Biol. Chem.** 274 (1999) 34459-34466.
44. Zuhorn, I.S., Kalicharan, R. and Hoekstra, D. Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. **J. Biol. Chem.** 277 (2002) 18021-18028.
45. Schnitzer, J.E., Allard, J. and Oh, P. NEM inhibits transcytosis, endocytosis, and capillary permeability: implication of caveolae fusion in endothelia. **Am. J. Physiol.** 268 (1995) H48-H55.