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Kinetics of Influenza Virus Fusion with the Endosomal and Plasma Membranes of Cultured Cells. Effect of Temperature

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Abstract. We performed a detailed kinetic analysis of influenza virus fusion with the endosomal and plasma membranes of Madin Darby canine kidney (MDCK) cells and provided a comparison of the kinetic parameters obtained for both cases at 20°C and 37°C. Using our mass action kinetic model, we determined that the fusion rate constant, *f*, for influenza virus with the endosomal membrane was 0.02 s^{-1} at 37°C and 0.0035 s^{-1} at 20°C. The analysis of the fusion kinetics of influenza virus with the plasma membrane yielded that the fusion rate constants were close to those deduced with the endosomal membrane. The systematic kinetic analysis performed in this study provides for the first time a biophysical support for studies on influenza virus-cell fusion where the acidic endosomal internal environment is simulated artificially by lowering the pH of the medium.

Key words: Influenza virus — Endocytosis — Membrane fusion — MDCK cells — Kinetic analysis

Introduction

Influenza virus is the most studied member of the orthomyxovirus family. This lipid-enveloped virus attaches to target cells' surface sialic residues [1, 20, 22] through its hemagglutinin (HA), a viral membrane glycoprotein [5, 8, 31]. After virus-cell binding, influenza virus enters the host cell by receptor-mediated endocytosis [10]. The low pH of the endosomal lumen, generated by a vacuolar proton ATPase, leads to a conformational change in the viral HA, including an extension of the N-terminal hydrophobic fusion peptide towards the inner endosomal membrane. The overall process culminates in the fusion of the viral envelope with the endosomal membrane and in the subsequent release of the viral RNA into the target cell cytosol [4, 5, 25, 30].

During the last years, a large number of studies were performed with the aim of characterizing many biochemical aspects regarding the binding and association of vesicles and viruses to cells, and the subsequent fusion between their membranes [7, 19, 21, 23, 24, 28]. However, a detailed quantification of the processes involved in viral entry that would permit the determination of the kinetic parameters of the adhesion and/or fusion steps has not yet been described and is indispensable for further analysis. Indeed, the results obtained from such studies may allow the development of strategies to prevent viral infection and may also be helpful in other research areas like gene therapy (relying on the uptake of viral and non-viral carriers).

In this context, we have developed a mass action kinetic model that provides the possibility to "uncouple" the kinetics of binding and fusion. The procedure has yielded the values for the rate constants of the aggregation and fusion processes, during virus-cell interaction, thus making it possible to obtain a more detailed description and prediction of the kinetics of the overall fusion process [12]. This model has also been employed to evaluate the kinetics of fusion between influenza virus and liposomes [15], of Sendai virus fusion with phospholipid vesicles, erythrocyte ghosts and cells [12, 13, 21] and of

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Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; HA, hemagglutinin; MDCK cells, Madin Darby canine kidney cells; R18, octadecylrhodamine B chloride.

influenza virus fusion with cells lacking endocytic capacity [3, 22]. By employing a similar formalism, we provided equations for the uptake of particles (liposomes) by cells via endocytosis [9, 14].

Recently, we performed a detailed kinetic analysis of the processes of binding and endocytosis of influenza virus (A/PR8/34) with Madin Darby canine kidney (MDCK) cells in culture, which are known to exhibit endocytotic capacity and to be infected by influenza virus following internalization by endocytosis [17]. In the present work we extended these studies by quantifying the subsequent fusion process. We present a kinetic analysis of influenza virus fusion with the endosomal and the plasma membranes of MDCK cells and provide a comparison of the kinetic parameters obtained for both cases at 20°C and 37°C.

Materials and Methods

Materials

Cells were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Antimycin A, NaN_3 and Sephadex G-25 were purchased from Sigma (St. Louis, MO), $C_{12}E_8$ was from Calbiochem (San Diego, CA) and octadecylrhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR).

Virus

Influenza virus, A/PR8/34 (H1N1) strain 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.

Viral Labeling

Viral preparations were labeled with the fluorescent probe octadecylrhodamine B chloride as previously described [6, 16–18, 22, 24]. 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 by the Sedmak assay [27].

Cells

MDCK cells were maintained in DME medium containing 10 mM HEPES and 1 g/l of sodium bicarbonate, supplemented with 10% fetal bovine serum, pH 7.4. The cells were grown in T-75 flasks under a 5% CO_2/95% 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 treatment for 10 min with a dissociation buffer (enzyme free). 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 (in mM) 110 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, 10 MES and 10 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 by Trypan blue exclusion and was routinely above 95%.

Fusion of R18-labeled Influenza Virus with MDCK Cells

Fusion was monitored continuously with the R18 fluorescence assay [3, 16, 18, 21–24] at 20°C and 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 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 [9]. Alternatively, cells were preincubated with 20 mM NH_4Cl for 15 min at 37°C, followed by incubation of the R18-labeled virus with treated cells for 30 min at 37°C or 20°C in the presence of this lysosomotropic agent. Then, the suspension (virus + cells) was sedimented by centrifugation at 4°C for 8 min at $180 \times g$ and the pellet was rapidly resuspended in 2 ml of Medium A. The monitoring of fluorescence increase was immediately initiated. Parallel experiments were carried out where, following sedimentation of the virus-cell suspension, the pellet was resuspended in 2 ml of medium A containing 20 mM of NH_4Cl. Fusion of influenza virus with the plasma membrane was triggered by lowering the pH of the external medium to 5 following virus preincubation with MDCK cells (5×10^6 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 to 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.

Binding and Cell Association

Fluorescently labeled influenza virus was incubated with MDCK cells (5.0×10^6 cells), in a final volume of 2 ml of Medium A for various times at 20°C or 37°C. The cells were preincubated for 30 min at 37°C with the following metabolic inhibitors: 1 μg/ml antimycin A, 10 mM NaF and 1 mg/ml sodium azide [9]. Antimycin A was solubilized in ethanol and was added to the cells to a final ethanol concentration of 1% (v/v) or less. The experiments were done in the presence of the same endocytosis inhibitors. After virus-cell incubation, 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). Percentages of 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.

Kinetic Analysis

Kinetics of virus fusion with the plasma membrane (after lowering the pH) was as previously described [3, 11, 23]. The analysis

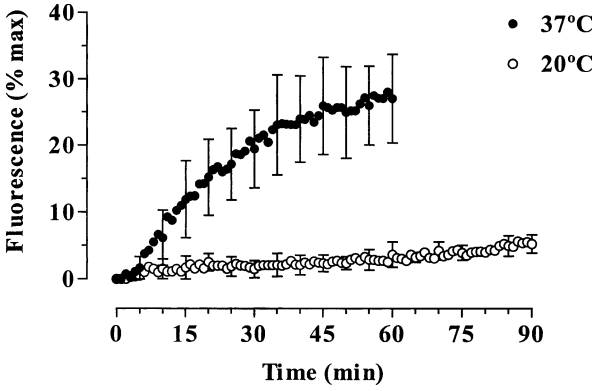

Fig. 1. Kinetics of lipid mixing of influenza virus with the endosomal membrane of MDCK cells. Influenza virus (2 μg viral protein) labeled with R18 was added to 5×10^6 MDCK cells in a final volume of 2 ml at pH 7.4, and incubated at 20°C (◯) and 37°C (●). Parallel experiments were performed in the presence of metabolic inhibitors of endocytosis as described in Materials and Methods and the values of fluorescence obtained were subtracted from those in the absence of the inhibitors. Experimental values are given as mean ± SD of three independent experiments performed in duplicate.

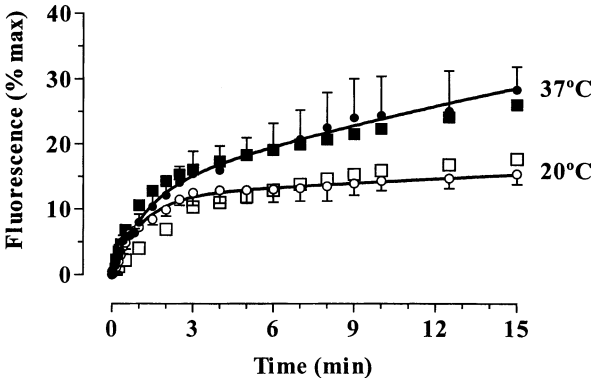

Fig. 2. Kinetics of lipid mixing of influenza virus with the endosomal membrane of MDCK cells. Influenza virus (2 μg viral protein) labeled with R18 was added to 5×10^6 of NH_4Cl-treated MDCK cells in a final volume of 2 ml at pH 7.4 and incubated at 20°C or 37°C for 30 min in the presence of NH_4Cl. The cells were then washed and resuspended in 2 ml of medium in the absence of NH_4Cl and fusion was monitored as a function of R18-fluorescence dequenching at 20°C and 37°C. Parallel experiments were performed, where after being washed the cells were resuspended in 2 ml of medium with NH_4Cl, and the values of fluorescence obtained were subtracted from those in the absence of NH_4Cl. Experimental values at 37°C (●) and at 20°C (○) are given as mean ± SD of three independent experiments performed in duplicate. The squares represent the values calculated by using the mass-action kinetic model for experiments performed at 37°C (■) and 20°C (□).

required a predetermination of the fraction of virus bound in the presence of metabolic inhibitors of endocytosis. In the analysis of the kinetics of virus fusion with the endosomes no delay was introduced. The analysis was based on an extension of a program for binding and endocytosis [14]. In this extension a vector (cyto (3600)) incorporates the amount of virus endocytosed at times 1, 2, ... (s). For each element in this vector, fusion kinetics proceeds as for the bound virus and the cumulative fused fraction of the virus is calculated at any time. As in Nir et al. [11], fusion kinetics was described in terms of two parameters, the rate constant of fusion, f (s^{-1}) and the rate constant of inactivation, γ (s^{-1}).

Results

The fluorescence dequenching of R18 has been used as a reliable indicator of virus-cell fusion in numerous systems [3, 16, 18, 21–24]. In the present study we used this assay to investigate fusion of influenza virus with the endosomal membrane of MDCK cells. The time course of R18 fluorescence dequenching during the incubation of influenza virus with MDCK cells at neutral pH is shown in Fig. 1. In order to account for some unspecific exchange of the R18 probe, we performed parallel experiments in the presence of metabolic inhibitors of endocytosis, and the values obtained for fluorescence increase were subtracted from those in the absence of the inhibitors. In these experiments the virus was allowed to interact with MDCK cells for 60 min at 37°C and for 90 min at 20°C at pH 7.4. As expected, the virus fuses with the cell endosomal membrane more extensively and with a larger initial rate at the higher temperature. At 37°C, a lag phase of approximately 5 min is apparent, which can be attributed to the time required for the first virus to reach the endosomes.

We examined the time course of virus-cell fusion by a mass-action kinetic analysis (*see* Materials and Methods). Using these experimental conditions it is hard to calculate precisely the fusion and inactivation rate constants. The difficulty arises from the fact that the percentage of endocytosed virus cannot be obtained by a mere subtraction of the percentage of bound virus after equilibrium is reached in the presence of metabolic inhibitors of endocytosis, from the percentage of virus that associates with the cells (both determined previously, *see* reference 24). To overcome this problem a different experimental approach was used.

MDCK cells were preincubated with NH_4Cl and the virus was then incubated with the cells in the presence of NH_4Cl for 30 min, as described in Materials and Methods. Under these conditions endocytosis occurs but the virus is unable to fuse with the endosomal membrane because the presence of NH_4Cl prevents acidification of the endosomal lumen. After that period of time the cells were washed and the fluorescence increase was followed for 15 min (Fig. 2). It should be noted that acidification of endosomes is readily restored upon removal of NH_4Cl, as demonstrated previously [2, 29]. In order to rule out the contribution of fluorescence increase due to nonspecific exchange of the R18 probe, parallel experiments were performed where, after being washed, the cells were resuspended in medium with NH_4Cl, and the values of fluorescence obtained were subtracted from

Table 1. Endocytosis, fusion and inactivation rate constants for the interaction of influenza virus with the endosomal membrane of MDCK cells

Temperature	Endocytosis rate constant[1] ε (s^{-1})	Fusion rate constant[2] f (s^{-1})	Inactivation rate constant[2] γ (s^{-1})
37°C	3.5×10^{-4}	0.02	0.003
20°C	2.2×10^{-4}	0.0035	0.003

[1]The estimated uncertainty in ε is 20% at 20°C and 40% at 37°C.
[2]The estimated uncertainties in f and γ are 30% and 50% at 20°C, and 50% and 60% at 37°C, respectively.

those in the absence of NH_4Cl. Therefore, the increase in fluorescence observed is due to fusion of the virus with the endosomal membrane and results from the virus that has associated with the cells during approximately 40 min (30 min incubation with the cells in the presence of NH_4Cl + 8 min during cell washing). This time is sufficient for the virus to reach the endosomes both at 20°C and 37°C [17], and consequently much higher initial rates of fluorescence dequenching (Fig. 2) are expected when compared to the ones observed when the fluorescence increase was followed with time since the first moment of virus contact with the cells, where no preincubation leading to endocytosis was applied (Fig. 1; note the difference in the time scale).

Using the results obtained in this set of experiments, we examined the time course of virus-cell fusion by the mass-action kinetic analysis (*see* Materials and Methods). The endocytosis (ε), fusion (f) and inactivation (γ) rate constants given in Table 1 resulted in fits to the curves in Fig. 2 shown by the squares. In this case the calculated values fit well the experimental values obtained over the 15 min incubation at both temperatures, although an exact fitting of the data at the shorter times is difficult.

In the majority of the studies on virus-cell fusion, the endosomal lumen is simulated artificially by lowering externally the pH of the medium. Thus, it is of interest to perform an analysis of the fusion kinetics of influenza virus with the plasma membrane of MDCK cells and compare the results with the ones obtained for virus fusion with the endosomal membrane. We started the analysis of influenza virus fusion activity towards the plasma membrane of MDCK cells by determining the percentage of viral binding and association to the cell plasma membrane. All experiments were performed in the presence of metabolic inhibitors of endocytosis. Table 2 shows the values obtained for binding and cell association at 20°C and 37°C. The values obtained in cell-association experiments when compared to those obtained for binding indicate that after acidification of the medium to pH 5, i.e., when influenza virus starts to fuse with the plasma membrane, more virus particles

Table 2. Influenza virus binding to and association with the plasma membrane of MDCK cells

Temperature	Binding[1]	Cell association[2]
37°C	57.5 ± 5.0	70.5 ± 8.9
20°C	50.0 ± 2.1	70.0 ± 4.8

Influenza virus (2 μg of viral protein) was added to 5×10^6 MDCK cells, in a final volume of 2 ml at pH 7.4 at the indicated temperatures in the presence of metabolic inhibitors of endocytosis. Experimental values are given as the mean ± SD of three independent experiments performed in duplicate.
[1]Influenza virus was incubated with MDCK cells for 10 min at 20°C and for 5 min at 37°C, at pH 7.4.
[2]Influenza virus was incubated with MDCK cells for 10 min at 20°C and for 5 min at 37°C, at pH 7.4, followed by a further incubation at pH 5 for 10 min at the same temperatures.

Table 3. Fusion and inactivation rate constants for the interaction of influenza virus with the plasma membrane of MDCK cells

Temperature	Fusion rate constant[1] f (s^{-1})	Inactivation rate constant[1] γ (s^{-1})
37°C	0.03	0.005
20°C	0.002	0.001

[1]The estimated uncertainties in f and γ are 20% and 40%, respectively.

are able to bind to the target membrane. This effect is more pronounced at 20°C. However, influenza virus fuses with the plasma membrane more extensively and with a larger initial rate at the higher temperature (Fig. 3). These results were analyzed by using the mass-action kinetic model (*see* Materials and Methods). The calculated values for the fluorescence increase, which employed the fusion (f) and inactivation (γ) rate constants given in Table 3, resulted in good fits, shown by the squares, to the curves in Fig. 3.

Discussion

Our mass-action model considers the fusion of influenza virus with the endosomal membrane as a sequence of steps: (1) binding, which includes on and off events; (2) endocytosis; (3) fusion with the endosomal membrane. Our study has enabled us to deduce the rate constant for virus fusion with the endosomal membrane on the basis of previous experiments and by modeling virus binding in the presence of metabolic inhibitors of endocytosis, as well as total virus-cell association in absence of the inhibitors [17]; we then considered explicitly the fusion of each endocytosed segment, which starts at a different time. In parallel we determined the fusion rate constant of the virus with the plasma membrane by first prebinding the virus to the plasma membrane at neutral pH,

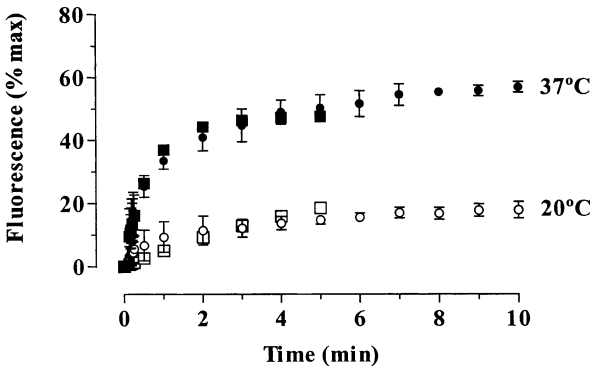

Fig. 3. Kinetics of lipid mixing of influenza virus with the plasma membrane of MDCK cells. Influenza virus (2 μg viral protein) labeled with R18 was added to 5×10^6 MDCK cells in a final volume of 2 ml, and R18 dequenching was monitored for 10 min at 20°C and 37°C at pH 5, following virus-cell preincubation at pH 7.4 for 10 min at 20°C and for 5 min at 37°C. Experimental values at 37°C (●) and at 20°C (○) are given as mean ± SD of three independent experiments performed in duplicate. The squares represent the values calculated by using the mass-action kinetic model for experiments performed at 37°C (■) and 20°C (□).

under a condition where fusion does not occur, then lowering the pH to 5, which results in immediate fusion accompanied by viral inactivation, as described before [3, 11, 24].

The results yield an interesting outcome: the rate constants describing the fusion of influenza virus with the plasma membrane (Fig. 3, Table 3) are essentially the same as those deduced from its fusion with the endosomal membrane (Fig. 2, Table 1). This result is rewarding, since the inner leaflet of the endosome originates from the outer leaflet of the plasma membrane. This similarity in the deduced rate constants of fusion was obtained both at 37°C and 20°C. Our results show an increase in the rate constants of fusion and endocytosis when going from 20°C to 37°C, the increase being more dramatic in the former case, and are in accordance with previous results on the fusion of the virus with the plasma membranes of several types of cells [3, 23].

In performing analysis of fusion kinetics of a virus with the endosomes, a critical element that has to be taken into account is that the amount of virus that has been endocytosed cannot be simply obtained by subtracting the amount of virus bound (in the presence of metabolic inhibitors of endocytosis) from the total amount of virus associated with the cells in the absence of inhibitors. Such a subtraction may yield a serious underestimate in the amount endocytosed. Our analysis [14, 17] has demonstrated (by calculations only) that initially most of the virus (or other particles, e.g., liposomes) associated with the cells is due to binding. As the overall amount of virus associated with the cells keeps increasing, the amount bound does not equal the equilibrium value (in the absence of endocytosis), but rather reaches a maximum, and then falls off. The explanation is that at equilibrium the on and off rates of virus binding to the cell are equal, whereas when endocytosis occurs the amount bound is reduced due to the endocytosis. Schnitzer et al. [26], who studied endocytosis of albumin-gold particles via caveolae, noted the occurrence of fewer albumin-gold particles at the cell surface after 30 min incubation than after 10 min incubation. They raised the possibility that the number of receptors and/or vesicular carriers may be diminished after ligand internalization. According to our analysis, while such a process can occur, it would result in a significantly lower rate of uptake at earlier times and in a plateau in the uptake by 30 min. A reduction in the amount of liposome binding from a maximum at 10 min incubation to a lower value at 30 min, while total uptake was increasing rather than approaching a plateau, was reported in [9] for murine macrophage-like cells in a monolayer.

The magnitude of the fusion rate constant is about an order of magnitude larger than that of the rate constant of endocytosis. This outcome is understandable, since the endocytosis rate constant reflects the composite rate of several steps, namely, the fusion of the plasma membrane with itself in the formation of the endocytotic vesicle, movement of the vesicle towards the interior of the cell, a process accompanied by a reduction in the pH in the interior of the vesicle, and vesicle fusion with another early endosome.

In summary, the results and analysis developed in this study indicate that the low pH-induced fusion of influenza virus with the plasma membrane of living cells constitutes a convenient model to study the fusion of the virus with endosomal membranes.

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