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Fusion activity of the influenza virus hemagglutinin does not require a transbilayer pH gradient

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Abstract

Following reports suggesting that membrane fusion mediated by the influenza virus hemagglutinin might be dependent on a pH gradient across a putative target membrane, we have designed experiments in which this issue could be addressed directly. Accordingly, we have prepared two populations of liposomes, both simulating the plasma membrane of target cells, but with the pH of the internal aqueous medium buffered either at pH 7.4 (physiological cytosol pH) or pH 5.0 (endosomal pH at which influenza virus displays maximal fusion activity). Monitoring fusion as the relief in self-quenching of the fluorescent probe octadecylrhodamine B chloride we have found that the internal pH of the target liposomes did not influence membrane merging as mediated by the influenza virus hemagglutinin, thus demonstrating that a transmembrane pH gradient is not required for the fusion process to take place. © 1997 Elsevier Science B.V.

Keywords: Influenza virus hemagglutinin; pH gradient; Liposome; Erythrocyte ghost

1. Introduction

Influenza virus is a lipid-enveloped virus that enters cells by receptor-mediated endocytosis. Access of viral RNA into the target cell cytosol occurs by fusion of the viral envelope with the membrane of an intracellular compartment (the endosome). This event is mediated by the viral envelope hemagglutinin (HA), and is triggered by conformational changes in the protein that take place at the acidic pH normally found in the endosomal lumen [for recent reviews see Refs. [1,2]].

However, recent publications have demonstrated that influenza virus entry into target cells at low pH is inhibited when the cell cytosol is acidified [3,4]. Similar observations have been made for Semliki Forest virus, a virus that also enters cells by receptor-mediated endocytosis [6,7]. It has, therefore, been proposed that the need for acidic pH in the entry of influenza virus (and possibly other viruses that share the same pathway) into target cells is more subtle, with the virus requiring not only an acidic (endosomal) environment per se, but a pH gradient across the target membrane [3,4,7]. Productive infection would thus result from the difference between the endosomal lumen pH (≈ 5) and the target cell cytosol pH (≈ 7). This type of specificity might be

Abbreviations: HA, influenza virus hemagglutinin; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; R18, octadecylrhodamine B chloride

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relevant in preventing virions from infecting non viable cells [5].

More importantly, the observations made with influenza virus led to the intriguing suggestion that the absence of a pH gradient between the endosome and cytosol resulted in the inhibition of the HA-mediated fusion step, possibly by reducing penetration of the fusogenic protein into the target membrane [3,4]. This question could not be settled by re-interpretation of previous data. Indeed, in all studies involving fusion activity of the influenza HA (either using intact viral particles, reconstituted HA in membranes, or cells that express HA at their surface) fusion is routinely triggered by lowering the pH of the reaction medium from pH $\approx$ 7.4 to pH $\approx$ 5.0. However, compartments sealed by target membranes for the virus (normally liposomes or cultured cells) have an internal aqueous medium which is at neutral pH. Therefore, by designing this type of experiments, one can always imagine the existence of a pH gradient across the target membrane.

To resolve this issue we have planned experiments in which the putative importance of a transmembrane pH gradient to the fusion activity of the influenza virus HA might be evaluated. For this purpose fusion was followed by the octadecylrhodamine (R18) dequenching assay [8–10], using liposomes as target membranes for intact virions. In order to simulate the outer monolayer of a target cell plasma membrane these model membranes were composed of the zwitterionic phospholipids PC and PE, and also included the sialic acid-containing ganglioside GD1a, a molecule known to act as a receptor for the virus [10,11,15,20]. Two populations of liposomes were prepared: one encapsulating an aqueous medium at pH 7.4, and one encapsulating exactly the same medium, but with its pH adjusted to 5.0. In this system virus-liposome fusion is triggered by lowering the pH of the reaction buffer from neutral to $\pm$ pH 5.0. However, only when liposomes with an internal pH of 7.4 are used (i.e., the normal procedure in this type of experiments) will there be a transmembrane pH gradient across the target membrane. To further pursue this issue we have carried out experiments using proton ionophores such as FCCP and nigericin, both of which dissipate pH gradients across membranes [19].

Since it has been shown that liposomes are not always equivalent to biological membranes in the study of viral fusion activity [10], experiments using erythrocyte ghosts (resealed in medium buffered at either pH 7.4 or pH 5.0) as targets for influenza virus were also performed.

2. Materials and methods

2.1. Virus, liposome and erythrocyte ghost preparation

Influenza virus, A/PR/8/34 (H1N1) strain, was grown for 48 h at 37°C in the allantoic cavity of 11-day-old embryonated eggs, purified by discontinuous sucrose density gradient centrifugation and stored at −70°C in phosphate buffered saline.

Liposomes (LUVs – large unilamellar vesicles) composed of PC and PE (Avanti Polar Lipids) in a 2:1 molar ratio, and containing 5 mol% of the ganglioside GD1a (Sigma), were prepared in 85 mM NaCl, 50 mM KCl, 1 mM EDTA, 10 mM Hepes, 10 mM Mes, 10 mM sodium citrate, buffered at pH 7.4 or 5.0 by the reverse-phase evaporation method as described [12]. The vesicles were sized through 0.1 μm polycarbonate filters and their concentration determined by a phosphate assay.

Human erythrocyte ghosts were prepared by the method of Steck and Kant [18]. Following hypotonic lysis the membranes were resealed in 85 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, 10 mM Mes, 10 mM sodium citrate, buffered at pH 7.4 or 5.0.

2.2. Viral fusion activity

Influenza virus was labeled with octadecylrhodamine B chloride (R18, Molecular Probes Inc., Eugene, OR) as described previously [8–10]. The final 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 min at room temperature. R18-labeled virus was separated from noninserted fluorophore by chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) using 150 mM NaCl, 10 mM Hepes, pH 7.4 as elution buffer. The protein concentration of the labeled virus was determined by the Lowry assay.

In all assays using lipid vesicles as target membranes R18-labeled influenza virus was injected into a cuvette containing liposomes of either population (100 nmol of LUV lipid per experiment) in a final volume of 2 ml. Fusion was monitored continuously at pH 5.0 using the fluorescence dequenching assay as described previously [8–10]. In some cases the virus was allowed to bind the target liposomes at neutral pH before acidification of the medium, and in other assays virus and liposomes were added directly to buffer at pH 5.0. All experiments were carried out at 37 or 20°C using 1 μg of viral protein/ml, and the fluorescence scale was calibrated such that the initial fluorescence of R18 labeled virus and LUV suspension was set at 0% fluorescence. The value obtained by detergent lysis after each experiment with Triton X-100, at a final concentration of 1% (v/v), was set at 100% fluorescence. Fluorescence measurements were performed in a Perkin–Elmer LS-50 luminescence spectrometer, with excitation at 560 nm and emission at 590 nm. The sample chamber was equipped with a magnetic stirring device, and the temperature was controlled with a thermostated circulating water bath. The initial rate of fluorescence dequenching was calculated in the first instants following the onset of fusion. The extent of fluorescence dequenching was measured after 5 min (37°C) or 10 min (20°C).

Experiments with human erythrocyte ghosts (resealed with medium at pH 7.4 or 5.0) were carried out in basically the same manner. In all cases 1 μg of viral protein/ml was added to 2 ml of buffer adjusted to pH 5.0 and containing 100 μg of target membrane protein/ml. Membrane fusion, as a function of R18 dequenching, was monitored for 5 min at 37°C.

3. Results and discussion

In a first batch of experiments influenza virus was added to either population of liposomes (with internal aqueous medium buffered at pH 7.4 or 5.0) at 37°C and neutral pH. After a short period (±1.5 min) to allow virus-liposome binding, the pH of the reaction medium was adjusted to 5.0, and HA-mediated membrane fusion followed by relief of R18 self-quenching (Fig. 1, Table 1). As is obvious from the results obtained, there is no significant difference in influenza virus fusion activity towards both types of target membranes. The presence of the proton ionophores FCCP and nigericin also had no visible effect on either the kinetics or the extent of membrane merging (Table 1).

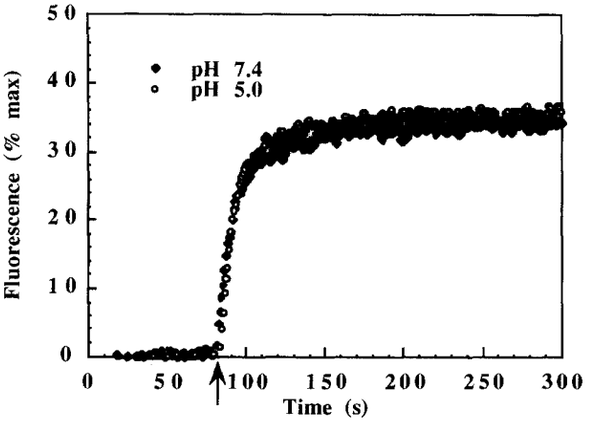

Fig. 1. Effect of the internal pH of target liposomes on the fusion activity of influenza virus. R18 labelled influenza virus (1 μg viral protein per ml) was added to 2 ml of buffer at 37°C and pH 7.4. Both populations of liposomes prepared in medium at pH 7.4 (F) or 5.0 (E) at a final concentration of 50 μM of lipid were used as target membranes for the virus. Following a short period to allow virus-liposome binding (1.5 min) the medium pH was adjusted to 5.0, and fusion followed as a function of R18 dequenching for 5 min. Curves represent typical experiments (for statistical significance see Table 1).

It should be noted that internal acidification of viral particles, either by proton ionophores or by the viral envelope M2 protein (a proton channel), has been shown to slightly increase the fusion activity of some strains of influenza virus, possibly due to a weakening in the interactions between transmembrane viral envelope proteins and the viral M1 protein, located at the interior of the virion [13,14]. However, this effect was not visible in our system.

Although the process is mediated by target membrane composition, membrane fusion activity of intact influenza virus is generally a very quick and efficient phenomenon at 37°C, and is essentially complete following the first few seconds after acidification (Fig. 1). Indeed, prolonging the virus-liposome incubation time at pH 5.0 up to 15 min did not significantly increase the fusion extent (not shown). It is, therefore, difficult to judge eventual small differences in experimental curves at this temperature. This

Table 1
Effect of a pH gradient across the target membrane and proton ionophores on the fusion activity of influenza virus towards ganglioside-containing liposomes

Conditions	Extent of fusion (% max.) [f]	Initial rate of fusion (% max./min.) [g]
37°C		
Control (Liposomes pH 7.4) [a]	37.7 ± 3.3	138.0 ± 14.3
Liposomes pH 5.0 [b]	38.1 ± 3.0	141.3 ± 15.1
FCCP [c]	38.2 ± 3.6	145.7 ± 9.7
Nigericin [d]	37.6 ± 2.0	149.4 ± 11.1
20°C [e]		
Control (Liposomes pH 7.4)	46.6 ± 3.2	24.8 ± 2.2
Liposomes pH 5.0	45.7 ± 2.7	24.4 ± 4.5

[a] Influenza virus (1 μg viral protein per ml) was added to 2 ml of buffer at 37°C and pH 7.4 containing liposomes prepared in medium at pH 7.4, at a final concentration of 50 μM of lipid. Following a short period to allow virus-liposome binding (1.5 min) the medium pH was adjusted to 5.0, and R18 dequenching followed for 5 min.
[b] Experiments carried out as in (a), but using liposomes prepared in medium at pH 5.0.
[c] Experiments carried out as in (a), but including 2 μM FCCP in the reaction medium.
[d] Experiments carried out as in (a), but including 0.5 μg/ml nigericin in the reaction medium.
[e] Influenza virus (1 μg viral protein per ml) was added to 2 ml of buffer at 20°C and pH 7.4 containing liposomes prepared in medium at pH 7.4 or 5.0 at a final concentration of 50 μM of lipid. Following virus-liposome binding (10 min) the medium pH was adjusted to 5.0, and R18 dequenching followed for 10 min.
[f] Fusion extent measured as a function of R18 dequenching after 5 min (37°C) or 10 min (20°C) at pH 5.0; results represent the average ± SD of 3–6 experiments.
[g] Initial rate measured in the first 10–15 s of the fusion process; results represent the average ± SD of 3–6 experiments.

prompted us to carry out parallel experiments at 20°C, since at temperatures below 37°C fusion is much slower, due to a lower mobility of viral fusion proteins [9,10,15].

Although the initial rate of membrane mixing at 37°C was much higher, the extent of fusion was larger at 20°C (Table 1), as was previously noted [10,15]. Indeed, this phenomenon has been described before with the same fusion system [15], and has been explained by an interplay between influenza virus fusion activity and viral inactivation, both of which take place at low pH. Thus, if the virus is challenged by low pH and cannot fuse with a target membrane (either by its absence, or by ineffective viral positioning) it is known to lose its membrane perturbing properties [9–11,16]. Both fusion and inactivation seem to share a common mechanism, both processes being reduced at lower temperatures [9,17]. The drop in the inactivation rate constant with temperature is greater than the drop in the fusion rate constant [9], which results in the virus fusing slower at low temperatures, but more extensively, since inactivation is less significant, although the physiological meaning of this effect remains open to debate [9,10].

However, experiments carried out using both populations of liposomes tested gave essentially the same results as those done at 37°C, i.e. the internal medium of the target membranes did not influence HA-mediated membrane merging (Table 1). It also made no difference if virus and liposomes were prebound at neutral pH prior to medium acidification, or if they were placed in contact in buffer already adjusted to pH 5.0 (not shown).

Similar observations were made when the same experimental procedure was carried out at 37°C using biological membranes (erythrocyte ghosts resealed in buffers at different pH values) as targets for the virus (Table 2).

Overall our results demonstrate that a pH gradient across the target membrane is not necessary for (and does not modulate) influenza virus fusion activity, contrary to what has been proposed [3,4]. An eventual block in virus infection following dissipation of

Table 2
Effect of a pH gradient across the target membrane on the fusion activity of influenza virus towards erythrocyte ghosts

Conditions	Extent of fusion (% max.) [c]
Control (Erythrocyte ghosts pH 7.4) [a]	27.1 ± 5.2
Erythrocyte ghosts pH 5.0 [b]	24.8 ± 4.3

[a] Influenza virus (1 μg viral protein per ml) was added to 2 ml of buffer at 37°C and pH 5.0 containing erythrocyte ghosts resealed in medium at pH 7.4, at a final concentration of 100 μg/ml target membrane protein, and R18 dequenching was followed for 5 min.
[b] Experiments carried out as in (a), but using erythrocyte ghosts resealed in medium at pH 5.0.
[c] Fusion extent measured as a function of R18 dequenching after 5 min; results represent the average ± SD of at least 3 experiments.

the pH gradient between the virus-containing endosome and cell cytosol cannot be explained by an arrest in membrane merging per se, but may be related instead to later steps in the process. These could include incorrect delivery of the nucleocapsid into the cytoplasm, lack of proper viral disassembly, or even unforseen effects of the procedures used to neutralize the pH gradient (cell cytosol acidification, ionophores and specific inhibitors of endosomal proton pumps) on the target cells themselves. However, it is clear that changes in the membrane fusion activity of the HA should not be postulated to explain alterations in overall viral infectivity, since correlation between these two processes may not be linear.

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