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INFLUENCE OF CALCIUM ON LIPID MIXING MEDIATED BY INFLUENZA HEMAGGLUTININ

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Abstract

We studied the influence of calcium on lipid mixing mediated by influenza hemagglutinin (HA). Lipid mixing between HA-expressing cells and liposomes containing disialoganglioside, influenza virus receptor, was studied at 37°C and neutral pH after a low-pH pulse at 4°C. With DSPC/cholesterol liposomes, calcium present after raising the temperature significantly promoted lipid mixing only when it was triggered by a short low-pH application. In case of DOPC/cholesterol liposomes, calcium promotion was observed regardless of the duration of the low-pH pulse. Calcium present during a short, but not long, low-pH application to HA-expressing cells with bound DSPC/cholesterol liposomes at 4°C inhibited subsequent lipid mixing. We hypothesize that calcium influences lipid mixing because it binds to a vestigial esterase domain of hemagglutinin or causes expulsion of the fusion peptide from an electronegative cavity. We suggest that calcium promotes the transition from early and reversible conformation(s) of low pH-activated HA towards an irreversible conformation that underlies both HA-mediated lipid mixing and HA inactivation.

Keywords

Membrane fusion; Influenza virus; Hemagglutinin-expressing cell; Liposomes; Calcium; Inactivation; Surface density

Membrane fusion is an ubiquitous process in nature, and it is important for many aspects of the life of the eukariotic cell [1–4]. Due to membrane fusion, enveloped viruses enter and infect cells, many cells fuse with each other (intercellular fusion), and in many cells, organelles fuse with each other or with the plasma membrane (intracellular fusion). According to the stalk-pore hypothesis [5,6], during the process of fusion, initially the outer monolayers of two fusion bilayers merge and form an intermediate named stalk. Expansion of stalk generates formation of the hemifusion diaphragm. At the next step of the fusion process, inner monolayers merge creating the fusion pore.

There are two classes of viral fusion proteins - class I and class II. However, some viral fusion proteins do not belong to either of these two classes [7]. There is evidence that both class I and

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class II viral proteins mediate fusion through the same stalk-pore pathway [8]. Hemagglutinin (HA)¹ is a class I membrane fusion protein that mediates the fusion of influenza virus with the target cell. The surface of influenza virus is covered with HA trimers. Each monomer consists of two subunits - HA1 and HA2 - linked by a disulfide bond. The HA1 subunit binds to sialic acid, the influenza virus receptor. After binding, the virus enters the cell using an endocytotic pathway. Initial, pre-fusion conformation of HA is metastable; this conformation can be compared to a loaded spring [9]. In this conformation, the amino terminal of HA2, called the fusion peptide, is buried in an electronegative cavity containing residues of both the HA1 and HA2 subunits. At low pH, HA undergoes a conformational change. Such a conformational change can also happen at neutral pH, because of denaturants or high temperature. During the initial steps of this conformational change, the fusion peptide leaves the cavity in which it was buried in the pre-fusion conformation. The final stable structure of the HA2 subunits represents the trimer of hairpins conformation. The energy of this conformational change is thought to mediate membrane fusion. *In vivo*, HA mediates fusion at low pH inside an endosome. Many important details of this process remain unknown.

Diverse cell biological and biochemical processes including fusion reactions are dependent on and controlled by calcium [10–24]. It was also shown that calcium can influence membrane fusion mediated by fusion peptides [25–27]. However, to the best of our knowledge the effects of calcium on HA-mediated fusion have remained unexplored. In [28] the influence of lipids of different spontaneous curvatures on the fusion of HA-cells and extruded liposomes containing cardiolipin was studied. In the absence of calcium, cardiolipin has a positive spontaneous curvature, while in the presence of calcium it has a negative spontaneous curvature. According to the prediction of stalk-pore hypothesis [5], in the inner monolayer lipids that possess positive spontaneous curvature are energetically favorable for fusion, while in the outer monolayer they are unfavorable for fusion. For the lipids that possess negative spontaneous curvature, the opposite is true. That is why it is not surprising that in the presence (but not in the absence) of cardiolipin, loading of liposomes with calcium inhibits cell-liposome fusion [28]. Calcium in the outside medium also promoted fusion of cells with cardiolipin-containing liposomes. However, surprisingly, calcium promoted such a fusion even when liposomes did not contain cardiolipin [28]. In this work we explore the effects of calcium in the outside medium on lipid mixing between HA-cells and cardiolipin-free liposomes.

Materials and methods

Materials

1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), cholesterol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (RhDPPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (RhDOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Disialoganglioside (G_{D1a}) from bovine brain, citric acid, neuraminidase (EC 3.2.1.18, type V, from *Clostridium perfringens*), and trypsin (EC 3.4.21.4, type IX, from porcine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol and sodium hydroxide were purchased from Mallinckrodt (Paris, KY). Sodium chloride, calcium chloride, and benzene were purchased from Fisher Scientific (Pittsburgh, PA).

¹*Abbreviations used:* DMEM, Dulbecco's modified Eagle medium; DOPC, dioleoyl phosphatidylcholine; DOPC-LS, DOPC/cholesterol liposomes; D-PBS, Dulbecco's phosphate-buffered saline; DSPC, distearoyl phosphatidylcholine; DSPC-LS, DSPC/cholesterol liposomes; DTT, dithiothreitol; FBS, fetal bovine serum; HA, influenza virus hemagglutinin; HA-cell, HA-expressing cell; HD, high density; LD, low density; LS, liposomes; PC, phosphatidylcholine; PDB, Protein Data Bank; PE, phosphatidylethanolamine; RBC, red blood cells; RhPE, rhodamine-labeled PE; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

HEPES and DTT were purchased from ICN Biomedicals, Inc. (Aurora, OH). Triton X-100 was purchased from Research Products International Corp. (Mount Prospect, IL). Dulbecco's phosphate-buffered saline (D-PBS) with Ca and Mg and D-PBS without Ca or Mg were purchased from Biofluids (Rockville, MD). Calcium chloride solution (1 M in water) and EGTA were purchased from Fluka (Buchs, Switzerland). EDTA was purchased from Quality Biological, Inc. (Gaithersburg, MD). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin–glutamine were purchased from Gibco BRL (Gaithersburg, MD).

Liposomes

Liposomes were prepared as described in [29]. In brief, solutions of lipids (total amount 1 μ mole) in benzene/methanol (95:5, vol:vol) were dried and then hydrated using HEPES buffer (140 mM sodium chloride, 10 mM HEPES, pH 7.4) to generate a 1 mM multilamellar liposome suspension. After five freeze-thaw cycles, the liposomes were extruded at 60°C (above the gel-to-liquid crystalline phase transition) 10 times through two polycarbonate Nucleopore filters (100-nm pore size) and one mesh spacer (Osmonics Inc., Minnetonka, MN) in a pressure extruder (Lipex Biomembranes, Vancouver, BC, Canada) to produce large unilamellar vesicles. After extrusion, liposomes were diluted in a 1:4 ratio with HEPES buffer. The size of extruded liposomes (i.e., ~100 nm in diameter) was verified by means of quasi-elastic light scattering on a "Coulter N4 Plus" particle sizer (Beckman Coulter, Inc., Fullerton, CA). The 100-nm liposomes were used in this and previous [28,30] studies because liposomes of this size are homogenous [31] and fusogenic [29,32].

Liposome compositions are stated as molar ratios between component lipids. All compositions included 5% fluorescent lipid probe and, if not stated otherwise, 5% disialoganglioside, which serves as a receptor for HA binding [33]. DSPC/cholesterol liposomes (DSPC-LS) had the following composition: 49.5% DSPC, 40.5% cholesterol, 5% RhDPPE, and 5% disialoganglioside. DSPC-LS without disialoganglioside had the following composition: 52.25% DSPC, 42.75% cholesterol, and 5% RhDPPE. DOPC/cholesterol liposomes (DOPC-LS) had the following composition: 49.5% DOPC, 40.5% cholesterol, 5% RhDOPE, and 5% disialoganglioside. The concentration of cholesterol in the liposomes (40.5 mol %) was chosen to mimic the composition typical for many biological membranes including RBC (~40 mol % [34]). Liposomes were always used on the day of extrusion.

Cells

CV-1 green monkey kidney cells were grown in DMEM supplemented with 10% FBS. CV-1 cells were infected 48 h before the experiment with SV40 recombinant viral vector containing Udorn HA-gene (influenza A/Udorn/72; H3 subtype) [35,36], a generous gift of Dr. Robert A. Lamb, Northwestern University, Evanston, IL. (Udorn strain has a very high degree of similarity, ~96%, to extensively studied X-31 strain). Slightly subconfluent monolayers of cells were incubated with SV40-HA stock at 37°C, as described in [37]. After a 1-h incubation, the viral suspension was diluted in a 1:6 ratio with DMEM complete medium. On the day of the experiment, cells were treated for 15 min at 37°C with trypsin (5 μ g/ml) and neuraminidase (1 unit/ml) in D-PBS with Ca and Mg and washed twice with this buffer. Then, cells were incubated with liposomes for 1 h at 4°C and washed five times, still at 4°C, to remove unbound liposomes. After that, cells were removed from the flasks with Ca- and Mg-free D-PBS containing both EDTA and EGTA at 0.5 mg/ml. EDTA- and EGTA-containing buffer was removed, and cells were washed with cold (4°C) HEPES buffer and then harvested with ~3 ml of the same buffer.

Lipid mixing between HA-cells and RBC

In some experiments, we studied HA-mediated lipid mixing in a well-characterized experimental system of HA-cells fusing with labeled human red blood cells (RBC). RBC were labeled with membrane dye PKH26 (Sigma Chemical Co., St. Louis, MO), and fusion experiments were performed as described earlier [6].

Lipid mixing between HA-cells and liposomes

600 μ l of the suspension of cells with bound liposomes ($\sim 10^5$ cells and $\sim 5 \times 10^9$ liposomes) were incubated at 4°C and pH 4.9 for different durations and then 500 μ l of this suspension were mixed in four-clear-sided methacrylate cuvette (Fisher Scientific, Pittsburgh, PA) with 1.5 ml of HEPES buffer, pH 7.4. As a result, our measurements were carried out at neutral pH. Fluorescence was monitored for at least 4.5 min at 37°C with an Aminco Bowman Series 2 luminescence spectrometer (Rochester, NY) with excitation at 530 nm and emission at 590 nm. The medium in the cuvettes was continuously stirred with a magnetic stirring device and thermostatted with a circulating water bath (Hoefer Scientific Instruments, San Francisco, CA).

Since our liposomes contained fluorescent lipid probe in a self-quenched concentration, lipid mixing upon fusion between liposomes and cells diluted the probe and increased the observed fluorescence. To quantify the rates and extents of lipid mixing for different conditions, we measured the fluorescence of the samples (F) as a function of time and normalized it to the fluorescence ($F_{\max}$) measured upon maximum dequenching by solubilizing membranes with 1 mM Triton X-100 at the end of each experiment. The percentage of lipid mixing was calculated as

$$\% \Delta F / \Delta F_{\max} = 100 \times (F - F_0) / (F_{\max} - F_0)$$

where F_0 is the fluorescence of the suspension of cells with liposomes not exposed to low pH at the onset of the measurements.

Each experiment presented was repeated at least two times, and all functional dependencies reported were observed in each experiment.

Measuring HA activation

In some experiments, we used SDS-PAGE and Western blotting to detect low-pH forms of HA as those with the S-S bond between the HA1 and HA2 subunits accessible to dithiothreitol (DTT) [38]. Since this bond is accessible only in low-pH forms, the percentage of HA with the DTT-reducible bond reflects the percentage of low pH-activated HAs. The assay was performed as described in [39].

Results

Calcium effects on HA-mediated lipid mixing and HA inactivation

We found 5 mM calcium chloride to increase the percentage of low pH-activated HAs at the surface of Udorn HA-cells observed after a 5-min application of pH 4.9 from 30% to 54%. Calcium chloride (5 mM) also promoted lipid mixing between X-31 HA-cells and bound PKH-labeled human erythrocytes treated with a 2-min pulse of pH 5.3 at 37°C from 2.6% to 10.8%.

To further elucidate the effects of calcium on HA-mediated lipid mixing, we treated Udorn HA-cells with bound liposomes with a low-pH pulse applied at 4°C in the absence of Ca^{2+} , and then we measured lipid mixing after raising the temperature to 37°C and simultaneously returning the pH to neutral either in the presence or in the absence of Ca^{2+} (when HA-cells with bound liposomes were not treated with low-pH pulse, in the absence or in the presence

of calcium, no lipid mixing was observed, data not shown). As seen in Fig. 1, Ca^{2+} applied at the time of raising the temperature to 37°C promoted lipid mixing at 37°C between cells and liposomes of both (DSPC/cholesterol and DOPC/cholesterol) tested compositions. For DOPC-LS significant Ca^{2+} promotion was observed regardless of the duration of the low-pH pulse (apparent high fluorescence background is due to the significant lipid mixing observed at 4°C [30]). In contrast, for DSPC-LS calcium significantly promoted lipid mixing at 37°C only if it was triggered by a relatively short (1–10 min) low-pH application. For long (40–60 min) durations of low-pH pulse, such a promotion was much less significant (Fig. 1A); note that a longer incubation at pH 4.9 lowered the extent of lipid mixing at 37°C and neutral pH in the absence of calcium [30].

The statistical analysis (mean extents and rates of fluorescence with standard deviations) emphasizes promotion of lipid mixing between HA-cells and liposomes (recorded after the incubation at 4°C and pH 4.9 for 10 min) by calcium. In the presence of 5 mM calcium after raising the temperature to 37°C , the lipid mixing extents (dequenching (%)) 3 min after raising the temperature) were on average $181.0 \pm 34.7\%$ of extents observed in the absence of calcium for 15 experiments with DSPC-LS, and $137.6 \pm 25.2\%$ for 4 experiments with DOPC-LS. Initial lipid mixing rates (dequenching (%) per minute, in the presence of 5 mM calcium after raising the temperature, were on average $356.7 \pm 134.4\%$ of initial rates observed in the absence of calcium for 15 experiments with DSPC-LS.

Both relatively high (5 mM, Fig. 1) and lower (0.5 mM, data not shown) concentrations of calcium present after raising the temperature promote lipid mixing between HA-cells and liposomes of both (DSPC/cholesterol and DOPC/cholesterol) tested compositions after the incubation at 4°C and pH 4.9 for 10 min.

One can hypothesize that influence of calcium on lipid mixing may be due to the disruption of the binding between sialic acid and HA1 subunit of hemagglutinin. However, promotion by 5 mM calcium chloride, after 10-min low-pH pulse, was also observed for the lipid mixing between HA-cells and ganglioside-free DSPC-LS (Fig. 2).

Calcium, added a few minutes after onset of lipid mixing between HA-cells and DSPC-LS, after incubation at pH 4.9 for 10 min, also promotes this lipid mixing (Fig. 3).

In contrast to the promotion of lipid mixing by Ca^{2+} added *after* the low pH application at 4°C , Ca^{2+} present *during* a short (1–10 min) low-pH application to HA-cells with bound DSPC-LS at pH 4.9 inhibited subsequent lipid mixing at 37°C and neutral pH (Fig. 4). In case of long (40–60 min) low-pH application to HA-cells with bound DSPC-LS at 4°C , such inhibition was not observed (data not shown).

To summarize, Ca^{2+} -effects on HA-mediated lipid mixing depend on the relative timing of the low-pH and Ca^{2+} applications and on the composition of the target membrane.

Identification of a putative Ca^{2+} -binding sites in HA

Assuming that calcium effects on HA-mediated lipid mixing and HA inactivation are due to direct binding of calcium cations to HA, we performed structural analysis to identify putative calcium-binding sites in HA. Primary sequence analysis showed that HA of the X-31 viral strain (which has an amino acid sequence very similar to the sequence of HA of the Udorn strain we studied) does not contain a calcium-binding EF-hand motif (Dr. Hiroshi Kawasaki, Yokohama City University, personal communication, 2004). However, there are many types of calcium-binding sites that are different from the EF-hand motif. For example, charge clusters often bind calcium [40]. In [40], two mixed charge clusters in the three-dimensional structure of hemagglutinin were described, one in the HA1 subunit and another in the HA2 subunit. In

order to find possible Ca^{2+} -binding sites, we examined the HA from the influenza X-31 strain (PDB entry code 1EO8 [41]) using the program WebFEATURE (web-accessible structural analysis software that permits the identification of calcium-binding sites in three-dimensional protein structures [42]); the three-dimensional protein structure of HA of the Udorn strain is not available. Although WebFEATURE suggests that the binding affinity of Ca^{2+} to HA2 is low, a potential Ca^{2+} -binding site within the vestigial esterase domain of HA1 [43] was revealed close to residues Asp⁶⁸-Val⁷⁸ and Ser⁹⁵-Tyr¹⁰⁰ (not far from the charge cluster in the HA1 subunit described in [40]), see Fig. 5.

For reasonably strong Ca^{2+} -binding sites WebFEATURE usually gives scores higher than 50. The maximal score observed for any Ca^{2+} -binding site in the X-31 HA, using WebFEATURE, was 59.25. The amino acid sequences of X-31 HA and Udorn HA around the putative calcium-binding site are almost identical (except Gly⁷⁸ in Udorn in place of Val⁷⁸ in X-31, but such a change, to less hydrophobic amino acid, should not decrease calcium binding). This putative calcium-binding site is located in the domain, which represents a vestige of the receptor-destroying enzyme active site of the ancestral protein similar to hemagglutinin-esterase-fusion (HEF) protein of influenza C virus [43]. We would like to note that putative calcium-binding site in HA is relatively weak. The maximal score, 59.25, that we obtained, using WebFEATURE, for Ca^{2+} -binding site in HA is higher than maximal score, 51.54, we found in a known calcium-binding protein hemagglutinin-neuraminidase from Newcastle disease virus (PDB entry code 1E8U [44]), but lower than maximal scores, 65.56, in mammalian synaptotagmin (PDB entry code 1DQV [45]), and 85.82, in mammalian calmodulin (PDB entry code 3CLN [46]).

We also studied the HA from X-31 strain using the program GG (a fast web-accessible algorithm able to detect calcium-binding sites in three-dimensional proteins structures [47]). The program GG found a putative calcium-binding site in HA1 at exactly the same place as WebFEATURE. The GG program suggests that four oxygen atoms represent the potential calcium ligands of this putative binding site: two of these oxygen atoms belong to Asp⁶⁸, one to Ser⁹⁵, and one to Tyr¹⁰⁰ (Fig. 5).

Discussion

Putative Ca^{2+} -binding sites in HA

Our studies showed the influence of calcium cations in the outside medium on influenza HA-mediated lipid mixing. Calcium present during short (but not long) incubations of HA-cells with bound DSPC-LS at 4°C and pH 4.9 inhibits subsequent lipid mixing between cells and liposomes that occurs at 37°C and neutral pH. Calcium present during such lipid mixing significantly promotes this lipid mixing in case of DOPC-LS (any durations of incubation of cells with such liposomes at 4°C and pH 4.9) and in case of DSPC-LS (short incubations only).

Calcium promotion of HA-mediated lipid mixing was observed not only for ganglioside-containing liposomes but also for liposomes that are ganglioside-free and, hence, lack acidic lipids. In addition, the outer membrane monolayer of neuraminidase-treated HA-cells is unlikely to contain significant amounts of acidic lipids. Therefore, influence of calcium on HA-mediated lipid mixing, most probably, does not involve electrostatic interaction of calcium cations and acidic lipids. Although it is still quite possible that calcium cations directly influence lipids, we proceed from assumptions that calcium effect on HA-mediated lipid mixing and HA inactivation reflects the interaction of calcium with HA. We performed structural analysis of HA using programs WebFEATURE and GG. These two programs use different approaches: GG uses a graph theory algorithm to find oxygen clusters, while WebFEATURE predicts the calcium-binding sites by taking into account multiple properties (such as chemical groups, secondary structures, and atom types for which high sensitivity and

selectivity have been demonstrated). Interestingly, both programs found a putative calcium-binding site at exactly the same part of the vestigial esterase domain from the HA1 subunit (Fig. 5).

It is not surprising that the Ca^{2+} cation was not observed in X-ray structure of the X-31 HA [41] because calcium-binding sites with relatively weak affinities are often not observed by X-ray crystallography [47].

We do not know exactly, how this putative calcium-binding site in the vestigial esterase domain of HA1 can influence HA-mediated membrane fusion. Not much is known about the role of HA1 in the fusion process. However, at least in some HA strains vestigial esterase domain may play a role in fusion. For example, the vestigial esterase domain from the HA1 subunit of A/Puerto Rico/8/34 [48,49] and A/South Carolina/1/18 strains [50] can be involved in fusion process. One can hypothesize that the vestigial esterase domain of HA1 of Udorn HA (used in our experiments) may play a role in membrane fusion as such domain of these two strains, and, hence, calcium-binding site in this domain can influence fusion.

Putative acceleration of HA conformational changes by calcium

Importantly, calcium promotes HA-mediated lipid mixing when conditions for such a lipid mixing are favorable, and inhibits lipid mixing when conditions for it are not favorable (low temperature). Putative acceleration of HA conformational changes by calcium can explain these phenomena. We hypothesize that calcium promotes the transition from early and reversible conformation(s) of low pH-activated HA towards an irreversible protein conformation that underlies both HA-mediated lipid mixing and HA inactivation [39,51].

One of the earliest stages of HA conformational change, caused by low pH, is the expulsion of the fusion peptide from the electronegative cavity in which it is buried in the pre-fusion conformation [52]. That is why we suggest that the influence of calcium on HA-mediated lipid mixing involves the expulsion of the fusion peptide from a negatively charged cavity. Perhaps Ca^{2+} cations directly or indirectly disrupt hydrogen-bonding interactions involving the residues of the fusion peptide, such as hydrogen bonds between fusion peptide residues and two aspartate residues (Asp¹⁰⁹ and Asp¹¹²) of the HA2 subunit [53]. These hydrogen bonds may be regarded as a clamp that prevents the HA conformational change which leads to membrane fusion. Hypothetical influence of calcium on these hydrogen bonds may be somewhat analogous to the interaction of calcium with synaptotagmin; that interaction presumably releases the block on membrane fusion during exocytosis [54,55]. If, as was proposed [56–58], fusion peptides of other viral fusion proteins are also buried in the native, pre-fusion state, it will be interesting to study the role of divalent cations in the disruption of hydrogen bonds involving fusion peptide residues of these fusion proteins and in the expulsion of these fusion peptides from cavities in which they are buried.

The two hypotheses - the first regarding the binding of calcium cations to the vestigial esterase domain of HA1, and the second regarding the calcium cation-facilitated expulsion of the fusion peptide from a negatively charged cavity - are not necessarily mutually exclusive. Binding of calcium to the vestigial esterase domain may change the conformation of HA1, and such a conformational change may lead to the interaction of the fusion peptide cavity with basic HA1 residues. This interaction may hypothetically facilitate the release of the fusion peptide from the cavity [50].

Calcium effects may depend on HA surface density

In [30] we described the fusion of HA-cells with DSPC-LS and DOPC-LS at 37°C and neutral pH after different durations of incubation at 4°C and pH 4.9. For DSPC-LS, half of fusion

intermediates inactivated during ~15 min of incubation at pH 4.9, and the other half remained stable and yielded fusion when temperature was raised. For DOPC-LS, ~55% of fusion intermediates yielded fusion after incubation at 4°C and pH 4.9 during 1 hour, while other ~45% remained stable and produced lipid mixing only after temperature was raised.

We suggested in [30] that even for the same composition of target membrane at 4°C influenza HA can form two types of fusion intermediates with different stabilities and propensities to mediate fusion. We called these intermediates HD-HA and LD-HA because, hypothetically, they correspond to high surface density and low surface density of HA molecules on the surface of HA-expressing membrane. At 4°C, the LD-HA intermediate inactivates in case of less fusogenic DSPC/cholesterol target membrane, and does not mediate fusion in case of more fusogenic DOPC/cholesterol target membrane. In contrast, the HD-HA intermediate does not inactivate in case of DSPC/cholesterol target membrane and mediates fusion in case of DOPC/cholesterol target membrane.

Analysis of calcium effects further emphasizes the distinction between the two types of fusion intermediates discussed in [30] as HD-HA and LD-HA intermediates. Calcium present during lipid mixing between HA-cells and DSPC-LS (or during incubation of cells with bound liposomes at low pH) significantly promotes (or, correspondingly, inhibits) lipid mixing *only in case of short low-pH applications*. These results may be interpreted the following way: calcium has no effect on HD-HA intermediates but, depending on the timing of application, either promotes or inhibits lipid mixing mediated by LD-HA intermediates. The irreversible stage of HA restructuring involves lateral interactions between adjacent proteins. Such restructuring is facilitated at high densities of reversibly-activated HA molecules [39,51] and at high HA surface densities (HD-HA) can not be further significantly promoted by calcium. In contrast, at lower HA densities calcium might promote the shift to irreversible conformation thereby increasing the surface density of HA molecules in such conformation.

To conclude, we found that if influenza HA-expressing cells with bound liposomes were incubated in the absence of calcium at 4°C and pH 4.9, calcium promotes significantly the subsequent lipid mixing between these cells and liposomes at 37°C and neutral pH for DSPC-LS (only in case of short incubations at low pH) and DOPC-LS (for any durations of incubation at low pH). Furthermore, we found that calcium present during incubation of cells with bound DSPC-LS at 4°C and pH 4.9 inhibits the subsequent lipid mixing between cells and liposomes at 37°C and neutral pH, but only in case of short incubations at low pH. We hypothesize that calcium promotes the transition from early and reversible conformation(s) of low pH-activated HA towards an irreversible protein conformation. Depending on conditions, such an influence of calcium can lead to either promotion or inhibition of HA-mediated lipid mixing. Possibly, calcium cations influence transition of HA molecules from early to reversible conformation(s) because they bind to a vestigial esterase domain of HA1 subunit of hemagglutinin and/or cause expulsion of the fusion peptide from a negatively charged cavity.

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Fig 1.

Lipid mixing at 37°C and neutral pH between HA-cells and DSPC-LS (A) or DOPC-LS (B) is dependent on the presence of calcium and on duration of incubation at 4°C and pH 4.9. (A) Lipid mixing after the incubation at 4°C and pH 4.9 for 10 min in the absence of calcium (*curve 1*), in the presence of 5 mM CaCl_2 after raising the temperature (*curve 2*). Lipid mixing after the incubation at 4°C and pH 4.9 for 60 min in the absence of calcium (*curve 3*), in the presence of 5 mM CaCl_2 after raising the temperature (*curve 4*). (B) Lipid mixing after the incubation at 4°C and pH 4.9 for 10 min in the absence of calcium (*curve 1*), in the presence of 5 mM CaCl_2 after raising the temperature (*curve 2*). Experiments with incubation at 4°C and pH 4.9 for 1 min, 30 min, 60 min, 90 min gave similar results.

Fig 2. Lipid mixing at 37°C and neutral pH between HA-cells and ganglioside-free DSPC-LS is dependent on the presence of calcium. Lipid mixing after the incubation at 4°C and pH 4.9 for 10 min in the absence of calcium (*curve 1*), in the presence of 5 mM CaCl₂ after raising the temperature (*curve 2*).

Fig 3.

Lipid mixing at 37°C and neutral pH between HA-cells and DSPC-LS recorded after the incubation at 4°C and pH 4.9 for 10 min: in the absence of calcium (*black*), in the presence of 5 mM CaCl₂ after raising the temperature (*red*), or CaCl₂ (5 mM) added 30 sec (*green*), 1 min (*yellow*), 2 min (*blue*), 3 min (*pink*), 4 min (*gray*) after raising the temperature.

Fig 4. Lipid mixing at 37°C and neutral pH between HA-cells and DSPC-LS recorded after the incubation at 4°C and pH 4.9 for 10 min: in the presence of 5 mM CaCl₂ after but not before raising the temperature (*curve 1*), in the presence of 5 mM CaCl₂ before and after raising the temperature (calcium was added, at 4°C, 5 min before the low-pH pulse, *curve 2*).

Fig 5. Putative Ca^{2+} -binding site in the vestigial esterase domain from the HA1 subunit. This figure was made by Discovery Studio 1.7 (Accelrys).