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The fusion of artificial lipid membranes induced by the synthetic arenavirus 'fusion peptide'

S.E. Glushakova [a], V.G. Omelyanenko [b,1], I.S. Lukashevitch [a], A.A. Bogdanov, Jr. [b,2], A.B. Moshnikova [c], A.T. Kozytch [c] and V.P. Torchilin [b]

[a] *Belorussian Scientific Research Institute of Epidemiology and Microbiology, Minsk (Belarus),* [b] *USSR Cardiology Research Center, Moscow (Russia) and* [c] *M.M. Shemiakin Institute of Bioorganic Chemistry, Moscow (Russia)*

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The fusing activity of the synthetic 23 amino-acid fragment (fusion peptide, FP) of the fusion protein of the Lassa arenavirus membrane was tested in a model liposomal system. The resonance energy transfer between two fluorescent phospholipid probes was monitored in order to detect dioleoylphosphatidylcholine liposome fusion induced by the peptide. Fusion rates were compared at different pH values, ionic strength and calcium concentrations. FP demonstrated fusing activity at pH 4.5–5.5, indicating that the protonated form of the FP is the active one. A transmembrane proton-gradient induced by acidification was not relevant to the fusion process, since its elimination with nigericin did not affect the FP-mediated fusion. Both Ca^{2+} (8 mM) and the increase of the ionic strength (1 M NaCl) inhibited liposome fusion. The efficacy of liposome fusion depended also on the lipd-to-lipid ratio. Non-linear dependence was observed at a saturation ratio of 10 mol lipid per mol peptide. A model of 'side insertion' is suggested, describing FP interaction with the membrane.

Introduction

Membrane fusion is assumed to be necessary for the penetration of the enveloped viruses into cells. This process is induced and performed by proteins of the viral coat [1]. For viruses penetrating cells via an endocytic pathway, the endosomal membrane serves as the target, and the fusion proceeds upon the acidification of intravacuolar content. Some data evidence pH-dependent conformational transitions in the fusion protein, which result in the exposure of hydrophobic amino-acid sequence fragment on the surface of protein globule [2]. Recently, the first direct proofs appeared that this hydrophobic peptide (called 'fusion peptide', FP) interacts with the target membrane and induces its fusion with the viral lipid membrane [3]. The mechanism of the process remains unexplained. The results obtained in studies performed with some toxins and viral proteins proved the principal similarity in the interaction of these proteins with both cellular and artificial membranes [4]. Some parameters of the process and its sensitivity to the microenvironment were also investigated [4].

The location of the FP in viral proteins may vary; a hydrophobic and highly conservative amino-acid sequence can be found on the terminus of the polypeptide chain (conformationally protected from the polar surroundings) or inside the protein molecule [5,6]. Recently, we have been able to predict the localization of the FP in the protein GP2 from arenaviruses. This was accomplished using the computer analysis of amino-acid sequences of the surface proteins of arenaviruses and developed criterion for the identification of the fusion proteins of the enveloped viruses [7]. The identification of the natural fusiogenic agents and elucidation of the mechanism of their action is necessary for the understanding of virus penetration and elaboration of prophylactic measures against viral infections.

Here we describe the results of our studies on the fusiogenic activity of a synthetic amphiphilic peptide containing 23 amino-acid residues and imitating the FP of Lassa virus. The experiments were performed on dioleoylphosphatidylcholine (DOPC) liposomes using

Corresponding author (Present address): V.P. Torchilin, Center for Imaging and Pharmaceutical Research, Massachusetts General Hospital-East, Bldg 149, 13th Street, Charlestown, MA 02129, USA.

[1] Present adress: Department of Pharmaceutics, University of Utah, Salt Lake City, Utah 84108, USA

[2] Present address: MR Pharmaceutical Program, MGH-NMR Center, Department of Radiology, Massachusetts General Hospital-East, Bldg 149, 13th Street, Charlestown, MA 02129, USA.

the method of resonance energy transfer between two fluorescent labeled lipids, energy donor and energy acceptor [8].

Materials and Methods

Peptide synthesis. Reagents and amino-acid derivatives from Reanal (Hungary), PRF (Japan) and Fluka (Germany) were used in the present study. Aminomethylated copolymer of styrene with 1 mol% of divinylbenzene (Bio-Rad, USA) was used as a carrier. Solvents were purified as in Ref. 9. The FP was obtained by solid-phase synthesis [10] on Biosearch 9600 (USA) synthesizer. The incorporation of the anchor group was performed with the use of preliminary synthesized *p*-hydroxymethyl phenyl acetamidomethyl (PAM)-derivative of the protected C-terminal amino acid [11]. BOC-group was used as a temporary protection for amino groups. The condensation of amino acids was performed with the use of activated hydroxy benzotriasole esters and symmetric anhydrides. The splitting of the peptide from the polymer and the removal of protecting groups was performed under the action of fluid HF with the addition of *p*-cresole and *p*-thiocresole. The peptide was extracted with 20% acetic acid and purified by gel-filtration and preparative reverse-phase HPLC. The structure of the FP was proved by analytical HPLC, amino-acid analysis and mass-spectrometry.

Liposome preparation. DOPC liposomes (DOPC from Sigma, USA) were prepared by the sonication of multilamellar liposomes in the ultrasound desintegrator (Ultratip, Lab-Line, USA). The buffer used contained 150 mM NaCl, 10 mM Tris-HCl and 1 mM EDTA (pH 7.9). Total lipid concentration was 1 mg/ml. Suspension volume was 0.2 ml. Sonication was performed in four cycles 5 min each with 1 min intervals, at 40 W and 0°C. The preparation obtained was then centrifugated for 15 min at $14000 \times g$ to precipitate large liposomes and lipid aggregates. The supernatant was used in the fusion experiments. According to the data of Coulter N4 Sub-Micron Particle Analyzer, the liposomes obtained had the average size of 200–250 nm.

Liposome fusion. The method of the resonance energy transfer [8] between fluorescent phospholipid derivatives was used to study the process of liposome fusion. *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-dioleoylphosphatidylethanolamine (NBD-PE) was used as the energy donor, and *N*-[lissamine-rhodamine B-sulfonyl]dioleoylphosphatidylethanolamine (Rh-PE) as the energy acceptor. Both NBD-PE and Rh-PE were from Molecular Probes (USA). Fluorescent labeled liposomes contained 1 mol% of NBD-PE and 0.5 mol% Rh-PE. Fluorescence intensity was measured using Hitachi 4100 spectrofluorimeter (Japan) at the excitation wavelength of 450 nm and the emission wavelength of 530 nm. The measurements were performed in thermostated 5×5 mm cuvette at 25 or 37°C. Liposome suspension (200 μl total) contained labeled and non-labeled liposomes in 1:10 weight ratio. Total lipid concentration was $7 \cdot 10^{-5}$ M. The FP was added into cuvette as 2 to 10 μl of the solution in dimethyl sulfoxide (DMSO). The fluorescence intensity of the mixture of the labeled and non-labeled liposomes immediately after mixing was taken as a zero value. The fluorescence intensity of the liposome mixture upon the addition of Triton X-100 up to 0.5 vol% was taken as 100%. To study the influence of pH on the liposome fusion process, the following buffer systems were used: 140 mM NaCl, 50 mM sodium acetate, 1 mM EDTA (pH 4.3 and 4.8) and 140 mM NaCl, 5 mM Tes, 1 mM EDTA (pH 5.3, 6.0 and 7.4). In separate experiments,, non-labeled liposomes were used for turbidity control. The input of scattered light was diminished by the use of glass 490 nm cut off filter.

To investigate the influence of a transmembrane proton gradient on the liposome fusion under the action of the FP, and the dependence of induced membrane fusion on the pH value, the following experimental procedure was used. The mixture of the labeled and non-labeled DOPC liposomes was added to the cuvette (thermostated at 37°C), containing 1 ml of an appropriate buffer plus 10 mM KCl and the fluorescence intensity recording was started. In several minutes, the suspension was additionally supplemented with 5 μl of alcohol solution of nigericin up to $5 \cdot 10^{-6}$ M. The mixture was incubated for 5 min and then FP was added to the system up to $5 \cdot 10^{-6}$ M. 5 min after FP addition, the medium was acidified to pH 4.8 with citric acid. The latter caused also the acidification of the intraliposomal compartment due to the presence of ionophore nigericin in the liposomal membrane. After 5 min the mixture was neutralized with the concentrated NaOH solution, and in several min the second portion of citric acid was added to transfer liposomes into the weakly acidic medium.

Results

In the present study, we have studied the lipid membrane fusing activity of the synthetic peptide which is the predicted FP of Lassa arenavirus (Fig. 1). The peptide is a fragment of the GP2 viral coat protein. It contains 23 amino-acid residues, has high degree of helical periodicity and is amphiphilic with rather high hydrophobicity (the hydrophobicity index according to Keith and Doolittle scale is 0.554, see Fig. 2) [7]. Besides, the structure of the peptide is highly conserved in the arenaviral family. It is located close to the N-terminus of GP2 protein, but according to the prediction made with the use of RAOARGOS computer

PREDICTED ARENAVIRAL GP2 FUSION PEPTIDES

Lassa	GGYCLTRWMLIEAELKCFGNTAV
LCM	GGYCLTKWMILAAELKCFGNTAV
Takaribe	GGYCLEKWMIVASELKCFGNTAI
Picinde	GGYCLEQWALIWAGIKCFDNTYM

Fig. 1. Amino-acid sequences of the predicted fusion peptides of viruses of Arenaviridae family (the regions with conservative amino-acid replacements are enframed).

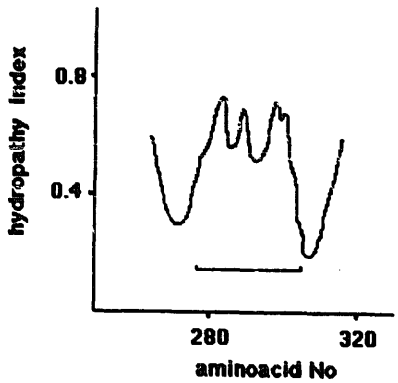

Fig. 2. The hydropathicity profile of the fragment of GP2 protein from Lassa virus. The analysis has been performed utilizing the computer program AASCALE [7]. The bar points the region corresponding to the fusion peptide. The abscissa shows the number of the appropriate amino-acid residue in the sequence of the protein-precursor of the viral glycoproteins; the ordinate shows the value of the hydrophobicity.

program [12] is situated inside the protein globule. As we have shown in Ref. 7, the N-terminus itself shares some features characteristic of viral FP. Nevertheless, the sequence starting from amino-acid residue No. 19 (the peptide used in our studies) seems to be be more promising both from the theoretical considerations and because of its channel forming properties in planar lipid bilayers [7].

The efficacy of the liposome fusion was estimated following the intensity of the energy donor fluorescence, which depends on the efficacy of the energy transfer within the donor-acceptor pair of fluorescent labeled lipid derivatives. The efficacy of the energy transfer is determined by the density of donor and acceptor molecules in the phospholipid bilayer of the liposomes, i.e., by the labeled-to-non-labeled lipid ratio. The decrease of the fluorescent lipid density upon the mixing of labeled and non-labeled liposomes can proceed because of two different processes, i.e., liposome fusion and lipid exchange between bilayers through the water phase. It was shown in Refs. 13 and 14 that for the donor-acceptor pair chosen the lipid exchange through the water phase does not proceed. Even the aggregation of liposomes made of DOPC/NBD-PE or DOPC/Rh-PE in the presence of poly(ethylene glycol) or Ca^{2+} ions does not provoke any decrease in the efficacy of the energy transfer [8]. Thus, in our experiments, the decrease in the efficacy of the energy transfer resulting in the increase of the energy acceptor fluorescence always serves as evidence of the liposome fusion.

The influence of pH and temperature on the liposome fusion by FP

Fig. 3 demonstrates the pH-dependence of the fluorescence changes upon the addition of FP (up to 10^{-5} M) to the mixture of the labeled and non-labeled DOPC liposomes. At neutral pH values, only slight fluorescent increase was found after 4 to 10 min, corresponding to 2–6% of the maximal possible fluorescence increase in the given system. The pH decrease caused fast and pronounced fluorescence increase. Zero and 100% fluorescence points have been determined preliminary in each case because of the dependence of the NBD-PE/Rh-PE pair fluorescence on pH. As shown in Fig. 3B, the increase in the initial rate of the fluorescence increase (the change in the fluorescence intensity during the first minute) was the most pronounced at pH values from 4.5 to 5.5.

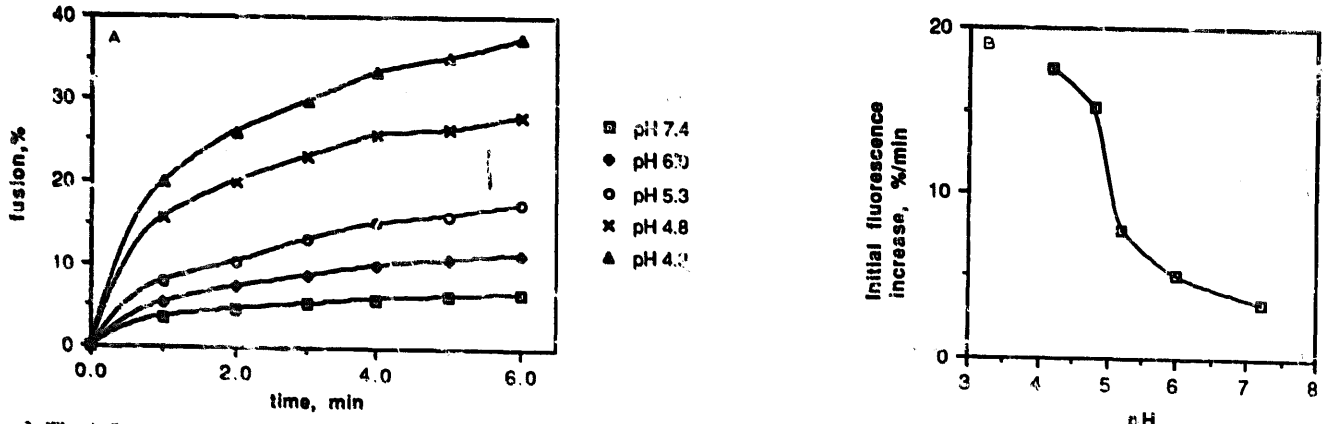

Fig. 3. The influence of pH values on the peptide-induced liposome fusion (A) (the fusion values are given as a % from the maximal possible fluorescence in each system and are estimated following the fluorescence increase); (B) shows the dependence of the initial rate of liposome fusion (given as the fluorescence increase in the system during the first minute of incubation) on pH.

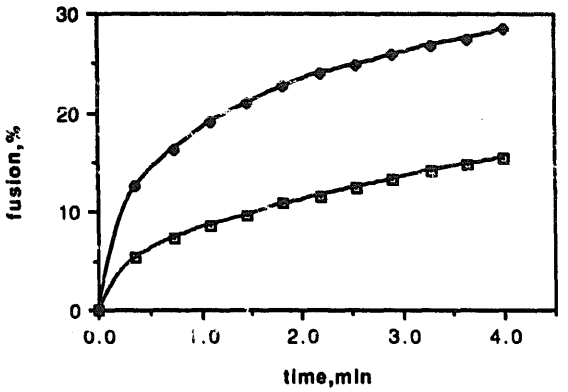

Fig. 4. The influence of temperature on the peptide-induced liposome fusion (estimated as the increase in NBD-PE fluorescence intensity). Squares, 25°C; diamonds, 37°C.

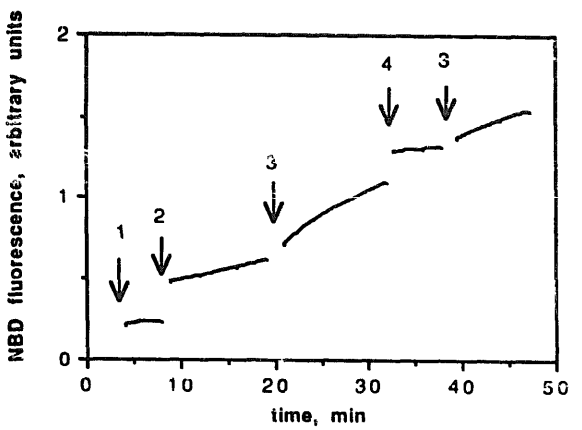

Fig. 5. The FP-induced fusion of nigericin-containing liposomes and its dependence on pH changes. Arrows point the addition of: 1, nigericin; 2, FP; 3, citric acid; 4, NaOH.

The fusion of liposomes by FP can be influenced by temperature. Fig. 4 demonstrates the changes in the fluorescence intensity of DOPC liposomes in the presence of 10^{-5} M FP at pH 4.3 and temperatures 25 and 37°C. The temperature increase from 25 to 37°C almost doubles the fluorescence intensity at the same observation times. Similar results were observed at all pH values tested.

It is worth mentioning that pH and temperature influences also the spontaneous liposome fusion. Depending on pH and temperature values, spontaneous fusion increased slightly but never exceeded 4% from maximal and was always subtracted from the fluorescence values used for the estimation of the peptide-induced liposome fusion.

DMSO used for the FP solubilization did not influence spontaneous liposome fusion and fluorescence values at the concentration used, i.e., from 1 to 5 vol% of the liposomal suspension.

Peptide-induced liposome fusion upon the elimination of the transmembrane proton-gradient and pH changes

The elimination of the transmembrane proton-gradient with nigericin does not influence the fusion action of the FP studied. We observed minor fusion-activity at neutral pH values and increasingly high fusion-activity upon medium acidification (Fig. 5). Besides,the fusion process can be repeatedly stopped by the pH shift to neutral values and reinitiaited by the medium acidification.

The influence of the lipid-to-peptide molar ratio on liposome fusion

The degree of fusion depends on the lipid-to-peptide molar ratio in the suspension. The results of fusion experiments involving increasing peptide concentrations from 10^{-8} to $5 \cdot 10^{-5}$ M are presented in Fig. 6. The experiments were performed at pH 4.3, 37°C and $7 \cdot 10^{-5}$ M lipid concentration. It means that the lipid-to-protein molar ratio in the system was varied from 7000 to 1.4. It is clearly seen that the efficacy of the liposome fusion increases with the lipid-to-protein ratio decrease. The analysis of the process performed in 4 min after its initiation (Fig. 6B) shows that at the lipid-to-peptide molar ratio about 10, the saturation of the membrane with the peptide takes place and the further increase in the peptide concentration affects the fusion extent only slightly. At the same time, the

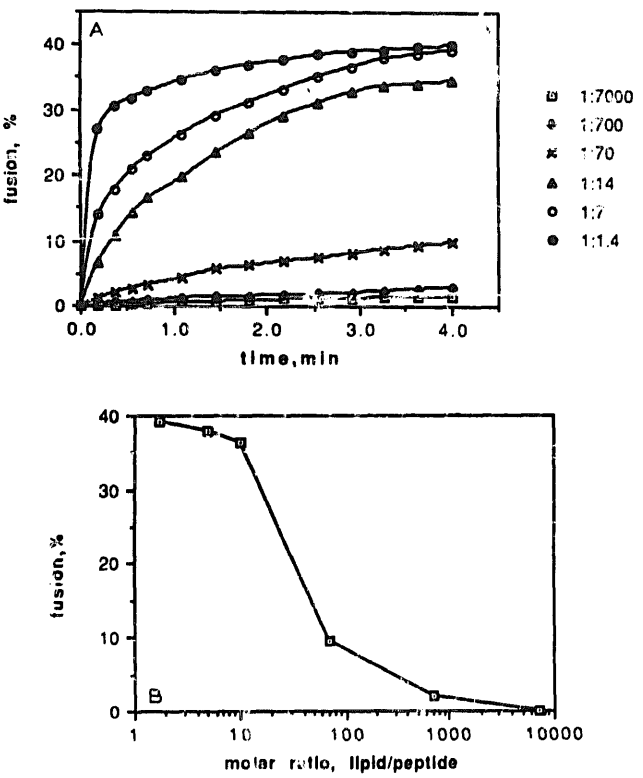

Fig. 6. The dependence of liposome fusion (estimated following the increase in NBD-PE fluorescence) on the lipid-to-peptide molar ratio (A). (B), The fusion in the system 4 min after the addition of the FP at different lipid-to-FP ratios (% from maximum possible, estimated following NBD-PE fluorescence)

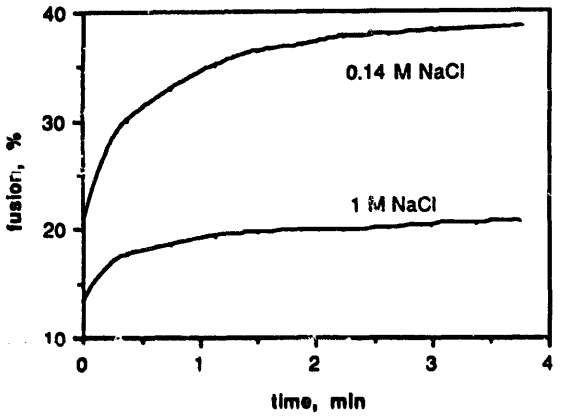

Fig. 7. The influence of the ionic strength on the peptide-induced liposome fusion (following the increase in NBD-PE fluorescence).

initial rates of the fusion within the used lipid-to-peptide ratios interval are quite different. Their values are much higher at low ratios (see curves 4–6 in Fig. 6A). It is also evident that at the saturating peptide concentrations and optimal fusion conditions (37°C, pH 4.3) the fusion reaction has a definite biphasic character (during the first min the fusion rate is 15–20-fold higher than afterwards).

The influence of the ionic strength and Ca^{2+} ions on the peptide-induced liposome fusion

The data on the ionic strength influence on the peptide-induced liposome fusion are presented in Fig. 7. The experiments were performed at 37°C and pH 4.3 with saturating peptide concentration ($5 \cdot 10^{-5}$ M) and liposomes made of neutral phospholipids. It turned out that the increase of ionic strength from 0.14 to 1.0 M (NaCl) decreases the efficacy of the FP-induced liposome fusion by 2-fold and somewhat diminishes the fusion rate.

Fig. 8 presents the data on the influence of Ca^{2+} ions on the peptide-induced liposome fusion. When liposomes made of DOPC are used, 8 mM Ca^{2+} decreases the efficacy of the liposome fusion at 37°C, pH 4.3 and saturating FP concentration.

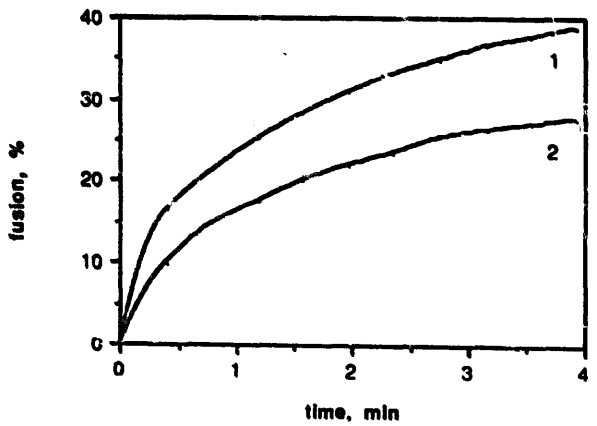

Fig. 8. The influence of Ca^{2+} ions on the peptide-induced liposome fusion (following NBD-PE fluorescence). 1, no Ca^{2+} ions in the buffer; 2, 8 mM Ca^{2+}.

Discussion

The data obtained in the present study demonstrate that the synthetic hydrophibic peptide (the analog of the FP of Lassa virus), possesses pH-induced fusion activity toward DOPC liposomes.

The critical importance of low pH values for the efficient membrane fusion by FP (Fig. 3) can be explained by several factors; (1) the protonation of the peptide facilitating electrostatic interaction between FP and liposomes; (2), pH-induced conformational changes in FP leading to the formation of its active form and (3), the formation of the transmembrane proton-gradient through the liposomal membrane.

At neutral pH values, FP carries two positive (arginine and lysine) and two negative (two glutamic acid residues) charges. Upon the medium acidification, the protonation of carboxylic groups of glutamic acid residues located close to each other in the central part of the FP molecule (Fig. 1) takes place. The pK' value of the ionized carboxylic group of the glutamic acid residue in poly(glutamic acid) molecule is 5.0 [15] and stays within pH interval of the drastic increase in the fusion activity of FP (Fig.3B). Therefore, the effect observed can be explained by the partial or complete neutralization of the negative charges on the FP molecule and the acquisition of the net positive charge by the peptide. The electrostatic interaction of FP with the membrane probably has certain (but not decisive) significance in the process of the peptide-induced liposome fusion (Figs. 7 and 8). The appearance of the transmembrane proton gradient in liposomes as a result of the acidification is not important for the fusion initiation, as its elimination with ionophore nigericin does not affect the fusion (Fig. 5).

In our opinion, the most important consequence of the medium acidification for FP-induced membrane fusion is the conformational rearrangement of the peptide itself. The 'acidic' conformation of the peptide makes it possible for FP to interact with the membrane in a fashion providing the membrane destabilization sufficient for the initiation of the fusion process. The abortion of the liposome fusion upon the medium neutralization and fusion re-initiation upon the re-acidification (Fig. 5) indicate the reversibility of the peptide conformational rearrangement and demonstrate that only protonated form of FP is the active and fusiogenic one.

The conformational rearrangements in the tertiary structure of viral fusion proteins upon the medium acidification were discovered in many viruses, which enter cell interior by endocytosis, in a fashion similar

to that of arenaviruses [16,17]. The complex pH-dependence of the liposome fusion under the action of synthetic FP of the influenza virus shown in Ref. 18 and FP of Lassa virus shown by us in the present study, indicates fine and sterically localized character of pH-induced conformational changes which are necessary for the fusion to proceed.

The FP studied exhibits activity in the wide range of lipid-to-peptide molar ratios (Fig. 6A,B). On the basis of the data obtained, one can not exclude the existence of the cooperative mechanism of FP action in the process of the membrane fusion. From the model studies where synthetic amphipatic GALA peptide was used, it is known that the leakage and fusion are induced by bilayer-incorporated peptide aggregates which form a channel-like pores [19]. It is possible that the fusion in our case was also initiated by similar oligomeric peptide aggregates. The aggregative properties of some other FP were revealed by electron microscopy [5] and by other physico-chemical methods (see Ref. 20 on the properties of melittin, a peptide from the bee venom).

The results obtained in the present study are very similar to the data on the fusion activity of the FP from the influenza virus in the model experiments [18]. Moreover, this data match well with the results obtained in the experiments on the fusion of natural and artificial membranes by some toxins and viruses which enter cells by endocytosis [21,22]. The common features of all the processes mentioned are pH- and temperature-dependent biphasic kinetics and a non-linear effect dependence on the FP concentration. This enables us to consider the predicted FP of arenaviruses as the real functional fragment of the appropriate viral protein which participates in the process of the virus penetration into the cell.

In some properties revealed in our studies, FP from Lassa arenavirus differs from other FPs and whole viral particles. This is the pronounced inhibitory effect of Ca^{2+} ions on the fusion activity of FP. The inhibiting action of Ca^{2+} ions on the toxin-, virus- or membranotropic-agent-induced membrane permeability for different ions, charged metabolites and proteins is well documented [23]. At the same time, this cation usually does not alter the membrane fusion caused with the same agents [24]. We suppose that in our particular case Ca^{2+} ions slow down the fusion by inhibition of the pH-induced FP conformational transition into the 'fusiogenic' conformation.

It should be noted that the length of the FP is quite sufficient for its complete penetration through the phospholipid bilayer assuming that the α-helix conformation of the FP is retained after its protonation and interaction with the membrane. We may hypothetize that the FP interacts with the lipid bilayer in a fashion similar to that of the FP from the viral coat protein. It is known that charged amino-acid residues are located in the sequence of GP2 protein immediately after the FP. Since FP sequence is a non-terminal one, we suggest the model of the 'side insertion' as the most probable one for the description of FP interaction with the membrane. According to this model the FP is incorporated into the external monolayer of the lipid bilayer as it was described for melittin [20]. Recently, Horth and co-authors [25] suggested the theoretically calculated model of the spatial orientation of FP of viral proteins in the membrane in the process of the fusion. They suppose that the functional location of the FP in the membrane is an inclined one with the appropriate angle of about 50°. The theoretical calculations were confirmed by experiments with virus mutants, when the replacement of amino acids in the FP for ones which should theoretically increase the angle of the FP incorporation into the membrane, caused the decrease in the fusion activity of the mutants. As we have already mentioned, one can not exclude the cooperative action of several FP in the membrane desorganization. The lack of the information about the structure of coat spike proteins of arenaviruses does not favour more definite conclusion.

Thus, the data demonstrated the ability of the synthetic peptide which has a homology with the functional domain of the native protein from the Lassa virus, to retain to a great extent the properties characteristic of complex biological structures, viral proteins and whole viruses.

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