Volume 227, number 2, 110–114 FEB 05452 January 1988

Membrane action of synthetic N-terminal peptides of influenza virus hemagglutinin and its mutants

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Received 22 October 1987; revised version received 13 November 1987

Synthetic peptides corresponding to the N-terminal of the cleaved hemagglutinin (HA2) of influenza virus induce an increase in conductance of planar phospholipid bilayers, and cause the release of encapsulated molecules from large unilamellar liposomes. Two mutant peptides, derived from hemagglutinins of mutant viruses with no or reduced fusion activity, do not alter the membrane conductance significantly. These observations support the hypothesis that influenza virus fuses with its target membrane by inserting the HA2 N-terminal into the membrane.

Membrane fusion; Influenza hemagglutinin; Planar bilayer; Liposome; Synthetic peptide

1. INTRODUCTION

Influenza virus is thought to infect its host cell by fusion with the endosome membrane, following endocytosis and acidification of the endosome lumen, thereby releasing its nucleocapsid into the cytoplasm [1–4]. The virus can also induce cell-cell fusion at mildly acidic pH [5,6], or fuse with target membranes such as liposomes and erythrocytes [7–10]. Fusion is mediated by the hemagglutinin (HA) glycoprotein of the viral envelope, but only after proteolytic cleavage of the protein into the HA1 and HA2 subunits [6,7,10–13]. At low pH, the HA molecule changes its conformation [14,15], binds to liposomes of various lipid composition [14,16,17], and induces hemolysis and liposome lysis [18]. The insertion of the N-terminal hydrophobic segment of the HA2 into the target membrane has been suggested to be the mechanism by which the glycoprotein induces the fusion of the viral envelope with the target membrane [4,7,10,13,14]. The observation that tetrapeptides corresponding to this segment inhibit the infectivity of influenza virus also supports the hypothesis that the HA2 N-terminal interacts with the cell membrane during fusion [19]. Here, we present the first direct evidence that the N-terminal peptide segment of HA2 can interact with phospholipid membranes in a manner sufficient to perturb the barrier properties of the membranes. We have investigated the interaction of synthetic peptides corresponding to the N-terminal sequence of influenza X:31 HA2 with planar bilayers and liposomes. We have found that the 7-amino acid peptide HA2.7 (table 1) induces discrete conductance spikes in planar Montal-Müller membranes and causes the leakage of encapsulated compounds from large unilamellar liposomes.

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2. MATERIALS AND METHODS

Solvent-free planar membranes were formed across a hole of approx. 80 μm diameter on a thin (25 μm) teflon septum by raising the level of phospholipid monolayers spread on aqueous media on both sides of the septum [20,21]. Membranes were made from soybean asolectin (type II-S, Sigma) purified according to Kagawa and Racker [22], or egg phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL). The aqueous medium was 100 mM KCl, 10 mM Hepes, pH 7.0. The

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conductance of the membranes was measured by using a conventional voltage clamp system. The signal was filtered with a low-pass filter of a band-width of 2 kHz, processed by a SONY PCM-F1, and stored on video recorder tapes [23]. The signal was continuously monitored on an oscilloscope and an oscillographic paper recorder (Hewlett Packard 7402A). The peptides were synthesized by Biosearch, Inc. (San Rafael, CA), and by Dr Andras Patthay (Biomolecular Resource Center, UCSF), using the Merrifield solid-phase technique [24] with an Applied Biosystems 430A peptide synthesizer. HPLC analysis indicated the purity to be 90%, and amino acid analysis revealed the expected composition. The peptides were suspended in the KCl/Hepes buffer at a concentration of 1 mg/ml and sonicated for 30 s using a probe sonifier.

Large unilamellar liposomes of an average diameter of 140 nm (determined by dynamic light scattering in a Coulter particle analyzer) were prepared by reverse-phase evaporation, followed by extrusion through polycarbonate membranes with a pore size of 100 nm [25,26]. The effect of peptides on liposome membrane permeability was determined by monitoring the leakage into the medium of co-encapsulated ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) and its collisional quencher DPX (*N,N'-p*-xylylenebis(pyridinium bromide) [27,28]. Both compounds were supplied by Molecular Probes (Eugene, OR). The liposomes contained 12.5 mM ANTS, 45 mM DPX, 10 mM Hepes, pH 7.0, and sufficient NaCl to adjust the osmolality to that of the medium (100 mM KCl, 10 mM Hepes, pH 7.0). Leakage results in the dilution of the compounds in the medium, and in an increase of ANTS fluorescence, since quenching by DPX is concentration-dependent. Egg phosphatidylcholine and bovine brain phosphatidylserine were obtained from Avanti Polar Lipids. Fluorescence was measured in an SLM-4000 fluorometer, using a Corning 3-68 high-pass filter, with the excitation wavelength at 360 nm. The fluorescence scale was set to 100% by lysing the vesicles (50 nmol lipid/ml) with 0.8 mM *n*-dodecyl octaethylene monoether.

3. RESULTS

3.1. *Hemagglutinin peptides induce conductance fluctuations in planar bilayers*

When HA2.7 was added to one side of a planar membrane, composed either of asolectin or phosphatidylethanolamine, discrete conductance spikes were observed (fig.1a). Decreasing the pH to 5 did not alter significantly the conductance behavior of the membranes. In some cases, superposition of conductance spikes gave rise to a macroscopic conductance increase (fig.1a). The applied voltage in the righthand section of trace a was reduced to 10 mV to be able to follow the increase in membrane conductance. In fig.2, the specific conductance, G, is plotted as a function of time on a bilogarithmic scale, and the data can be fitted with a straight line. The relationship $G = G(0)t^n$ suggests that the conductance is determined by the aggregation of several (n) molecules of peptide, if the concentration of monomers in the membrane increases linearly with time, and the aggregation process is very fast with respect to the incorporation process [29,30]. The current-voltage characteristics revealed an overlinearity when negative voltages were applied to the chamber opposite that containing the peptide, i.e. the *trans* side (fig.3). This was particularly evident immediately after the addition of the peptide when the ionic conductance was lower. At higher permeability the I-V characteristics tended to become more symmetric, with the overlinearity evident at both positive and negative voltages.

3.2. *Mutant peptides do not alter the membrane conductance significantly*

Gething et al. [31] have recently produced influenza virus mutants by site-directed mutagenesis of the hemagglutinin. Fusion activity of the virus was eliminated when glutamic acid was substituted for the glycine at the N-terminus of HA2. The efficiency of fusion was reduced, and the pH threshold was raised, when glutamic acid was substituted for the glycine at position 4. We have examined the effects on planar membrane conductance of 7-amino acid peptides corresponding to these mutant proteins. The first of the mutant peptides, HA2.7mu1 (table 1), was essentially ineffective in causing conductance fluctuations or an overall increase in conductance, although occasional spikes were observed (fig.1b). The second mutant peptide, HA2.7mu4, was much less effective than the wild type peptide, but did cause conductance spikes (fig.1c). The percent of time, τ, that the membrane current in the presence of the peptides was higher than the steady-state value is shown in table 2.

Addition of HA2.17 to planar membranes at the

Table 1

N-terminal peptides of influenza virus hemagglutinin HA2 used in this study

Peptide	Amino acid sequence
HA2.7	Gly-Leu-Phe-Gly-Ala-Ile-Cys
HA2.7mu1	Glu-Leu-Phe-Gly-Ala-Ile-Cys
HA2.7mu4	Gly-Leu-Phe-Glu-Ala-Ile-Cys
HA2.17	Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Glu-Gly-Cys

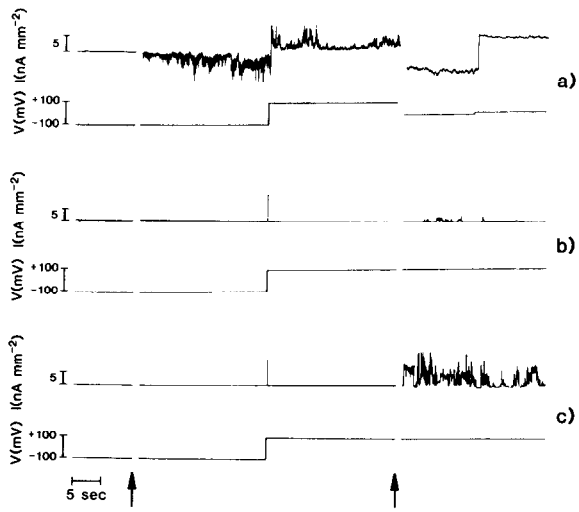

Fig.1. Typical current traces of asolectin membranes in the presence of peptides from hemagglutinin and its mutants. Peptides were added to the chamber on one side of the membrane (the *cis* side) at 50 μg/ml (left-hand arrow). (a) HA2.7; (b) HA2.7mu1; (c) HA2.7mu4. The initial applied voltage, represented by the lower trace in each section, was always −100 mV; the voltage was periodically inverted. The sign of the voltage is that of the *trans* compartment (not containing the peptide) with respect to the *cis* side. Note that the magnitude of the applied voltage in the righthand section of trace a is 10 mV. The behavior of the current immediately after the addition of the peptide (lefthand arrow) and after (a) 8 min, (b) 55 min, and (c) 11 min (righthand arrow) is shown.

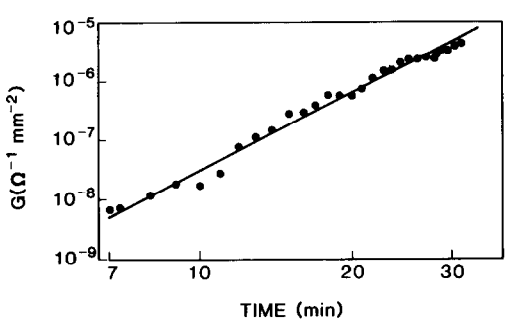

Fig.2. The specific conductance of a planar bilayer composed of asolectin, as a function of the time after the addition of 50 μg/ml HA2.7, plotted on a bilogarithmic scale. The straight line obtained by the best fit of the experimental points indicates that the specific conductance increases as a power of time (n = 4.3). The applied voltage was 100 mV.

same molar concentration as the HA2.7 produced no change in the membrane conductance in three experiments over several hours of recording; in one experiment a τ value of 1.2% was obtained over a total observation time of 3260 s. To ensure that the membrane would have responded to the insertion

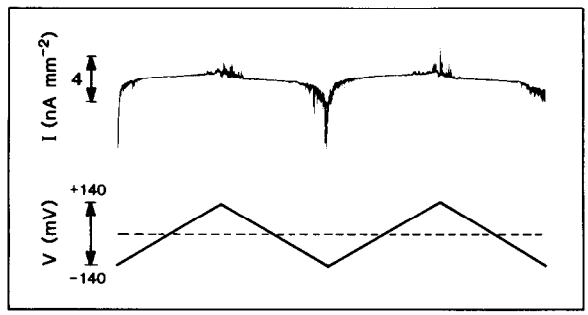

Fig.3. Current response to a continuous cycled voltage from −140 to −140 mV, recorded in the presence of 50 μg/ml of HA2.7.

of the HA2.17, HA2.7 was added to the chamber at the end of the observation period and invariably caused a conductance increase.

3.3. *Hemagglutinin peptides cause the release of aqueous contents from liposomes*

Addition of HA2.7 to a suspension of liposomes composed of asolectin or phosphatidylserine/phosphatidylcholine (1:1) induced the release of encapsulated ANTS/DPX, whereas HA2.7mu1 and HA2.7mu4 were essentially ineffective. The initial rate of release from phosphatidylserine/phosphatidylcholine liposomes in the presence of 20 or 50 μg/ml HA2.7 was 3.1 and 9.0% maximal fluorescence/min, respectively. In contrast, with 50 μg/ml HA2.7mu4 or HA2.7mu1 the initial rate was 0.1 and 0.05% maximal fluorescence/min, respectively. Consistent with the planar bilayer experiments, these observations indicate that the permeability barrier of the liposome membrane was disrupted by the interaction of the HA2.7 with the lipid bilayer.

Table 2

The percent of time, τ, the membrane current is higher in the presence of peptides, than the steady-state value observed in their absence

Peptide	τ (% time)	Observation time (s)
HA2.7	75.5	5310
HA2.7mu1	1.6	5445
HA2.7mu4	2.8	6000

The peptide concentrations were 50 μg/ml. The data were derived from experiments performed at an applied voltage of 100 mV. Experiments performed at different voltages produced similar results

4. DISCUSSION

Our observations with planar bilayers indicate that synthetic peptides corresponding to the N-terminal hydrophobic sequence of the hemagglutinin of influenza virus cause transient perturbations of the phospholipid bilayer structure, allowing for the conductance to increase momentarily and return to the steady-state level. The perturbations may be caused by the lateral and head-to-tail self-aggregation of the peptides in the membrane. The conductance fluctuations and increase may not be specific for hemagglutinin N-terminal peptides, since it is quite likely that a combination of equally hydrophobic amino acid residues could produce similar results. Since repetitive, step-like conductance transitions typical of single channels were not observed, the increase of the baseline current in the presence of the peptide is likely to be caused by the extensive insertion of the peptides into the membrane and the local disorganization of the lipid bilayer structure.

The different efficiency of peptides tested can be explained by the lower hydrophobicity of the 17-amino acid peptide and of the two mutants, in comparison with that of HA2.7. According to the hydrophobic scale of Eisenberg et al. [32], HA2.7 has a hydrophobicity of 0.79 per amino acid, while the two mutants and HA2.17 present hydrophobicities of 0.61 and 0.48, respectively. We should note that the inability of HA2.17 to affect conductance changes does not necessarily mean that it does not interact with the membrane. The N-terminal segment of HA2.17 could have inserted into the membrane without causing a conductance increase. The rest of the peptide could be resting in the aqueous phase, because of its lower hydrophobicity, and thus may not be able to penetrate the bilayer. If the conductance increase is caused by the self-aggregation of several peptides, as proposed above, it is possible that the inserted segment is not able to associate with other peptides in the membrane. The binding of 16- and 20-amino acid peptides from influenza B/Lee/40 to sonicated phosphatidylcholine vesicles has been reported [33]. On the other hand, when HA2.17 is initially dissolved in a guanidine-ethanol mixture and added to phospholipid vesicles, it does cause extensive lysis (unpublished), suggesting that the conformation of the peptide in water is not particularly conducive to alteration of membrane conductance.

The current-voltage asymmetry observed immediately after the addition of HA2.7 can be interpreted as a potential-driven incorporation of the peptide into the membrane, particularly favored at high negative voltages. Peptide molecules crossing the lipid bilayer would tend to balance the peptide concentration difference between the two sides of the membrane, giving rise to a progressive symmetrization of the conductance increase and the overlinear behavior.

The insertion of the peptides into the membrane occurs at pH 7. This observation suggests that once the hydrophobic N-terminal of HA2 is exposed as a result of the pH-induced conformational change of the hemagglutinin, it is prone to insert into the target membrane, if the latter is physically accessible. The inability of one of the mutant viruses (mu1) to fuse [31] may thus be explained by our observation that the N-terminal peptide derived from this mutant (HA2.7mu1) cannot interact with target membranes. The low efficiency of fusion of the second mutant is also consistent with the low frequency of interaction of its N-terminal peptide with planar membranes (table 2). How the penetration of the N-terminal of HA2 into the target membrane mediates membrane fusion is not known. We should note that the conformation of the N-terminal peptide in the intact protein may be different from that of the free peptide in solution, and that correlations such as we have obtained may not always hold.

Low pH-induced fusion of influenza virus with erythrocyte ghosts can be arrested by restoration of the pH to neutral and re-initiated by reducing the pH a second time [8]. Assuming that the low pH-induced conformational change of HA is irreversible [14,18], continuous protonation of the acidic groups in the N-terminal region of HA2 would appear to be necessary for fusion. However, the above observation on virus-ghost fusion may also be explained if fusion is mediated by the process of conformational change of HA2 when the pH is lowered, and not by the final equilibrium conformation of the protein at low pH. Thus, when the pH is returned to neutral, HA2 molecules in the irreversible low-pH conformation would not be able to induce fusion. If the HA2 molecules of a population of virus particles had not undergone

the conformational change during the initial low-pH treatment, these virus particles may have been responsible for the fusion reaction observed when the pH was lowered a second time.

Ohnishi and Murata [34] have reported that a 20-amino acid peptide from influenza A/PR/8/34 induces fusion of small unilamellar phosphatidylcholine vesicles at low pH. The sequence of this peptide is different from that of HA2.17, with a glycine substituted for asparagine in position 12 and a threonine substituted for glutamic acid at position 15. In contrast, the 16-amino acid peptide from influenza B/Lee/40 does not induce fusion of sonicated palmitoyloleylphosphatidylcholine vesicles, whereas the 20-amino acid peptide has pH-independent membrane fusion activity [33]. HA2.17 from influenza X:31, however, does not mediate the fusion of small unilamellar phosphatidylcholine vesicles, at pH 7 or 5 (Düzgüneş, N. and Parente, R., unpublished). Thus, minor variations in peptide length and composition appear to influence the fusogenic activity of peptides derived from influenza hemagglutinin.

Acknowledgements This research was supported by the Consiglio Nazionale delle Ricerche (Italy), grants from the UCSF School of Medicine (MSC-25) and Academic Senate, a Grant-in-Aid from the American Heart Association, and Grant GM-28117 from the National Institutes of Health. We are indebted to M. Montal, S. Oiki and S. Nir for helpful discussions, and J. Fedor for technical assistance.

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