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Fusion of Sindbis virus with model membranes containing phosphatidylethanolamine: implications for protein-induced membrane fusion

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The pH-induced fusion of Sindbis virus with model lipid membranes containing phosphatidylethanolamine has been studied using a quantitative fluorescence technique. The headgroup and acyl chain domains of the lipids have been altered systematically to determine their effect on fusion. Unsaturated phosphatidylethanolamines (PE) have been found to promote fusion, either by themselves, or in combination with phosphatidylcholines (PC). Cholesterol added to a mixture of unsaturated PE and PC was also shown to increase the extent of viral fusion. The results of these studies have been interpreted in terms of a tentative model for the molecular aspects of the target membrane which are necessary for viral fusion. In this model, the target membrane must have a sufficiently-sized domain containing poorly hydrated lipids which are capable of existing in a non-bilayer arrangement.

Introduction

Sindbis virus is a simple enveloped virus which is capable of using its membrane with a membrane of the host cell. For togaviruses such as Sindbis, this process is thought to occur in the acidic environment of the endosome, resulting in the extrusion of the viral capsid into the cytoplasm of the host cell, where viral replication can begin [1,2]. The surface of Sindbis virus is covered by protruding viral spikes, each of which is thought to be composed of a heterodimer of the two envelope proteins (E1 and E2) which are responsible for the biological functions of cell binding and membrane fusion [3].

Both Sindbis and the closely related Semliki Forest virus have also been shown to be capable of interacting with model membranes at low pH in vitro [4,5]. In addition to this demonstrated pH-dependence of the membrane fusion phenomenon mediated by these viruses, there also appears to be a dependence on the lipid makeup of the target membrane. Thus, both Sindbis and Semliki Forest virus appear to require the presence of cholesterol in a target membrane for their successful low pH interaction [4,5]. While this cholesterol dependence has been characterized, its significance is not yet well understood [6].

Abbreviations: egg PE_t, phosphatidylethanolamine made by transphosphatidylation of egg PC; $PMME_t$, phosphatidylmonomethylethanolamine; $PDME_t$, phosphatidyldimethylethanolamine; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles.

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The present study attempts to identify lipid parameters of a target membrane which are important for the pH-dependent fusion of Sindbis virus. The identification of sufficient lipid factors may eventually enable deductions regarding the actual molecular mechanism of the viral protein-mediated membrane fusion process – details of which are currently unknown. The targets for viral fusion are large unilamellar vesicles (LUV) in which the lipid composition and vesicle size are well defined. The phospholipids investigated have been restricted to phosphatidylethanolamines (PE) and phosphatidylcholines (PC) in order to keep the headgroup charge invariant. The acyl chain compositions have been chosen such that all lipids exist in the liquid-crystalline phase at the temperature of the experiments. The effects of added cholesterol in the target membrane have also been determined. These target LUV, coupled with a sensitive fluorescence technique to monitor membrane fusion, constitute a simple model system with which to investigate details of this protein-mediated membrane fusion process.

Materials and Methods

Virus

Sindbis virus (SV) was obtained from the MIT Cell Culture Center, and was purified on sucrose gradients [7]. Sindbis virus which had been pelleted with cold water [8] was labeled with octadecyl rhodamine (R-18) essentially according to the method of Hoekstra et al. [9]. Typically, 1 mg of R-18 labeled virus was prepared at 0.5–1 mg of viral protein/ml in 10 mM Hepes/0.15 M NaCl/1 mM EDTA (pH 7.4), and was stored by quick-freezing aliquots in liquid nitrogen. R-18 SV prepared in this way was stable for months. Repeated freezing and thawing was avoided, since it was found to decrease eventually the degree of fusion obtained.

Virus containing variable, known amounts of R-18 was prepared by incubating variable amounts of R-18 with ^{125}I-labeled (using 'Iodogen', Pierce Chemical) virus. The mole ratio of R-18 to virus was determined from the R-18 and viral concentrations in aliquots of the R-18 labeled virus. R-18 concentrations were determined by solubilizing the labeled virus in ethanol and measuring the R-18 absorbance *, and viral concentrations were determined using the specific activity of the virus. It should be noted that the R-18 moiety was found to interfere with standard protein [10] and lipid assays [11] as well as liquid scintillation counting.

Labeled virus lacking spikes was prepared to demonstrate the importance of intact viral spikes for membrane fusion as well as to demonstrate the non-exchangeability of the R-18 probe in the absence of membrane fusion under the experimental conditions of the fusion assay. For these controls, R-18 Sindbis virus was treated with pronase as described [12], purified by pelleting with cold water, and resuspended in a buffer of 5 mM Tris/0.15 M NaCl/1 mM EDTA (pH 7.4).

Lipids

Egg phosphatidylcholine (egg PC), egg phosphatidylethanolamine (egg PE), egg phosphatidylethanolamine made by transphosphatidylation of egg PC (egg PE_t), monomethyl egg phosphatidylethanolamine ($PMME_t$), dimethyl egg phosphatidylethanolamine ($PDME_t$), and dilauroylphosphatidylethanolamine (DLPE) were obtained from Avanti Polar Lipids. It should be noted that egg PE_t, $PMME_t$, and $PDME_t$ were synthesized starting with egg PC, and therefore have identical acyl chain compositions. The purity of the unsaturated lipids was checked by TLC, both before and after the fusion experiments. The purity of egg PC was checked using an eluant of chloroform/methanol/water (65 : 25 : 4, v/v). The purities of egg PE and egg PE_t were checked using a solvent system of chloroform/methanol/ammonia/water (90 : 54 : 5.7 : 5.4, v/v), and gave a single ninhydrin- and iodine-positive spot. Both lipids are estimated to be greater than 99.9% pure relative to other lipids by TLC. Dimyristoylphosphatidylcholine (DMPC) was used as obtained from Calbiochem. All lipids were stored under argon at −20 °C.

* An absorption coefficient for R-18 in ethanol was determined at 557.5 nm by drying a film of R-18 in vacuo over P_2O_5 to constant weight on a Cahn 31 microbalance and then dissolving it in a volumetric amount of ethanol. The absorption coefficient found in this way was $84.25 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$.

Other chemicals

Octadecyl rhodamine B, chloride salt (R-18) was obtained from Molecular Probes. Triton X-100 was from Calbiochem. All other chemicals were of the highest grade commercially available.

Preparation of LUV

Multilamellar vesicles were found to be unsuitable for the fluorescence studies due to the excessive amount of background scattering. To minimize light scattering and also avoid possible fusion artifacts arising from detergents or organic solvents, large unilamellar vesicles were prepared by extrusion through polycarbonate membranes (Nucleopore) under nitrogen using an 'Extruder' (Lipex Biomembranes, Vancouver) [13]. LUV were prepared to have a nominal diameter of 1000 Å. In brief, a dried lipid film was resuspended to approx. 10 mg/ml in a buffer of 5 mM Mes/1 mM EDTA (pH 7.4). The resuspended lipid was passed through two filters in the extruder ten times at a temperature above the gel-liquid crystalline phase transition of the lipid. Dispersions of the LUV were stored under argon and were used within one day of their preparation. Multilamellar liposomes made with lipids such as egg PE are unstable at 37°C due to the formation of macroscopic hexagonal H_{II} aggregates [14]; these unilamellar vesicles did not exhibit this same instability. In fact, egg PE LUV could be kept at 37°C for hours with absolutely no changes either in their physical characteristics, i.e., they remained as true colloidal dispersions, or in their ability to support viral fusion.

Fusion assay

The fusion assay makes use of the relief of fluorescence quenching of the R-18 probe incorporated into the viral envelope as it becomes diluted into the total lipids of the fused virus-LUV membrane. The validity of this probe as an assay for membrane fusion events has been documented both in lipid vesicle and viral systems [9,15]. It has been shown to be non-exchangeable through the aqueous phase and does not undergo collisional transfer between membranes [9,15]; the non-exchangeability of the probe in the present experiments was not altered by low pH, as was demonstrated in control experiments in which fusion did not occur (not shown) using R-18 labeled LUV (prepared by adding R-18 to LUV in a manner exactly analogous to that used to prepare R-18 Sindbis virus) or protease-treated R-18 Sindbis virus together with fusion-competent target LUV.

Fig. 1 illustrates a typical fusion assay in which 10 μl of LUV at approx. 5 mg of lipid/ml were added to 850 μl of buffer (5 mM Mes/1 mM EDTA (pH 7.4)) in a stirred fluorescence cuvette in a Perkin-Elmer 650-10S fluorescence spectrophotometer. Temperature was controlled to within 0.1 Cdeg by a circulating water bath (Lauda K-2). R-18 Sindbis virus (5–10 μl at 0.5–1 mg of viral protein/ml) was added next, and following equilibration, a precalibrated amount of 0.1 M HCl was added to reduce the pH from 7.4 to 5.4. Finally, after the fluorescence had reached a plateau value (defined as 10 min after the addition of acid), 100 μl of a solution of 10% Triton X-100 was added to solubilize all membranes and thereby achieve maximal dilution of the probe. In general, the moles of LUV lipid exceeded the moles of viral lipid by at least a factor of 30. This corresponds to about a 10-fold excess of LUV over virus. The molar ratio of LUV to virus was varied over an order of magnitude to ensure that it was not limiting the measured degree of fusion.

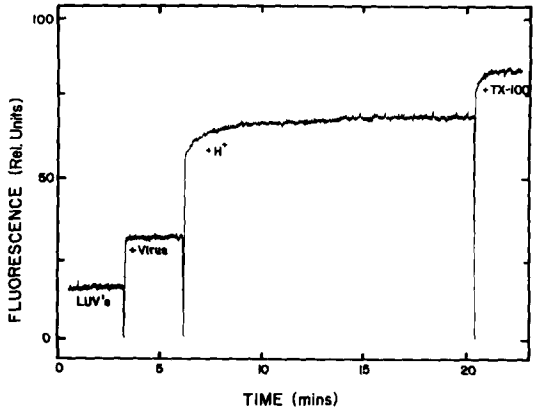

Fig. 1. Kinetics of fusion of R-18 labeled Sindbis virus with LUV of egg PE at 37°C. Fluorescence intensities at 590 nm are given in relative units, and trace an actual experiment. Additions of R-18 Sindbis virus, acid, and Triton X-100 to the LUV are indicated. Unless noted otherwise, the initial and final pH values for this experiment and all others are 7.4 and 5.4, respectively. Use of the fluorescence intensity changes to calculate the fusion index is described in Materials and Methods.

Individual batches of virus and LUV were checked for internal consistency of results. Occasionally, virus which had been frozen and thawed more than a few times, or LUV which had been kept (under argon) for more than a few days were found to give a degree of fusion which was lower than normal. Therefore, virus was thawed at most two times, and LUV were not used more than one day after their preparation.

Quantitation of fusion

The extent of fusion of R-18 labeled virus and target LUV was quantitated by comparing the surface densities of the R-18 probe before and after the pH was lowered to 5.4 to initiate fusion. In these studies, calculation of the surface density of the probe assumes that all the R-18 resides in the outer leaflet of the virus or the fused virus-LUV combination. This assumption is thought to be reasonable based both on the method used to incorporate the probe into the virus (see above), and on the relatively higher degrees of quenching found for equivalent probe concentrations (in total lipid) in virus as compared to pure lipid vesicles (SUV of egg PE and egg PC, data not shown).

Sindbis virus has an outer lipid bilayer radius of 258 Å [16]; fusion with one LUV of 1000 Å diameter should result in a combined surface area of the outer leaflet which is 4.75-times larger than the surface area of the viral outer leaflet alone. Successive fusion events between the fused virus-LUV and additional LUV will result in proportionately larger combined surface areas. Using these calculated increases in surface area, a 'fusion index' f, describing the number of LUV with which a particular virus has fused, can be defined

$$f = a\left(\frac{\sigma_0}{\sigma_f} - 1\right) \tag{1}$$

where σ_0 and σ_f are the initial (before acidification) and final (after acidification) surface densities, respectively, of the R-18 probe, and $a = 0.267$, as determined from a linear regression analysis of the predicted values of f and σ_0/σ_f. Thus a fusion index of 0 indicates that no fusion has occurred, while f values of $1, 2, \ldots$, imply that on the average, each virus has fused with $1, 2, \ldots$, LUV.

The surface density of the R-18 probe was related to its degree of fluorescence quenching by constructing a standard curve using samples of labeled virus that had been prepared with well-defined R-18 contents. Fig. 2 shows the standard curve obtained using virus prepared with R-18 contents ranging from 0.1 to 4 mole percent in total viral lipid, i.e. 0.2 to 8 mole percent in the outer leaflet. As Fig. 2B illustrates, the relationship between probe quenching and surface density for the labeled virus is non-linear, unlike that of the probe in egg PE or egg PC SUV, which was found to be linear (data not shown). To extract surface densities (both σ_0 and σ_f) of the probe from experimentally-determined degrees of fluorescence quenching, Q, the data used to construct the standard curve were fit empirically to an equation of the form

$$Q(\sigma) = A + B \ln \sigma \tag{2}$$

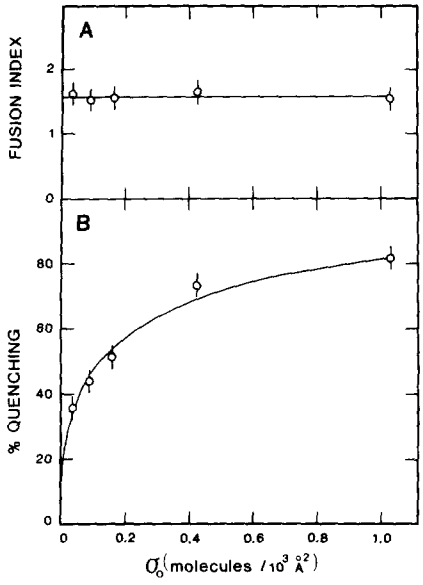

Fig. 2. (A) Fusion indices, and (B) fluorescence quenching, of Sindbis virus samples prepared with variable amounts of R-18, expressed as the initial surface density of the probe (σ_0) in the outer viral leaflet. The points represent averaged experimental data, and the curve represents the fit to the data obtained using $Q(\sigma) = A + B \ln \sigma$. The fusion indices in (A) were obtained by using the standard curve given in (B) to analyze fusion experiments in which egg PE LUV were used as targets (see Materials and Methods for additional details). All measurements were made at 37 °C. Error bars reflect the limits of experimental reproducibility.

The values of A and B were determined from a linearization of the Q versus σ data, and were found to be 81 and 14.4, respectively. Thus, given the experimental values of the fluorescence quenching before and after acidification (Q_0 and Q_f), the corresponding surface densities (σ_0 and σ_f) can be obtained from Eqn. 2; these values then can be inserted into Eqn. 1 to calculate the fusion index. When this procedure is used to calculate the final surface densities, σ_f, for the R-18 virus preparations used to establish the standard curve (i.e., with different initial R-18 contents and known initial surface densities, σ_0), Fig. 2A shows that the resulting fusion indices are essentially independent of the initial R-18 content of the virus. This procedure has been used to calculate the fusion indices in these studies.

The experimental values of the initial and final fluorescence quenching, Q_0 and Q_f, which are required to determine the surface densities using Eqn. 2 were calculated from the fluorescence values before and after acidification relative to the fluorescence value obtained in the total absence of quenching, viz., the value obtained after the addition of Triton X-100 (see Fig. 1). All values of fluorescence were corrected for dilution and buffer scattering. The fluorescence values before and after acidification were also corrected for the light scattering (corrected for dilution) of the LUV. Since the LUV were always present in large molar excess relative to virus (at least a 10-fold excess), and since the dimensions of the two particles are roughly the same, the light scattering contribution of the virus to the total observed fluorescence intensity at 590 nm was neglected. R-18 virus by itself, i.e. in the absence of added LUV, resulted in a very small fusion index (< 0.1) which is most probably the result of light scattering due to viral aggregation; the reported fusion indices are not corrected for this factor.

Degradation of R-18

Hydrolysis of the rhodamine headgroup of the R-18 probe during the fusion reaction would generate rhodamine B (RHO) and result in an apparent relief of fluorescence quenching which could be interpreted as fusion. The stability of the R-18 probe was therefore investigated under the conditions of the fusion reaction. A large scale fusion reaction was carried out between R-18 labeled virus and egg PE LUV which was in every way identical to those carried out with much less virus. Following fusion, the solution was extracted three times with 1 ml of $CHCl_3/CH_3OH/0.1$ M HCl (60 : 32 : 8, v/v); extractions of known amounts of R-18 and RHO resulted in extraction efficiencies (per extraction) of greater than 99% for either compound. Using a sensitive TLC protocol, no RHO could be detected in the extract, and gave an upper limit for the degradation of R-18 to RHO of 0.22%. In a typical fusion reaction, the total relief of fluorescence quenching of 0.22% of the R-18 would result in a fluorescence change which is within the experimental noise limits, and thus cannot affect the results obtained.

Relative quantum yields of R-18

Since differences in the quantum yields of the R-18 moiety in Triton X-100 and in lipids would influence the apparent degree of fusion, the relative quantum yields of R-18 in lipid and Triton X-100 were determined experimentally. Sonicated vesicles (SUV) of egg PE and egg PC containing less than 0.1 mole percent R-18 in lipid (the quenching of less than 0.3 mole percent R-18 in either egg PC or egg PE SUV was not detectable) were prepared using standard techniques [12]. Quantitation of the R-18 absorbance and fluorescence of these SUV in the absence and presence of Triton X-100 gave relative quantum yields of R-18 in egg PC, egg PE and Triton X-100 of 1.01, 1.06, and 1.00, respectively, normalized to the quantum yield in Triton X-100. Since the experimental uncertainty in the observed fluorescence values is estimated at 2.5–4%, these observed differences in relative quantum yields are not expected to influence the results detectably. The experimental fluorescence values therefore were not corrected for the relative quantum yields of R-18 (see also Ref. 9).

Results

In order to quantitate the relative abilities of target lipid vesicles to support the low-pH fusion of Sindbis virus, it was necessary to establish the nature of the relationship between the R-18 content of the virus and the resulting degree of flu-

orescence quenching. As noted in Materials and Methods, this relationship was found to be distinctly non-linear (Fig. 2B). Based on the quenching versus mole fraction curves obtained using pure egg PE and egg PC SUV (data not shown, see also Ref. 9), such non-linearity would be expected at relatively high surface densities of the probe. The surface densities noted in Fig. 2 were calculated based solely on the geometry of the virus, and did not take into account the surface area actually taken up by viral lipid and protein. Thus, one possible reason for the non-linearity of this plot is that the surface area actually accessible to the probe is less than the calculated area.

To validate the use of the quenching versus surface density curve in quantitating viral fusion, fusion indices were calculated from fusion experiments between egg PE LUV and virus which had been prepared with variable (known) amounts of R-18. If the calculational method is correct, such experiments should result in identical fusion indices for all the R-18 Sindbis virus preparations used, since the target LUV are the same in each case. The results of these experiments are given in Fig. 2A, and demonstrate that the fusion index can be appropriately calculated using the experimentally-determined viral quenching versus probe surface density curve (Fig. 2B). Furthermore, these results indicate that apparent surface densities of the probe up to 1 molecule/10^3 Å^2 do not significantly influence the fusion ability of the virus.

The kinetics of membrane fusion between enveloped viruses and model liposomes have been described for a togavirus, Semliki Forest [5], a paramyxovirus, Sendai [9,15,17], and a myxovirus, influenza [9,18–20]. Fig. 1 indicates that the kinetics of the low pH induced fusion of Sindbis virus with PE-containing LUV are relatively fast, and occur on a time scale similar to that seen for Semliki Forest virus [5] and influenza virus [9,18–20].

Togavirus interaction with model membranes is also known to be pH dependent, occurring only at pH values approaching those of the endosome [4,5]. Fig. 3 indicates that R-18 labeled Sindbis virus fuses with a model membrane with an acidic pK. Fusion of R-18 labeled Sindbis virus was also found to be absolutely dependent on the presence of intact viral spikes, since fusion experiments

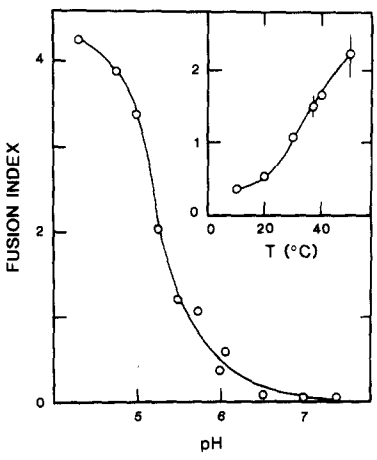

Fig. 3. pH dependence of R-18 labeled Sindbis virus fusion with egg PE LUV at 37 °C. Inset: Temperature dependence of fusion of R-18 Sindbis virus with egg PE LUV.

with pronase-treated R-18 Sindbis virus and fusion competent LUV resulted in virtually no membrane fusion (data not shown). Together with the observations that fusion is not detected either between virus competent vesicles at pH 7.4 (see, for example, Fig. 1) or between R-18 containing LUV and fusion competent vesicles at pH 5.4 (data not shown), these observations confirm that this technique is indeed a true measure of membrane fusion [9,15].

One additional characteristic of the fusion of R-18 labeled Sindbis virus with model membranes that can be noted is its temperature dependence. Fig. 3 shows that the fusion index increases markedly with temperature over the range 10 to 50 °C; this result is very similar to that obtained previously for Semliki Forest virus using a different fusion assay [5]. All fusion experiments have been carried out at 37 °C to eliminate variability from this factor. The choice of physiological temperature for these experiments also ensures that the interpretation of the results will not be complicated by the presence of gel-phase lipids, since at 37 °C, all the lipids employed in this study are in the liquid-crystalline phase.

To address the question of the importance of the headgroup of the target lipids in viral membrane fusion, four lipids were compared which differed only in the number of methyl groups on the nitrogen atom of the headgroup; headgroup

charges and acyl chain compositions were the same for all four lipids. Thus, phosphatidylethanolamine was used which was prepared from egg phosphatidylcholine by transphosphatidylation (designated as egg PE_t); the acyl chain compositions of egg PE_t and egg PC are therefore identical. The mono- and dimethyl derivatives of egg PE_t, namely $PMME_t$ and $PDME_t$, respectively, were also compared for their fusion competence. Fig. 4 compares the abilities of these four lipids to support the pH-dependent fusion of Sindbis virus. The fusion index is seen to be markedly dependent on the number of methyl groups on the headgroup of the lipid, decreasing significantly upon the replacement of each hydrogen atom of the phosphatidylethanolamine headgroup by a corresponding methyl group.

To find out whether or not the degree of unsaturation in the acyl chain correlated with the ability of the lipid to support viral fusion, this parameter was varied in two related sets of experiments. In one set of experiments, viral fusion was compared using mixtures of PC and PE headgroups as part of either a saturated or an unsaturated lipid model system. Fig. 5A shows that using LUV made up exclusively of unsaturated PC and PE, viz. egg PC and egg PE_t, the degree of viral fusion is strikingly dependent on composition; significant fusion occurs only above

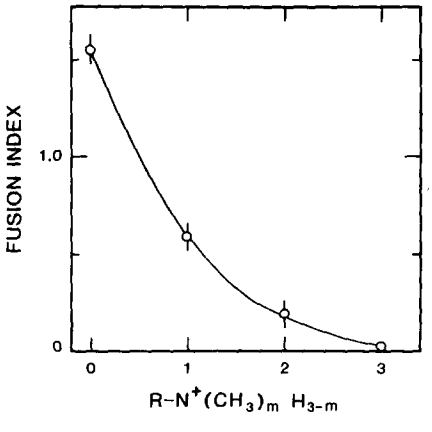

Fig. 4. Variation of fusion of R-18 Sindbis virus with numbers of methyl groups (m) on the headgroup of the target lipid at 37 °C; the acyl chain composition of all lipids is identical to that of egg PC. Error bars reflect limits of experimental reproducibility.

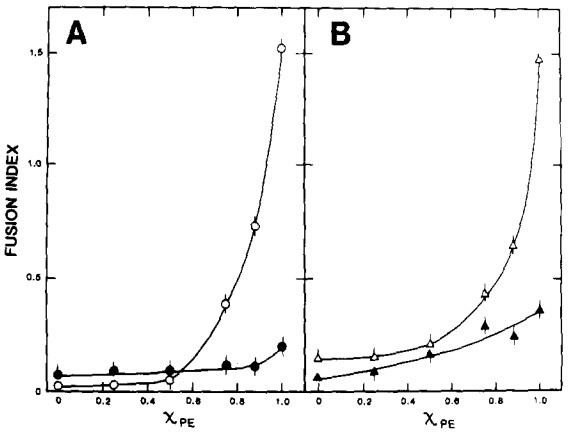

Fig. 5. Variation of fusion of R-18 Sindbis virus with mole fraction of PE in PC-PE mixtures at 37 °C. (A) Unsaturated lipids egg PC and egg PE_t (○) and saturated lipids DMPC and DLPE (●). (B) Mixtures of saturated and unsaturated lipids: DMPC with egg PE (△) and egg PC with DLPE (▲). Error bars reflect limits of reproducibility using different preparations of LUV and R-18 Sindbis virus.

50 mole percent egg PE_t. These results thus extend those obtained using LUV of pure egg PE_t and egg PC (Fig. 4). In direct contrast to these results using unsaturated lipids, however, Fig. 5A also shows that for mixtures of PC and PE in which the acyl chains are saturated, viz. DMPC and DLPE, the degree of viral fusion is uniformly much lower and, in fact, is essentially independent of the headgroup composition. Thus, at corresponding headgroup compositions, the unsaturated lipid system supports much more viral fusion than the saturated lipid system.

A second set of experiments, designed to provide additional insight into the role of the acyl chain domain in viral fusion, confirms and extends the results of Fig. 5A. Fig. 5B shows that compared to the unsaturated lipid system of Fig. 5A, the substitution of a saturated PC for an unsaturated PC does not result in significant changes in the fusion competence of the target vesicles. Fig. 5B also shows, however, that the substitution of a saturated PE for an unsaturated PE of the unsaturated lipid system of Fig. 5A significantly decreases the ability of the target vesicle to support viral fusion. In fact, the inclusion of a saturated PE in an otherwise unsaturated lipid system produces results similar to those seen

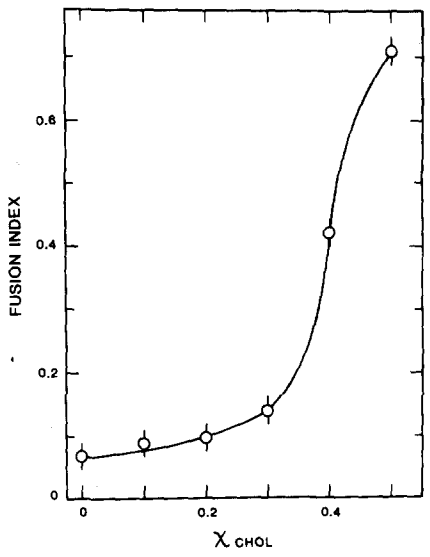

Fig. 6. Variation of fusion of R-18 Sindbis virus with mole fraction cholesterol in egg PC/egg PE LUV at 37°C. The egg PC/egg PE mole ratio is constant at 1:1. Error bars reflect limits of reproducibility.

in Fig. 5A in which all lipids were saturated.

Previous studies have noted that the interaction of togaviruses with model membranes is characterized by a dependence on the presence of cholesterol in the target membrane [4,5]. To investigate further the influence of cholesterol on Sindbis virus fusion, target membranes were prepared in which increasing amounts of cholesterol were added to an equimolar mixture of egg PC and egg PE, i.e., an unsaturated lipid model system. As already noted in Fig. 5A, an equimolar mixture of egg PC and egg PE_t displays minimal fusion activity at 37°C; (egg PE and egg PE_t support roughly equal amounts of fusion; Fig. 5). Fig. 6 shows that as the mole fraction of cholesterol is increased in the egg PC/egg PE mixture, the fusion index increases significantly at constant temperature, from less than 0.1 at zero mole fraction cholesterol to about 0.7 at 0.5 mole fraction cholesterol.

Discussion

These studies have tried to define better the lipid properties of a target membrane which support the low-pH induced fusion of Sindbis virus. With respect to lipid headgroup parameters, these experiments have attempted to evaluate the effects of relatively minor changes in the structure of the headgroup on the ability of the lipid to support viral fusion. Headgroup charge has been kept constant so that the importance of structural factors can be assessed; the differences between choline and ethanolamine headgroups have been emphasized. Perhaps the most important consequence of the structural difference between choline and ethanolamine headgroups which is relevant to this study is their individual hydrogen-bonding abilities. The PE headgroup is known to be involved in intermolecular hydrogen-bonding interactions with adjacent PE molecules, while PC headgroups are not [21]. As a result of this difference in hydrogen-bonding abilities, the PC headgroup is well-hydrated with electrostricted water molecules [22] while the PE headgroup is much less hydrated [21,23].

The differences in hydration between choline and ethanolamine headgroups, in turn, have consequences for the physical properties of the corresponding lipid bilayers. With respect to fusion, hydration of the headgroup domain is thought to present a large repulsive barrier to the close approach of two lipid bilayers [24]. Conversely, the dehydration of the lipid headgroup domain by agents such as polyethylene glycol is thought to be an important prerequisite for the fusion of lipid bilayers in general [25]. Thus, one would predict that bilayers made up of PE would be intrinsically more susceptible to fusion events than those made up of PC; this has in fact been found to be true in pure lipid model systems [26,27].

It is clear from Fig. 4 that with respect to Sindbis virus fusion, the detailed structure of the headgroup domain is indeed important. The progressive substitution of methyl groups for the protons of the nitrogen atom on the ethanolamine headgroup results in stepwise decrements in the ability of the lipid to support viral fusion. Given that the headgroup charge and acyl chain domains of the series of lipids egg PE_t, $PMME_t$, $PDME_t$, and egg PC are identical, this experiment shows conclusively that relatively small alterations in the structure of the headgroup can result in large differences in the ability of the lipid to support virally-mediated membrane fusion. These dif-

ferences could be the result of headgroup size, i.e., a steric factor, or headgroup hydration, or both.

If headgroup hydration or size determined whether or not a particular lipid could support viral fusion, one would predict that all PE would be equivalent in this regard. However, if one compares the fusion indices of EPC bilayers containing either egg PE_t or DLPE (Fig. 5, A and B), one sees that at equivalent mole fractions of the PE component, the egg PC/egg PE_t LUV support substantially more viral fusion than egg PC/DLPE LUV. While the overall degree of unsaturation in the bilayer may play some role (compare, for example, the fusion indices of DLPE in the presence of either DMPC or egg PC), the most important factor appears to be the degree of saturation of the acyl chains of the PE component. Thus, in contrast to the inability of a saturated PE to support significant fusion, an unsaturated PE supports fusion in the presence of either a saturated or an unsaturated PC.

The observation that unsaturated PE supports much more viral fusion than PE containing saturated acyl chains thus suggests that non-headgroup parameters are also important in protein-induced membrane fusion. Since all PE is in the liquid-crystalline phase in these experiments, differences in packing parameters would be expected to be minimal. One property of PE, however, which does correlate with the degree of saturation of the acyl chain is its ability to enter into non-bilayer phases. In particular, unsaturated PE can exist in the hexagonal H_{II} phase [14,28,29], at least in multilamellar lipid systems. Saturated PE apparently enters the hexagonal phase much less readily [14,21,22].

From Fig. 5, it would appear that fusion depends critically on some property or properties of unsaturated PE. In fact, if the maximal value of the fusion index is assigned unit probability, the experimental data of Fig. 5 for LUV containing an unsaturated PE can be fit quite well (not shown) using an equation with the form

$$P(n) \approx X^n \tag{3}$$

where P is the probability of forming a domain consisting of n PE molecules in a bilayer with X mole fraction of unsaturated PE. This analysis predicts that a minimum grouping of about 5 PE molecules is required in the target membrane before significant viral fusion can occur. Using a value of 75 $Å^2$ for the surface area of an unsaturated PE [23], this gives an approximate domain size of 400 $Å^2$. Such a treatment assumes ideal mixing behavior of the individual components, which experimental evidence supports for PC and PE in the liquid-crystalline phase [30].

The hypothesis that a domain of critical size made up of lipids with specific molecular characteristics is needed in the target membrane to support viral fusion is reinforced by the experiments involving target LUV containing cholesterol (Fig. 6). Cholesterol shares with unsaturated PE the characteristic of being poorly hydrated and the ability to stabilize (possibly due to its cone-like shape [31,32]) non-bilayer structures in mixed lipid systems composed of unsaturated PC and PE [30,33]. In liquid-crystalline, mixed PE/PC systems, cholesterol does not appear to associate preferentially with either phospholipid [30]. Thus, if a specialized domain of poorly-hydrated lipids with non-bilayer propensities is necessary for fusion, one would predict that cholesterol, when its smaller surface area is taken into account, should serve the same purpose(s) in fusion as an unsaturated PE.

A comparison of Figs. 5 and 6 indicates that, starting with an equimolar mixture of egg PE and egg PC, the addition of 40 mole percent cholesterol increases the fusion index by about half as much (to 0.4) as an additional 40 mole percent egg PE (fusion index of 0.8 at 90 mole percent egg PE). Put another way, it takes twice as much cholesterol as egg PE on a mole basis to achieve the same degree of fusion enhancement. This observation thus is consistent with the hypothesis of a required critical domain in the target bilayer, since due to its smaller molecular area of about 38 $Å^2$ [34], each cholesterol would contribute approximately half the area to such a 'fusion-active' domain as an unsaturated PE molecule (75 $Å^2$; [23]).

The differential abilities of unsaturated and saturated PE to enter the hexagonal phase has been rationalized according to a 'molecular shape' hypothesis [35,36]. According to this interpretation, lipids with small headgroups relative to their acyl chain domains (such as unsaturated PE and

cholesterol), possess a dynamic cone shape, and are well-suited in a steric sense to participate in hexagonal structures. Conversely, lipids with larger headgroups relative to their acyl chain domains, such as PC (and presumably saturated PE [35]), do not fit well into the inverted micellar arrangement characteristic of the hexagonal phase, and therefore stabilize bilayer phases [35,36]. As a consequence of their distinctive phase preferences, hexagonal phase lipids such as unsaturated PE have been hypothesized to be important for the formation of intermediate lipid structures along the pathway of bilayer fusion [31,33,37,38]. Freeze-fracture evidence consistent with the presence of these inverted micellar regions, or 'lipidic particles', has been found in several lipid model systems undergoing membrane fusion [37,39–41].

Direct evidence for the involvement of the hexagonal phase in viral fusion is not apparent from the present studies. The transition from lamellar to hexagonal phases in multilamellar systems of egg PE is very cooperative; at 25 °C the lipid is in a bilayer, while at 30 °C it is in the hexagonal phase [14]. The observed temperature dependence of fusion of labeled Sindbis virus with egg PE (see Fig. 3) does not exhibit such a sharp, cooperative transition behavior. However, it should be noted that the unknown effects of temperature on the ability of the virus to fuse may obscure the effects of temperature on the target lipids. In addition, it should be noted that these results cannot preclude the involvement of some fraction of the total lipids in transient inverted micellar structures during fusion.

In summary, the present studies have used a sensitive fluorescence assay coupled with well-defined target vesicles as a model system with which to investigate details of the pH-dependent fusion of Sindbis virus. The results of these studies illustrate that characteristics of both the headgroup domain and the lipid as a whole play a role in virally-mediated membrane fusion. Taken together, they indicate that the fusion of Sindbis virus correlates strongly with the presence in the target membrane of a sufficiently-sized domain of membrane components (phospholipid and/or cholesterol) which have the related properties of relatively poor headgroup hydration plus the ability to exist in non-bilayer configurations. One may further speculate that such a domain would facilitate the close approach of virus to the target membrane (due to its lack of hydration), while at the same time providing membrane components which could take up (transient) non-bilayer configurations in the zone of initial contact and membrane fusion.

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