Probing the Mechanism of Fusion in a Two-Dimensional Computer Simulation

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ABSTRACT A two-dimensional (2D) model of lipid bilayers was developed and used to investigate a possible role of membrane lateral tension in membrane fusion. We found that an increase of lateral tension in contacting monolayers of 2D analogs of liposomes and planar membranes could cause not only hemifusion, but also complete fusion when internal pressure is introduced in the model. With a certain set of model parameters it was possible to induce hemifusion-like structural changes by a tension increase in only one of the two contacting bilayers. The effect of lysolipids was modeled as an insertion of a small number of extra molecules into the *cis* or *trans* side of the interacting bilayers at different stages of simulation. It was found that *cis* insertion arrests fusion and *trans* insertion has no inhibitory effect on fusion. The possibility of protein participation in tension-driven fusion was tested in simulation, with one of two model liposomes containing a number of structures capable of reducing the area occupied by them in the outer monolayer. It was found that condensation of these structures was sufficient to produce membrane reorganization similar to that observed in simulations with "protein-free" bilayers. These data support the hypothesis that changes in membrane lateral tension may be responsible for fusion in both model phospholipid membranes and in biological protein-mediated fusion.

INTRODUCTION

Despite significant progress in the identification of fusion proteins and understanding the details of their function, an understanding of the mechanism of biological membrane fusion is still far from complete. A number of different hypotheses have focused on the question of how conformational changes in proteins are coupled to the profound rearrangement of lipid molecules associated with the formation of the initial fusion pore (reviewed in Bonnafous and Stegmann, 2000; Lentz and Lee, 2000; Ruysschaert and Epand, 1999; Zimmerberg and Chernomordik, 1999). Computer simulations of fusion seem to be useful tools to study lipid and protein rearrangements at the molecular level, but even a nanosecond-long atomic resolution simulation of a lipid bilayer with 50–100 lipid molecules requires several months of supercomputer time (Pastor, 1994). A two-dimensional model of lipid bilayers developed several years ago (Chanturiya, 1997) still remains the only computer model suitable for practical experimentation on the possible mechanisms of a large-scale bilayer rearrangement during membrane fusion. Although this model is schematic and does not allow direct extrapolation to measurable macroscopic parameters of real lipid bilayers, it is still useful for the rough evaluation of different fusion mechanisms.

The majority of theoretical works on membrane fusion are focused on bilayer curvature-related effects in the immediate vicinity of the point of fusion (Kozlov and Markin, 1983; Siegel, 1999; Kozlov and Chernomordik, 1998). However, the possibility of the involvement of distant lipid molecules in the unification of the membrane in the contact area is now receiving more attention (Safran et al, 2001; Garcia et al, 2001). In the present work we use a two-dimensional (2D) model, with minor modifications, to study several aspects of purely lipidic fusion that were not tested in previous studies, and thus confirm the viability of this approach for fusion studies. We also modified the model to test the hypothesis that changes in protein conformation may be coupled to fusion via the membrane lateral tension mechanism that has been suggested to explain calcium-induced fusion in different systems (Chanturiya et al., 2000).

MODEL DESCRIPTION

General principles of the 2D bilayer model

The basic principles used for all modifications of a 2D lipid bilayer model were described in detail previously (Chanturiya, 1997). Instead of constructing an atomic resolution model bilayer with most of the multitude of interactions between individual atoms, we have designed a very simplistic 2D projection of a phospholipid bilayer. After numerous test runs, a minimum set of parameters was found that allows maintenance of stable bilayers that mimic the known basic features of real lipid bilayers. Lipid molecules were represented by rigid, rod-like structures with only three interacting points in each "molecule," one located on a "hydrophilic head" and two others located on a "hydrophobic tail" (Fig. 1 *A*). Interactions between points in model molecules were assumed to consist of attraction and repulsion forces, described in terms of energy. In all simulations described here (except those with "protein"-containing bilayers) we used

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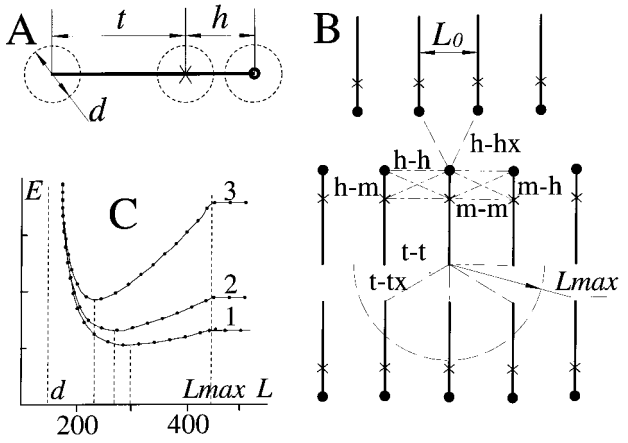

FIGURE 1 A schematic representation of the two-dimensional model of lipid molecules and forces between them. (*A*) A rod-like lipid molecule has three points, one representing the head of the molecule, one representing the tail, and one in between (further referred to as a middle point). A circle around each of these points reflects the physical dimensions of the molecule. Molecular parameters used in the model were $h = 100$, $t = 300$, $d = 80$. (*B*) A model bilayer with a contacting monolayer of another bilayer is shown. Interactions calculated for molecules in the bilayer are represented by dashed lines, and letters indicate the type of interaction. The initial distance between molecules, L_0, was set to 300 and the plateau of energy begins at $L_{max} = 450$ in the simulations described in this paper. (*C*) Profiles of in termolecular energies used in the model: $K_{xx} = 0.007$ (1), $K_{xx} = 0.011$ (2), $K_{xx} = 0.022$ (3), and $W_{xx} = 1.0$ for all three curves. Energy (E) and distance (L) are given in abstract units. d represents the width of the molecule as in *A*.

the same abstract equations as in the previous study to produce a simple energy function with a minimum and plateau:

$$E = E_a + E_r = KL + d/(L - d)$$
$$E = E(L_{max}); \qquad \text{if } L > L_{max} \tag{1}$$

Where E_a is the energy of attraction; E_r is the energy of repulsion; L is the distance between interacting points, and d is the absolute minimum distance (represents the physical size of the molecule). K is a variable parameter, which determines the position of energy minimum (Fig. 1 *C*). L_{max}, the absolute maximum distance, was introduced as the simplest way to create a plateau in the energy function, which can be expected for molecules separated by some distance. These equations are a 2D analog of the expression used to describe surface free energy in three-dimensional (3D) lipid bilayers (Marsh, 1996) with linear distance replacing the area occupied by lipid molecule. The total energy of a molecule in a bilayer E_T is described by the following expression:

$$E_T = E_{hh} + E_{hhx} + E_{hm} + E_{mh} + E_{tt} + E_{ttx} \tag{2}$$

Where $E_{xx} = E(K_{xx})*W_{xx}$ and xx indicates the type of interaction with an adjacent molecule in the same monolayer (head/head (hh), head/middle (hm), middle/head (mh) and tail/tail (tt)) and hhx and ttx represent interactions of head/head and tail/tail, respectively, between molecules in different monolayers (Fig. 1 *B*). In preliminary experiments with models it was found that middle-tail interactions do not significantly affect the behavior of model bilayers. For this reason, and to increase the speed of calculations, *mt* indexed parameters were excluded. A second variable parameter, W_{xx}, was introduced as a multiplexer of energy function to vary the relative weight of different interactions. This parameter was set to the highest value for head-head interactions and to 5–10 times lower values for tail-tail and midpoint interactions (a similar ratio was suggested for lateral lipid-lipid interactions in real bilayers (Gavrisch and Holte, 1996)).

Tension increase in bilayers was modeled as an increase of only one parameter, K_{hh}. An increase of K_{hh} shifts the position of energy minimum to shorter distances between heads in the monolayer and is equivalent to the increased membrane lateral tension induced by polyvalent ions in real bilayers (MacDonald, 1988). Because it is assumed that headgroups in the area of bilayer contact are less hydrated than the headgroups outside the area of contact, K_{hh} in that region was set to lower values.

Computational method

Molecules were placed in starting positions tail to tail, in a linear or circular order. These two types of structures represent 2D analogs of planar bilayers and vesicles. To increase the speed of computation, coordinates and dimensions of molecules were defined by integer-type variables, while floating-point variables were used only for energy calculations. To minimize errors associated with float-integer conversion, head-to-tail distance of the molecule was set to 400 units. This molecule size, in principle, allows putting two circular bilayers having a diameter-to-thickness ratio up to 14–16 into a 32,000 × 32,000 modeling space. In a majority of the simulations we used circular bilayers with a diameter-to-thickness ratio of 6–8 (corresponding to 30-40-nm diameter lipid vesicles). An energy minimization algorithm with ±1 unit step along the x and y axes for head and ±0.2° angle steps for tail directions was used.

After equilibration for ~500 computation cycles bilayers were placed "in contact," i.e., within a distance smaller than L_{max}, and allowed to reorganize spontaneously. For every $5 \times N$ (where N is the total number of molecules in simulation) computation cycle, the current position of each molecule, scaled to fit 640 × 480 pixel window, was displayed. When significant changes in the area of interest were found, the image was captured into a bitmap file using Windows 98 print screen utility. The distance between the selected molecules and changes in the total system energy (E_s) were recorded for further analysis of the molecule rearrangements. The average of pairs of lipid head-to-head distances was calculated for n, $n + 1$, and n, $n - 1$ molecules in different positions. For molecules away from the area of contact this parameter was found to be close to the average distance calculated for the whole pool of molecules. For molecules within the contact area it was more variable, and depended mainly on the proximity to the breaking point. The total system energy for all molecules in the simulation was calculated using the following equation:

$$E_S = \sum_{n=1}^{N} E_T(n)/N \tag{3}$$

Where N is the total number of molecules in simulation.

Simulations were performed with a program written in C++ on a computer based on the Pentium-II, 450 MHz processor.

Planar bilayer with tension

Static tension in linear bilayers was introduced by fixing the positions of four molecules on the edges of the bilayer and by increasing the distance between them during bilayer generation. Molecules in the bilayers created under such conditions have higher than normal energy because they were separated by a distance longer than the distance that corresponds to the energy minimum. Tension in the model bilayer was characterized by "fractional displacement", defined as the percentage increase in the initial distance between molecules in the bilayer with respect to the distance that corresponds to the energy minimum.

Internal pressure in the vesicle

An equivalent of vesicle internal pressure was introduced as an additional energy component (dE_p). dE_p increases or decreases when a molecule is moved to a position that causes a reduction or increase, respectively, in the area inside the circular structure. dE_p was calculated only for the headgroups of molecules in the inner monolayer using the following algorithm. For "j" molecule sampled during the elementary cycle of calculations the area of the triangle with vertexes defined by the positions of j, $j + 1$, and $j - 1$ molecules was calculated before ($S0$) and after ($S1$) the move. For this small area change dE_p was assumed to change proportionally to the area change:

$$dE_p = K_p(S0 - S1) \quad (4)$$

Insertion of additional molecules into monolayer

The effect of lysolipids on membrane fusion (Chernomordik et al., 1993) was modeled by inserting a number of additional lipid molecules into either the contacting or distal monolayer. Features assigned to these additional molecules were the same as those for lipids already in the bilayers. Molecules were inserted initially such that their heads were protruding outside the monolayer by ~1/4 of molecule length. During the simulation these molecules spontaneously moved into a position of energy minimum, between original molecules.

Fusion with tension in only one of the two contacting monolayers

A minor modification of the original model, with the ability to selectively increase the head-head attraction parameter in one monolayer, allowed modeling of bilayer reorganization induced by tension in only one monolayer. Instead of a single value of K_{hh} for both interacting structures, we introduced separate parameters K_{hh1} and K_{hh2} for the first and second structure. Only K_{hh2} was increased in the course of this simulation. Structure 2 was always a liposome, and structure 1 was either a liposome or a planar bilayer.

Protein-mediated fusion

It has been suggested (Pantazatos and MacDonald, 1999; Chanturiya et al., 2000) that certain conformational changes in fusion proteins may lead to protein removal from the outer monolayer of fusing membranes or a reduction in their area of occupation in the outer monolayer. When this happens, voids are generated that must be filled by lipid molecules. This results in an increase in the area per lipid in that monolayer of the membrane, and thus an increase in the membrane lateral tension. This may lead to fusion by a mechanism that is similar to calcium-induced fusion of purely lipidic molecules, but with a sensitivity to a stimulus determined by the nature of the protein. This hypothesis was tested using the simulation model described here. Parameters used for the lipid molecules were the same as the ones used in simulations of purely lipidic bilayers. Protein molecules capable of reducing the area occupied by them in the outer monolayer were represented by structures (referred to hereafter as proteins) having a length equal to that of the lipid and width equal to the width of two lipid molecules. These proteins were actually homologous to lipid dimers, and like regular lipids have three points of interactions (in the headgroup region, tail region, and 1/4 deep from the headgroup region) with neighboring lipid molecules on each side (e.g., 6 points total). Interactions of these points with neighboring lipid molecules were described by the same set of equations as interactions between lipid molecules. Parameters of these "lipid/protein" interactions and of interactions between lipid molecules were unchanged in the course of simulations. Condensation of proteins was induced at some point during simulations by increasing attraction forces between three pairs of points on opposite sides of the protein molecule.

RESULTS

Effect of internal pressure on fusion

While previous experiments successfully demonstrated the applicability of 2D simulation to model hemifusion of bilayers (Chanturiya, 1997), the question about the ability of this model to simulate complete fusion was left open. A number of experimental results on liposome/BLM fusion point out that vesicle internal pressure may be responsible for the conversion of hemifusion into complete fusion (Cohen et al., 1984; Chanturiya et al., 1997). Here we tested this hypothesis using the model modified to permit the introduction of internal pressure as described above. When we introduced vesicle internal pressure into the model, breaking of the outer monolayer occurred, followed by breaking of the inner monolayer in the same region (Fig. 2 *B*). This effect was observed in a relatively narrow range of K_p (2-3). Higher values resulted in breaking of both monolayers in several places, different from the region of contact and/or complete destruction of the bilayer in the region of contact.

Corresponding changes in head-head distances for molecules in different locations and changes in system energy are presented in Fig. 3. For molecules far from the contact region in both monolayers, internal pressure effectively reduces or even reverses reduction in head-head distances (Fig. 3, *A* and *B*), resulting in higher lateral tension in the bilayer compared to simulations without internal pressure (Fig. 2 *A*). In contrast, molecules close to the breakpoint of the membrane undergo transient ups and downs in head-head distances, but eventually condense to approximately the same head-head distances unrelated to the value of the pressure parameter.

Effect of incorporation of additional molecules into membranes

Additional molecules were inserted either into the contacting monolayers or the distal monolayers of two interacting bilayers. These additional molecules were assigned the same parameters as other molecules in the bilayers, and hence no shape-related effects were present. Results from this simulation are shown in Fig. 2 *C*. No signs of monolayer breaking were seen during 4000 cycles of computation, giving a similar effect on fusion as the experimental incorporation of lysolipids (Chernomordik et al., 1995). In a control experiment carried out with the same parameters but without the introduction of extra molecules, both contacting monolayers broke at cycle 1150 and an expanded zone of hemifusion was formed at cycle 3500 (Fig. 2 *A*). Similar results were observed even with a lower fraction of extra molecules than was used in Fig. 2 *C*. Having extra lipids at

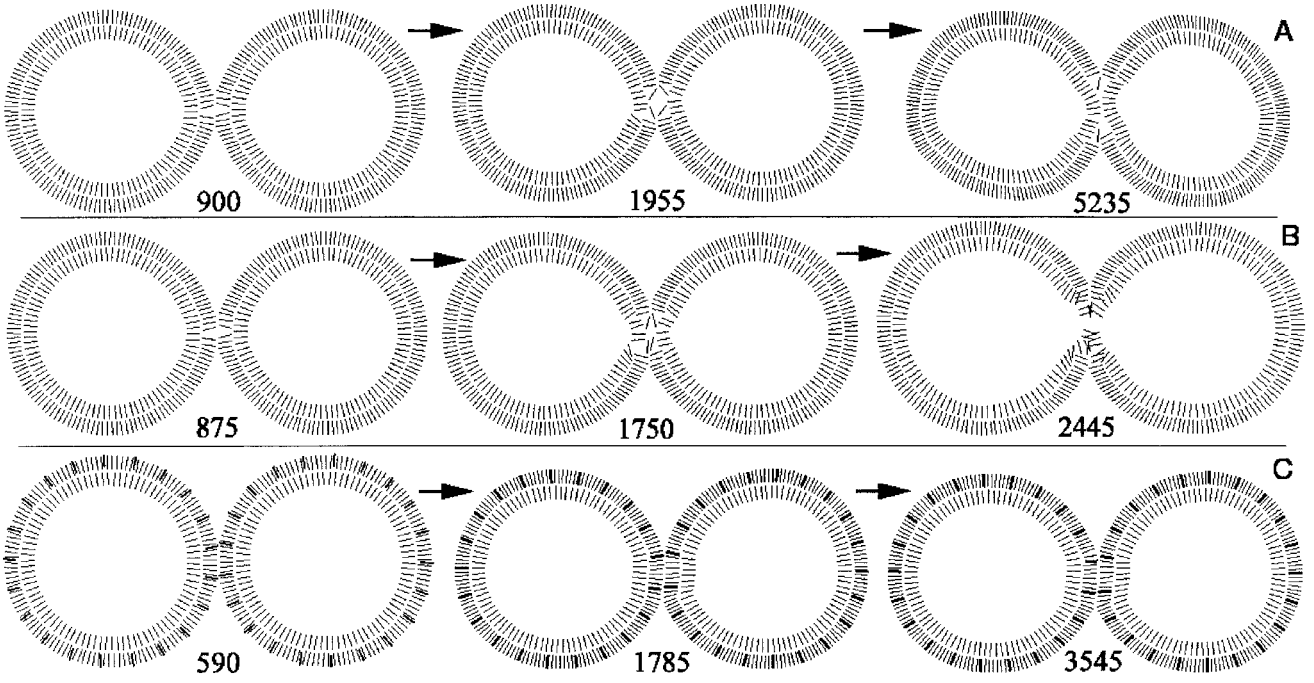

FIGURE 2 "Hemifusion," "fusion," and inhibition of fusion of two-dimensional bilayers. Two circular bilayers containing 216 molecules each were allowed to equilibrate over a period of 550–570 cycles, and then placed in contact with a simultaneous increase of K_{hh} to 0.022 and K_{hhx} to 0.011. (*A*) Results of a simulation carried out with zero internal pressure ($K_p = 0$). A structural defect became visible at cycle number 875 and grew in size, allowing molecules from the distal monolayer to come into contact, leading to hemifusion. (*B*) Results of a simulation where the internal pressure (K_p) was increased from zero to 2.5 at cycle 700. This resulted in the formation of a "trilamellar structure" (cycle 1750), the breakage of distal monolayers, and the formation of a "fusion pore" at cycle 2445. (*C*) Results from a simulation with the same parameters as in *A*, but with the incorporation of additional molecules into the contacting monolayers. Additional molecules (*thick lines*) were inserted into the outer monolayer (cycle 590) and simultaneously head-head attraction parameters were increased to the same values that normally resulted in bilayer reorganization similar to that shown in *A*. Numbers under the structures show the number of cycles at a given moment. The number of computational cycles shown here and further is normalized by dividing the actual number of cycles by the number of molecules in the simulation. All simulations began with a standard set of parameters: $K_{hh} = K_{mm} = K_{tt} = 0.007$, $K_{hm} = 0$, and $W_{hh} = 1.0$ $W_{mm} = 0.2$, $W_{mh} = 0.5$, and $W_{tt} = W_{ttx} = 0.1$.

a ratio of ~1/9 to 1/8 to original lipids resulted in complete inhibition of hemifusion, whereas with extra lipids at a ratio of 1/11 to 1/10 contacting monolayers broke, but molecules on the edges were separated by only three to four times the initial distance at cycle 5500.

Corresponding changes in system energy and intermolecular distance are shown in Fig. 3 *C*. Incorporation of additional molecules caused an initial increase in system energy, led to a reduction of head-head distance, and eventually led to a reduction of both energy (compare Fig. 3, *A* and *C*) and lateral tension in the outer monolayer, inhibiting hemifusion. Insertion of the same number of extra molecules into distal monolayers did not result in any inhibition of bilayer fusion (data not shown).

Insertion of additional molecules into circular bilayers under the conditions used to simulate an internal hydrostatic pressure gave significantly different results. Outer monolayer insertion prevented normal hemifusion and fusion, but did not block the process completely. Breakage of monolayer continuity and connection (while distorted) of the internal contents of vesicles (data not shown) was observed even in the presence of extra molecules.

Fusion induced by increased tension in only one of two contacting bilayers

In these simulations we used the same protocol (with $K_p = 0$) as used previously for both structures, but limited the increase in K_{hh} to only one. As in all other simulations, head-head attraction parameters (K_{hh} and/or W_{hh}) for the molecules in the area of contact were set lower than outside of this area. With K_{hh} and W_{hh} in the contact area set to one-half of the respective values outside the region of contact, it was possible to get breaking of both monolayers. The monolayer with an increased K_{hh} always broke first. The structure of bilayers in the region of contact was different from that observed for bilayers with tension in both monolayers. As a result of breaking in several points within the region of contact, an analog of an "inverted micelle" (Rand, 1981; Siegel, 1993) was formed (Fig. 4 *A*).

When a planar bilayer under tension was placed in contact with liposomes, no breakage of the contacting monolayer was observed unless K_{hh} was increased for the liposome. If the internal pressure of the liposomes is increased along with an increase in K_{hh}, breakage of both bilayers in the area of contact,

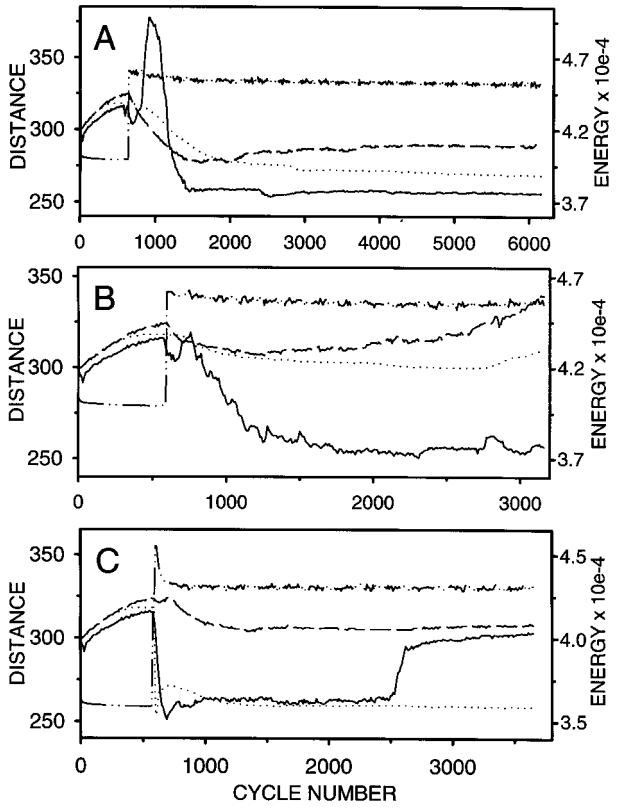

FIGURE 3 Changes in system energy and intermolecular distances during the course of simulations. Experimental conditions and panel labeling (*A*, *B*, and *C*) are the same as in the legend for Fig. 2. Head-head distances were calculated for selected molecules in different positions as described in the Methods section. Changes in intermolecular distance for molecules in the outer monolayer in the area of contact (*solid line*), molecules in the outer monolayer far from the area of contact (*dotted line*), and molecules in the inner monolayer far from the area of contact (*dashed line*) are shown under conditions where hemifusion (*A*), fusion (*B*), and inhibition of fusion (*C*) were observed. For molecules located far from the area of contact, changes in head-head distances were more or less reproducible and similar to the average for the whole pool of molecules. The time course of head-head distance for molecules in the contact area varied significantly, and depended on the molecule's position relative to the breakpoint in the monolayer. The dash-dot-dash line represents changes in the system energy E_S.

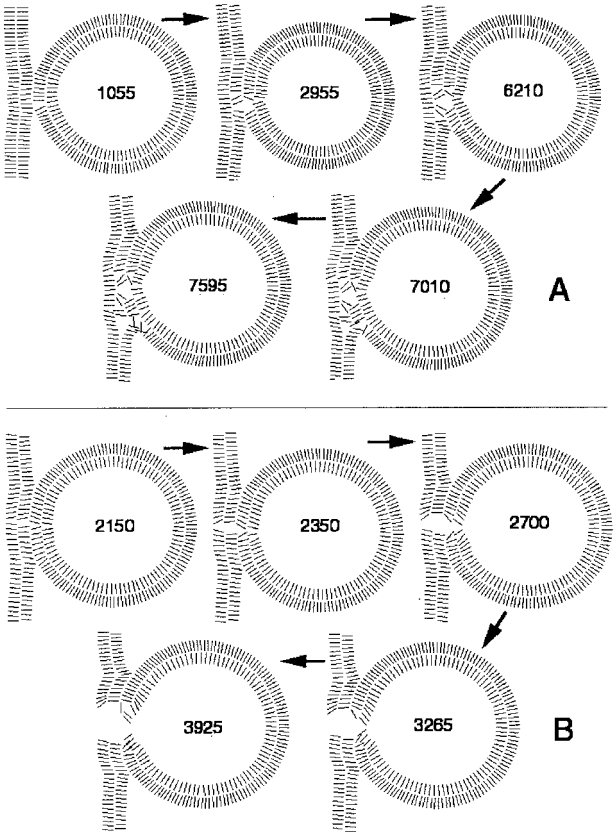

FIGURE 4 "Hemifusion" and "fusion" induced by an increase in tension in one of the two contacting bilayers. In both cases bilayers (planar and circular) were equilibrated over the period of 550 cycles and then placed in contact. At this point head-head attraction was increased for the "liposome" only, to the same value as in previously described simulations. The linear bilayer (only part is shown) and liposome contain 120 and 216 molecules, respectively. (*A*) Linear bilayer without tension. Compared to a similar experiment with tension in both structures, it took significantly more time to get similar size structural defects in the area of contact. Note the formation of the inverted micelle in the area of contact after the internal pressure is increased (at cycle 6215). (*B*) A planar membrane in this case was formed with tension (fractional displacement 1.1) and forces in the liposome included internal pressure ($K_p = 2$).

i.e. fusion, was observed (Fig. 4 *B*). Similar simulation results were also obtained for two liposomes when K_{hh} was increased in only one of them (data not shown).

The model is not sufficiently sophisticated to correctly reorient the lipids on the edges of pores upon breakage of a monolayer or to provide for an accurate simulation of the pore size. It loses connection with physical realities when the lipid molecules are separated by distances that exceed L_{max}.

Protein-mediated fusion

Linear bilayers with "protein" molecules inserted in only one of the monolayers were found to reduce their overall length and bend in response to "condensation" of the protein molecules. However, when we placed two bilayers, one containing proteins, either as linear with circular or both circular in contact, and attempted to induce fusion by reducing the area occupied by protein, no fusion-like structural changes could be observed. Similar results were obtained on varying different model parameters, even when protein molecules were present in both structures. Condensed protein molecules become separated from adjacent lipid molecules and the protein-containing bilayer eventually disintegrated into a number of separate lipidic fragments and independent protein molecules (data not shown).

To achieve fusion induced by a conformational change of a protein it was necessary to introduce some modifications to the model. First, we increased the energy cost of mono-

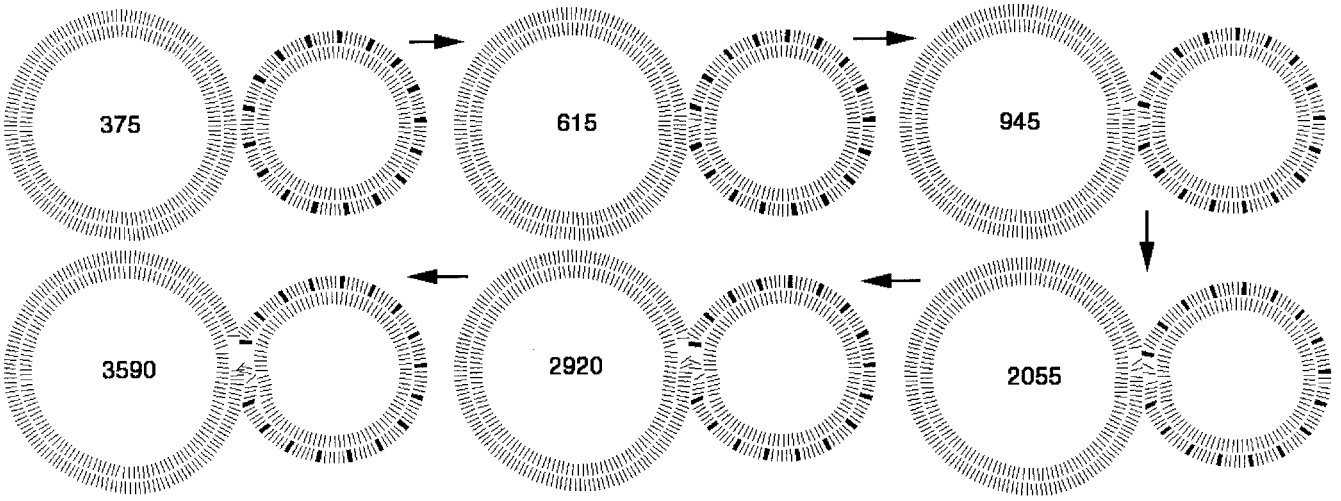

FIGURE 5 "Hemifusion" induced by condensation of "protein" molecules incorporated into the outer monolayer of circular bilayer. Bilayers were equilibrated over a period of 375 cycles and then placed in contact. Lateral dimensions of protein molecules were gradually reduced by ~30% over the period of 1000 cycles starting at cycle 500. Forces between lipid molecules were unchanged. The small vesicle consists of 178 lipid and 17 protein molecules (thick lines) and the bigger vesicle has 275 lipid molecules.

layer stretching by replacing the energy functions described by Eq. 1 with others that provide a sharper increase in the system energy with changes in distance.

$$E_a = -K/(L - d); \qquad E_r = d/(L - d)^2 \tag{5}$$

Second, to give lipid molecules more time to follow new lipid/protein boundaries, we increased the time during which the protein lateral dimension was reduced to new values. To achieve this, we substituted the instant increase of attraction forces within protein molecules, with a linear increase in these forces (determined by values of K_{xx} for protein) over the period of 300+ cycles. These two modifications resulted in a breakage of monolayers in the area of contact in response to "protein condensation" (Fig. 5). Corresponding changes in the protein area and distances between lipid molecules are shown in Fig. 6. The first transient increase in head-head distance between lipid molecules close to the monolayer breakpoint correlates with the beginning of the decrease in the size of protein molecules. The following decrease begins when the monolayer breaks and molecules close to the edge are getting freedom to condense. The reason for the second transient distance increase is less clear and is possibly related to the movement of the molecules out of the area of contact. In contrast to situations shown in Fig. 3, *A* and *B*, there was no reduction in head-head distances between molecules far from the contact area in this case. An opposite effect, an increase in head-head distances between lipid molecules in the outer monolayer in response to protein condensation, generates forces that pull lipids apart in the area of membrane contact.

DISCUSSION

Experiments with a simplified 2D model of a phospholipid bilayer presented here demonstrate the viability of this approach for studying the basic principles of the early stages of membrane fusion. We have demonstrated that two major experimental observations in fusion of protein-free membranes, hemifusion in the absence of internal pressure

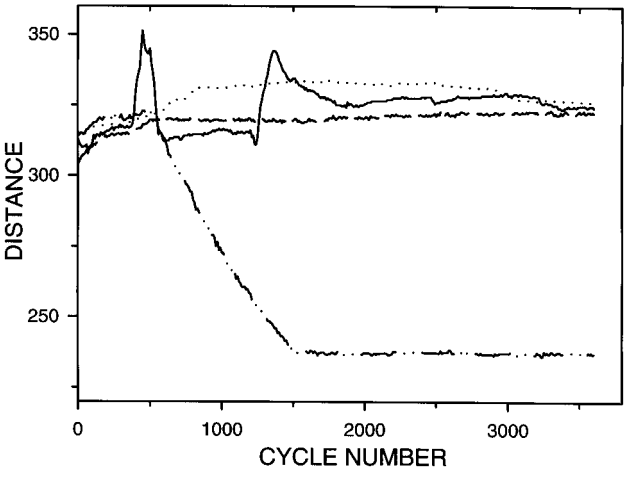

FIGURE 6 Changes in protein size and interlipid distances during the course of simulations. Experimental conditions were the same as described for Fig. 5. *Dash-dot-dash line*, lateral dimension of a protein molecule; *dashed line*, head-head distances between lipid molecules in the inner monolayer far from the area of contact; *solid line*, head-head distances between lipid molecules in the outer monolayer in the area of contact; *dotted line*, head-head distances between lipid molecules in the outer monolayer far from the area of contact.

(Chanturiya et al., 1997) or complete fusion in the presence of internal pressure (Cohen et al., 1984), can be reproduced by this model.

We have also demonstrated that inhibition of fusion by lysolipids (which implies the insertion of extra molecules into the contacting monolayers) could be reproduced, but as a result of a mechanism completely insensitive to the shape of lysolipid molecules (Chernomordik et al., 1993). The universal inhibition of fusion in various systems by lysolipids is a well-known phenomenon. It is assumed that this effect is due to the specific molecular shape of lysolipid molecules. They have a relatively large polar headgroup area compared with the smaller hydrophobic area due to their having only one hydrophobic tail. The effect of this shape is to increase a preference for curvature toward a micelle, and for this reason the presence of lysolipids in a bilayer should increase the energy cost of the initial highly curved fusion intermediate, "stalk" (Kozlov and Markin, 1983) formation. However, our data indicate that lysolipids may inhibit fusion simply by insertion into the contacting monolayers, relaxing the tension required for fusion. Interestingly, the fraction of extra molecules required to inhibit hemifusion in simulation experiments (9–12%) is quite close to the fraction of lysolipids required to inhibit fusion in real membranes (13%, Chernomordik et al., 1995). These three results support the idea that tension increases in contacting phospholipid membranes is the primary reason for membrane breakage that leads to fusion.

The model also allowed us to investigate the possibility of tension-driven fusion in situations not as well-studied experimentally. It demonstrated the possibility of inducing breakage in both contacting monolayers even when an increase in lateral tension occurs only in one. Earlier it was shown that for vesicle/vesicle fusion, no aqueous content mixing or complete fusion was detected unless both membranes were perturbed in a way that increased tension (Lee and Lentz, 1997). However, when we performed experimental measurements on calcium-induced membrane mixing of vesicles, we observed fusion events similar to the results predicted by this computer model (Fig. 7). Our experimental design was based on the fact that at low concentrations (a few millimolar), calcium ions strongly interact with negatively charged phospholipid headgroups, but not with neutral phospholipids. If one of two contacting membranes bathed in a calcium-containing solution is charged and the other is not, then tension should develop in the first membrane only. With one of two membranes labeled with fluorescent lipid dye, fusion can be monitored by the established technique of fluorescent dye dequenching or by visual observation of dye redistribution.

The ability to induce fusion of two membranes by modification of only one of them is interesting for two reasons. First, it is important in the design of optimal systems for targeted drug delivery where the target, the cell membrane, obviously cannot be modified to fit optimal fusion requirements. Second, since some data suggest that even in many cellular systems all the necessary fusion machinery is located in only one membrane (Vogel et al., 1992; Chanturiya et al., 1999), an understanding of how this can occur is important to achieve an understanding of biological membrane fusion mechanisms.

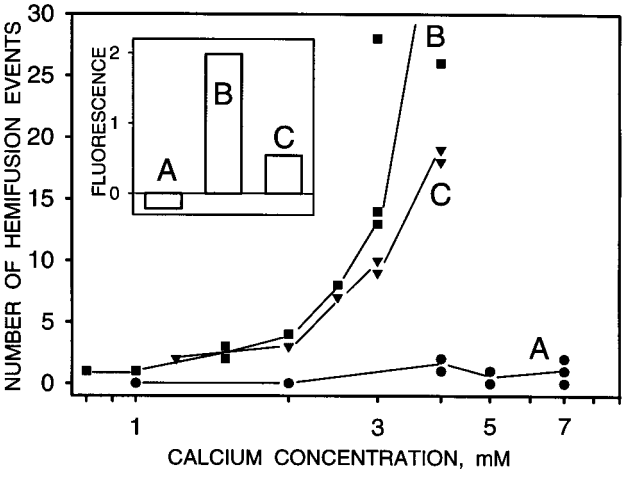

FIGURE 7 Calcium-induced fusion of charged and noncharged phospholipid membranes. *Main panel*: fusion of liposomes to planar phospholipid membrane (BLM). *Circles*, neutral liposomes to neutral BLM; *squares*, charged liposomes to charged BLM; *triangles*, charged liposomes to neutral BLM (from Chanturiya et al., 2000). *Inset*: fusion of liposomes in suspension induced by the addition of 5 mM calcium (*A*) between neutral liposomes; (*B*) between charged liposomes; (*C*) between charged and neutral liposomes (original data: liposomes were made by extrusion through 100 nm polycarbonate filters in 0.1 M KCl. Charged liposomes were prepared from DOPS and 7.5 wt % rhodamine PE, and neutral liposomes were prepared from DOPC).

Our experiments with modeling tension-driven, protein-mediated fusion demonstrated that this mechanism is, in principle, possible. Lateral condensation of a limited number of specific molecules in the outer monolayer was sufficient to break both contacting monolayers (Fig. 5), which is a necessary precondition for membrane fusion to occur. However, it was also found that to get to this point, our model needed to be significantly more fine-tuned than in a seemingly similar case when tension was increased purely with packing changes in the lipidic monolayer, as occurs with calcium ion binding. While an exact analysis of differences in bilayer reorganization for lipid- and protein-induced tension is not possible, an approximate analysis of these two situations might be helpful. For purely lipidic bilayers we modeled the induction of fusion very similar to that by the binding of divalent ions to a negatively charged membrane. The increase in head-head attraction force (K_{hh}) between lipid molecules outside of the area of contact is greater than that between the molecules within the area of contact. This leads to a differential change in the head-head distance for molecules in these two areas. Such differential changes in attraction forces are justified by the assumption

that ionic charges in the area of membrane contact are distributed between two membranes, and thus have lesser effect on counter charges in the membrane compared to ions located outside of this area of contact. This assumption is yet to be tested in real systems, but modeling experiments have not resulted in fusion when forces in the contact region, determined by the K_{hh} value, were the same as forces outside the region. This means that the increase in system energy by itself is not sufficient to cause breakage of a bilayer. Only when a gradient of energy is present can lipid molecules flow out of the contact region and initiate fusion (Chanturiya, 1997; Safran et al., 2001). With increased separation, attraction forces finally become insufficient to hold molecules together and monolayers break. There is a difference between this situation and the situation when tension is induced by condensation of a limited number of molecules located outside of the area of membrane contact. In the latter case we do not have an increase in K_{hh} for lipid molecules next to the area of contact. These molecules are not active participants in tension development, but rather mediators of energy delivery to this area. As shown in Fig. 6, protein condensation increases the separation between lipid molecules in contrast to the decrease in interlipid distance when K_{hh} is increased (Fig. 3). It still produces forces that attempt to pull apart molecules in the area of contact, but these forces are weaker than the forces that result from the increase of head-head attraction energy. They were not sufficient to break monolayers when a simple linear dependence of attraction energy versus distance was used in the original model. Only when we replaced it with Eq. 5, producing a nonlinear dependence of energy on distance, does it become possible to overcome the sum of reaction forces and break the monolayer in the area of contact.

Taken together with results of model simulations, the analysis indicates that while tension-driven fusion may be induced by protein condensation, this mechanism is not as effective as one that involves condensation of the whole lipid monolayer. It seems likely that tension development due to protein condensation may work in tandem with other protein mediated effect(s) in fusion, such as creation of a hydrophobic defect in the area of contact (Bentz, 2000) or destabilization of the target membrane by a fusion peptide.

It is important to remember that the behavior of a 3D system should certainly be different from the 2D model used here. It is possible that tension-driven fusion can be completed in a 3D system without additional assumptions. There are two major differences between 2D and 3D model bilayers that can make formation of the initial 3D pore easier. First, in a 3D model system more than two molecules will participate in the delivery of energy to the break point in the monolayer. Second, molecules in a 3D system have more freedom to move in the monolayer, and this may also change the results significantly. A 3D model based on similar principles as described above is currently under development. Because both the number of molecules and the number of interactions calculated would be higher in a 3D system, we expect about a two-order increase in computational time will be required. However, even 200–500 h of desktop computer time per experiment is still acceptable, and may be reduced with the use of more powerful machines.

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REFERENCES

Bentz, J. 2000. Membrane fusion mediated by coiled coils: a hypothesis. *Biophys. J.* 78:886–900.

Bonnafous, P., and T. Stegmann. 2000. Membrane perturbation and fusion pore formation in influenza hemagglutinin-mediated membrane fusion. A new model for fusion. *J. Biol. Chem.* 275:6160–6166.

Chanturiya, A. N. 1997. Fast two dimensional computer simulation of bilayer hemifusion. *J. Biomol. Struct. Dyn.* 15:547–553.

Chanturiya, A. N., L. V. Chernomordik, and J. Zimmerberg. 1997. Flickering fusion pores comparable to initial exocytotic pores occur in protein-free phospholipid bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 94: 14423–14428.

Chanturiya, A., P. Scaria, and M.C. Woodle. 2000. The role of membrane lateral tension in calcium-induced membrane fusion. *J. Membr. Biol.* 176:67–75.

Chanturiya, A. N., M. Whitaker, and J. Zimmerberg. 1999. Calcium-induced fusion of sea urchin egg secretory vesicles with planar phospholipid bilayer membranes. *Mol. Membr. Biol.* 16:89–94.

Chernomordik, L., A. Chanturiya, J. Green, and J. Zimmerberg. 1995. The hemifusion intermediate and its conversion into complete fusion: regulation by membrane composition. *Biophys. J.* 69:922–929.

Chernomordik, L. V., S. S. Vogel, A. A. Sokoloff, H. O. Onaran, E. A. Leikina, and J. Zimmerberg. 1993. Lysolipids reversibly inhibit (Ca^{2+}), GTP- and pH-dependent fusion of biological membranes. *FEBS Lett.* 318:71–76.

Cohen, F. S., M. H. Akabas, J. Zimmerberg, and A. Finkelstein. 1984. Parameters affecting the fusion of unilamellar phospholipid vesicles with planar bilayer membranes. *J. Cell Biol.* 98:1054–1062.

Garcia, R. A., S. P. Pantazatos, D. P. Pantazatos, and R. C. MacDonald. 2001. Cholesterol stabilizes hemifused phospholipid bilayer vesicles. *Biochim. Biophys. Acta.* 511:264– 270.

Gavrisch, K., and L. L. Holte. 1996. NMR investigation of non-lamellar phase promoters in the lamellar phase state. *Chem. Phys. Lipids.* 1:105–116.

Kozlov, M. M., and L. V. Chernomordik. 1998. A mechanism of protein-mediated fusion: coupling between refolding of the influenza hemagglutinin and lipid rearrangements. *Biophys. J.* 75:1384–1396.

Kozlov, M. M., and V. S. Markin. 1983. Possible mechanism of membrane fusion. *Biofizika.* 28:255–261.

Lee, J., and B. R. Lentz. 1997. Outer leaflet-packing defects promote poly(ethylene glycol)-mediated fusion of large unilamellar vesicles. *Biochemistry*. 36:421–431.

Lentz, B. R., and J. K. Lee. 2000. Poly(ethylene glycol) (PEG)-mediated fusion between pure lipid bilayers: a mechanism in common with viral fusion and secretory vesicle release? *Mol. Membr. Biol.* 16:279–296.

MacDonald, R. C. 1988. Mechanism of membrane fusion in acidic lipid-cation system. In Molecular Mechanisms of Membrane Fusion. S. Okhi, editor. Plenum Press, New York.

Marsh, D. 1996. Lateral pressure in biomembranes. *Biochim. Biophys. Acta.* 1286: 186–223.

Pantazatos, D. P., and R. C. MacDonald. 1999. Directly observed membrane fusion between oppositely charged phospholipid bilayers. *J. Membr. Biol.* 170:27–38.

Pastor, R. W. 1994. Molecular dynamic and Monte Carlo simulations of lipid bilayers. *Curr. Opin. Struct. Biol.* 4:486–492.

Rand, R. P. 1981. Interacting phospholipid bilayers: measured forces and induced structural changes. *Annu. Rev. Biophys. Bioeng.* 10:277–314.

Ruysschaert, J., and R. M. Epand. 1999. Role of the N-terminal peptides of viral envelope proteins in membrane fusion. *Adv. Drug. Deliv. Rev.* 38:233–255.

Safran, S. A., T. L. Kuhl, and J. N. Israelachvili. 2001. Polymer-induced membrane contraction, phase separation, and fusion via Marangoni flow. *Biophys. J.* 81:659–666.

Siegel, D. P. 1993. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* 65:2124–2140.

Siegel, D. P. 1999. The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion. *Biophys. J.* 76:291–313.

Vogel, S., L. Chernomordik, and J. Zimmerberg. 1992. Calcium triggered fusion of exocytotic granules requires proteins in only one membrane. *J. Biol. Chem.* 267:25640–25643.

Zimmerberg, J., and L. V. Chernomordik. 1999. Membrane fusion. *Adv. Drug. Deliv. Rev.* 38:197–205.