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The Role of Membrane Lateral Tension in Calcium-Induced Membrane Fusion

A. Chanturiya, P. Scaria, M.C. Woodle
Genetic Therapy Inc., Synthetic Vector Group, 9 West Watkins Mill Rd., Gaithersburg, MD 20878, USA

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Abstract. Calcium-induced fusion of liposomes was studied with a view to understand the role of membrane tension in this process. Lipid mixing due to fusion was monitored by following fluorescence of rhodamine-phosphatidyl-ethanolamine incorporated into liposomal membrane at a self-quenching concentration. The extent of lipid mixing was found to depend on the rate of calcium addition: at slow rates it was significantly lower than when calcium was injected instantly. The vesicle inner volume was then made accessible to external calcium by adding calcium ionophore A23187. No effect on fusion was observed at high rates of calcium addition while at slow rates lipid mixing was eliminated. Fusion of labeled vesicles with a planar phospholipid membrane (BLM) was studied using fluorescence microscopy. Above a threshold concentration specific for each ion, Ca^{2+}, Mg^{2+}, Cd^{2+} and La^{3+} induce fusion of both charged and neutral membranes. The threshold calcium concentration required for fusion was found to be dependent on the vesicle charge, but not on the BLM charge. Pretreatment of vesicles with ionophore and calcium inhibited vesicle fusion with BLM. This effect was reversible: chelation of calcium prior to the application of vesicle to BLM completely restored their ability to fuse. These results support the hypothesis that tension in the outer monolayer of lipid vesicle is a primary reason for membrane destabilization promoting membrane fusion. How this may be a common mechanism for both purely lipidic and protein-mediated membrane fusion is discussed.

Key words: Membrane — Lipid — Calcium — Tension — Fusion — Fluorescence — Microscopy

Introduction

Fusion of lipid membranes occurs in a variety of important biological processes such as nerve signal transmission, fertilization, secretion, protein trafficking, and viral infection. An understanding of fusion mechanism has potential value for progress in treatment of medical anomalies involving these processes as well as development of new methods of targeted drug delivery including gene delivery. Despite identification and characterization of a number of proteins participating in fusion (Bullough et al., 1994; Wessenhorn et al., 1997; Weber et al., 1998; Christoforidis et al., 1999), it is still not clear how the profound rearrangement of lipid molecules occurs during membrane fusion and moreover it is not clear what precise roles the proteins play. Various models of protein-mediated membrane fusion range from the purely proteinacous initial connection between membranes to mixed lipid-protein structures (Monk & Fernandes, 1992; White, 1993). Recently, similar models were suggested for both viral (Wessenhorn et al., 1997) and exocytotic membrane fusion (Weber et al., 1998). According to these models, several identical proteins assemble at the circumference of the future fusion site and create a membrane configuration favorable for fusion of the two lipid bilayers. However, it is not known if the proteins, while clearly important in fusion, directly participate to rearrange the lipids or function at an earlier stage of the complex process (Mayer, Wickner & Haas, 1996). Model membranes (liposomes and planar bilayers) devoid of protein are able to fuse in response to same stimulus, calcium ions or pH, that induce protein mediated fusion, though with less sensitivity (Papahadjopoulos et al., 1977; Portis et al., 1979; Duzgunes et al., 1985).

It seems likely that biological membrane fusion shares a common physicochemical mechanism active with pure lipid membranes but fine-tuned and regulated by the incorporated proteins. This hypothesis predits that the fundamental mechanism is identical whether protein is present or not but is modulated by the proteins when present. However, testing this hypothesis requires a good understanding of the fusion in the absence of proteins.

Correspondence to: A. Chanturiya

Three major hypotheses exist for the best-studied case of pure lipid membrane fusion, calcium-induced fusion of charged lipid membranes. According to an early hypothesis, fusion is the result of a calcium induced phase separation of different lipids in the membrane (Papahadjopoulos et al,. 1977). A later hypothesis proposed cross-linking of the two membranes by calcium ion binding (Portis et al., 1979) as the cause of fusion. A third hypothesis suggests that lateral tension created by asymmetrical calcium binding to the lipid head groups leads to fusion (Ohki & Duax, 1986; MacDonald, 1988; Helm, Israelishvili & McGuiggan, 1989). This last hypothesis is especially interesting since it is relatively easy to imagine how this mechanism could be enhanced or regulated by incorporation of specific fusion proteins over the course of evolution.

In this study experiments were performed to discriminate between the tension-driven and the other two fusion mechanisms for the calcium-induced fusion of lipid membranes. The experiments were designed with the following considerations. According to the first two hypotheses, fusion depends primarily on specific calcium-bound states and thus the final calcium concentration. The tension-driven fusion should also depend on the rate of calcium concentration increase because slow addition of calcium should allow stress dissipation. Additionally, the lateral tension mechanism is unique in predicting a requirement for asymmetrical calcium binding to the outer lipid monolayer and a dependence on vesicle but not BLM surface charge. Studies performed to distinguish between these hypotheses are reported here. The results obtained support the lateral tension mechanism for calcium-induced membrane fusion but not the other two mechanisms.

Materials and Methods

Vesicle Preparation

For vesicle-vesicle fusion experiments, extruded vesicles were prepared using the standard techniques. A chloroform solution with 5 mg of lipid was dried under vacuum, hydrated by adding 0.5 ml of Buffer I (100 mM KCl, 10 mM TES, pH-7.4), and extruded 15 times through a 100 nm polycarbonate filter. Two types of vesicles were prepared; one with 50/50-wt% of 1,2 Dioleoyl-sn-Glycero-3-Phospho-L-Serine (DOPS) and 1,2 Dioleoyl-sn-Glycero-3-Phospho-Ethanolamine (DOPE), and another with 50% DOPS, 42.5% DOPE and 7.5% Rhodamine Phosphatidylethanolamine (RhPE). The same technique was used to prepare 50/50-wt% DOPE/DOPS vesicles containing 200 mM calcein in the internal aqueous phase used in the leakage experiments. For vesicle-BLM fusion experiments, vesicles were prepared with 20-wt% RhPE with either egg phosphatidylcholine (PC) or phsophatidylserine (PS) or with 55-wt% PC and 25-wt% egg phosphatidylethanolamine (PE) by a sonication-freeze-thaw technique (Cohen et al., 1984). A chloroform solution with 5 mg of lipid was dried under vacuum, hydrated by adding 0.5 ml of Buffer II (100 mM KCl, 10 mM MES, 1 mM EDTA, pH-6.5), sonicated, and freeze/thawed twice in a glass tube.

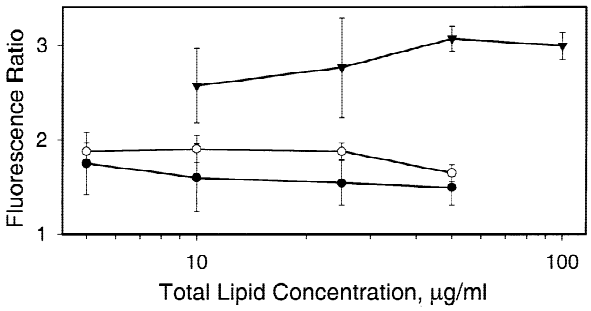

Fig. 1. Dependence of vesicle fluorescence on RhPE concentration in membrane. Each point represents ratio of fluorescent intensities for two different RhPE concentrations in vesicle membrane. ● - 1.88 *vs.* 3.75%; ○ - 3.75 *vs.* 7.5%; ▼ - 7.5 *vs.* 15%. X axis shows total amount of lipids in vesicle suspension.

30 μl portions of this suspension were frozen again and kept at −20°C. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Vesicle-Vesicle Fusion Assay

Membrane fusion was monitored by measuring fluorescence of suspension containing vesicles with self-quenched RhPE and vesicles lacking RhPE. While this technique alone does not allow discrimination between hemifusion, mixing of lipids just in the outer monolayers, and complete fusion, mixing of both monolayers and aqueous contents (Chanturiya, Chernomordik & Zimmerberg, 1997), it was sufficient for our goal to measure either phenomenon. For simplicity we will use the word "fusion" throughout the paper to specify either complete or hemifusion. For the assay, 15 μl of DOPS/DOPE and 15 μl of DOPS/DOPE/RhPE vesicle suspension were mixed with 1.17 ml of Buffer I in 1 cm spectrofluorometer cuvette and the fluorescence was measured with 540 nm excitation and 590 nm emission wavelengths. Fusion was triggered by injection of 40 μl of 120 mM $CaCl_2$ solution into the cuvette through a thin polyethylene tube from a 50 μl Hamilton syringe either as rapidly as possible, referred to here as instantly, or at a given rate using a syringe pump. In some experiments vesicles were made permeable to calcium by addition of the calcium ionophore A23187 from 2.5 mM stock solution in dimethylsulfoxide (DMSO) to give a final concentration of 31 μM. Although DMSO did not show any effect on fusion, the same volume (15 μl) of DMSO was added to vesicles in control experiments.

The relationship between the degree of RhPE dequenching and extent of fusion was estimated using the following assumptions. Upon fusion, RhPE in one vesicle can mix with the lipid of the other vesicle. If the fusion is between a labeled vesicle and an unlabeled one, this results in RhPE dilution and fluorescence dequenching. If these vesicles are of similar size the maximum dilution that the dye undergoes is twofold. Dilution induced dequenching was modeled by fluorescence measurements on vesicle preparations containing some total amount but different RhPE wt-%, 15.0, 7.5, 3.75 and 1.88. The ratio of fluorescence for the twofold different samples is shown in Fig. 1. This difference in fluorescence should correspond to the fluorescence increase resulting from a complete fusion event. Note that the fluorescence ratio is only weakly dependent on the total lipid concentration in suspension, indicating only minor interference from light scattering. For all subsequent experiments, vesicle containing 7.5 wt% RhPE were

used for which a twofold increase in fluorescence corresponds to complete lipid mixing.

VESICLE-BLM FUSION

BLMs were formed across a 120 μm hole in Teflon partition by the Montal-Mueller (Montal & Mueller, 1972) technique. The chamber was milled from Teflon and placed between the objective and condenser lenses of a custom made video fluorescence microscope, similar to the one described previously (Chanturiya et al., 1997). Membranes were formed in Buffer II from 1% solution (wt/vol) of diphyantoyl phosphatidylcholine (DPhC), PS or PS/PE in hexane. A fresh vial of frozen vesicles was used each day. A glass pipette filled with a diluted vesicle suspensoin, fitted on a hydraulic micromanipulator, was used to inject the vesicle suspension towards the BLM.

Results

VESICLE-VESICLE FUSION

Dependence on Calcium Addition Rate

Dependence of fusion on the rate of calcium addition was studied by injection of calcium chloride solution into the vesicle suspension while monitoring the fluorescence in real time. Figure 2A shows the fluorescence intensity changes at various rates of calcium addition. The maximum fluorescence increase was observed when calcium was added instantly giving a twofold increase corresponding to complete lipid mixing. Decreasing the rate of calcium addition reduced fluorescence maximum, indicating reduced lipid mixing. To make sure that differences in fluorescence were not caused by RhPE photobleaching or by errors in sample preparation, vesicles were lysed by detergent Triton X-100 (TX-100) at the end of each experiment. In all experiments fluorescence intensities after detergent addition ("indefinite" dilution and complete dequenching of RhPE) were similar.

Another possibility, that the observed reduction in maximum fluorescence is the result of calcium-induced formation of large vesicle aggregates that do not stay in suspension, was tested by measuring the total amount of RhPE in suspension over the course of calcium addition. Vesicles were mixed in the usual proportion with Buffer I in a 6 well tissue culture plate and placed atop of magnetic stirrer. Each well contained 12 ml of suspension and a stirring bar. Calcium solution was injected into all wells at 25 μM/min. 40 μl samples were taken from the central part of each well at 20 to 30 min intervals. Fluorescence intensity of these samples was measured after mixing with 0.4 ml of Buffer I and 30 μl of 2% TX-100. Loss of fluorescence attributable to a decrease in the amount of lipid in suspension was observed only near the end of calcium injection indicating that this effect is unlikely to interfere significantly with fusion that occurs earlier (Fig. 2*A*, filled).

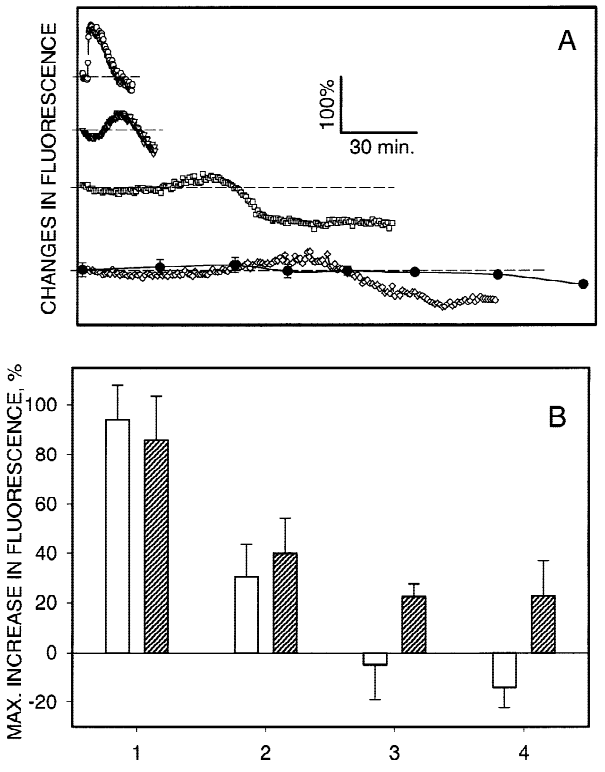

Fig. 2. Effect of the rate of calcium concentration increase and vesicles permeability to calcium on vesicle-vesicle fusion. (*A*) Time course of RhPE fluorescence at different rates of calcium addition. In all experiments 4 mM $CaCl_2$ was added either instantly (○); or at rates of 250 μM/min (▽); 50 μM/min (□), and 25 μM/min (◇). At the end of each experiment addition of 30 μl of 2% TX-100 caused sharp increase of fluorescence to the level proportional to the total amount of RhPE in the cell (*not shown*). Results from independent control experiment for vesicle precipitation/aggregation are marked by filled circles (mean ± SE for 5–6 experiments). Data are presented as a ratio of current intensity of fluorescence to the fluorescence in the beginning of experiment. (*B*) Dependence of maximum RhPE fluorescence on vesicles permeability to calcium at different rates of calcium addition. 1 –instant addition of 4 mM $CaCl_2$, 2–4-addition of same amount of $CaCl_2$ at rates of 250, 50, and 25 μM/min. Open bars - for A23187 permeabilized vesicles, filled bars - control vesicles (mean ± SE for 3 experiments).

Calcium Asymmetry

Dependence of the fusion on asymmetry of calcium binding was studied using vesicles made permeable to calcium by the addition of calcium ionophore A23187 to the vesicle suspension. In the absence of ionophore, calcium can bind only to the outer monolayer lipid head groups, at least initially, with inner head group exposure only possible later due to vesicle leakage. Addition of the ionophore allows exposure of the inner monolayer head groups to calcium, which leads to symmetrical monolayer condensation, as long as the rate of calcium addition is slower than the ion transport rate. As the rate

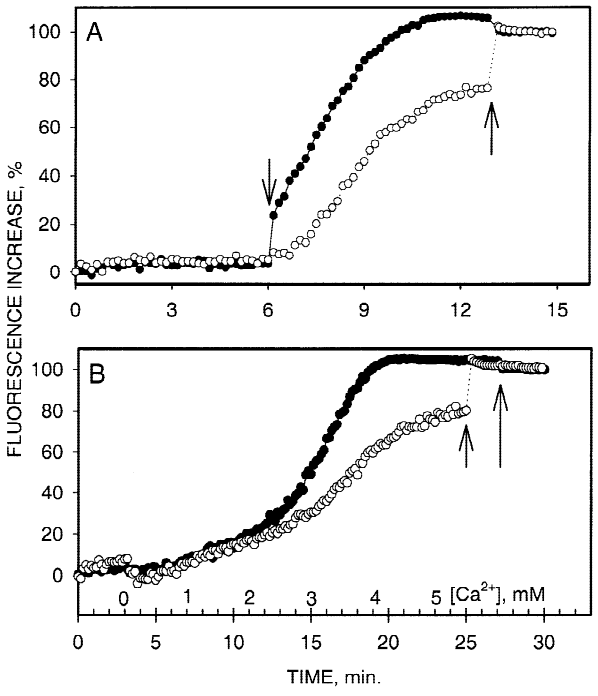

Fig. 3. Effect of vesicle permeability to calcium on calcium induced leakage of vesicles. (*A*) Instant addition of 4 mM $CaCl_2$ at the moment indicated by a descending arrow. (*B*) Gradual increase of $CaCl_2$ concentration from 0 to 5 mM at the rate 0.25 mM/min. Same symbols for both panels: ● - Control, ○ - in presence of 30 μM A23187. Ascending arrows indicates addition of TX-100.

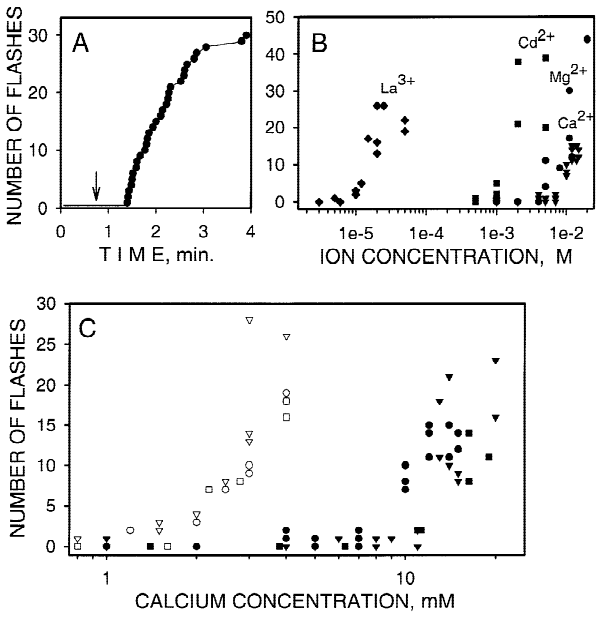

Fig. 4. Fusion of vesicles to the BLM. (*A*) Calcium-triggered fusion of PC/PE/RhPE vesicles applied to DPhC BLM prior to calcium addition. Each point represents an individual fusion event detected as a flashlike (due to dequenching) spread of fluorescent lipid from the vesicle to the BLM. An arrow indicates the moment of 20 mM $CaCl_2$ addition. (*B*) Concentration dependencies of vesicle/BLM fusion for different cations. Vesicles were applied to BLM bathed in the Buffer II supplemented with different di- or tri- valent cations. ▼- calcium, ● - magnesium, ■ - cadmium, ◆ - lanthanum. (*C*) Effect of membrane charge on fusion of: PS vesicles to DPhC BLM (○), PS vesicles to PS BLM (▽), PS vesicles to 80%PS/20%PE BLM (□), PC vesicles to DPhC BLM (●), PC vesicles to PS BLM (▼), PC vesicles to 80%PS/20%PE BLM (■). In panels *B* and *C* each point represents the total number of fusion events registered within 2.5 min after vesicle injection.

of calcium addition exceeds ion transport, asymmetry of head group exposure increases. The percent change in fluorescence is shown in Fig. 2*B* as a function of calcium addition rate (at the same rates as used in Fig. 2*A*). These results show no significant effect of the calcium ionophore on increase in fluorescence at the two fastest rates of calcium addition. In contrast, at the two slowest rates of calcium addition the inclusion of A23187 completely inhibited the fluorescence increase.

The effect of calcium access to the inner monolayer also was studied using vesicle leakage. Aqueous content mixing assays on small vesicles are not suitable for studies of fusion induced by slow addition of calcium because of a rapid leakage (Lentz et al., 1992). However, calcium-induced leakage is an indicator of significant perturbations of the bilayer structure and can be used for testing the effect of tension-modifying factors. The effect of calcium access to the inner monolayer on content leakage was determined from experiments with water-soluble dye, calcein, encapsulated in vesicles. Calcium was added either instantly (Fig. 3*A*) or gradually (Fig. 3*B*), to vesicle suspension with or without ionophore. The presence of the ionophore reduces the rate of fluorescence increase and thus the rate of leakage. To insure that the slower fluorescence increase in presence of ionophore is actually due to lower leakage and not to an effect of A23187 on calcein, vesicles were lysed at the end of the experiment.

VESICLE-BLM FUSION

Di- and Trivalent Ion Triggering

Vesicle fusion with planar bilayer membranes was studied using video microscopy to monitor fluorescence of individual vesicles. In the presence of calcium, vesicles can either hemifuse (in the absence of osmotic gradient) or fuse completely (when osmotically stressed) (Chantiuriya et al., 1997). When vesicles are applied to the BLM in calcium-free solution a number of them become "docked" but remain intact for a long period of time or until calcium addition triggers fusion (Fig. 4*A*) visualized as fluorescence flashes due to dye dequenching. Magnesium and other di- or trivalent cations were also found to be capable of inducing fusion. Their efficiency was compared by measuring the cumulative number of

flashes produced in the first 2.5 min after addition as a function of ion concentration (Fig. 4*B*). Each cation tested shows a characteristic threshold concentration required for fusion. The threshold concentration for the divalent ions is much higher than that of the trivalent ion tested, lanthanum. For the divalent cations, threshold concentration follow the order $Ca^{2+} \approx Mg^{2+} > Cd^{2+}$.

Effect of Vesicle and BLM Charge

The dependence of threshold calcium concentration on vesicle and BLM charge was studied to distinguish a tension-related mechanism from phase separation and ion-induced membrane bridging mechanisms. The cumulative number of flashes produced in a defined time period as a function of calcium concentration was measured with either charged or neutral vesicles and three different compositions of BLM: neutral (DPhC), charged (PS), and bi-component charged (PS/PE). The results, shown in Fig. 4*C*, indicate that the threshold calcium concentration required to induce fusion is dependent only on charge of vesicle and not on the BLM charge or heterogenity. It was about 12 mM for neutral PC vesicles and 3 mM for negatively charged PS vesicles.

Inhibition by Calcium Pretreatment

Fusion of vesicles in suspension can be inhibited by the calcium ionophore permeabilization combined with a slow rate of calcium addition and studies were performed to determine if a similar effect occurs with vesicle-BLM fusion. Since vesicles bound to the BLM can be easily washed off by solution stirring, treatment of bound vesicles with the ionophore was not possible. Instead, dilute vesicle suspensions were treated with the calcium ionophore A23187 at 96 μM concentration in an Eppendorf tube and then applied to the BLM bathed in solution containing 20 mM $CaCl_2$. The permeabilized vesicles fuse to BLM with a significantly lower probability compared to control vesicles. For both control and A23187 permeabilized vesicles number of flashes over a period up to 20 hr remained unchanged (*data not shown*). When permeabilized vesicles were incubated with 20 mM $CaCl_2$ for different periods of time prior to adding to the BLM, the number of flashes decreased considerably after approximately two hours and completely disappeared after about 6 hr of incubation. This inhibition was found to be reversible; treatment with 20 mM EDTA to remove free calcium restored vesicle's ability to undergo calcium-dependent fusion (Fig. 5).

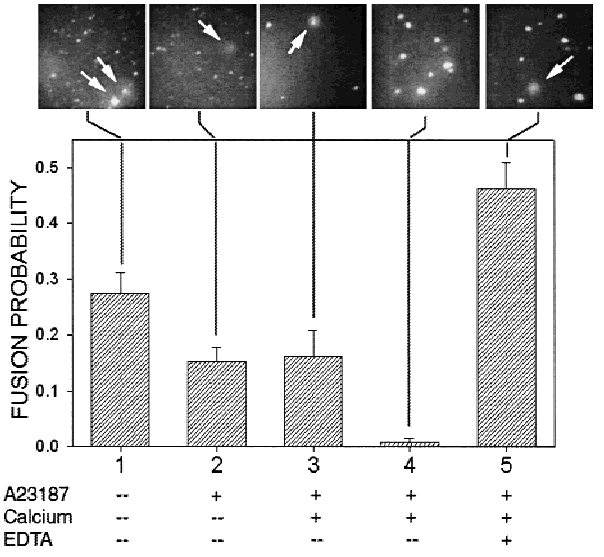

Fig. 5. Reversible inhibition of calcium triggered fusion by calcium pretreatment of vesicles. Images above the bar chart show typical appearance of BLM with bound vesicles at different stages of treatment. Arrows indicate fusing vesicles. Due to significant variation in the number of vesicles bound to BLM in these experiments, fusion probability was used as a measure of fusion. It was defined as a number of flashes divided by the total number of bound vesicles in the beginning of the experiment. 1, control vesicles; 2, calcium permeable vesicles; 3 and 4, 2–3 and 6–12 hr incubation with calcium; 5, 3–4 hours after chelation of calcium.

Discussion

A continuing question concerning biological membrane fusion is whether it shares the same mechanism that is operative in pure lipid membrane fusion. Resolving this question requires a good understanding of the fusion in the absence of proteins, the aim of this report. Several hypotheses exist to explain pure lipid membrane fusion, as it has been observed in model systems such as calcium-induced vesicle fusion. Experiments reported here were designed to distinguish between a dynamic lateral tension driven mechanism and others based on a static calcium binding that induces phase separation or bridge between the two membranes. The results obtained from these studies strongly support the tension-driven mechanism for membrane fusion but not the other two hypotheses, based on the following analysis.

Studies were performed to measure calcium-induced vesicle-vesicle fusion, as a function of calcium addition rate. According to the static hypotheses, fusion depends primarily on specific bound states and thus the final calcium concentration. In contrast, the tension-driven fusion also should depend on the rate of increase of calcium concentration since the membrane tension should dissipate over time. In tension-driven fusion, binding of calcium to the outer monolayer of a vesicle produces the forces that attempt to reduce separation between lipid head groups in the monolayer. However, fast condensation of the outer monolayer is impossible, as it requires a reduction in both the internal volume of the vesicle and in the area of the inner monolayer. Reduced internal

volume requires either compression of the water inside the vesicle or its diffusion through the membrane. This result in a significant lateral tension when calcium concentration increases faster than water diffusion can relieve the stress. Slow calcium addition reduces tension developed in the outer monolayer (*see* Appendix). The water diffusion alone, however, cannot completely relax the tension because of lower lateral compressibility of inner monolayer. When the rate of water diffusion is not limiting, providing the vesicle interior with access to calcium should allow condensation of both monolayers and thus eliminate all lateral tension. As we found, fusion decreases with decreasing rate of calcium addition (Fig. 2*A* and *B*). Furthermore, fusion is diminished (Fig. 2*B*) by inclusion of the calcium ionophore A23187, which also reduces calcium induced membrane destabilization (Fig. 3). In this situation, however, the other two hypotheses would predict fusion to occur.

Results obtained from vesicle-BLM fusion studies demonstrated that liposomes can be "docked" on BLM in the absence of calcium and elevation of calcium above threshold concentration triggers fusion (Fig. 4*A*). The threshold concentration vary for the different cations tested ($Ca^{2+} \approx Mg^{2+} > Cd^{2+} > La^{3+}$; Fig. 4*B*) and correlate with their ability to increase tension in lipid monolayers ($Ca^{2+} \approx Mg^{2+} > La^{3+}$) (Ohki > Duax, 1986) or to induce condensation of the planar membrane ($Cd^{2+} > La^{3+}$) (Chanturiya & Nikolishina, 1994).

A priori, it is clear that charge of membrane will greatly affect the cation concentration required for fusion, irrespective of which mechanism is operative. However, considering the distinctive differences between phospholipid vesicle and planar membrane, we designed an experiment to distinguish tension-related effects from effects of phase separation or formation of transmembrane calcium complexes. For the latter two effects, the threshold concentration should equally depend on the composition of both membranes. In contrast, tension-related effects should depend solely on the vesicle composition for the following reasons. First, the mechanism responsible for calcium induced tension in a closed spherical vesicle, cannot work with the BLM because the BLM is planar and separates two open volumes. Second, even the "solvent-free" BLMs have a significant excess of lipids dissolved in hydrocarbon in the torus area (Chanturiya, 1996) and calcium induced tension can be relaxed when lipid molecules from this reservoir incorporate into bilayer. Taking this into account, one can expect that if the tension mechanism is operative, only the charge of the vesicle membrane will affect the threshold calcium concentration. In contrast, if either phase separation or transmembrane calcium complexes are responsible for fusion, then the charge of both membranes should equally affect fusion. As we found, the threshold calcium concentration required for fusion, depends on charge of the vesicles and not on that of the BLM (Fig. 4*C*). This, again, rules out a significant role of phase separation or transmembrane calcium complex formation in fusion. Finally, the inhibition of vesicle/BLM fusion by incubation of A23187 permeabilized vesicles with calcium, and its reversal by a further treatment with EDTA to remove the calcium, again shows the dependence of fusion on asymmetry of calcium binding. The reasoning described above is that an asymmetrical calcium binding creates lateral tension while a symmetrical binding does not. When permeabilized vesicles were exposed to the external calcium for a long time the conditions for tension dissipation were fulfilled. This again supports the hypothesis that calcium-induced lateral tension is critical for fusion but not the static binding mechanisms.

Is tension in one of two fusing membranes, sufficient for fusion? This question is particularly important for designing optimal systems for targeted drug delivery. It has been shown recently (Shangguan, Alford & Bentz, 1996) that influenza virus-liposome fusion is almost insensitive to the properties of target (liposome) membrane. Our results, fusion of charged vesicles to neutral BLM and reversible inhibition of fusion by calcium treatment of ionophore permeabilized vesicles, indicate that tension in one membrane may be sufficient. On the other hand, it has been shown (Lee & Lentz, 1997) that vesicle aqueous content mixing was observed only when the outer leaflets of both contacting vesicles are perturbed in a way that increase lateral tension. One explanation for this discrepancy is that the results obtained here using lipid mixing do not discriminate between hemifusion and complete fusion and complete fusion may have different requirements. It is worth mentioning that biological fusion systems appear to have the fusion "machinery" locatd either in one (Vogel, Chemomordik & Zimmerberg, 1992) or in both (Weber et al., 1998) fusing membranes.

The time required for stress to dissipate appears to vary with vesicle size. The results with small vesicles (~0.1 μm) used for vesicle-vesicle fusion and large vesicles (~1 μm) used for vesicle-BLM fusion suggest that minutes (Fig. 2) or hours (Fig. 5), respectively, are required for tension dissipation. A potential explanation is that the slow dissipation is a result of low permeability of membranes for water (Finkelstein, 1976), and/or cooperative nature of bilayer reorganization upon calcium binding (Chanturiya, 1997). Since the present work did not discriminate between hemifusion and complete fusion (actually for osmotically balanced large vesicles used in experiments with BLM, most of the dye transfer events should be attributed to hemifusion (Chanturiya et al., 1997)), another point to consider is the relevance of these data to complete fusion. However, recent studies on purely lipidic (Chanturiya et al., 1997), protein con-

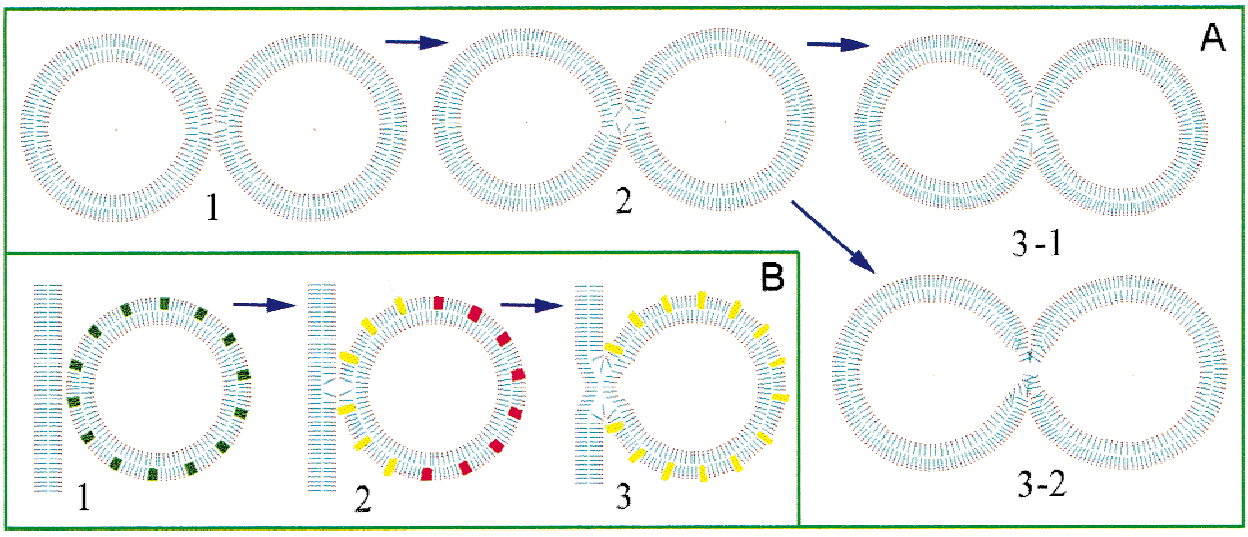

Fig. 6. Fusion of protein-free bilayers and the proposed mechanism for protein-mediated fusion. (*A*) Upon an increase in head group attraction between molecules in the outer monolayer of the vesicles (which is supported by the unaffected inner monolayer) the tension that is developed leads to breakage of this monolayer in the area of contact (1). A structure similar to 'stalk' (Kozlov & Markin, 1983) is formed (2), which can later expand into a zone of hemifusion (3-1), or, when internal pressure is included in the model, form an analog of the fusion pore (3-2). (*B*) Protein molecules, (only part immersed in outer monolayer is shown) initially (1) in relaxed state (green), become stressed (red) and condense (yellow) in response to changes in the environment. As in the case of purely lipidic membranes, the outer monolayer breaks in the region of contact (2). While lipid and protein molecules slide over the inner monolayer relaxing the stress, initial connection between vesicle and target membrane expands and allows the unification of distal monolayers (3) that can be followed by pore formation (not shown). Panel *A* represent snapshots of the actual computer simulation of vesicle fusion (Chanturiya, 1997). To create panel *B*, molecules were manipulated manually to illustrate a possible mechanism not yet tested by simulation model.

taining (Kemble, Danieli & White, 1994), and hybrid systems (Melikyan, White & Cohen, 1995), provide strong evidence that hemifusion is the first and necessary step for complete fusion. In addition, computer simulations of fusion (Chanturiya, 1997) indicate that tension in the outer monolayer may lead to either hemifusion or complete fusion depending on the properties assigned to the internal volume of model vesicle.

How can membrane proteins participate in fusion if fusion is driven by lateral tension? As it was proposed recently (Pantazatos & MacDonald, 1999) a conformational change in membrane protein may result in the withdrawal of these proteins from the outer monolayers of fusing membranes which in turn can lead to development of tension due to the increase in separation between lipid molecules. Alternatively, calcium or pH-induced conformational changes in membrane proteins may result in a decreased area they occupy in the outer monolayer of the vesicle. If this happens, the resulting tension-induced fusion will be similar to the fusion of purely lipidic membranes but with the sensitivity to stimulus determined by the protein (Fig. 6). Since the number of fusion proteins in the viral envelope or cellular membrane is quite significant, a relatively small decrease in their area may create sufficent tension in the lipid monolayer to induce fusion.

This model does not rule out the possibility that proteins in the area of contact may perform other specific functions in fusion. Close apposition of fusing membranes, lipid head group dehydration, and destabilization of the bilayer in the area of contact are all necessary for fusion (Rand & Parsegian, 1986) and numerous studies show that fusion peptides and proteins are involved in creation of these conditions (Pecheur et al., 1999). However, while lowering the energy barrier for fusion may be sufficient in model systems, it may not be enough for fusion in highly regulated biological systems.

Importantly, the lateral tension mechanism proposes that the main energy for membrane rearrangement is derived from molecules that can be distant to the site of fusion. Thus it can explain a number of experimental results not well accommodated by other mechanisms. For example, in viral fusion, the dependence of fusion on hemagglutinin surface density (Chernomordik et al., 1998; White, 1993) is in natural agreement with this mechanism. For exocytotic fusion systems, which are geometrically similar to the vesicle/BLM model, both the dependence of exocytosis on the rate of calcium concentration increase (Knight & Baker, 1982; A. Chanturiya, *unpublished data*) and the reversibility of calcium induced changes in isolated cortical granules (Whalley & Whitaker, 1988) parallels our experimental results. Finally, the inhibition of fusion by lysolipids (Chernomordik et al., 1993) in many cases can be explained simply

as the relaxation of stress in the outer monolayer due to lysolipid incorporation. Consequently, the results of these studies strongly support a lateral lipid tension as the underlying mechanism for calcium-induced membrane fusion and potentially for biological membrane fusion processes.

References

Bullough, P.A., Hughson, F.M., Skehel, J.J., Wiley, D.C. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* **371:**37–43

Chanturiya A.N. 1996. A comparative study of planar lipid membranes formed by Montal-Mueller and Mueller-Rudin techniques. *Membrane and Cellular Biology* **13:**235–240

Chanturiya, A.N. 1997. Fast two dimensional computer simulation of bilayer hemifusion. *J. of Biomolecular Structure and Dynamics* **15:**547–553

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

Chanturiya, A.N., Nikoloshina, H. 1994. Correlations between changes in membrane capacitance induced by changes in ionic environment and the conductance of channels incorporated into bilayer lipid membranes. *J. Membrane Biology* **137:**71–77

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

Chernomordik, L.V., Frolov, V.A., Leikina, E., Bronk, P., Zimmerberg, J. 1998. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion and lipidic pore formation. *J. Cell Biol.* **140:**1369–1382

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

Christoforidis, S., McBride, H.M., Burgoyne, R.D., Zerial, M. 1999. The Rab5 effector EEA1 is a core component of endosome docking. *Nature* **397:**621–625

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

Duzgunes, P.A., Straubinger, R.M., Baldwin, P.A., Friend, D.S., Papahajopoulos, D. 1985. Proton-induced fusion of oleic acid-phosphatidylethanolamine liposomes. *Biochemistry* **24:**3091

Finkelstein, A. 1976. Water and nonelectrolyte permeability of lipid bilayer membranes. *J. Gen. Physiol.* **68:**127–135

Helm, C.A., Israelishvili, J.N., McGuiggan, P.M. 1989. Molecular mechanism and forces involved in the adhesion and fusion of amphiphilic bilayers. *Science* **246:**919–922

Holz, R., Finkelstein, A. 1970. The water and nonelectrolyte permeability induced in thin lipid membranes by polyene antibiotics nystatin and amphotericin B. *J. Gen. Physiol.* **56:**125–145

Hustler, D., Paasche, G., Dietrich, U., Zschoring, O., Gutberlet, T., Gavrisch, K., Arnold, K. 1999. Investigation of phospholipid area compression induces by calcium-mediated dextran sulfate interaction. *Biophys. J.* **77:**879–887

Kemble, G.W., Danieli, T., White, J. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* **76:**383–391

Knight, D.E., Baker, P.F. 1982. Calcium dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J. Membrane Biology* **68:**107–140

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

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

Lentz, B.R., McIntyre, G.F., Parks, D.J., Yates, J.C., Massenburg, D. 1992. Bilayer curvature and certain amphipaths promote poly(ethylene glycol)-induced fusion of dipalmitoylphosphatidylcholine unilamellar vesicles. *Biochemistry* **31:**2643–2653

MacDonald, R.C. 1988. Mechanisms of membrane fusion in acidic lipid-cation system. *In:* Molecular Mechanisms of Membrane Fusion. S. Ohki, editor. pp. 101–112. Plenum Press, New York

Mayer, A., Wickner, W., Haas, A. 1996. Sec-18p (NSF) driven release of Sec17p (α-SNAP) can precede docking and fusion of yeast vesicles. *Cell* **85:**83–94

Melikyan, G.B., White, J.M., Cohen, F.S. 1995. GPI-anchored influenza hemagglutin induces hemifusion to both red blood cells and planar bilayer membranes. *J. Cell Biology* **131:**1–13

Monk, J.R., Fernandes, J.M. 1992. The exocytotic fusion pore. *J. Cell Biol.* **119:**1395–1404

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Ohki, S., Duax, J. 1986. Effect of cations and polyamines on segregation and fusion of phosphatydilserine membranes. *Biochim. Biophys. Acta* **861:**177–186

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

Paphadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jakobson, K., Poste, G., Lazo, R. 1977. Studies on membrane fusion. III. The role of calcium-induced phase changes. *Biochem. Biophys. Acta* **465:** 579–598

Pecheur, E.L., Sante-Marie, S., Bienventie, A, Hoekstra, D. 1999. Peptides and membrane fusion: toward the understanding of the molecular mechanism of protein induced fusion. *J. Membrane Biol.* **157:**1–17

Portis, A., Newton, C., Pangborn, W., Papahadjopoulos, D. 1979. Studies on the mechanism of membrane fusion; Evidence for intermembrane Ca^{2+}-phospholipid complex, synergism with Mg^{2+} and inhibition by spectrin. *Biochemistry* **18:**780–790

Rand, R.P., Parsegian, V.A. 1986. Mimicry and mechanism in phospholipid models of membrane fusion. *Annu. Rev. Physiol.* **48:**201–212

Shangguan, T., Alford, D., Bentz, J. 1996. Influenza virus-liposome lipid mixing is leaky and largely insensitive to the material properties of target membrane. *Biochemistry* **35:**4956–4965

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

Weber, T., Zemelman, V., McNew, J.A., Wetermann, B., Gmachi, M., Parlati, F., Soliner, T.H., Rothman, J.E. 1998. SNARE pins: minimal machinery for membrane fusion. *Cell* **92:**759–772

Wessenhorn, W., Dessen, A., Harrison, S.C., Skehel, J.J., Willey, D.C. 1997. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387:**426–430

Whalley, T., Whitaker, M. 1998. Exocytosis reconstituted from the sea urchin egg is unaffected by calcium pretreatment of granules and plasma membrane. *Bioscience Reports* **8:**335–343

White, J. 1993. Membrane fusion. *Science* **258:**917–924

Appendix

The surface area of a spherical vesicle cannot be reduced without a corresponding reduction in the vesicle volume. Since water is virtually incompressible, a reduction in volume will require transfer of certain amount of water across membrane. Calcium binding without surface area reduction creates lateral tension in the lipid monolayer where calcium has bound. To keep tension close to zero, the rate of calcium-induced surface area and volume reduction should correspond to the rate of volume reduction due to water diffusion. This condition is described by the following equation:

$$\Phi_{wD} = \Phi_{wCa} \quad (1)$$

Where: Φ_{wD} − Diffusion flow of water; Φ_{wCa} − water flow results from Ca^{2+}-induced condensation of vesicle membrane.

Using a number of assumptions we can roughly estimate the rate of calcium concentration increase required to fulfill this equation. For a 100 nm diameter (d) vesicle membrane area is $S = \pi d^2 = 3 \times 10^{-10}$ cm^2 and volume is $V = \pi d^2/6 = 5 \times 10^{-16}$ cm^3. ϕ_{wD} through the membrane of given area can be found from the following equation (Finkelstein, 1976)

$$\Phi_{wD} = P_{Dw} \times S \times \Delta C, \quad (2)$$

Where: P_{Dw} is permeability coefficient and ΔC is the difference in water concentratoins on both sides of membrane.

Since water is incompressible ΔC can only be the result of water substitution by other molecules or ions on opposite sides of the membrane. For this reason we can assume ΔC to be of an order of 1 mM/l or 10^{-6} M/cm^3. Assuming $P_{Dw} = 10^{-4}$ cm/sec (actual value for DOPE/DOPS membrane is not known but for other lipid mixtures it is in the range of 10^{-5}–10^{-3} (Holz & Finkelstein, 1970), we can get estimated value of $\Phi_{wD} = 10^{-20}$ M/sec. Assuming that reduction in the area of vesicle membrane due to calcium binding is of the order of 5% (Hustler et al., 1999) and that small changes in the sphere volume are roughly equal to corresponding changes in the area of a sphere, we can estimate the volume of water that need to be removed from the vesicle (ΔV) in order to keep membrane tension at minimum. $\Delta V = 2.5 \times 10^{-17}$ cm^3 or 1.4×10^{-18} M H_2O. To fulfill the condition set by Eq. 1 this amount of water should be transferred through vesicle membrane with the rate $\Phi_{wCa} = \Phi_{wosm} = 10^{-20}$ M/sec or over the time of about 23 min.