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REVIEW

PEG as a tool to gain insight into membrane fusion

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Abstract Thirty years ago, Klaus Arnold and others showed that the action of PEG in promoting cell–cell fusion was not due to such effects as surface absorption, cross-linking, solubilization, etc. Instead PEG acted simply by volume exclusion, resulting in an osmotic force driving membranes into close contact in a dehydrated region. This simple observation, based on a number of physical measurements and the use of PEG-based detergents that insert into membranes, spawned several important areas of research. One such area is the use of PEG to bring membranes into contact so that the role of different lipids and fusion proteins in membrane fusion can be examined in detail. We have summarized here insights into the fusion mechanism that have been obtained by this approach. This evidence indicates that fusion of model membranes (and probably cell membranes) occurs via severely bent lipidic structures formed at the point of sufficiently close contact between membranes of appropriate lipid composition. This line of research has also suggested that fusion proteins seem to catalyze fusion in part by reducing the free energy of hydrophobic interstices inherent to the lipidic fusion intermediate structures.

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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Fundamental questions of membrane biophysics

It was not long after statement of the Fluid Mosaic Model of membrane structure by Singer and Nicolson (1972) and the elucidation of the basic dynamics of a bilayer (McConnell 1978) that membrane biophysicists began to wonder about two fundamental questions. First, given that a very stable and competent bilayer can be formed from only one species (phosphatidylcholine), what are the roles of the myriad lipid species that compose biological membranes? Second, how do cellular membranes join or break into offspring membranes, in other words what is the mechanism of membrane fusion and of its cognate process, membrane fission? Much progress has been made during the past 30 years towards answering both questions, although a detailed mechanism of membrane fusion and how proteins catalyze it remains to be established. This review addresses progress toward revealing this mechanism, with special emphasis on the use of poly(ethylene glycol) (PEG) to reveal this mechanism.

Early studies of membrane fusion mechanism

In the early years of thinking about membrane fusion, a debate raged over what constituted fusion, primarily because the methods used to promote fusion (calcium and other divalent cations (Papahadjopoulos et al. 1976c), detergents (Papahadjopoulos et al. 1976a), and bilayer phase transitions (Papahadjopoulos et al. 1976b; Suurkuusk et al. 1976) also triggered considerable leakage of membrane contents. Indeed, two methods (divalent cations and detergents) could produce non-lamellar or non-physiological lamellar structures.

In about 1975, a Biophysical Discussions (sponsored by the Biophysical Society) engendered a spirited discussion that led to an agreed-upon definition of the fusion process: mixing of entrapped contents between two-membrane-enclosed aqueous compartments accompanied by mixing of membrane components (lipids and likely proteins) but with little accompanying leakage of entrapped contents. This definition led to a dramatic increase in efforts to define the mechanism of fusion. A great deal of effort focused on fusion triggered by calcium or other di- or trivalent cations in bilayers formed from acidic lipids, primarily phosphatidylserine membranes [reviewed by Bentz and Ellens (1988)]. These studies established three stages of ion-induced fusion: (1) membrane aggregation, (2) close-contact, and (3) membrane merger, but were unable to distinguish membrane merger from contents mixing, except in one instance (Wilschut et al. 1985). The inability of these studies to distinguish between steps of the fusion process and identify fusion intermediates was due to the fact that di- or trivalent cations induced all three stages simultaneously, making kinetic analysis difficult and separation of intermediates impossible. Furthermore, the multiple effects of Ca^{2+} or other cations on acidic lipid bilayers (tight binding, dehydration, phase separation or non-lamellar phase formation, increased surface tension, depolarization, reduction in membrane fluidity, and formation of tight inter-bilayer complex) made it impossible to isolate the effects that were critical to inducing fusion. By the early and mid-1980s, there was a need for a different model system for examining fusion.

PEG-mediated fusion

PEG has long been used to fuse cells (Ahkong et al. 1975; Kao and Michayluk 1974). Because of this, a half dozen labs began investigating in the 1980s the mechanism by which PEG induced fusion [admirably reviewed in Boni and Hui (1987)]. Several mechanisms were suggested for how PEG accomplished this, including (1) increasing surface tension, (2) absorbing to and cross-linking bilayers, (3) altering the structure and dielectric properties of bulk water, (4) altering the molecular order of the bilayer at the point of contact, (5) producing volume-exclusion-induced aggregation and dehydration, (6) induction of non-bilayer structures, (7) acting as a detergent to disrupt bilayer structure, (8) inducing phase separation that destabilizes the bilayer, (9) producing compressive and then, upon dilution, expansive osmotic stress on membrane vesicles, and (10) containing impurities that disrupted membranes. These proposals and their origins were previously reviewed in detail (Lentz 1994). Most of these proposed mechanisms did not hold up to scrutiny. Early work did show that some of the effect of PEG was due to impurities, but we have routinely purified PEG 6000 before use, and it is still effective in triggering fusion (Lentz et al. 1992). The work of Klaus Arnold and colleagues provided crucial information in ultimately defining the mechanism by which PEG promoted membrane fusion. PEG was known to aggregate membranes, as demonstrated quantitatively by electron microscopy (Saez et al. 1982) and X-ray scattering (Boni et al. 1984). Some explained this according to surface absorption and cross-linking. Arnold showed that low molecular weight PEG covalently attached to an alkyl chain that would insert into bilayers (the detergent C12E8), pushed bilayers apart (Arnold et al. 1980), rather than drawing them together. This was extended to phosphatidylethanolamine labeled with PEG-2000 attached to the head group (Kasbauer et al. 1997). This suggested that absorption of PEG to membrane surfaces was not likely to explain PEG-mediated membrane aggregation, as was confirmed by the observation that PEG could be separated from lipid vesicles by a dialysis membrane and still drive membranes into closer contact (Boni et al. 1984). Ruby MacDonald (1985) then showed that vesicles would still fuse when aggregated by PEG in this manner. In addition, Arnold demonstrated that water was excluded from regions of contact between PEG-aggregated vesicles using NMR (Arnold et al. 1985) and electrophoretic mobility (Arnold et al. 1990). A theoretical treatment supported these experiments by showing that surface exclusion would be expected to provide a membrane aggregating attraction (Evans and Needham 1988), as was then confirmed by direct experiment (Kuhl et al. 1996). Today we know that, aside from producing a thermodynamic force driving close contact between membranes, PEG also promotes fusion via a positive osmotic pressure that likely helps stabilize fusion intermediates (Malinin et al. 2002). This osmotic effect is not required to trigger fusion, so that the main effect of PEG on membrane vesicles is volume-exclusion aggregation of membranes and dehydration in areas of contact.

PEG-mediated fusion as a model for cell membrane fusion

Establishing basic tools and properties of PEG-mediated fusion

The observations of Arnold and others made it seem to me that PEG-mediated fusion might be ideal for

studying the mechanism of fusion. This was primarily because PEG had only one major effect on membrane vesicles: bringing them into close contact resulting in a degree of dehydration at the point of contact. It does not produce significant membrane depolarization. It produces increases in membrane packing, phase separation, or non-lamellar structures to the extent that dehydration can lead to these consequences, meaning that these effects are minimal if one confines interest to low PEG concentration. It does not produce an irreversible tight inter-bilayer complex, as does Ca^{2+}. Instead, inter-bilayer spacing decreases with PEG concentration in a reversible fashion; an observation that was widely used to define what was originally called the ''hydration potential'' between bilayers but then came to be understood as due to membrane fluctuations as well as water structure (McIntosh and Simon 1994). This means that PEG can be used to bring membranes to a controlled inter-bilayer distance, and the role of other variables in triggering or promoting fusion can be evaluated.

Because fusion is a dynamic process, its mechanism cannot be revealed by static methods, although studies of lipid mesomorphic phase behavior have yielded useful insights into possible structures for fusion intermediates (Siegel et al. 1989; Siegel and Epand 1997; Yang and Huang 2003). This was recognized in early studies of Ca^{2+}-induced fusion that introduced fluorescence assays for contents mixing and leakage (Ellens et al. 1984; Wilschut et al. 1980) and membrane lipid mixing (Struck et al. 1981). In the mid-1980s, we began to develop new methods and adapt the existing methods (Burgess et al. 1991; Lentz et al. 1997; Malinin et al. 2001; Malinin and Lentz 2002; Parente and Lentz 1986) to examine the kinetics of membrane fusion induced by PEG. These assays allowed us to establish many of the characteristics of PEG-mediated fusion and to show that it was surely due to membrane fusion as defined at the Biophysical Discussion meeting. These characteristics are summarized here:

- Close bilayer approach was sufficient to trigger inter-bilayer lipid exchange between large, unilamellar vesicles (LUVs), but a critical PEG concentration seemed needed for fusion. The activation energy for lipid transfer at a sub-fusion PEG concentration was reduced by PEG by 10 kcal/mol, while the activation entropy was about 10 eu ($\Delta\{T\Delta S\}$ ~ 6 kcal/mol) less negative in the presence of PEG (Wu and Lentz 1991). However, the rate of lipid transfer increased dramatically at a PEG concentration at which fusion and contents leakage was observed (Burgess et al. 1991). This PEG concentration appears to create a special inter-bilayer complex that favors rapid lipid mixing and fusion.
- The concept of a critical inter-bilayer complex was reinforced when we found that the threshold PEG concentration at which fusion was first observed occurred at the same inter-bilayer distance in membranes of very different compositions, roughly 5 ± 1 Å between phosphate scattering centers (Burgess et al. 1992; Haque et al. 2001b). This is so close that there can be no free water and even very few water molecules between bilayers at the point of fusion.
- LUVs were found to fuse only at fairly high PEG concentration (20–30 wt%), although small amounts of amphipathic ''impurities'', acyl chain unsaturation, and removal of small amounts of lipid from contacting bilayers significantly lowered the threshold for fusion (Arnold et al. 1985; Burgess et al. 1991; Lee and Lentz 1997b; Lentz et al. 1992; Talbot et al. 1997; Wu et al. 1996). As others had shown early on (Suurkuusk et al. 1976), PEG-mediated fusion was also promoted by membrane curvature.
- PEG-mediated fusion was normally accompanied by some leakage, and very high PEG concentration led to rupture of LUVs (Massenburg and Lentz 1993). However, threshold concentrations of PEG were found to promote content mixing in the absence of any measurable leakage for small, unilamellar vesicles (SUVs) (Evans and Lentz 2002; Talbot et al. 1997), making it clear that fusion of bilayers brought into close contact by PEG is not always mechanistically linked to leakage.
- Finally, lipid movement from outer to inner leaflets occurs in SUVs during fusion but as a consequence of vesicle size increase and not as an intrinsic part of the fusion mechanism (Lentz et al. 1997). The rate of *trans*-bilayer movement mimics the rate of fusion pore formation and not the faster process of initial contents transfer (Evans and Lentz 2002). This means that pores that allow contents transfer through the stalk structure are fundamentally different from the final stable fusion pore. It also argues that fusion intermediates must leave non-contacting monolayers intact, making early suggestions of inverted micelle intermediates unlikely (Hui et al. 1981; Siegel 1984).

A mimic of biomembrane fusion

Having established the basic parameters of PEG-mediated fusion, we asked next whether this type of

fusion might be a good mimetic for biomembrane fusion. The essential kinetic characteristics of cell membrane and viral membrane fusion were established in the early 1990s through studies of Ca^{2+}-triggered release of mouse mast cells (MCs) (Oberhauser et al. 1992; Spruce et al. 1990) and pH triggered fusion of vesicular stomatitis virus (VSV) with cells (Clague et al. 1990) or fusion of cells expressing influenza virus hemagglutinin with erythrocytes (Spruce et al. 1991; Tse et al. 1993; Zimmerberg et al. 1994). Upon stimulation, both types of fusing system exhibited a rapidly opening transient conductance pore that could close and reopen several times before stabilizing into a full fusion pore. These transient conductance pores appeared slightly before rapid mixing of membrane components as followed by lipid dyes (Zimmerberg et al. 1994). Movement of soluble fluorescent dyes between fusing compartments corresponds to large and stable jumps in cell capacitance occurred a few to many seconds later (Oberhauser et al. 1992; Zimmerberg et al. 1994). Mixing of membrane components in the absence of a stable pore is termed *hemifusion*. These studies demonstrate that preliminary or transient pores occur in the hemi-fused state.

We were fortunate in the late 1990s to stumble onto a vesicle system that could be fused by PEG in a fashion that demonstrated the formation of a semi-stable initial intermediate. These vesicles were composed of a mixture of dioleoyl-phosphatidylcholine (DOPC) and dilinolenoyl-phosphatidylcholine ($DC_{18:3}PC$) (85/15) and were sonicated to a diameter of 45 nm (Lee and Lentz 1997a), roughly twice that of standard SUVs but 40% that of LUVs. PEG led to aggregates containing roughly 4–6 vesicles whose fusion was triggered by 17.5 wt% PEG. Lipid mixing occurred immediately (~10 s) upon aggregation by 17.5 wt% PEG, along with a small amount of proton movement (measured using a proton-sensitive dye HPTS—see Lee and Lentz (1997a) for details) between vesicles. Mixing of larger trapped solutes (Tb^{3+} and dipicolinic acid {DPA}—(Lee and Lentz 1997a)) and the remaining trapped protons occurred after a lag of ~100–180 s on a slower timescale (~150 s). A second, very small component of lipid mixing (corresponding to mixing of non-contacting membrane leaflets) occurred during contents mixing. Remarkably, the first step (demonstrated by mixing of contacting leaflet lipids and minimal proton movement) was reversible if PEG was diluted below aggregating concentrations, while progression beyond this step led irreversibly to fusion even if PEG was removed. The physical model resulting from these observations is shown in Fig. 1, which also illustrates how the behavior of PEG-triggered fusion of model membrane vesicles is highly analogous to fusion of MC granules triggered by Ca^{2+}, or of hemagglutinin expressing cells (IV) or of VSV triggered by pH drop. This analogy depends on identifying the slight proton movement that we observed early in the fusion process with the flickering pores observed early in hemagglutinin-mediated cell–cell fusion or mast cell–vesicle fusion. The original interpretation offered for the flickering pores was formation of protein pores that then gave way to final fusion pores (Spruce et al. 1991; Zimmerberg et al. 1994). However, comparable flickering pores have been observed during osmotic force-mediated fusion of vesicles to planar lipid bilayers (Chanturiya et al. 1997), justifying our identification of early proton movement with flickering pores.

I note that PEG-mediated fusion of other model membrane systems does not produce the same clear demarcation between intermediate states as we observed in this one type of vesicle preparation. However, all the other systems that we have characterized produce time courses of contents mixing, lipid mixing, and leakage that have one or two exponential terms and that can be described globally by the same three-step, sequential kinetic model (G. Weinreb and B.R. Lentz, submitted for publication):

$$\text{Aggregate}\,(A) \xrightarrow{k1} \mathrm{I}_1 \xrightarrow{k2} \mathrm{I}_2 \xrightarrow{k3} \text{Fusion pore (FP)}. \qquad (1)$$

The rate constants that describe these three observables also account for the time courses of right angle light scattering and pyrene excimer to monomer fluorescence ratio that monitors the appearance of fusion intermediates (Malinin and Lentz 2002). Note that this kinetic model is consistent with the physical model illustrated in Fig. 1. Thus, the same model describes quantitatively the PEG-triggered fusion of many model membranes as well as qualitatively viral and secretory vesicle fusion.

The analogy between PEG-mediated fusion and biomembrane fusion is enhanced when we consider the activation energies for the individual steps in the fusion process. The activation energy of lipid mixing in viral fusion is quite large [~42 kcal/mol (Clague et al. 1990)], while the activation energy of stable pore formation in mast cell is smaller [~23 kcal/mol (Oberhauser et al. 1992)]. Using a crude kinetic analysis, we estimated the activation energies of PEG-triggered lipid mixing (stable pore formation) as 35–37 kcal/mol and of contents mixing as 21–22 kcal/mol (Lee and Lentz 1998). Despite the crude kinetic analyses used, this is a remarkable correspondence. Even more remarkable is the thermodynamic makeup of the free energy barriers to the three steps in the process. The first two steps

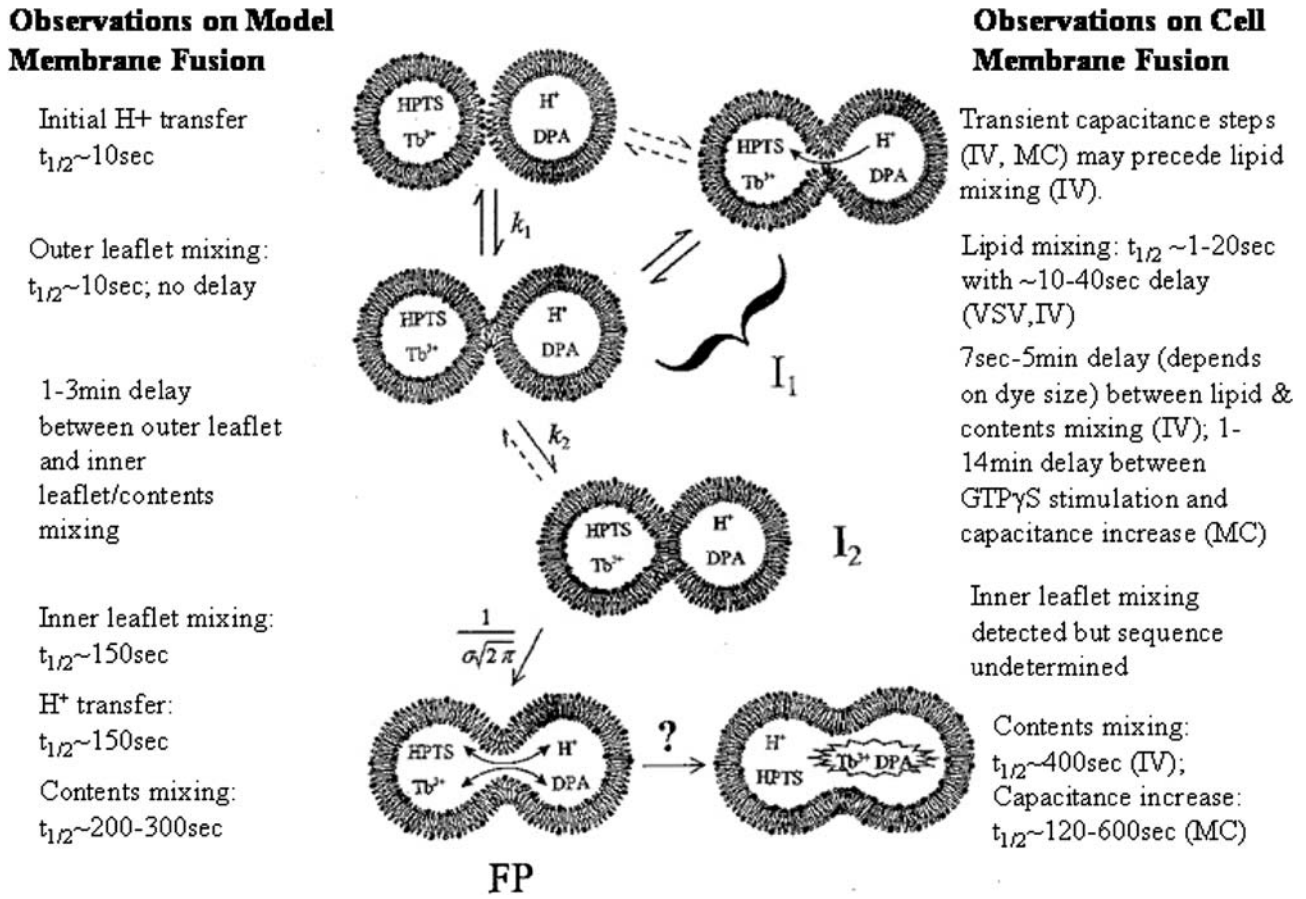

Fig. 1 Mechanism of PEG-mediated fusion of model membrane vesicles adapted from Lee and Lentz (1997a). HPTS is a proton-sensitive fluorescent dye, *DPA* dipicolinic acid, *MC* mast cell, *IV* influenza virus hemagglutinin, *VSV* vesicular stomatitis virus. The first intermediate (I_1) corresponds to the stalk (Leikin et al. 1987) and the second intermediate (I_2) to the *trans*-monolayer contact [TMC or hemi-fused diaphragm (Siegel 1999)]

($A \rightarrow I_1$, $I_1 \rightarrow I_2$) have enthalpic barriers that are lowered considerably by a positive entropy of activation (Lentz and Lee 1999). The third step ($I_2 \rightarrow FP$) is dominated by enthalpic terms, as commonly expected. Recently, we analyzed fusion of a highly fusogenic vesicle system (70% DOPC plus 30% dioleoyl-phosphatidylethanolamine {DOPE} SUVs) using the three-intermediate model (Eq. 1) and found that steps 1 and 2 were similarly favored by a positive entropy of activation, but that the last step (fusion pore formation) was severely impeded by a large negative entropy of activation (E. Haque & B.R. Lentz).

PEG-fusion yields insights into fusion mechanism

Lipidic versus proteinacious intermediate

Membrane biophysicists have sought the molecular nature of biomembrane fusion for more than thirty years. In this time, two conflicting hypotheses have evolved to explain very different observations. One, based on electrophysiological measurements on patch-clamped cells (Lindau and Almers 1995), is the *proteinacious pore hypothesis.* This contends that the initial fusion pore has the conductance properties of a single proteinacious channel. This model, popular among neurobiologists, presumes that the initial protein pore components somehow dissipate into the lipid bilayer following the fusion event. Recent mutational studies of the *trans*-membrane domain (TMD) of the synaptic fusion protein syntaxin (SX) claim to support this hypothesis and propose that several SX TMDs may form the initial pore (Han and Jackson 2005; Han et al. 2004). The alternative hypothesis is the *lipidic pore hypothesis*. It contends that the fusion pore derives from non-lamellar lipid structures and proceeds through an initial partially fused structure in which lipids mix although aqueous compartments do not. As noted above, the partially fused state is termed *hemifusion*. A large number of observations support this view for viral systems (Chernomordik and Kozlov

2005). In addition, recent evidence is that hemifusion also occurs during fusion in a model system containing mutated fusion proteins from the yeast secretory system (Xu et al. 2005) and even for fusion between isolated yeast vacuoles (Reese et al. 2005). Our recent study of fusion between neuronal SNARE-joined liposomes showed that the presence of SNARE proteins promoted formation of an initial lipid-mixed intermediate (Dennison et al. 2006) as has another study of a model system containing neuronal SNARE proteins (Giraudo et al. 2005). Thus, it seems most likely that lipidic intermediates account for fusion in biological fusion events as different as viral fusion and synaptic release.

The stalk model

The lipidic pore hypothesis is most often expressed in terms of the *''stalk model''*. This proposes specific structures for the intermediates that lead to a fusion pore. After examining bilayers supported on mica sheets (Chernomordik et al. 1987), the *stalk* structure was proposed to consist of merged contacting or *trans* monolayers and unfused *cis* monolayers (Leikin et al. 1987), as illustrated in Fig. 1. Siegel proposed a ''modified stalk model'' in which another type of intermediate structure, the *trans*-membrane contact (TMC) (see Fig. 1) might also be energetically possible (Siegel 1999). Our kinetic studies of PEG-mediated vesicle fusion established two intermediate states (Lee and Lentz 1997a), which we suggest in Fig. 1 may be the stalk and TMC of the modified stalk model. We refer to this as the *two-intermediate stalk model*.

The earliest support came from observations that lysophosphatidylcholine (lyso-PC) inhibits secretory fusion (Chernomordik et al. 1993), model membrane fusion (Chernomordik et al. 1995), and viral fusion (Chernomordik and Zimmerberg 1995) and promotes hemifusion in a way that can be rationalized based on the material properties of the presumed stalk intermediate. The problem with this line of reasoning is that lyso-PC can locate in different leaflets of a bilayer and certainly in different parts of the proposed intermediate structures. In some locations, it would inhibit, and in others it might promote fusion. Clearly additional tests were needed.

The stalk intermediate is generally viewed as unfavorable relative to a planar bilayer. This is due both to its highly curved monolayers (unfavorable *bending free energy*) and to packing inconsistencies between its non-lamellar geometry and lamellar bilayers. However, when one compares the energy of a stalk intermediate with that of a closed spherical vesicle of minimal radius (SUV), the stalk is only slightly unfavorable, and the unfavorable free energy comes not from the bending free energy but from the lamellar/non-lamellar packing inconsistencies (Fig. 2) (Malinin and Lentz 2004). The unfavorable packing free energy reflects the inability of non-lamellar geometry to join smoothly with the lamellar regions of the membrane, although the Helfrich treatment of membrane material properties assumes a smooth surface. This unfavorable free energy is termed the *hydrophobic interstice free energy*. Gruner et al. (1988) have termed this effect ''hydrocarbon packing constraints'' and note that it ''competes effectively with the spontaneous curvature in determining mesomorphic behavior''. This unfavorable free energy can be described alternatively in terms of a ''*void*'' free energy (Siegel 1993) or in terms of the free energy needed to *tilt* lipid molecules relative to the local monolayer normal (Kozlovsky and Kozlov 2002). We have made this free energy proportional to the shaded ''void volumes'' shown in Fig. 3, both because this approach is conceptually and computationally simpler and because it suggests a possible explanation for the role of fusion proteins, as discussed below. Although any model based on lipid material properties is undoubtedly a gross over-simplification for the actual molecular events involved in lipidic pore formation,

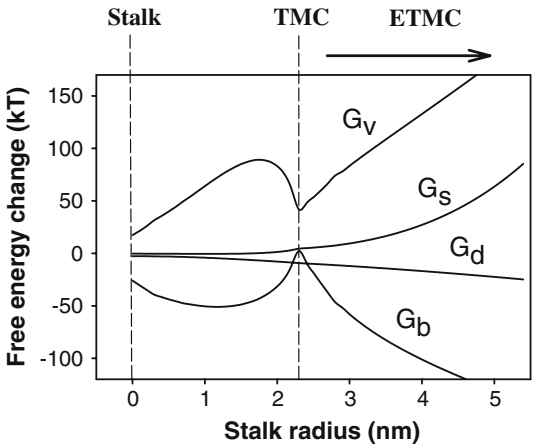

Fig. 2 Major contributions to the free energy of the intermediate structures presumed by the two-intermediate stalk model to constitute the fusion pathway are shown. G_v is the interstice free energy, G_s the expansive (or compressive) free energy, G_d the ''depletion'' (PEG-induced water depletion next to the membrane) free energy, and G_b the bending free energy. *TMC* is the *trans*-membrane contact described by Siegel (1999) (I_2 in Fig. 1), while ETMC is an extended *trans*-membrane contact in which the *TMC* diaphragm is stretched. Adapted from Malinin and Lentz (2004)

this simple approach, based on the modified, two-intermediate, stalk hypothesis, produced an estimate for the activation free energy consistent with our earlier estimate (Lentz and Lee 1999). Because we modeled closed vesicles, we were able for the fist time to compare changes in the calculated free energy profile for fusion to observed effects of osmotic gradients (Malinin et al. 2002), membrane curvature (Haque and Lentz 2004; Talbot et al. 1997), and long-chain hydrocarbons (Haque and Lentz 2004; Malinin et al. 2002) on PEG-mediated fusion (Malinin and Lentz 2004). The ability to account for several experimental observations added to the evidence that our kinetics studies already provided in support of the modified stalk hypothesis. Shortly after our calculations appeared, another calculation of the free energy profile of fusion intermediates was published, but from the point of view of a simple course grain model and using field theory to derive free energies of the same presumed intermediate states (Katsov et al. 2004). This work produced qualitatively the same reaction profile as we obtained by a very different approach (macroscopic material properties of lamellar structures). The agreement of these two results gives extra credence to the two-state stalk model.

Two recent observations provide dramatic further support for the stalk model. Using X-ray scattering, Huang and co-workers observed, under extreme dehydration and elevated temperature, a stable rhombohedral lipid phase consisting of hexagonally packed stalk structures (Yang and Huang 2003). Most recently, Zampighi et al. (2006) observed well-defined stalk intermediates at active zones of the pre-synaptic membrane. Thus, lipids can form stalk-like structures under appropriate conditions, and these structures are observed in the context of biomembrane fusion. Despite a great deal of support for the modified stalk model, there is still room for further testing and, in particular, a need to explain how experiments claimed as support for the proteinacious pore model (Han and Jackson 2005; Han et al. 2004) might be explained by the stalk model.

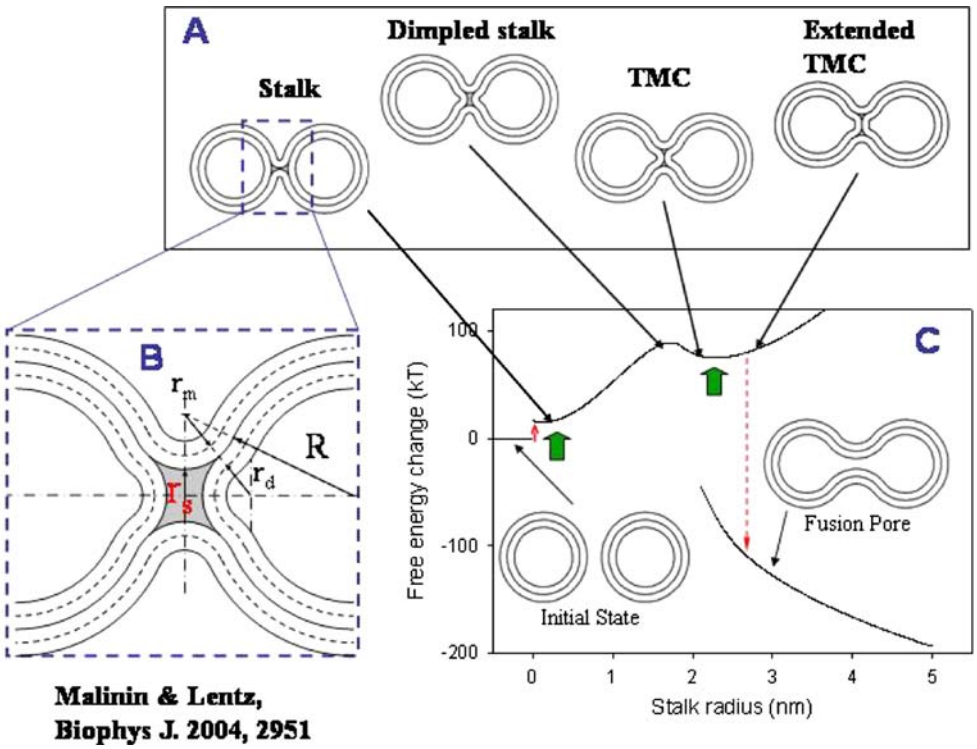

Fig. 3 Membrane reorganizations associated with the "stalk" model. **a** Stages of membrane fusion. **b** Blow-up of the contact region between two membranes in the stalk. *R* is the vesicle radius to the inter-leaflet plane, stalk radius (r_s) is the distance between the stalk axis and the apparent inner surface of the stalk (**b**). The marginal radius (r_m) and the dimple radius (r_d) are constrained by energy minimization. **c** The free energy for the fusion reaction varies with the stalk radius, the assumed reaction coordinate. Evolution of the fusion intermediates shown in (**a**) is a topologically continuous process that we can model. Formation of the stalk and the pore are topologically discontinuous event (shown by *dashed red arrows*) that we cannot model. The calculations predict two observed intermediates indicated by minima at the two *green arrows*. Correspondence between the presumed fusion stages and the energy profile is depicted by *black arrows*

Protein machines work on lipid materials

PEG-mediated fusion allows one to explore the mechanism by which fusion proteins might catalyze fusion. PEG can bring model membranes into sufficiently close contact that they will fuse or be on the brink of fusion (Burgess et al. 1992). This is likely one of the roles of fusion proteins in vivo (Chernomordik and Zimmerberg 1995; Lentz et al. 2000; Sutton et al. 1998). By performing this task with PEG, we are free to investigate the other contributions of fusion proteins by monitoring changes in rates and extents of content mixing and lipid mixing associated with the presence of whole fusion proteins or peptides derived from them. This also provides a means for detecting alterations in the mechanism of fusion (after analysis with the two-intermediate model (Weinreb and Lentz 2006)). Using this approach, we have found that the fusion peptide and *trans*-membrane domains (TMDs) of fusion proteins both catalyze fusion (Dennison et al. 2002; Haque and Lentz 2002; Haque et al. 2001a). In all cases that we have examined, the effects of these membrane-contacting regions of fusion proteins were: (1) to enhance the rate of formation of the initial intermediate (I_1), (2) to increase somewhat the probability of pore formation in the I_1 state (unstable or flickering pore), and (3) to increase the rate of conversion of intermediates to stable fusion pore (major effect). In all cases, addition of hexadecane [known to partition to and stabilize interstices (Chen and Rand 1998)] overrode the effects of peptides, suggesting that at least some of the effect of fusion proteins is to stabilize hydrophobic interstices with their membrane-inserted regions.

Quite a few papers [reviewed in Haque et al. (2005)] suggest that the *fusion peptides of type-1 viruses* disrupt bilayer surface packing to produce rupture and fusion. However, we have recently shown that the fusion peptides of Influenza hemagglutinin (HA) or HIV gp41 do not disrupt membrane surface packing at reasonable surface occupancies (Haque et al. 2005). Others propose that viral fusion peptides impart membrane curvature strain that leads to fusion (Colotto and Epand 1997; Epand and Epand 1994). Our results suggest the alternative possibility that fusion peptides fill space in the bilayer interior to lower the interstice free energy and thus the barrier to conversion of stalk to TMC and/or fusion pore (Haque et al. 2005; Haque and Lentz 2002; Haque et al. 2001a).

Several papers have stressed the importance of viral fusion protein TMDs in fusion pore formation (Armstrong et al. 2000; Cleverley and Lenard 1998; Langosch et al. 2001; Melikyan et al. 1997, 1999). We showed that the VSV TMD promotes PEG-triggered fusion when it contains a helix-breaking motif but blocks fusion when it contains greater helix content (Dennison et al. 2002). A mutant fusion protein having the more rigid TMD also blocks VSV infection in vivo (Cleverley and Lenard 1998). The fact that this fusion-promoting ability was overridden by hexadecane (interstice-filler) suggests that the flexible helix of the native TMD may be required to adapt to the non-lamellar geometries of fusion intermediates so that it can fill interstice space and lower free energies of barriers between intermediate states (Dennison et al. 2002). Our current hypothesis is that this is the role of fusion protein TMDs.

Syntaxin (SX) is one of three neuronal *SNARE proteins* [SX, synaptobrevin (SB), and SNAP-25 (SN25)] thought to anchor synaptic vesicles to cell membranes via a four-helix bundle (the *SNARE complex*) (Jahn et al. 2003). Mutations in the TMD of SX also influence fusion (Han and Jackson 2005; Han et al. 2004). Several in vitro reconstitution studies of the SNARE complex have used samples prepared by removal of detergent from fully solubilized protein–lipid mixtures (detergent reconstitution) at high (1/150 to 1/20) protein/lipid ratios (Fix et al. 2004; Nickel et al. 1999; Schuette et al. 2004; Weber et al. 1998). On the basis of measurements of membrane mixing, these all conclude that the SNARE complex is sufficient to trigger fusion. However, this view has been questioned (Bowen et al. 2004; Szule et al. 2003), and a recent article shows that the claim that SNARE complex is sufficient to trigger fusion depends on the method of SNARE reconstitution into membranes (Chen et al. 2006). We recently showed that only vesicles having low surface densities of SNAREs can retain contents. As a result, very slow mixing of lipids between SNARE-linked vesicles reported at higher surface densities cannot be shown to be due to fusion, since the vesicles under these conditions do not retain trapped contents (Dennison et al. 2006). We reconstituted these proteins in a unidirectional fashion and at a low protein/lipid ratio (1/2,000 to 1/500) by adding protein in detergent to preformed vesicles (constitutive reconstitution, see Fig. 4) and then using PEG to hold these vesicles in contact so that a stable and parallel SNARE complex could form between them (see Fig. 4). This complex does not trigger fusion. In this model system, SNARE complex nonetheless promotes fusion triggered by PEG, as did incorporation of either SX or SB alone into membranes, without formation of a SNARE complex (Dennison et al. 2006). Thus, SX and SB are both sufficient to *promote* PEG-triggered fusion, but the SNARE complex alone did not bring membranes

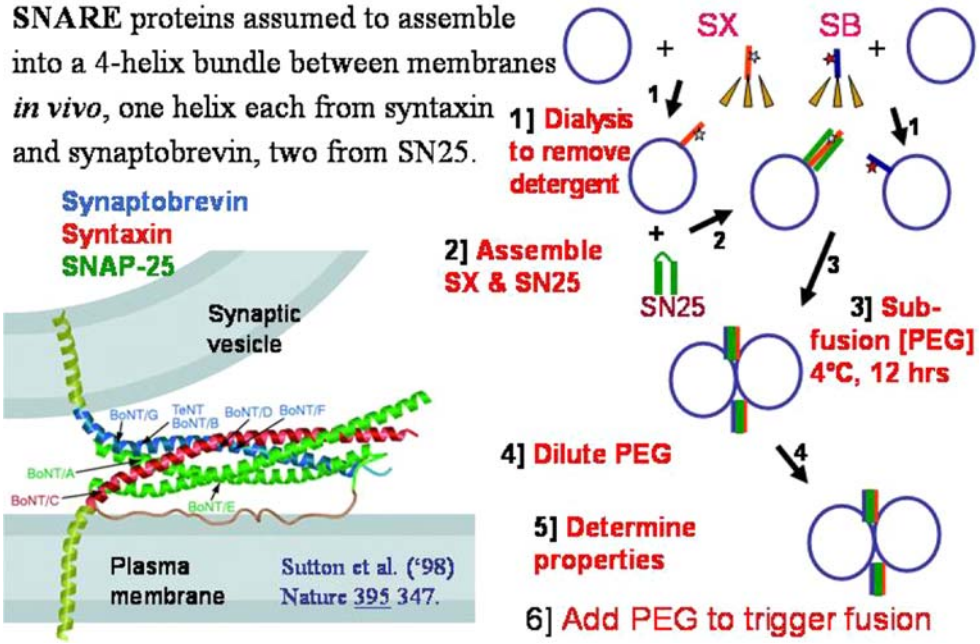

Fig. 4 *Left* Model for how the four-helix bundle of SNARE proteins is thought to anchor synaptic vesicles to the plasma membrane (Sutton et al. 1998). While it is generally believed that the SNARE four-helix bundle holds the vesicle and target membranes in juxtaposition in vivo, this bundle structure has been demonstrated only for soluble forms of these proteins in vitro (Poirier et al. 1998; Sutton et al. 1998). Although a parallel bundle is shown, both parallel and anti-parallel bundles form between soluble and membrane-associated SNARES (Weninger et al. 2003), while only parallel complexes form between constitutively reconstituted membrane vesicles (Dennison et al. 2006). *Left* Cartoon illustrating our method for assembling a SNARE complex between continuatively reconstituted model membrane vesicles so that membrane integrity and sidedness are maintained (Dennison et al. 2006; Saez et al. 1982)

into sufficiently close contact to *trigger* fusion; PEG was needed to drive contact sufficient for fusion. We suspect that SX and/or SB have flexible-helix TMDs that also promote fusion by stabilizing hydrophobic interstices. We are testing this hypothesis using PEG-triggered fusion.

Conclusions

Ever since Klaus Arnold and others showed PEG to drive membrane vesicles together by simple volume exclusion, it has proven to be an important tool in efforts to uncover the mechanism of membrane fusion. It provides the researcher with control over membrane contact and its concentration defines the exact inter-bilayer separation. Since inter-membrane contact is a pre-requisite for fusion and PEG creates this, the researcher is free to examine the role of other factors in triggering or promoting fusion. This simple bit of polymer physical chemistry has proven invaluable in revealing possible mechanisms by which fusion proteins catalyze fusion in biomembranes.

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