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Membrane fusion is a central event for a wide variety of biological processes, such as transport of cargo through the secretory and endocytic pathways, organelle inheritance, neurotransmission, and viral entry into host cells, among others. Hence, understanding the mechanism(s) of physiological membrane fusion is a subject of wide biological interest, and extensive studies using theoretical and experimental approaches have been devoted to this goal (1). It is generally believed that fusion proceeds through a “stalk intermediate” involving a merger of the proximal leaflets of the two bilayers. This intermediate may lead directly to formation of a fusion pore followed by expansion of the pore, but it may also arrest, at least transiently, to yield what is known as a “hemifusion intermediate.” In addition, electrophysiological studies of neurosecretion have suggested that fusion pores can open and close quickly, a phenomenon called “flickering,” and that fusion pore formation can be followed by fast membrane fusion leading to separation of the two membranes (known as “kiss-and-run”) (2). Because of the fleeting nature of these intermediates and events as well as of potential alternative phenomena, their detailed study using defined reconstituted systems is very challenging. An ingenious method described by Yoon et al. (3) in this issue of PNAS now provides a powerful tool that can yield unprecedented information on membrane fusion at the single-vesicle level.

The authors developed this method to study membrane fusion induced by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Proteins from this family play a key role in membrane traffic through all steps of the secretory and endocytic pathways by forming tight four-helix bundles that bring two membranes together, which is believed to cause mechanical force on the membranes to catalyze fusion (4). Lipid mixing induced by SNARE proteins has been extensively studied in bulk solution by using reconstituted proteoliposomes (5), and some studies have also analyzed SNARE-mediated fusion of proteoliposomes to planar bilayers (6). However, the first approach is based on ensemble-averaged measurements that yield no information on the kinetics of fusion at the individual vesicle level. The information on single-vesicle fusion events provided by the second approach is limited because fluorophores included in the vesicles to monitor lipid mixing inevitably diffuse away in the large planar bilayer upon fusion.

The clever technique designed by Yoon et al. (3) overcomes this problem by monitoring fusion between single-vesicle pairs, which offers a closed system that does not allow infinite dilution of the fluorophors upon fusion. The basis for the approach is summarized in Fig. 1. Vesicles containing one of the SNARE proteins is labeled with a fluorophore that does not allow infinite dilution of the fluorophors upon fusion. The diagram at the top illustrate the experimental approach used by Yoon et al. (3) to study SNARE-mediated fusion between donor vesicles containing green fluorescent lipids and acceptor vesicles containing red fluorescent lipids and biotinylated lipids. The latter allow attachment of the acceptor vesicles to a quartz surface coated with PEG-containing neutravidins (small blue ellipses). The emission intensities in arbitrary units expected for the green and red channels in the different intermediates, as well as the resulting FRET efficiencies, are illustrated below the diagrams. (D) The diagram represents a transient fusion pore that allows only limited lipid mixing between the distal bilayer leaflets and then closes quickly. Hence, the distal leaflets are represented in orange and light green to illustrate the partial lipid mixing in the resulting intermediate (E), which is represented as a hemifusion intermediate but could also have a different nature. For further details, see text.
Remarkably, the large number of time traces of individual fusion events that were recorded reveals a wide variety of behaviors. In some traces, full lipid mixing was observed without any intermediates. In other traces, the appearance of a plateau with half of the FRET efficiency corresponding to full fusion provides strong evidence for the formation of a hemifusion intermediate (Fig. 1C). The fact that these traces eventually evolve to full fusion suggests that this state is a true intermediate in the fusion pathway rather than a dead-end. Interestingly, the hemifusion intermediate is followed in some cases by one or more additional plateaus with a FRET efficiency that is intermediate between those of hemifusion and full fusion (Fig. 1E). This observation may arise from formation of a transient fusion pore that closes quickly and hence does not allow full mixing of the distal bilayer leaflets (Fig. 1D and E); this behavior is characteristic of a flickering fusion pore. Moreover, abrupt decreases in red and green emission intensity without a change in FRET efficiency (Fig. 1G) were observed in some cases after these flickering events or after full fusion but not at or before the hemifusion intermediate. These observations indicate that the donor vesicle was detached from the acceptor vesicle quickly after fusion, suggesting the occurrence of kiss-and-run events. Finally, it is also worth noting that the FRET efficiency increased gradually to the hemifusion level in some traces, which may arise from formation of multiple intermediates or from flickering events before hemifusion but may also reflect a disorganized lipid-mixing process.

The studies of Yoon et al. (3) were performed with vesicles containing a high protein-to-lipid ratio, which is necessary for efficient lipid mixing in this reconstituted system. Because highly inefficient or no lipid mixing is generally observed at lower protein densities, there is still considerable debate as to whether SNARE proteins truly constitute minimal fusion machineries and whether the lipid mixing observed at high protein densities reflects the events that occur during physiological membrane fusion (4). However, regardless of this debate, it is quite remarkable that Yoon et al. were already able to observe diverse behaviors that are consistent with hemifusion, fusion pore flickering, and kiss-and-run events in this simple reconstituted system. Further research will be necessary to confirm fully the occurrence of at least some of these phenomena, but the importance of the design from Yoon et al. goes far beyond the results described in this work. Hence, the use of the same overall experimental approach with soluble fluorophors trapped inside the vesicles will allow further exploration of the flickering and kiss-and-run events. Contents mixing and potential leakiness during membrane merger, which are critical criteria to demonstrate physiological membrane fusion, can also be investigated by this approach. It can also be expected that constant technological improvements will make it possible to perform these experiments with higher sensitivity and time resolution, which will facilitate statistical analyses of the data and may reveal additional phenomena. Furthermore, this powerful tool will now allow investigating in detail and at the single-vesicle level how the SNAREs cooperate with additional proteins that control membrane fusion in different membrane compartments, and this approach can also be used to study other types of membrane fusion such as that mediated by viral protein. The future of research on membrane fusion is increasingly bright.