# The 51st Annual Meeting and Symposium of the Society of General Physiologists

## *Mechanisms of Secretion*

(ORGANIZED BY GEORGE J. AUGUSTINE and THOMAS F.J. MARTIN)

The plasma membrane defines the spatial extent of a cell. The plasma membrane is not, however, an invariant entity as the membrane shape and area vary continuously. Specifically, the cellular events that underlie neurotransmitter release, as well as endocrine and exocrine secretion, depend on a continuous turnover of membrane components that involve the fusion (and subsequent retrieval) of intracellular vesicles and granules with the plasma membrane. Despite common elements (e.g., the fusion of two lipid bilayers into one), the molecular basis of secretion differs among cell types, and a given cell type may secrete different compounds using different mechanisms.

The generality and specificity of the secretory process was highlighted at The 51st Annual Meeting of the Society of General Physiologists, which took place in Woods Hole, MA, September 4–6, 1997. George J. Augustine (Duke University Medical Center) and Thomas F.J. Martin (University of Wisconsin) organized the symposium on Mechanisms of Secretion, which covered secretion in a variety of cell types and provided insights into the common elements and the differences of secretion. The attendance was high, with more than 300 scientists in the audience, which led to lively discussions that put things into perspective for the expert and nonexpert alike.

The endpoint of the secretory process is the fusion of two lipid bilayers, which leads to the mixing of two aqueous compartments (Fig. 1 *A*). Bilayer fusion remains enigmatic, as important intermediate (transition) states remain elusive. The fusion is likely to involve a hemifusion intermediate (Fig. 1 *B*), in which the two adjacent monolayers of the vesicle and the target bilayer become continuous (F. Cohen, J. Zimmerberg). This hemifusion stage can, in principle, be identified by the exchange of lipid markers between the two monolayers. Because the "outer" monolayers are continuous, the hemifusion stage provides an attractive mechanism to ensure that there is no uncontrolled spillage of vesicular contents into the cytoplasm (Cohen). It remains unclear, however, whether hemifusion is a prerequisite for fusion. If it is an important intermediate step, it must be transient (Zimmerberg). If there is detectable exchange of lipid markers before the formation of fusion pores, which signals the creation of (transient) connections between the aqueous compartments, then the hemifusion stage is a dead end. Possibly because the hemifusion diaphragm has become so stable that it could expand and thereby cre-

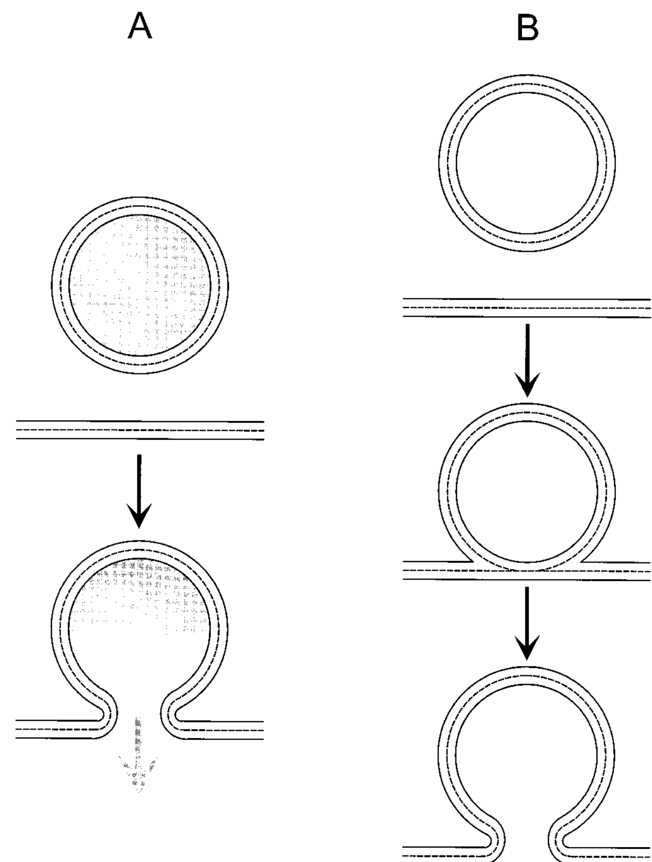


FIGURE 1. Schematics of secretion fusion. (*A*) When the secretory vesicle empties its contents into the extracellular space, the two lipid bilayers fuse. (*B*) The bilayer fusion may involve a hemifusion state in which the outer monolayer of the vesicle becomes continuous with the *cis* monolayer of the target membrane. The inner monolayer of the vesicle and the *trans* monolayer of the target membrane remain separate until the fusion is complete. (The figure is drawn with no regard for the lipid packing problem in the hemifusion state.)



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ate a monolayer–monolayer contact of sufficient length to allow for measurable lipid exchange.

Bilayer fusion is triggered and controlled by proteins. In some systems (e.g., influenza hemagglutinin), this protein control arises from a "simple" pH-dependent change in the structure of the fusion-inducing molecule. In other systems (e.g., the avian leukosis retrovirus), binding of a viral envelope glycoprotein to a plasma membrane receptor resembling the low density lipoprotein (LDL) receptor triggers a conformational change that occurs at neutral pH and exposes the fusion peptide (J. White). In either case, the fusion is triggered when hydrophobic/amphipathic α-helical protein segments are exposed in a manner where they can interact with, and destabilize, the target bilayer.

The major protein components involved in secretion probably have been identified, but the mechanisms by which they function remain elusive. The difficulties arise in part because the fusion process is so effective/fast: the synaptic delay in the giant synapse of the squid is only 200 μs (R. Llinás)—meaning that all the kinetic steps, including the actual bilayer fusion, must be even faster. The proximal trigger for neurotransmitter secretion is $Ca^{2+}$ influx via N- or P/Q-type calcium channels. The $Ca^{2+}$ influx occurs in localized domains, which are stationary over >30 min, as demonstrated by Llinás using engineered aequorin species. In some cells (e.g., hair cells from the inner ear), $Ca^{2+}$-activated potassium channels are incorporated into these domains, as demonstrated by the ability of fast, mobile $Ca^{2+}$ buffers (e.g., BAPTA) to decouple potassium channel activation from $Ca^{2+}$ influx (W. Roberts).

The central role of $Ca^{2+}$ in secretion arises because $Ca^{2+}$ affects several steps in the secretory cycle, which involves the fusion of a vesicle (or granule) with the plasma membrane followed by retrieval of the excess membrane and the replenishment of the readily releasable pool of vesicles or granules (Fig. 2). Important questions that need to be considered in this cycle are: the vesicle origin and dynamics, the proteins that are important in the different steps, the $Ca^{2+}$ and ATP dependence of each step, and the relationship between calcium channels and the release sites.

To complicate matters, the cycle differs (at least in detail) between neuronal and neuroendocrine cells. In neuronal cells, $Ca^{2+}$ influx is through N- or P/Q-type calcium channels, which triggers a fast secretion by secretory vesicles that are retrieved rapidly and recycled. In neuroendocrine and non-neuronal cells, $Ca^{2+}$ influx is through L-type calcium channels, which triggers a relatively slow secretion by secretory granules that are derived from the *trans* Golgi network and retrieved by a conventional endocytic pathway. Not surprisingly, the $Ca^{2+}$ dependence of the fusion step differs among different cell types; e.g., ribbon synapses in retinal bipolar cells (R. Heidelberger) and adrenal

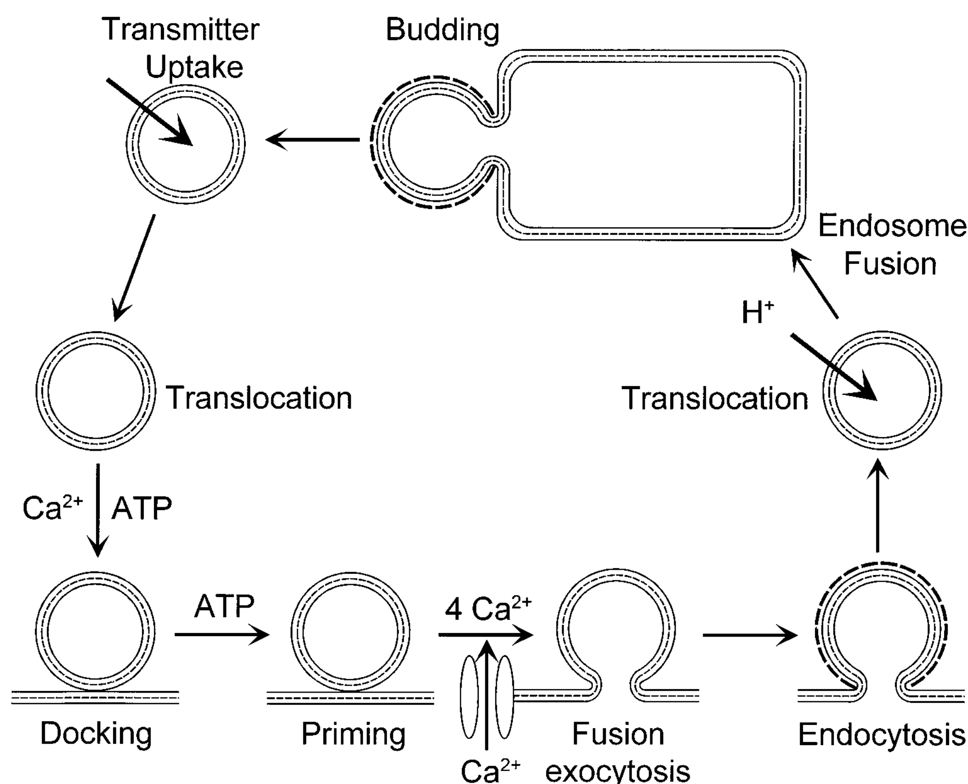


FIGURE 2. The secretion cycle. The figure is based on schematic representation by a number of speakers during the symposium.



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chromaffin cells (E. Neher). It remains unclear, however, whether these differences reflect qualitative differences in the molecular machinery or more subtle differences in the cooperativity of the process.

To define the role of $Ca^{2+}$ in the fusion event per se, Heidelberger and Neher used caged $Ca^{2+}$, released by flash photolysis, to trigger the event, and monitored the fusion by the consequent capacitance increase. In bipolar cells (Heidelberger), the fusion rate varies as $[Ca^{2+}]^4$ (for $[Ca^{2+}] < \sim 30$ μM)—indicating a highly cooperative process (as first demonstrated by Dodge and Rahamimoff in 1967). This work is described more fully in an article in this issue of the Journal. In chromaffin cells (Neher), the rate of fusion varies as $[Ca^{2+}]^3$.

In addition to the immediate effect on vesicle-membrane fusion, $Ca^{2+}$ also is involved in the slower transfer of vesicles to their release sites close to the plasma membrane, which varies as a linear function of $[Ca^{2+}]$. Membrane retrieval is dependent on ATP hydrolysis, and MgATP has an important role in the maturation of the vesicles (upstream of the $Ca^{2+}$-induced fusion). The physiological vesicle fusion, triggered by $Ca^{2+}$ influx through calcium channels, was modeled based on the flash photolysis results (Neher). The analysis shows that most of the release sites (and the readily releasable vesicles) must be within 200–300 nm of the calcium channels (Neher). Approximately 10% of the release sites (or vesicles) appear closely associated with calcium channels, indicative of considerable higher-order organization at the release sites. Molecular evidence for such organization was provided by S. Mochida (speaking in a New Ideas/New Faces session), who presented evidence for direct binding of syntaxin to N-type calcium channels. The binding is $Ca^{2+}$ dependent, and the release could be triggered by mechanical transmission of voltage-dependent conformational changes in the calcium channel (as in skeletal muscle excitation–contraction coupling) or the local $[Ca^{2+}]$ increase.

The keynote speaker, J. Rothman, provided a masterful account of the proteins involved in vesicle budding and maturation, as well as the control and triggering of the vesicle fusion event. Studies in a wide variety of systems have shown a remarkable degree of similarity in these processes. Vesicle budding is driven by the formation of a protein coat, which is assembled by a family of GTPases related to ARF (ADP ribosylation factor). The vesicle targeting is determined by v-SNAREs (also called synaptobrevin or VAMP, vesicle-associate membrane protein), which are integral membrane proteins that are incorporated into the budding vesicle, and that react with their cognate t-SNAREs (complexes of syntaxin and SNAP-25, 25-kD synaptosomal-associated protein) in the target membrane. The COOH termini of the SNAREs are prenylated and the $NH_2$-terminal sequences of both v- and t-SNARE proteins are predicted to fold as coiled-coils, which could suggest that the SNAREs interact by forming such structures. Vesicle priming, to become fusion competent, involves a complex that sediments at 20S and is composed of the v-/t-SNARE complex, which sediments at 7S, plus NSF (NEM-sensitive fusion protein) and SNAP (soluble NSF attachment protein). (The 7S/20S nomenclature dates back to the initial characterization of these complexes.) There is no evidence for NSF, SNAP, or the SNAREs being $Ca^{2+}$-binding proteins. That role is fulfilled by synaptotagmin, which is an integral component of the SNARE/NSF/SNAP complex. The structure of this complex was determined by cryoelectron microscopy, which, in combination with fluorescence resonance energy transfer studies, show that the NSF covers the coiled-coil SNARE assembly. The need for ATP hydrolysis during vesicle maturation and docking raises the possibility that NSF, which has ATPase activity, must be released before the vesicle's becoming fusion competent. This in turn suggests that the fusion is triggered by an unwinding/dissociation of the v-/t-SNARE coiled-coil, which is triggered by $Ca^{2+}$ binding to synaptotagmin. In this model, the final triggering does not involve any biochemistry (making or breaking of covalent bonds), but a destabilization of the apposed monolayers by the amphipathic α-helices in the $NH_2$ termini of the SNAREs, which would lower the energy barrier for forming the hemifusion intermediate. This would explain why the bilayer fusion can be as fast as it is.

The structure and dynamics of the protein assemblies involved in fusion was the topic of many additional presentations. The structure of the SNARE complex (syntaxin, SNAP-25, plus synaptobrevin) was described by P. Hanson (speaking in a New Ideas/New Faces session). In other presentations in the same session, the role of synaptotagmin as a $Ca^{2+}$ sensor was examined by R. Sagi-Eisenberg, who showed that synaptotagmin increases the $Ca^{2+}$ sensitivity of secretion in mast cells, and by M. Ohara-Imaizumi, who showed that $Ca^{2+}$-induced triggering was dependent on the C2A domain of the protein. The related C2B domain of synaptotagmin binds to synaptic vesicle protein 2, which is related to the 12-transmembrane-segment family of transporters with sequence similarity to the $H^+$-ATPases (S. Bajjalieh, also speaking in this New Ideas/New Faces session).

S.-C. Hsu (from R. Scheller's laboratory) reported that syntaxin indeed forms a parallel coiled-coil with v-SNARE (or synaptobrevin). The assembly of this syntaxin/v-SNARE complex is inhibited by n-sec1 (a neuronal protein related to yeast sec1). R. Jahn described combined electrophysiological/molecular biological studies on leech neurons, which form stable synapses in primary culture. Using this preparation as assay, one can define the functional role of different proteins by ex-



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ploiting their sensitivity to the neurotoxic bacterial neurotoxins, which are proteases that attack specific proteins involved in secretion. Tetanus toxin, for example, proteolyzes leech synaptobrevin and blocks transmitter release. In other studies, the structural features of the proteins was determined using 2-D NMR, which showed that the SNAREs have considerable α-helical content, which in the case of the t-SNARE complex of syntaxin and SNAP-25 increases when the complex forms from its constituents; consistent with the formation of a coiled-coil between syntaxin and v-SNARE.

Two other families of proteins that are important in the secretion cycle (Fig. 2) are the Rab GTPases, whose function is controlled by Rab binding proteins, such as Rabphilin-3A. G. Lonart (from T. Südhof's laboratory) described the role of Rab3A and provided yet another example of the knockout of an important protein, with little effect on cell function because of compensatory increases in a related protein (in this case Rab3C). G. Augustine described a number of proteins involved in the synaptic vesicle cycle, including Rabphilin-3A and the SNARE proteins using the squid giant synapse as assay system. The former work is described more fully in an article (see pp. 243–255) in this issue of *The Journal*. The SNARE proteins were investigated using microinjection of peptides derived from the proteins in the SNARE complex. A general finding was that peptides that perturb the SNARE proteins invariably inhibit synaptic vesicle fusion (transmitter release). When the electrophysiological studies were complemented by electron microscopic examination of the presynaptic terminals, the peptides had differential effects. SNAP-derived peptides, for example, depleted the number of vesicles at the plasma membrane, but the remaining vesicles are docked. Based on experiments using a series of peptides and toxins with specific targets among the SNARE proteins, it was suggested that (*a*) formation of the 7S v-/t-SNARE complex precedes that of the 20S complex (the 7S complex plus NSF and SNAP proteins); (*b*) the SNAREs are not important for docking per se, that the subsequent priming depends on the complete SNARE complex; and (*c*) the 20S complex may be involved in membrane retrieval after the fusion event.

Genetics is becoming an increasingly important tool in the study of secretion. One full session of the symposium focused on the use of genetics to identify the proteins involved in secretion and to begin to understand the functional roles of these proteins. Currently, three major experimental systems are used for this purpose: yeast, *C. elegans*, and *Drosophila*. Each of these systems has its own advantages and disadvantages (as summarized by T. Schwarz). For example, even though relatively little of the *Drosophila* genome sequence is known, functional assays in *Drosophila* tend to be superior to those in yeast or nematodes. B. Ganetsky described the secretory process in *Drosophila*, using the combined approaches of forward genetics, reverse genetics, and biochemical analysis to study the role of NSF in the secretory process. Mutations in NSF underlie all known alleles of the *Drosophila* comatose phenotype, which is due to a defect in vesicle fusion. M. Nonet described the characteristics of several secretory mutants in *C. elegans*. Syntaxin mutants display a complete absence of synaptic transmission, synaptobrevin mutants have no evoked responses, but synaptotagmin mutants survive (meaning they must have some regulated synaptic activity). Importantly, the synaptobrevin mutants have a phenotype similar to that seen with exposure to tetanus toxin, but the effects of the synaptobrevin mutagenesis cannot be reversed by coexpression of synaptobrevin that has been engineered to be resistant to tetanus toxin. This raises the question of whether there are other targets for the toxin. In a more detailed molecular analysis, all SNARE mutations that affect synaptic transmission are on the contact surface of the coiled-coil that stabilizes the complex—a result that provides additional support for the notion that fusion could be triggered by the unwinding of the v-/t-SNARE complex.

One of the strengths of the genetic approach is the ability to screen for suppressors; for example, one suppressor of syntaxin mutants is the gene for calmodulin kinase II. P. Novick summarized work on the functional relationships among proteins in the exocytotic pathway in yeast. The experimental approach is to use genetic tools available in yeast in combination with immunoprecipitation to derive information on the function of several of the sec gene products; e.g., sec3 as a spatial landmark defining sites of exocytosis and sec18 as having a role in coat disassembly.

It has been known for many years that there are different pools of secretory vesicles; the identities and relationships among these vesicle pools was another major topic of the symposium. R. Holz summarized some major issues currently being investigated, including the nature of the "readily releasable" pool of vesicles, the role of MgATP in "priming" vesicles for fusion, the roles of $Ca^{2+}$ in both early and late steps in the exocytotic pathway, and the role of phosphoinositides. L. Elferink presented studies on the functional domains of synaptotagmin, with focus on the C2A domain and its role in $Ca^{2+}$-dependent phospholipid binding. T. Martin summarized work on the $Ca^{2+}$-dependent activator protein (CAPS) and the role of phosphoinositides in the docking and ATP-dependent priming of large dense core vesicles (LDCVs). The transfer of phosphate from ATP to phosphatidylinositolphosphate in cytoplasmic monolayers of the LDCVs to produce $PIP_2$ appears to be an important event in priming of LDCVs



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and, surprisingly, the distribution of $PIP_2$ in the intracellular monolayer of the plasma membrane is punctate and closely related to the LDCVs. Even more surprisingly, CAPS antibodies inhibit the $Ca^{2+}$-dependent secretion of norepinephrine (from LDCV) but not of glutamate (from small clear synaptic vesicles) from the same cell population. This may provide some insights into the differences between neuroendocrine and neural secretion; it certainly constitutes a good example of the remarkable degree of control of the secretion process.

The generality of the budding/fusion processes that was emphasized by Rothman was underscored in a very different context by R. Steinhardt, who described how cells survive microdissection injury. Experimental studies on sea urchin eggs and 3T3 cells strongly suggest that distinct (immediate, fast, and slowly fusing) pools of vesicles are necessary for resealing these cells after injection injury. Interestingly, the resealing in unfertilized eggs is quite different from that in fertilized eggs, which behave similarly (in this respect) to 3T3 cells. The resealing process (in fertilized eggs and 3T3 cells) is blocked by botulinum toxins A, B, and C, as well as by tetanus toxin, which provides considerable evidence that the protein machinery involved in resealing is similar to that involved in secretion. Perhaps the secretory pathway originally evolved as a healing mechanism.

W. Almers described a novel method for visualizing only vesicles that are extremely close to the plasma membrane. The method, total internal reflectance (TIR) spectroscopy, is based on low angle illumination and total internal reflection that produces an evanescent wave, which penetrates only a small distance into the *trans* medium. If a cell is on a support that has a high refractive index, it is possible to illuminate a distance of a few hundred nanometers into the cytoplasm. Using this method, it is possible to visualize, in real time, the approach of vesicles to the plasma membrane and the release of their content. This provides unparalleled insights into the dynamics of the docking and priming processes. Docking is a reversible process, meaning that granules can withdraw from the membrane without emptying their contents into the extracellular space. Consistent with this observation, only ~10% of the docked vesicles are primed, with readily releasable contents. The approach and withdrawal of the vesicles appear to be directed (nondiffusive) processes, which stops when the cells are ATP depleted. The molecular identity of the motors remains unknown, but movement is blocked by 2,3 butanedione monoxime, an inhibitor of the myosin ATPase. The remarkable power of TIR spectroscopy should help bridge the gap between morphology, biochemistry, and physiology.

Physiologically, secretion depends on the retrieval of the vesicle membrane that has fused with the plasma membrane (the last session of the symposium was devoted to vesicle recycling). E. Lafer introduced the major models of vesicle recycling: first, "Kiss and Run;" second, the Heuser/Reese model; third, clathrin-dependent bulk internalization; and fourth, clathrin-independent bulk internalization. J. Heuser presented a comprehensive review of the use of electron microscopy for the study of synaptic vesicle exocytosis and recycling, including both recent and older studies. He reviewed several important technical limitations that could affect the interpretation of the results (effects of ice crystals, fixation artifacts) and discussed the evidence in favor of a clathrin-dependent membrane retrieval mechanism at the frog neuromuscular junction. P. DeCamilli described his recent work on accessory factors in clathrin-coated vesicles, including dynamin, amphiphysin, and synaptotagmin. A growing body of information on structure and function of these proteins, as well as the phenotypes of various organisms with mutations in these proteins, is producing an increasingly detailed picture of the life cycle of the clathrin-coated vesicle, although the mechanism of vesicle formation is far from complete. C. Artalejo concluded the symposium with a presentation of her data on rapid membrane retrieval in chromaffin cells, as determined by capacitance changes. After norepinephrine release, she has found that the membrane capacitance decreases with three time scales (0.3, 3, and >10 s). The most rapid retrieval event supports the Kiss and Run model and may be a major mechanism of retrieval at low levels of stimulation.

A traditional feature of the symposia organized by The Society of General Physiologists is the New Ideas/New Faces sessions, where the speakers are chosen by the organizers based on the free abstracts submitted to the meeting. This is, indeed, where the new ideas are presented—usually by young investigators. In addition to the presentations mentioned above, the following individuals spoke in two New Ideas/New Faces sessions: E. Levitan, on the dynamics (mobility and release of contents) of secretory granules labeled with green fluorescent protein; T. Ryan, on optical studies of quantal vesicle recycling; Y. Ushkaryov, on the stimulation of secretion by latrotoxin via a G protein-coupled receptor; and M. Bittner, on the cloning of a novel $Ca^{2+}$-independent receptor for α-latrotoxin.

The challenge for the future will be to bridge the gaps that exist among the various ways that we study secretion. The challenge is considerable: we may know the names of the proteins that are involved; we also may know the gross topology of the interactions among these proteins. But we are only beginning to identify the interacting domains, and there are large gaps in



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our understanding of the energetics and dynamics of their interactions. Finally, despite the existence of attractive model systems, it is not known how the proteins involved in fact promote the fusion of two bilayers. These issues were clearly identified in the presentations and the subsequent discussions, and there were several exciting hints about how we should go about resolving some of the uncertainties, many of which are central in cell physiological research. There are good reasons to be optimistic about the future.

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