Lipidic Antagonists to SNARE-mediated Fusion*

Received for publication, February 24, 2006, and in revised form, August 2, 2006. Published, JBC Papers in Press, August 3, 2006, DOI 10.1074/jbc.M601778200

Thomas J. Melia¹, Daoqi You², David C. Tareste, and James E. Rothman

From the Department of Physiology and Cellular Biophysics, Columbia University, College of Physicians and Surgeons, New York, New York 10032

SNARE proteins mediate the fusion of lipid bilayers by the directed assembly of coiled-coil domains arising from apposing membranes. We have utilized inverted cone-shaped lipids, antagonists of the necessary membrane deformation during fusion to characterize the extent and range of SNARE assembly up to the moment of stalk formation between bilayers. The inverted cone-shaped lipid family of acyl-CoAs specifically inhibits the completion of fusion in an acyl-chain length-dependent manner. Removal of acyl-CoA from the membrane relieves the inhibition and initiates a burst of membrane fusion with rates exceeding any point in the control curves lacking acyl-CoA. This burst indicates the accumulation of semi-assembled fusion complexes. These preformed complexes are resistant to cleavage by botulinum toxin B and thus appear to have progressed beyond the "loosely zipped" state of docked synaptic vesicles. Surprisingly, application of the soluble domain of VAMP2, which blocks SNARE assembly by competing for binding on the available t-SNAREs, blocks recovery from the acyl-CoA inhibition. Thus, complexes formed in the presence of a lipidic antagonist to fusion are incompletely assembled, suggesting that the formation of tightly assembled SNARE pairs requires progression all the way through to membrane fusion. In this regard, physiologically docked exocytic vesicles may be anchored by a highly dynamic and potentially even reversible SNAREpin.

Each membrane fusion event culminates in an organized assault on membrane integrity, rupturing two apposed bilayer surfaces in the process of fusing their lipid leaflets and mixing their respective contents. Not surprisingly, such an event is thermodynamically unfavorable, with the size of the energy barrier being in part a function of the intermediate structures sampled by the two membranes during the fusion event. The most successful model of these intermediate structures is the "stalk" model of membrane fusion (1–3) (see Fig. 1A), which predicts interaction of the apposed bilayers, followed by a transient hemi-fused state (the stalk), where the outer monolayers have merged, whereas the inner monolayers remain separated. Rupture of the stalk allows the formation of a pore (possibly with a "prepore" intermediate) that subsequently expands to complete the fusion of the bilayers.

In the course of stalk formation, interacting membranes must pass through transient intermediates that are highly curved. In a curved membrane the surface area to volume demands of the lipids on either side of the membrane are different. Manipulation of the lipidic composition of one leaflet of the membrane(s) can enhance or prevent certain types of curvature. Cone and inverted cone-shaped lipids have been used in a large number of pure lipid and viral-mediated fusion studies to introduce specific blocks or bursts to membrane bending and to allow an examination of the early stages of membrane fusion (4, 5) (see Fig. 1B).

The major proteins involved in overcoming the energetic barriers of membrane fusion are known for a wide variety of biological processes, most notably for enveloped viruses and intracellular trafficking. However, the molecular mechanisms employed to manipulate the lipid surfaces, the intermediate states sampled by both proteins and lipids, and the precise molecular organization of the fusion machinery remain largely unknown.

SNARE³ proteins are uniquely suited to biophysical characterization of the fusion event. They have a relatively simple architecture consisting of a transmembrane region, a SNARE domain, and at most one other presumably regulatory domain. This simplicity coupled with available in vitro fusion assays (6–8) has allowed a thorough characterization of the regions/residues that are essential for fusion. Furthermore, the machinery necessary for fusion is segregated into two membranes, which allows for the individual manipulation of the fusion components. The binding and zipping of coiled-coil domains derived from SNAREs on apposing membranes, SNAREpin assembly, provides the energy necessary to drive membrane fusion. This assembly process also includes at least one partially assembled intermediate in which the most membrane distal portions of the coiled-coil interact but zipping is incomplete (9–11). The extent of assembly and the degree of reversibility for this, or these, intermediates remains unknown.

We have previously used peptides derived from the SNAREs to isolate and describe an intermediate assembly stage during the neuronal SNARE-mediated fusion of reconstituted liposomes (9). Here we combine the use of SNARE-targeted fusion inhibitors and a battery of cone and inverted cone-shaped lipids

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Physiology and Cellular Biophysics, Columbia University, 1150 Saint Nicholas Ave., New York, NY 10032. Tel.: 212-851-5569; Fax: 212-851-5570; E-mail: tm2176@columbia.edu.

2 Present address: Genomics Core Facility, Dept of Molecular Biology, Memorial Sloan Kettering Cancer Center, 430 E. 67th St., New York, NY 10021.

3 The abbreviations used are: SNARE, soluble NSF attachment protein receptors; IC-lipid, inverted cone-shaped lipid; VAMP, vesicle-associated membrane protein; OA, oleic acid; BSA, bovine serum albumin; FRET, fluorescence resonance energy transfer; PC, phosphatidylcholine.
Lipidic Antagonists to SNARE-mediated Fusion

to characterize the extent of complex formation in the SNARE-pit when lipid membrane curvature becomes limiting.

EXPERIMENTAL PROCEDURES

Materials—Acyl-CoAs and oleic acid were purchased as powder from Sigma. Myristoyl-CoA displayed lot-dependent activities, including one preparation that activated fusion (lot number 22K70301); therefore only the several lots that inhibited fusion were used in this manuscript. Phospholipids were purchased in CHCl₃ from Avanti. Peptides derived from the membrane-proximal portion of the SNARE domain on VAMP2 were synthesized by the Microchemistry Core Facility of the Memorial Sloan-Kettering Cancer Center. The VAMP2-specific antibody 69.1 was purchased from synaptic systems.

Protein and Liposome Preparation—An N-terminally deleted syntaxin 1A, in which the first 150 amino acids were removed (comprising the whole of the N-terminal regulatory domain) was co-expressed with His₆-SNAP-25 and purified as described (6). t-SNARE complex containing His₆-SNAP-25 and syntaxin 1A lacking the transmembrane domain (Tsol), VAMP2-His₆, and His₆-VAMP2 lacking the transmembrane domain (Vsol) were each expressed and purified as described (9).

Fusion proteins of SNAREs with N-terminal fluorescent protein tags were generated as follows. ECFP-VAMP2 was cloned into the NcoI-XhoI sites on pET14b to form pYDQ1, which codes for a protein with a C-terminal His₆ tag. The coding sequence begins with the first methionine of enhanced cyan fluorescent protein (ECFP) and contains the entire sequence of the fluorescently labeled lipids. Because the bulk of the volume in the assay derives from t-SNARE liposomes (45–40 Å², a typical 35-nm liposome will have ~9000 lipids and ~11 t-SNAREs or ~90 v-SNAREs. Botulinum B and tetanus toxin light chains were bacterially expressed from plasmids pBN13 and pO7-7, respectively, and purified via C-terminal His₆ tags.

Lipid Mixing Fusion Assay—The lipid mixing assay is a measure of fluorescence dequenching from NBD (nitrobenzoxadiazole)/rhodamine lipid pairs concentrated in the “donor” liposome population and diluted after fusion with “target” liposomes. The assay was run on a Spectramax plate reader essentially as described (9). t-SNARE liposomes were preincubated with buffer A (25 mM HEPES, pH 7.4, 100 mM KCl, 10% glycerol), Vsol, peptides, and/or lipidic molecules including the acyl-CoAs and lysolipids for 7 min at 37 °C prior to addition of the v-liposomes and initiation of the assay. Acyl-CoAs and oleic acid (OA) were prepared as aqueous suspensions in buffer A and added as <10% of the assay volume. Lyso-lipids were prepared as an emulsion in ethanol by bath sonication and added as ~5% of the assay volume. Fatty acid-free BSA (Sigma) was prepared in buffer A immediately before use. The addition of material during an assay (buffer A, BSA, Vsol) sometimes caused a temporary decrease in the fusion rate caused by temperature fluctuations in the plate reader. In addition, when BSA was added to samples containing inhibitory lipidic molecules, an immediate, moderate decrease in fluorescence was routinely observed resulting from the sudden removal of the lipidic antagonists and consequent concentration (and quenching) of the fluorescently labeled lipids. Because the bulk of the volume in the assay derives from t-SNARE liposomes (45 μl versus 5 μl of v-SNARE and 5–10 μl of other compounds), we typically diluted the acyl-CoA into the t-liposome fraction. However, we also conducted numerous experiments in which the acyl-CoA was premixed with both the v- and the t-liposomes and observed identical results.

Acyl-CoAs inhibit fusion by wild-type syntaxin 1A (data not shown) just as effectively as fusion by syntaxin 1A lacking the auto-inhibitory N-terminal domain (14). We have used the N-terminal deletion variant throughout this manuscript, except where otherwise noted.

The results are presented as percentages of detergent signal, which is a normalization of the fusion-dependent fluorescence to the maximal fluorescent signal elicited with 0.4% dodecyl-maltoside (Roche Applied Science) (8).

Fusion rates were calculated from the maximal slope of the assay rather than the initial slopes to avoid effects from the occasional temperature-dependent lag. Inhibitory and stimulated rates were normalized to control samples (samples without any added activating or inhibiting substances) after first subtracting the background drift observed in Vsol-inhibited samples to give: (experimental rate − background rate)/(control rate − background rate) × 100.

Fluorescence Resonance Energy Transfer (FRET) Assay—Liposome assays were performed as described for the lipid mixing assay above, except that the liposomes contained no fluorescent lipids. Instead, the proteins were directly modified with ECFP (VAMP2) or EYFP (SNAP-25). In both cases, the fluorescent...
proteins were added at the N termini of the SNARE proteins. When either of these proteins was tested in a lipid mixing assay, we could still observe an increase in fluorescence over time, suggesting that the N-terminal tags did not prevent fusion. The quantitative analysis of the fusion extent in these cases, however, was limited because of the high background fluorescence imposed by the fluorescent protein tags.

The samples were excited with 433-nm light at 37 °C in a PerkinElmer Life Sciences luminescence spectrometer (LS50B). Full emission spectra were recorded (for example, see Fig. 6A) after a 2-h incubation, from which the peak values of ECFP and EYFP emission were determined. Liposomes mixed in the presence of Vsol (to inhibit SNARE complex formation) elicited a spectrum that is identical to liposomes that are solubilized and proteolyzed (to liberate the fluorescent tag and thus eliminate any chance of FRET), indicating that the Vsol control is an accurate indicator of the No FRET base-line condition.

FRET signal was measured as the change in the ratio of emission at 525 nm versus 480 nm upon protein complex formation. For time-dependent changes in FRET, the fluorescence intensity at these two wavelengths was recorded every minute in a Spectramax plate reader. The absolute intensity values differed somewhat between the Spectramax and PerkinElmer Life Sciences fluorimeters, such that the 525/480 ratio in any given experiment can only be compared with other experiments on the same machine.

RESULTS

Acyl-CoA-mediated Inhibition of SNARE Fusion—Inverted cone-shaped lipids (IC-lipids) are amphiphilic molecules having a large hydrophobic head group area as compared with the cross-sectional area of their hydrophobic tails. When applied to the outer leaflet of fusing membranes, they inhibit the type of hourglass curvature thought to be reminiscent of the stalk and fusion pore structure (Fig. 1, A and B). As such, they provide an avenue for blocking membrane fusion at or just before the formation of the stalk. To investigate intermediates in SNARE-mediated fusion, we screened a variety of IC-lipids for their ability to inhibit fusion in our reconstituted liposome assay.

All of the compounds that elicited some degree of inhibition are listed in Table 1. Lysophosphatidylethanolamine (lyso-PE) molecules having acyl-chains of either 14 or 16 carbons resulted in only a moderate level of inhibition. We could also consistently observe some inhibition by both lyso-PC and lysophosphatidylserine (lyso-PS); however, we avoided further experimentation with these molecules because of their greater propensity toward liposome solubilization at high concentrations (15). In a similar study, Chen et al. (15) recently showed that lyso-PC could elicit ~50% reduction in the rate of lipid mixing at concentrations between 100 and 300 μM. At higher concentrations, they observed an increase in fluorescence dequenching, signifying solubilization. We observed similar phenomena, with some compounds (like lyso-PC) exhibiting a plateau in inhibitory concentration. Indeed, a significant difficulty in characterizing IC-lipid behavior with our system is that the total lipid concentration is very high (~1.5 mM), requiring high concentrations of inhibitor. Not surprisingly, detergent molecules like Tween 20 were also unusable. One class of molecules that worked well and did not result in liposome lysis is the family of fatty acyl-CoAs (Fig. 1C).

Acyl-CoAs are the single hydrocarbon chain substrates used in fatty acid synthesis and protein acylation. Within the cell, the free acyl-CoA population is very low (0.1–200 nM) (16), and their known effects on trafficking or fusion are limited to their

**TABLE 1**

| Inverted cone-shaped lipid | Maximal inhibition of fusion | IC<sub>50</sub> μM |
|---------------------------|----------------------------|------------------|
| Lauryl-CoA (C12)          | 14 ± 3                     | 167              |
| Myristoyl-CoA (C14)       | 79 ± 11                    | 137 ± 34         |
| Palmitoyl-CoA (C16)       | 99 ± 5                     | 151 ± 17         |
| Stearoyl-CoA (C18)        | 101 ± 6                    | 129 ± 11         |
| Lyso-PC (egg yolk)        | 36 ± 21                    | 67               |
| Lyso-PE (C14-PE)          | 30 ± 19                    | 90               |
| Lyso-PE (C16-PE)          | 28 ± 24                    | 65               |

**FIGURE 1.** Membrane intermediates during fusion. A, a simplified cartoon of the stalk model of membrane fusion, in which the contacting (outer) leaflets of the two membranes fuse first to form a hemi-fused stalk structure. Rupture of the stalk leads to a fusion pore. In each of these intermediate structures, the membranes are believed to be highly curved. Eventually the pore expands, and the vesicle membrane completely collapses into the target membrane. B, magnification of the fusion pore (boxed in A). The formation of highly curved regions of the fusion pore or stalk structure is enhanced by the addition of different lipidic structures on the outer (cone-shaped, black triangles) or the inner (inverted cone-shaped, white triangles) leaflets of the membranes. C, structures of the inverted cone, palmitoyl-CoA, and the cone, oleic acid.
direct manipulation of the acylation state of various proteins (17, 18). Structurally, however, the acyl-CoAs resemble other IC-lipids, and their reduced propensity to lyse liposomes makes them an especially attractive tool for our in vitro analysis. To test their effects on fusion, acyl-CoAs were preincubated with t-SNARE liposomes for 7 min prior to the addition of v-liposomes and initiation of the assay. Acyl-CoAs (200 μM) inhibit SNARE-mediated lipid-mixing in an acyl chain length-dependent manner (A). % Detergent Signal indicates the fraction of fluorescent dequenching observed because fusion as compared with the maximum obtained in the presence of 0.4% dodecylmaltoside. B, the acyl-CoAs inhibit fusion in a concentration-dependent manner with half-maximal fusion occurring at ~180 μM (bottom axis) or roughly 10 mol % total lipid (top axis). Each measurement is shown in triplicate, and the plot is representative of three experiments. C12 is lauryl-CoA, C14 is myristoyl-CoA, C16 is palmitoyl-CoA, and C18 is stearoyl-CoA.

The apparent IC_{50} of inhibition is approximately the same for all three chain lengths and similar even for two lengths of lysophosphatidylethanolamine (lyso-PE) (Table 1). Interestingly, previous studies focusing on lyso-PC demonstrated an inhibitory effect when the lipid antagonist was present at >10 mol % total lipid. In comparison then, an apparent IC_{50} of ~150 μM would be predicted in our 1.5 mM total lipid liposome assay, very similar to the measured values.

A hallmark of IC-lipid-mediated inhibition is reversibility by removal of the IC-lipids. BSA has as many as five independent sites for binding free fatty acids or fatty acyl moieties (21, 22); therefore we tested whether adding BSA during a fusion reaction could overcome the inhibition. The addition of BSA to a C16-inhibited fusion reaction caused an increase in the rate of fusion within 3 min of BSA application (Fig. 3A). After BSA addition, the rate of fusion in the inhibitor-containing sample was greater than at any point during the inhibitor-free control assay, suggesting that liposomes bound through partially assembled trans-SNARE complexes had accumulated in the presence of the inhibitor (Fig. 3). BSA had no effect on control (inhibitor-free) assays. Importantly, the acyl-CoAs are not activating an additional subpopulation of liposomes. The final level

FIGURE 2. Acyl-CoA is a concentration-dependent, reversible inhibitor of SNARE fusion. Acyl-CoAs were preincubated with t-SNARE liposomes for 7 min prior to the addition of v-liposomes and initiation of the assay. Acyl-CoAs (200 μM) inhibit SNARE-mediated lipid-mixing in an acyl chain length-dependent manner (A). % Detergent Signal indicates the fraction of fluorescent dequenching observed because fusion as compared with the maximum obtained in the presence of 0.4% dodecylmaltoside. B, the acyl-CoAs inhibit fusion in a concentration-dependent manner with half-maximal fusion occurring at ~180 μM (bottom axis) or roughly 10 mol % total lipid (top axis). Each measurement is shown in triplicate, and the plot is representative of three experiments. C12 is lauryl-CoA, C14 is myristoyl-CoA, C16 is palmitoyl-CoA, and C18 is stearoyl-CoA.

FIGURE 3. Semi-assembled SNARE complexes accumulate during acyl-CoA inhibition. A, palmitoyl-CoA (200 μM) inhibition is reversed by the addition of BSA (190 μM). Palmitoyl-CoA was present at start of experiment (squares); BSA was added at 62 min (filled symbols). B, normalized fusion rates in the presence of 200 μM inhibitor (black bars) or inhibitor followed by 190 μM BSA addition (white bars) are plotted. Inset, fusion rates are normalized to maximal BSA-induced fusion rate in the presence of constant (200 μM) stearoyl-CoA and varying concentrations of BSA.
of fluorescence dequenching is the same in samples with and without IC-lipids, suggesting that the same numbers of liposomes proceed through fusion.

For convenience, most of our experiments were conducted by adding acyl-CoA to liposomes immediately before running the fusion assay. To establish that acyl-CoA was working within the plane of the membrane (rather than from an excess in solution), we preincubated liposomes with acyl-CoA and then refloated those liposomes over a nycodenz gradient to remove free IC-lipid. These treated liposomes were much less fusogenic than untreated and rapidly fused after BSA treatment, indicating that the acyl-CoA is absorbed into and active upon the membrane surface (data not shown).

The recovery rate is dependent upon the incubation time of the inhibitor lipid prior to BSA addition. For stearoyl-CoA-inhibited reactions, this rate of recovery continued to increase up to 45 min, indicating that new complexes continue to form over that time period (data not shown). The concentration of BSA needed to elicit 80% of the maximal recovery from 200 μM C16 was 72 μM (Fig. 3B, inset); therefore each fatty acid–free BSA was likely binding two or three molecules of acyl-CoA. The burst of fusion following BSA addition lasted only ~10 min, after which the rate of fusion matched the control sample. Thus, all accumulated trans-SNARE-bound liposomes likely completed fusion within 10 min. From these collected results, we can conclude that a rate-limiting step for SNARE-mediated fusion of liposomes involves formation of the initial trans-SNARE complex, possibly including the organization of a higher order structure, prior to the development of a highly curved membrane surface.

**Accumulated trans-SNARE Complexes Are Only Partially Assembled—trans-SNARE complexes form when the coiled-coil domains from SNAREs anchored in separate membranes come together. The precise organization of these complexes and the intermediate states sampled during their assembly are largely unknown. A popular hypothesis posits directed zippering of the coiled-coil domains of v- and t-SNAREs from their membrane distal N termini to their membrane proximal C termini to pull the membranes together. A variety of in vivo and in vitro results have been interpreted to support this model (9–11), but it remains controversial. For example, from single-molecule FRET experiments, a one-step assembly was proposed (23). Two similar electrophysiological studies of exocytosis from SNARE coiled-coil domain mutants also failed to produce a consensus model, with one group supporting the zipperping hypothesis (24), whereas a second group favored a largely stochastic assembly process followed by a partial disassembly during pore-opening/expansion (25). Thus, the trans-SNARE state might be expected to have any of a number of SNARE surfaces exposed in our trapped intermediate. We next explored whether the lipid-inhibited complexes exhibited a degree of incomplete assembly (or partial disassembly) by utilizing a number of probes of SNARE-mediated fusion described previously.

There is only one other example of a stable fusion intermediate for reconstituted SNARE liposomes. When liposomes are incubated together at 4 °C, SNAREpin formation is kinetically trapped (Ref. 8 and Fig. 4A). Warming to 37 °C results in a burst of fusion as the preformed trans-SNARE complexes proceed through to fusion. These preformed complexes are largely resistant to subsequent addition of Vsol, indicating that they have assembled past the point of reversibility (because Vsol will occupy the same space as liposome-embedded VAMP2).

When acyl-CoA-inhibited reactions are challenged with Vsol early in the assay, subsequent recovery by BSA is completely abolished (Fig. 4B). Surprisingly, Vsol is equally effective even when added just minutes before BSA (Fig. 4C), demonstrating that the preassembled complexes accumulated over the previous hour are fully susceptible to Vsol inhibition. This suggests that these complexes are loosely zipped/assembled and that the t-SNARE is still accessible to the competing soluble VAMP2. Likewise, the assembled complexes remain sensitive to Tsol, demonstrating similar accessibility of the loosely zipped VAMP2 (data not shown). Thus, although both 4 °C and acyl-CoA stabilized intermediates appear to have progressed...
beyond the rate-limiting step of the assay, the 4 °C blocked complex is closer to completion.

In vivo, synaptic vesicles that respond on a subsecond time scale to calcium influxes make up the readily releasable pool. The existence of a preformed SNARE complex has been inferred from the observation that this pool is resistant to neurotoxins that bind the membrane-distal region of the VAMP2 SNARE domain but remain vulnerable to membrane-proximally targeted toxins (10). We have used the same strategy to assess the extent of SNARE complex formation in the presence of a lipidic block. Tetanus and botulinum B toxins bind at opposite sides of the central conserved arginine (R56) but cleave after the same glutamine (Glu76) (Fig. 5A). Bacterially expressed recombinant light chains from the membrane-distally targeted tetanus toxin and the membrane-proximally targeted botulinum B toxin each completely inhibited fusion when preapplied to VAMP2 liposomes (Fig. 5B). The application of either toxin downstream of an acyl-CoA block did not prevent or significantly alter subsequent BSA rescue of the fusion reaction (Fig. 5C). Even when the toxins were present at a molar excess over total SNARE, no effect on fusion recovery was observed (data not shown). Thus, the SNARE complex formed in the presence of acyl-CoA is completely resistant to toxin cleavage.

We previously showed that peptides derived from the VAMP2 SNARE domain can be used to evaluate the extent of zipping (9). A peptide corresponding to the VAMP2 amino acids 29–56 (V29–56), making up roughly the N-terminal half of the VAMP2 SNARE domain, inhibits fusion prior to v-/t-SNARE complex formation. The C18-inhibited complexes are insensitive to V29–56 (Figs. 5D) (10), consistent with a state of partial assembly involving the N-terminal halves of the SNARE domains.

A peptide derived from the C-terminal half of VAMP2 and comprising amino acids 57–92 (V57–92) accelerates fusion by inducing conformational changes over the membrane-proximal half of the t-SNARE complex, syntaxin 1A/SNAP-25 (9). We next asked whether stimulating these conformational changes could overcome the lipidic block. Fusion in the pres-
ence of V57–92 is still largely inhibited by C18 (Fig. 5E). Removal of the block, with BSA, again leads to a burst of fusion, and Vsol still abrogates the BSA-mediated relief. Thus, even with the stimulatory peptide present, the trans-SNARE complex cannot fully assemble if the membranes cannot bend.

**FRET Indicates Accumulation of trans-SNARE Assemblies during Lipidic Block**—The burst of fusion following BSA-mediated removal of the lipidic inhibitors indicates the accumulation of SNAREs at an intermediate state of assembly. These complexes are resistant to challenge with neurotoxins or the N-terminal VAMP2 peptide, suggesting that the epitopes for these reagents are buried in a multi-protein complex. Given the simplified composition of our reconstituted assay, these complexes must either be v-/t-SNARE assemblies (trans-SNAREs) or homomultimers of v- or t-SNAREs (most probably in the plane of the liposome membrane). A straightforward way to establish actual contact between v- and t-SNAREs is FRET.

We next generated v-SNAREs with fluorescent protein domains at the N terminus of VAMP2 (ECFP) and t-SNAREs composed of full-length syntaxin 1A and a fluoroscence modified SNAP-25 (with an N-terminal EYFP) to monitor the time-dependent protein association via FRET. In the presence of the stimulatory V57–92 peptide, FRET between t- and v-liposomes is highly efficient (Fig. 6A). We chose to work under these conditions, which present the most stringent test of SNARE assembly during the lipidic block.

FRET between v- and t-liposomes develops with or without C18 (Fig. 6B), indicating the accumulation of trans-SNARE complexes in both cases. FRET develops quickly, faster than the lipid-mixing signal of comparable experiments, consistent with previous observations that interactions of the N termini of the SNAREs is fast in our liposome assay (9). The IC-lipid-induced block to lipid mixing must therefore be downstream of at least partial trans-SNARE assembly. Interestingly, after 1 h, the C18-containing samples exhibit slightly less total FRET (~22%) than those without IC-lipid. The subsequent addition of BSA leads to a burst of FRET, eliminating most of this difference; however, the magnitude of this burst is much smaller than that observed for lipid-mixing, suggesting that most of the available SNAREs had already entered into trans-SNARE complexes during the block. What does the burst signify then? A likely possibility is that this signal arises from cis-SNARE complexes forming inside the liposomes, as they complete fusion, and as such, would represent a real time content mixing assay. Application of Vsol prior to BSA blocks the burst. Vsol does not cause a decrease in FRET, however, demonstrating that the preassembled SNARE complexes are not fully reversible. Rather, these complexes contain an exposed epitope, such that Vsol association prevents the final stages of assembly without completely displacing the membrane-embedded VAMP2.

**Membrane Deformation Limits the Rate of SNARE-mediated Fusion**—The accumulation of partially zipper SNARE intermediates and the burst of fusion following the removal of IC-lipids indicate that a rate-limiting step is the formation of these intermediates. The suggestion that these complexes are reversible in the presence of acyl-CoA indicates that completion of assembly is dependent upon the passage through a highly curved membrane intermediate. If assembly is directly affected by the membrane curvature, and assembly is rate-limiting, we expect that factors that enhance membrane curvature will enhance assembly and thus accelerate fusion. To test this prediction, we introduced cone-shaped lipid agonists to our fusion reaction.

The cone-shaped lipid OA stimulates fusion in a concentration-dependent manner (Fig. 7). The half-maximal activation is observed at 140 μM OA or ~9 mol % of the total lipid, similar to the IC_{50} values of the various IC-lipids. OA stimulation is completely blocked by BSA (Fig. 7B), and a titration reveals that each BSA binds ~3 molecules of OA (data not shown). OA enhances the rate of fusion by as much as 400%, indicating that the energetic barrier imposed by straining to curve the membrane is also acting as a rate-limiting barrier. Higher levels of activation could not be observed because higher concentrations...
Lipidic Antagonists to SNARE-mediated Fusion

A mechanistic description of the membrane fusion event must include intermediates in the process and the respective roles played by lipids and proteins. To that end, we have characterized a novel class of IC-lipid inhibitors, the acyl-CoAs, which we have applied to the fundamental question of SNARE-mediated fusion. Every characterized fusogenic system, whether protein-mediated or purely lipidic, is sensitive to the addition of inverted cone lipids. It would then have been very surprising if SNARE-mediated fusion proved the exception (26). The effects of IC-lipids demonstrate the central role of membrane curvature in the fusogenic process and provide evidence that the membrane intermediates, to the extent that they can be known, are similar across all fusogenic fields. The acyl-CoAs should prove to be useful fusion antagonists over a wide range of fusogenic systems, including and especially those in which high concentrations of lipid are necessary.

One caveat with IC-lipid studies is the possibility that the observed effects are not on membrane curvature but instead are a direct effect on the fusogenic protein itself (4). It is very difficult to rule out a direct lipid-protein interaction, but two lines of evidence suggest that what we are observing is a membrane curvature modification. First, the IC_{50} concentrations across a wide range of potential IC-lipids are similar, indicating that mol % of total lipid is a critical driving force. Thus, when C18-acyl-CoA is added at 0.7 mol % of total lipid, there is almost no effect on fusion, even though the C18-acyl-CoA is in a 10-fold excess over t-SNARE. Second, when the acyl-CoA is restricted to the outer leaflet, the inhibitory activity can be overcome by including an equal concentration of a cone-shaped lipid.

The single best characterized attribute of the SNARE family is the ability to form highly stable four-helix coiled-coils, the cis-SNARE complex (27–29). The directed assembly of these complexes gives rise to membrane fusion in a variety of in vitro assays (6–8, 30), confirming the essential role of these proteins in fusion. We have now demonstrated that the assembly of SNAREs into an Vsol-resistant complex requires extensive membrane curvature and possibly fusion. Furthermore, the rate of fusion in our reconstituted system is limited by both the rate of SNAREpin assembly and by the rate at which membranes can be induced to curve, demonstrating that the two processes are tightly linked.

What might be the physiological significance of this intermediate? The readily releasable pool of synaptic vesicles is pre-docked to the synaptic membrane. When the SNARE disassembly capacity of NSF is reduced, more readily releasable vesicles accumulate (31), indicating that SNAREs are an essential determinant of the size of the pool. Furthermore, fusion from this pool of vesicles is completely blocked by the action of SNARE-proteolyzing neurotoxins and, in particular, those neurotoxins targeting the membrane-proximal region of the SNARE domain (10). Taken together, these results indicate a complex that is at least semi-assembled and is often modeled as being on the brink of fusion, perhaps having already restructured the membrane. Reversibility of this complex was first postulated by Scheller and co-workers (32), who suggest that in the “primed” state, SNAREs may be in an assembly/disassembly equilibrium, in anticipation of an appropriate trigger. The absence of toxin sensitivity in our assay suggests that SNAREs can zip up significantly beyond the predocked assembly stage of synaptic membranes and still present a binding site for Vsol. Our population FRET results do not lend any support to the notion of a fully reversible complex, but ultimately single-molecule FRET studies will be needed to capture transient uncouplings (33). By coupling final assembly of the SNAREpin to membrane fusion, the trans-SNARE complex can remain a malleable dynamic structure right up until the last moments at which the membrane becomes committed to lipid mixing.

In some instances fusion from the readily releasable pool does not lead to complete membrane mixture, culminating instead in a reversible fusion pore. These kiss and run events involve a transient fusion pore with a lifetime sufficient to release most or all of the vesicle contents. Subsequently, the vesicle “unfuses,” pinching off to be available for reloading and another round. An intriguing mechanistic question sur-
rounding these events remains: how the vesicle becomes “unSNAREd.” Previous in vitro evidence has demonstrated that trans-SNARE pairs are not substrates for NSF recycling (34), and in any event the time scale of kiss and run appears to be too short for NSF binding and SNARE unfolding. Thus, an important avenue for further study is whether SNARE complex assemblies remain dynamic up to the point of fusion pore formation, with irreversible cis-SNARE pairs representing the end state of a fully dilated fusion pore.

The ability to preferentially isolate a single intermediate in membrane fusion will open the study of SNARE-mediated fusion to a variety of novel biophysical techniques. In addition, it will be possible to put the role of regulatory proteins, particularly those that activate the fusion complex and provide the calcium-trigger for regulated exocytosis, into context with regards to SNARE activation, SNARE assembly, and membrane curvature.

REFERENCES
1. Kozlov, M. M., and Markin, V. S. (1983) Biofizika 28, 242–247
2. Gingell, D., and Ginsberg, L. (eds) (1978) Problems in the Physical Interpretation of Membrane Interaction and Fusion, pp. 791–833, Elsevier Science Publishers B.V., Amsterdam
3. Tamm, L. K., Crane, J., and Kiessling, V. (2003) Curr. Opin. Struct. Biol. 13, 453–466
4. Gunther-Ausborn, S., Praetor, A., and Stegmann, T. (1995) J. Biol. Chem. 270, 29279–29285
5. Chernomordik, L. V., Vogel, S., Sokoloff, A., Onaran, H. O., Leikina, E. A., and Zimmerberg, J. (1993) FEBS Lett. 318, 71–76
6. Fix, M., Melia, T. J., Jaiswal, J. K., Rappoport, J. Z., You, D., Sollner, T. H., Rothman, J. E., and Simon, S. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7311–7316
7. Hu, C., Ahmed, M., Melia, T. J., Sollner, T. H., Mayer, T., and Rothman, J. E. (2003) Science 300, 1745–1749
8. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998) Cell 92, 759–772
9. Melia, T. J., Weber, T., McNew, J. A., Fisher, L. E., Johnston, R. J., Parlati, F., Mahal, L. K., Sollner, T. H., and Rothman, J. E. (2002) J. Cell Biol. 158, 929–940
10. Hua, S. Y., and Charlton, M. P. (1999) Nat. Neurosci. 2, 1078–1083
11. Xu, T., Rammer, B., Margittai, M., Artalejo, A. R., Neher, E., and Jahn, R. (1999) Cell 99, 713–722
12. Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A., and Tsien, R. Y. (2001) J. Biol. Chem. 276, 29188–29194
13. Scott, B. L., Van Komen, J. S., Liu, S., Weber, T., Melia, T. J., and McNew, J. A. (2003) Methods Enzymol. 372, 274–300
14. Parlati, F., Weber, T., McNew, J. A., Westermann, B., Sollner, T. H., and Rothman, J. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12565–12570
15. Chen, X., Arac, D., Wang, T. M., Gilpin, C. J., Zimmerberg, J., and Rizo, J. (2005) Biophys. J. 90, 2062–2074
16. Faergeman, N. J., and Knudsen, J. (1997) Biochem. J. 323, 1–12
17. Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V., and Rothman, J. E. (1989) Cell 59, 95–102
18. Pfanner, N., Glick, B. S., Arden, S. R., and Rothman, J. E. (1990) J. Cell Biol. 110, 955–961
19. Powell, G. L., Grothusen, J. R., Zimmerman, J. K., Evans, C. A., and Fish, W. W. (1981) J. Biol. Chem. 256, 12740–12747
20. Chernomordik, L. V., Leikina, E., Frolow, V., Bronk, P., and Zimmerberg, J. (1997) J. Cell Biol. 136, 81–93
21. Curry, S., Mandelkow, H., Brick, P., and Franks, N. (1998) Nat. Struct. Biol. 5, 827–835
22. Gelamo, E. L., Silva, C. H., Imasato, H., and Tabak, M. (2002) Biochim. Biophys. Acta 1594, 84–99
23. Zhang, F., Chen, Y., Su, Z., and Shin, Y. K. (2004) J. Biol. Chem. 279, 38668–38672
24. Nagy, G., Kim, J. H., Pang, Z. P., Matti, U., Rettig, J., Sudhof, T. C., and Sorensen, J. B. (2006) J. Neurosci. 26, 632–643
25. Arac, D., Chen, X., Khant, H. A., Ubach, J., Ludtke, S. J., Kikkawa, M., Johnson, A. E., Chiu, W., Sudhof, T. C., and Rizo, J. (2006) Nat. Struct. Mol. Biol. 13, 209–217
26. Rizo, J. (2003) Nat. Struct. Biol. 10, 417–419
27. Fasshauer, D., Eliason, W. K., Brunger, A. T., and Jahn, R. (1998) Biochemistry 37, 10354–10362
28. Poirier, M. A., Xiao, W., Macosko, J. C., Chan, C., Shin, Y.-K., and Bennett, M. K. (1998) Nat. Struct. Biol. 5, 765–779
29. Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) Nature 395, 347–353
30. Schuette, C. G., Hatsuzawa, K., Margittai, M., Stein, A., Riedel, D., Kuster, P., Konig, M., Seidel, C., and Jahn, R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2858–2863
31. Lonart, G., and Sudhof, T. C. (2000) J. Biol. Chem. 275, 27703–27707
32. Chen, Y. A., Scales, S. J., and Scheller, R. H. (2001) Neuron 30, 161–170
33. Bowen, M. E., Weninger, K., Ernst, J., Chu, S., and Brunger, A. T. (2005) Biophys. J. 89, 690–702
34. Weber, T., Parlati, F., McNew, J. A., Johnston, R. J., Westermann, B., Sollner, T. H., and Rothman, J. E. (2000) J. Cell Biol. 149, 1063–1072