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Arachidonic Acid Allows SNARE Complex Formation in the Presence of Munc18

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Summary

SNARE complex formation underlies intracellular membrane fusion in eukaryotic organisms; however, the factors regulating the SNARE assembly are not well understood. The neuronal SNARE complex is composed of synaptobrevin2, SNAP-25, and syntaxin1, the latter being under tight control by the cytosolic protein Munc18. We found that the inhibition of syntaxin1 by Munc18 both in nerve terminals and in defined in vitro reactions can be overcome by specific detergents. This serendipitous finding led us to screen biologically relevant fatty acids, revealing that unsaturated arachidonic and linolenic acids can stimulate Munc18-regulated SNARE complex formation in a direct manner. The direct effect of arachidonic acid on the syntaxin1/Munc18 complex suggests a mechanism for the activation of the SNARE assembly pathway and provides a lead for the further investigation of fatty acids that may regulate SNARE-mediated membrane fusion in eukaryotes.

Introduction

Membrane fusion in neurons and neuroendocrine cells is dependent on vesicular synaptobrevin2 (also known as vesicle-associated membrane protein, VAMP2) and the plasma membrane syntaxin1 and SNAP-25 [1, 2]. The three proteins form the thermodynamically stable, SDS-resistant ternary SNARE complex that drives the fusion of vesicles with the target membrane both in vivo and in vitro [3–7]. This complex consists of four amphipathic α helices: one each from syntaxin1 and synaptobrevin2, and two from SNAP-25 [5]. Strict temporal and spatial control of membrane fusion is essential for neuronal physiology and may involve the direct regulation of each individual SNARE protein [8]. Highly purified neuronal SNAREs freely form the ternary SNARE complex in solution, indicating that regulation of their assembly would require additional cellular factors to prevent uncontrolled fusion of synaptic vesicles [4]. Genetic studies in model organisms revealed that the neuronal protein Munc18, also known as nSec1, and its homologs throughout the eukaryotic kingdom act as major regulators of exocytosis [9–12]. A mechanistic function for Munc18/nSec1 was suggested by studies showing that the cytosolic protein binds tightly to syntaxin1 and "locks" it in a closed conformation, thereby blocking any subsequent SNARE complex assembly [13–16]. Genetic studies, however, argued that Munc18 and its homologs play both negative and positive roles in the processes leading to membrane fusion [11, 17–20]. The impasse between the in vivo and in vitro findings [21] has recently been explained. In *S. cerevisiae* lacking a Munc18 ortholog, Vps45p, its cognate syntaxin—Tlg2p—is drastically downregulated through rapid proteasomal degradation, suggesting that Munc18 proteins act as chaperones for their cognate syntaxins [22]. In addition, in the absence of Munc18, syntaxin1 is retained in intracellular membrane compartments, while Munc18 coexpression results in syntaxin1 transport to the plasma membrane [23, 24]. Reanalysis of the Munc18 null mice confirmed a reduction of syntaxin levels, providing an explanation for the reduced levels of exocytosis observed [17]. It is likely, therefore, that Munc18 transports newly synthesized syntaxin molecules, in an inhibited state, to the sites of exocytosis, where syntaxin would be released from Munc18 inhibition to engage its partner SNAREs–SNAP-25 and, eventually, vesicular synaptobrevin.

Binding of Munc18 to syntaxin1 is of high affinity, and the crystal structure of their heterodimeric complex has recently been solved [14, 25]. This structure revealed that Munc18 envelops the syntaxin1 molecule in such a conformation that the SNARE partners can no longer reach the syntaxin's SNARE amphipathic helix [25]. The key question therefore arises: what molecular mechanisms exist in the neuron to relieve syntaxin1 from the Munc18-imposed inhibition? Several studies centered on the phosphorylation of Munc18 and suggested a role for protein kinase C and cdk5 in the modulation of syntaxin1 binding by Munc18 [26–28]. However, PKC phosphorylation can only occur when Munc18 is free of syntaxin1, arguing against direct disruption of the syntaxin1/Munc18 complex by this enzyme. Similarly, the cdk5 phosphorylation site lies buried at the center of the syntaxin1/Munc18 complex, according to the crystal structure [25], making it impossible for the kinase to reach it when Munc18 is bound to syntaxin1. Munc13 has been shown to bind to the free syntaxin through the same binding site as Munc18, likely regulating the pool of free, activated syntaxin [29]. Therefore, while these factors may regulate the pools of free Munc18 and syntaxin, it is possible that another as yet unidentified factor is required to cause the actual disruption of the syntaxin1/Munc18 complex.

In our study, we probed the availability of syntaxin1 in isolated nerve terminals, also known as synaptosomes, to better understand the regulation of ternary SNARE complex formation. In conjunction with in vitro assays, which employed four highly purified proteins, our synaptosomal experiments indicated that a pool of inhibited syntaxin1 is under direct control by Munc18 and that this Munc18-imposed control can be overcome by specific detergents. Further analysis of biologically important fatty acids revealed that arachidonic acid, a soluble messenger commonly released from the phospholipid membrane through the action of phospholipases, fulfils such a role with a potency in the micromolar range. Our data provide a mechanism for

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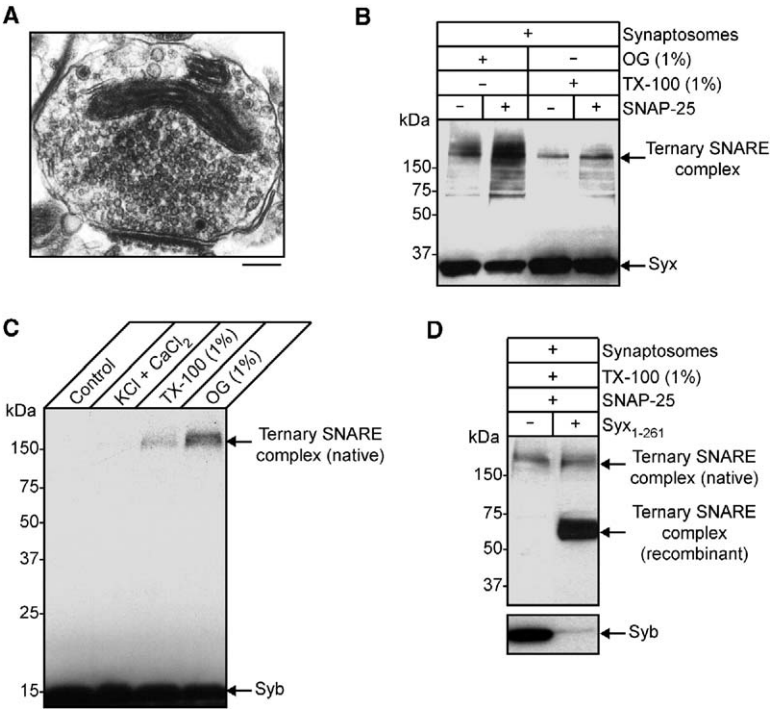


Figure 1. Analysis of SNARE Complex Formation in Rat Brain Synaptosomes

(A) An electron micrograph image of an isolated synaptosome. The scale bar indicates 150 nm.

(B) Addition of an excess of SNAP-25 to synaptosomes in the presence of 1% OG, but not 1% Triton X-100, leads to a dramatic increase in SNARE complex formation in a 30 min reaction. Western immunoblotting was performed with an anti-syntaxin1 antibody.

(C) Differential effect of 1% OG and 1% Triton X-100 on the ability of synaptic SNAREs to form the ternary complex. Reaction as in (B), but without the addition of exogenous SNAP-25. Stimulation of intact synaptosomes with 30 mM KCl, 2 mM Ca^{2+} produces a very weak SNARE complex signal compared to the SNARE assembly taking place upon dissolution of membranes by either detergent. Western immunoblotting was performed with an anti-synaptobrevin antibody.

(D) Addition of both recombinant syntaxin1 (aa 1–261) and SNAP-25 drives almost all synaptosomal synaptobrevin2 into SNARE complexes. Chemiluminescence in the upper and lower panels was recorded at different exposure times. The ternary complex formed with recombinant Syx_{1-261} migrates faster than the native one in the SDS gel, characteristic of SNARE proteins lacking their transmembrane regions. Western immunoblotting was performed with an anti-synaptobrevin2 antibody.

the activation of the SNARE assembly pathway, suggesting a direct role for fatty acid metabolism in Munc18-regulated SNARE function.

Results

Native, SDS-resistant ternary SNARE complex can be visualized by Western immunoblotting with specific anti-SNARE antibodies [6]. To assess the overall ability of native syntaxin1 to form the ternary SNARE complex in a mass reaction, we added freshly isolated synaptosomes (Figure 1A) to reaction tubes containing an excess of SNAP-25 in the presence of either *n*-octyl-β-D-glucopyranoside (OG) or Triton X-100. We used these two detergents to allow SNAP-25 to gain access to the intracellular syntaxin1 and also to release synaptobrevin from vesicular membranes [4]. The soluble brain-purified SNAP-25 was added to ensure a sufficient quantity to drive as much syntaxin1 as possible into the ternary SNARE complex. Figure 1B shows that addition of an excess of SNAP-25 in 1% OG, but not 1% Triton X-100, resulted in a robust increase in formation of a "slow-migrating" [30], SDS-resistant ternary SNARE complex. This OG-dependent increase in SNARE complex formation was also detectable in the absence of an excess of SNAP-25 (Figure 1B). Next, SNARE complex assembly, in the absence of added SNAP-25, was followed with a monoclonal anti-synaptobrevin antibody that readily detects the ternary SNARE complex [4]. Again, the significant increase in SNARE complex assembly was specific for OG, indicating that one of the SNAREs exhibits differential sensitivity to the two detergents. Interestingly, synaptosome stimulation by the addition of 30 mM KCl in the presence of 2 mM Ca^{2+}, which triggers norepinephrine release [31] (and data not shown), results in a barely detectable increase in SNARE complex formation, probably because only a limited amount of SNARE complex underlies neurotransmitter release in vivo. To probe the overall ability of endogenous synaptobrevin to form the ternary SNARE complex, we added an excess of SNAP-25 alone or together with the recombinant cytoplasmic part of syntaxin1 (aa 1–261) in the presence of Triton X-100. The recombinant syntaxin1 used in this reaction lacked its transmembrane region, and, therefore, the resulting ternary complex migrated as a 60 KDa band in the SDS gel; this is in full agreement with previous observations [30]. The addition of the recombinant syntaxin1 led to an almost full transition of the monomeric synaptobrevin into the SDS-resistant ternary SNARE complexes (Figure 1D), indicating that nerve terminal proteins inhibit neither endogenous synaptobrevin nor the added SNAP-25. Since it is addition of syntaxin that fully allows the SNAREs to form the ternary complex, we concluded that the limited SNARE engagement observed in the synaptosomal experiments may stem from the insufficient level of active endogenous syntaxin1.

Next, we analyzed whether in our preparation of synaptosomes the inhibition of the native syntaxin1 is due to its association with Munc18. It was previously demonstrated by employing chemical crosslinking that the two proteins, in brain membranes or dense vesicles, comigrate in a crosslinked product [32, 33]. Indeed,

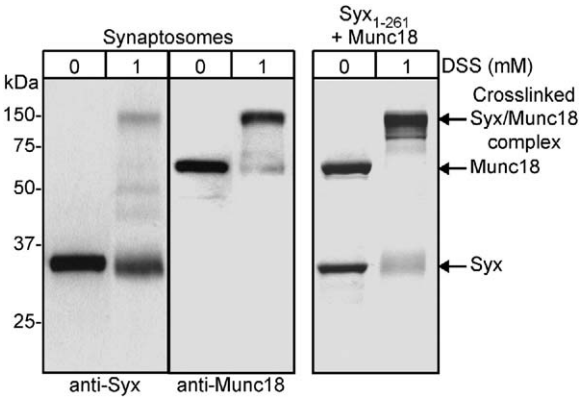


Figure 2. A Pool of Syntaxin1 Is Tightly Associated with Munc18 in Isolated Nerve Terminals

Synaptosomal proteins crosslinked with 1 mM disuccinimidyl suberate (DSS) were analyzed by Western immunoblotting with the indicated antibodies (left). A purified syntaxin1/Munc18 preparation, upon crosslinking with 1 mM DSS, gave rise to a band with similar migration properties to that seen in crosslinked synaptosomes; Coomassie staining (right).

treatment of nerve terminals with 1 mM DSS resulted in the appearance of a band that was crossreactive to both anti-syntaxin1 and anti-Munc18 antibodies and had an apparent molecular mass of 140 kDa (Figure 2). The robust consumption of Munc18 into the crosslinked product with native syntaxin1 indicates that a large proportion of Munc18 in synaptosomes is bound to syntaxin1. This result is supportive of Munc18 residing in a complex containing the native syntaxin1, but does their interaction involve other factors inside the nerve terminals? This question was valid since the sum of the two proteins is below the apparent molecular weight of 140 kDa for the major crosslinked product detected both in the Yang et al. study [32] and here. We purified native Munc18 from Triton X-100-solubilized brain material by using recombinant syntaxin1 as a bait [13] and analyzed the complex by SDS-PAGE, followed by MALDI-TOF mass-spectrometry of individual bands. Only two protein bands representing syntaxin1A and Munc18a were detectable in the purified complex (Figure 2, and data not shown). Significantly, addition of 1 mM DSS to the syntaxin1/Munc18 preparation resulted in efficient transition of both proteins into a 140 kDa band, matching the migration properties of the crosslinked product in synaptosomes. Since structural studies demonstrated an equimolar complex of syntaxin1 and Munc18 [25], the delayed migration of their crosslinked product can be explained by the anomalous behavior of chemically modified proteins [34]. It is thought that intra- and intermolecular covalent crosslinks reduce the amount of SDS bound per protein molecule, leading to slower than expected migration [35]. Regardless of the migration properties of the crosslinked product in 12% Bio-Rad Ready gels, our results are consistent with the idea that a pool of endogenous syntaxin and Munc18 is tightly associated [15].

Does native Munc18 inhibit assembly of syntaxin1 into the SDS-resistant ternary SNARE complex, and is this regulation sensitive to the presence of OG? To address this, a highly pure syntaxin1/Munc18 equimolar complex, with no contamination by free syntaxin1, was required. Since the crosslinking experiment of pure proteins (Figure 2, right panel) indicated the presence of free syntaxin1, we developed a further purification step by using an anti-syntaxin1 (clone HPC-1) monoclonal antibody attached to Sepharose beads. These beads specifically removed the free syntaxin1, whereas syntaxin1 "covered" by Munc18 was not recognizable by this antibody and was collected in the flow-through (Figure 3A). Protein estimation with the Sypro Orange quantitative protein stain confirmed that the molar ratio in the purified syntaxin1/Munc18 complex is 1:1, in agreement with the crystal structure of the syntaxin/Munc18 assembly [25]. The high purity of this syntaxin1/Munc18 preparation allowed a simple, direct assay of syntaxin1 availability for SNARE complex formation by Coomassie staining. Syntaxin1, when mixed with an excess of both SNAP-25 and synaptobrevin, readily formed the SDS-resistant ternary SNARE complex in a 30 min reaction. The consumption of the monomeric syntaxin1 into the ternary SNARE complex was clearly evident (Figure 3B). In contrast, when syntaxin1/Munc18 was added to the other two SNAREs, formation of the SNARE complex was fully inhibited. Evidently, Munc18 acts as a potent inhibitor of ternary SNARE complex formation.

The chance finding in synaptosomes that the inhibition of native syntaxin1 is sensitive to OG led us to test the effect of OG on ternary SNARE complex assembly in our defined reactions. Pure syntaxin1/Munc18 was mixed with the other two SNAREs in the presence of 1% TX-100 or 1% OG and was incubated for 30 min. Triton X-100 had no effect on the Munc18 inhibition of syntaxin1, whereas the presence of 1% OG relieved this inhibition, allowing for ternary SNARE complex formation (Figure 4). Therefore, the highly purified syntaxin1/Munc18 exhibits the same sensitivity to OG as the inhibition of native syntaxin1 in synaptosomes, providing further evidence that this endogenous syntaxin1 is under regulation by Munc18.

Detergents are obviously not the cellular factors that act in vivo, but can they reveal the properties of the endogenous molecule(s) regulating SNARE complex formation? OG consists of a glucose molecule linked to a medium-length hydrocarbon chain. Triton X-100, on the other hand, contains 9–10 oxyethylene units (CH_2CH_2O). Since OG has a clear effect on Munc18-regulated SNARE assembly, we analyzed the pyranoside family of detergents containing different headgroups and different length hydrocarbon chains for their ability to relieve the inhibition of syntaxin1 in our defined assay. The recombinant ternary SNARE complex migrates during SDS-PAGE very close to Munc18 (Figure 4), and, therefore, for the simplicity of presentation, we followed the ternary SNARE complex formation by Western immunoblotting with an anti-syntaxin1 antibody. The pyranoside detergents were used initially above their critical micelle concentrations (CMC), which are inversely proportional to the length of their hydrophobic tail. Figure 5A shows that the SNARE complex formation in the presence of Munc18 exhibited an apparent dependence on the chain length, with

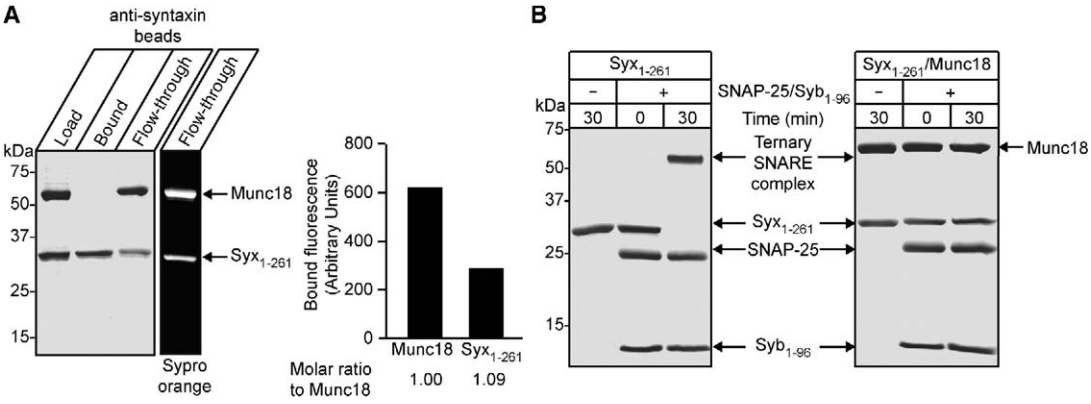


Figure 3. Syntaxin1 in an Equimolar Syntaxin1/Munc18 Complex Is Potently Inhibited for Ternary SNARE Complex Formation

(A) Separation of Munc18 bound syntaxin1 from the free syntaxin1 by using anti-syntaxin1 (clone HPC-1) Sepharose beads, which can bind only the free syntaxin1. Coomassie-stained gel (left). Sypro Orange staining of the flow-through material (center) was quantified and is of a 1:1 molar ratio (right).

(B) Free syntaxin1 exhibits full transition into the SDS-resistant ternary SNARE complex in the presence of an excess of SNAP-25 and synaptobrevin, while syntaxin1 in complex with Munc18 is completely inhibited. Coomassie-stained gels.

long hydrocarbon compounds being ineffective. However, our screen of glucopyranoside detergents at one fixed concentration (0.5%) revealed that it is medium-length hydrocarbon chains that are most potent in relieving Munc18 inhibition (Figure 5B). Importantly, OG was still effective at this submicellar concentration (17 mM), suggesting that the ability to form micelles is not essential, and that the hydrocarbon chains may act as individual soluble molecules to relieve the Munc18 inhibition of syntaxin1. Screening of middle-to-long saturated fatty acids at a concentration of 1 mM revealed that decanoic and dodecanoic fatty acids were most effective (Figure 5C). The latter screen conclusively showed that the glucose headgroup of OG does not play any role in activation of syntaxin1 in the presence of Munc18.

In addition to saturated fatty acids, the cell contains a multitude of biologically active soluble, unsaturated fatty acids [36]. Arachidonic acid, carrying four unsaturated bonds, is a common constituent of phospholipid membranes and is released into the cytosol by the action of a number of phospholipases to regulate cellular events [37]. Since arachidonic acid, due to its unsaturated bonds, can adopt a folded hairpin conformation resulting in a size similar to the middle-chain fatty acids [38] and has been implicated in vesicle fusion [39, 40], we investigated whether arachidonic acid can act directly at the step of Munc18-regulated SNARE complex formation. Remarkably, arachidonic acid triggered SNARE complex formation at submillimolar concentrations (Figures 6 and 7A), thereby exceeding the potencies of the pyranoside detergents and saturated fatty acids. Analysis of other unsaturated fatty acids with varying lengths of carbon chain and degree of unsaturation (depicted schematically in Figure 6) showed that arachidonic acid had the strongest effect among the mammalian fatty acids analyzed. However, linolenic acid, an omega-3 fatty acid of plant origin, exhibited a similar pronounced effect on Munc18-regulated SNARE complex formation. Titration of the arachidonic acid revealed that it was able to elicit SNARE complex formation below 100 μM, in the absence of any other cellular factors (Figure 7A). The fully saturated analog, arachidic acid (C20:0), was not capable of inducing SNARE complex formation, demonstrating an importance for the unsaturated bonds that determine the specific structural properties of arachidonic acid.

It was recently reported that pardaxin, which stimulates arachidonic acid release, leads to increased formation of the SNARE fusion complex in PC12 neuroendocrine cells [41]. The direct effect of arachidonic acid on the activation of syntaxin in the presence of Munc18 (Figure 7A) prompted us to test whether factors that release arachidonic acid from synaptic membranes can upregulate the endogenous syntaxin molecule. We tested the syntaxin ability for SNARE engagement in synaptic membranes treated with agents implicated in arachidonic acid release, in the presence of brain-purified SNAP-25. Following a 20 min incubation at 37°C,

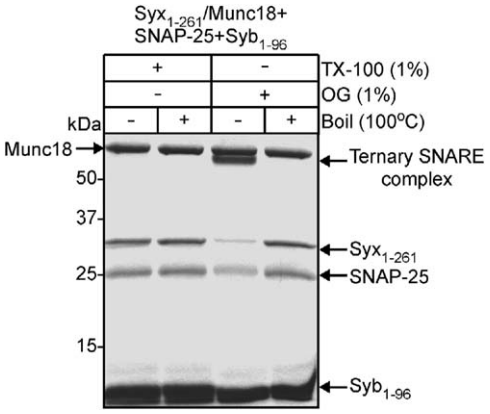
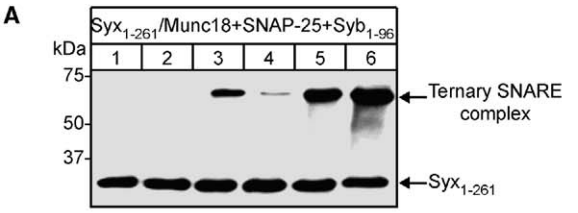
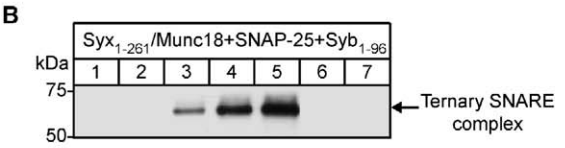


Figure 4. Addition of OG, but Not Triton X-100, Leads to Transition of Munc18-Regulated Syntaxin1 into the SDS-Resistant SNARE Complex

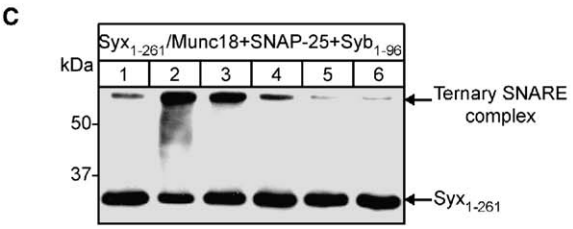
The stoichiometric Syx_{1-261}/Munc18 complex was mixed with an excess of SNAP-25 and synaptobrevin in the presence of 1% Triton X-100 or 1% OG. The 30 min reaction was analyzed by SDS-PAGE, followed by Coomassie staining. Brief boiling of the samples prior to SDS-PAGE results in disruption of the SDS-resistant ternary SNARE complex.



Lane	Detergent	Hydrophobic chain length	Concentration (>CMC) w/v
1	None		
2	n-Dodecyl-ß-D-maltopyranoside	12	0.5%
3	n-Decyl-ß-D-maltopyranoside	10	0.1%
4	n-Dodecanoyl sucrose	12	0.02%
5	n-Nonyl-ß-D-glucopyranoside	9	0.03%
6	n-Octyl-ß-D-glucopyranoside	8	0.8%



Lane	Detergent	Hydrophobic chain length	Concentration w/v
1	None		
2	n-Dodecyl-ß-D-maltopyranoside	12	0.5%
3	n-Decyl-ß-D-maltopyranoside	10	0.5%
4	n-Nonyl-ß-D-glucopyranoside	9	0.5%
5	n-Octyl-ß-D-glucopyranoside	8	0.5%
6	n-Heptyl-ß-D-glucopyranoside	7	0.5%
7	n-Hexyl-ß-D-glucopyranoside	6	0.5%



Lane	Saturated fatty acid	Hydrophobic chain length
1	n-Octanoic acid	8
2	n-Decanoic acid	10
3	n-Dodecanoic acid	12
4	n-Tetradecanoic acid	14
5	n-Hexadecanoic acid	16
6	n-Octadecanoic acid	18

Figure 5. Effect of Members of the Pyranoside Detergent Family and Saturated Fatty Acids on SNARE Complex Formation in the Presence of Munc18

(A) The detergents listed in the table were analyzed at the indicated concentrations, all above CMC, for the ability to overcome the Munc18 inhibition of syntaxin1. Western immunoblot with an anti-syntaxin1 antibody.

(B) The effect on SNARE complex formation of pyranoside detergents at 0.5% concentration. Western immunoblot with an anti-syntaxin1 antibody.

(C) Middle-chain saturated fatty acids (1 mM) are able to overcome Munc18 inhibition of syntaxin1. Western immunoblot with an anti-syntaxin1 antibody.

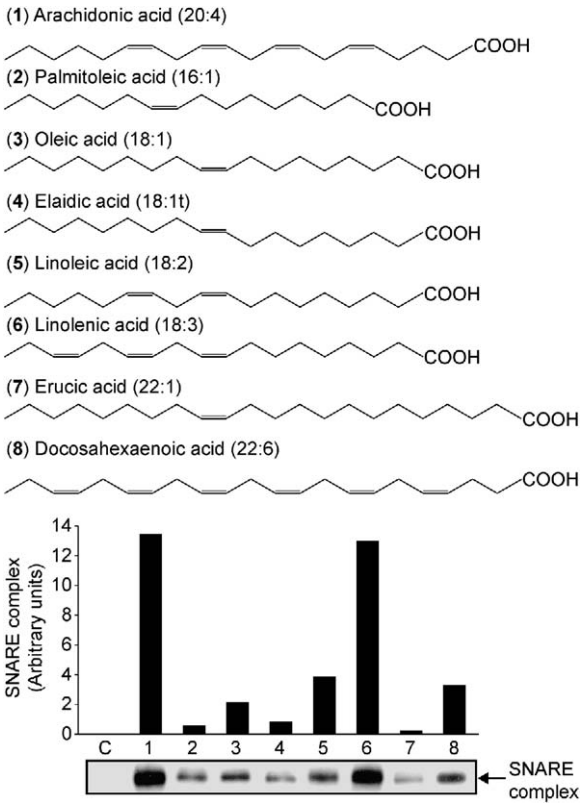


Figure 6. Specific Action of Arachidonic and Linolenic Acid on Munc18-Regulated SNARE Complex Formation

Soluble, unsaturated fatty acids were tested at a 500 μM concentration for their effect on formation of the ternary SNARE complex by Syx_{1-261}/Munc18. Western immunoblot with an anti-syntaxin1 antibody. The control reaction, C, was performed in the absence of fatty acids.

synaptobrevin in all reactions was released from vesicular membranes by the addition of 1% Triton X-100—an efficient way to test syntaxin function in a mass reaction. First, melittin that was implicated in activation of endogenous phospholipases [42] caused a robust increase in the heat-sensitive SNARE complex when added to synaptic membranes (Figure 7B). The faster migration of the SDS-resistant SNARE complex in this biological preparation compared to that in intact synaptosomes (Figure 1) was likely due to a decrease in the content of complexin, a small cytosolic protein that impacts on SNARE complex migration [30]. Further, phospholipase A_2 purified from either bovine pancreas or bee venom was capable of SNARE activation in synaptic membranes, suggesting that release of endogenous fatty acids does indeed play a role in ternary SNARE complex formation. Critically, 20 min treatment of synaptic membranes with 200 μM arachidonic acid was sufficient to increase SNARE complex formation, supporting a role for this ubiquitous unsaturated acid in relieving Munc18 inhibition of the endogenous syntaxin (Figure 7B).

Discussion

Both cytosolic and secretory phospholipases, that release fatty acids from the sn-2 position of membrane

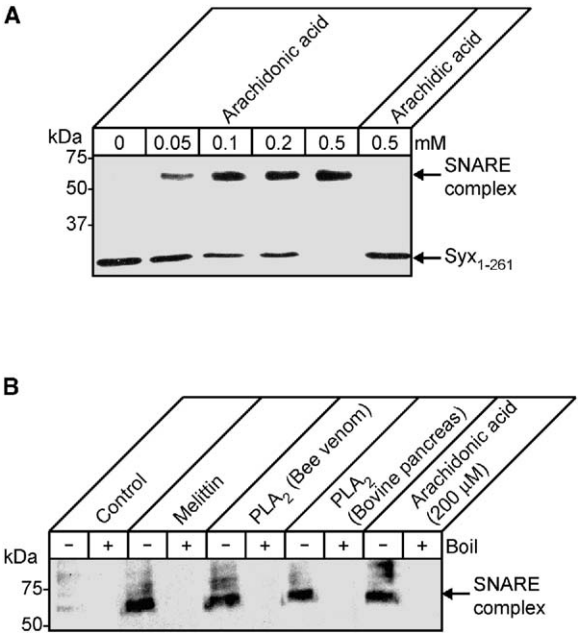


Figure 7. Arachidonic Acid Exhibits a Dose-Dependent Effect on SNARE Complex Formation and Is Capable of SNARE Activation in Synaptic Membranes

(A) Titration of the effect of arachidonic acid on SNARE complex formation. Western immunoblot with an anti-syntaxin antibody. Arachidic acid is unable to relieve the inhibition of syntaxin1 by Munc18.

(B) Treatment of synaptic membranes with agents enhancing arachidonic acid metabolism potentiates SNARE complex formation. Synaptic membranes were preincubated for 20 min in the presence of brain-purified SNAP-25 and the indicated agents. SNARE complex formation was then triggered by the addition of Triton X-100, which releases vesicular synaptobrevin to form the SDS-resistant SNARE complex, allowing for efficient readout of the SNARE assembly. Western immunoblot was performed with an anti-synaptobrevin antibody.

phospholipids have been implicated in the potentiation of neurotransmitter and hormone secretion for more than two decades [37, 43–45]. A wide variety of animal venoms has been found to utilize secretory phospholipase A_2 to cause unregulated secretion [40, 46]. Electrophysiological studies suggested that arachidonic acid, or its metabolites, can enhance synaptic transmission as a retrograde diffusible messenger in hippocampal neurons [47]. The proposal that it is specifically arachidonic acid that acts to stimulate release mechanisms relied originally on pharmacological manipulations but was later substantiated by studies based on direct application of this compound [47–49]. However, uncertainty with regards to any subsequent metabolism of the applied arachidonic acid in experimental tissues, through the well-characterized multiple enzymatic pathways [36], remains. Furthermore, the use of whole cells does not permit definitive dissection of the arachidonic acid action with regard to its possible effectors. Calcium channels were originally proposed as targets for phospholipase action in neurosecretion [50], and a recent study suggested a role for the actin cytoskeleton in taipoxin-stimulated vesicle release [51]. As a second messenger, arachidonic acid likely has several cellular targets to gear a cell for a particular response. The introduction herein of an assay employing a defined set of four pure proteins, all with a critical function in neurotransmission, now uncovers an unanticipated function for arachidonic acid. Our study reveals a fundamental step in the pathway of SNARE-mediated vesicle fusion that is targeted, specifically and directly, by arachidonic acid—activation of syntaxin in the presence of Munc18.

Syntaxin1 resides in the plasma membrane, which is rich in arachidonic acid-containing phospholipids. Since average cellular concentrations of free arachidonic acid have been estimated to be in the micromolar range, the concentrations needed to relieve Munc18 inhibition of membrane bound syntaxin are likely achievable in the immediate vicinity of the sites of phospholipase's action [52]. Importantly, enhancement of neurotransmitter release from synaptosomes [48] correlates well with the concentration required for the release of the Munc18 inhibition of SNARE complex formation (Figure 6). Soluble arachidonic acid can be enzymatically converted into a variety of metabolites such as prostaglandins, leukotriens, endocannabinoids, and others [36, 53, 54]. Although we did not analyze these metabolites in the current study, it is possible that some of these molecules may well exceed the potency observed for arachidonic acid in relieving the Munc18 inhibition of syntaxin1.

The crystal structure of the recombinant equimolar syntaxin/Munc18 complex has been solved, providing insights into how these two proteins interact [25]. Our results show that purified syntaxin1 and Munc18 can be crosslinked into the same product, as is observed in synaptosomes. In addition, the differential effects of detergents on SNARE behavior in synaptosomes are replicated by pure SNARE proteins in the presence of Munc18. These findings provide further evidence that the native, endogenous syntaxin is controlled by Munc18. The ability of detergents to influence syntaxin1/Munc18 function may have important implications for previous Munc18 investigations employing a variety of detergents. For example, we found that CHAPS, a detergent previously used in protein kinase C phosphorylation reactions of Munc18 [26], is itself able to relieve Munc18 inhibition of syntaxin1, although to a lesser degree than OG (data not shown). It is therefore important that future mechanistic studies of SNARE/Munc18 function carefully consider the use of particular detergents.

The vesicle fusion pathway consists of a cascade of protein-protein and protein-lipid interactions. Our assay with the purified SNARE and Munc18 proteins revealed a direct mechanism for the activation of the membrane fusion machinery involving arachidonic acid. It will now be essential to understand exactly how arachidonic acid can help to relieve syntaxin1 inhibition by Munc18 at an atomic level. Arachidonic acid may bind to hydrophobic surfaces on Munc18 or syntaxin1 and destabilize the syntaxin1/Munc18 complex, initiating a sequence of further events. As the local elevation in arachidonic acid is naturally transient, additional factors would be required to prevent the rebinding of Munc18 to syntaxin1 for further SNAP-25 and vesicular synaptobrevin engagement. Free Munc18 can be phosphorylated by protein kinase C and cdk5, preventing its reas-

sociation with syntaxin [26, 27]. Among many potential partners of syntaxin1, Munc13 is known to bind to the same site on syntaxin1 as Munc18 [29] and would be a good candidate to block Munc18 reassociation. These mechanisms would enable engagement of the plasma membrane syntaxin and SNAP-25—a critical step in the SNARE assembly pathway underlying vesicle fusion [55].

Significance

Neuronal communication relies on the release of neurotransmitters from nerve endings. Three SNARE proteins—vesicular synaptobrevin and the plasma membrane syntaxin1/SNAP-25 heterodimer—mediate the release process by forming an α-helical bundle between the two opposing membranes. The sustainable nature of neurotransmission requires tight control over the three proteins, which in solution freely form the ternary SNARE complex. The plasma membrane syntaxin1 is regulated by a cytosolic protein, Munc18, which binds syntaxin1 with high affinity and equimolar stoichiometry. We investigated the status of syntaxin1 in isolated nerve terminals and now show that a pool of endogenous syntaxin1 is in association with Munc18 and is incapable of forming the ternary SNARE complex. The syntaxin/Munc18 interaction exhibited high sensitivity to specific detergents, leading us to test the impact of naturally occurring fatty acids on its regulation. Our investigation of fatty acids revealed that middle-chain-saturated fatty acids can relieve syntaxin from Munc18 inhibition, allowing for formation of the SDS-resistant SNARE complex. Arachidonic acid, a long fatty acid with four *cis* double bonds that can adopt hairpin conformation, was more potent than any of the saturated hydrocarbon chains analyzed. Although the finding of arachidonic acid as a regulator of SNARE function could not have been anticipated, it is in good agreement with the observed effect of this molecule on vesicle exocytosis in a variety of systems. Among other unsaturated fatty acids, ω-3 linolenic acid, commonly used in commercially available health supplements, was able to potently stimulate formation of the ternary SNARE complex in the presence of Munc18. Our observations suggest a novel, to our knowledge, mechanism for the activation of the SNAREs, implicating fatty acid metabolism in the direct control of the membrane fusion machinery.

Experimental Procedures

Preparation of Proteins and Synaptosomes

Plasmids encoding glutathione S-transferase (GST) fusion proteins syntaxin1A (amino acids 1–261) and synaptobrevin2 (amino acids 1–96) were described previously [4]. Recombinant proteins, purified on glutathione Sepharose beads (GE Healthcare), were washed with buffer A (20 mM HEPES [pH 7.0], 100 mM NaCl, 2 mM EDTA), followed by elution with thrombin (Sigma). Eluted proteins were further purified by gel filtration on a Superdex 200 column (GE Healthcare) equilibrated in buffer A. SNAP-25 was purified from bovine brain cortex as described previously [4], followed by removal of *n*-octyl-β-D-glucopyranoside by dialysis into buffer A. Native Munc18 was isolated from bovine brain on GST-Syx_{1-261} glutathione Sepharose beads as previously described [13]. The syntaxin1/Munc18 complex was released from the glutathione Sepharose beads by cleavage with thrombin. To remove free Syx_{1-261}, the thrombin-eluted Syx_{1-261}/Munc18 mixture was incubated for 1 hr at 4°C with an anti-syntaxin1 antibody (clone HPC-1, Sigma) covalently coupled to CNBr Sepharose beads (GE Healthcare). Protein bound to the anti-syntaxin1 beads and the flow-through material were analyzed by SDS-PAGE in 12% Ready gels (Bio-Rad) and Coomassie or Sypro Orange staining. The fluorescent Sypro Orange signal was imaged by using a ChemiDoc XRS system (Bio-Rad) and quantified with Quantity One software (Bio-Rad).

SNARE Complex Formation Reactions

For the analysis of ternary SNARE complex formation in vitro, purified SNARE proteins (1 μg of each) were incubated alone or with the syntaxin1/Munc18 dimeric complex (3 μg) in buffer A for 30 min at 24°C. Detergents (Sigma) were included in the reactions at the concentrations specified. Fatty acids (Sigma) were dissolved in methanol and added at the concentrations indicated; methanol alone was used as a control. Ternary SNARE complex formation was observed by SDS-PAGE by using 12% Bio-Rad Ready gels and either Coomassie staining or Western immunoblotting. The chemiluminescence signal was analyzed with a ChemiDoc XRS system and quantified with Quantity One Software.

Syntaxin1 Regulation in Synaptosomal Preparations

Synaptosomes from rat forebrain were prepared and imaged by electron microscopy as described previously [31, 56]. Norepinephrine release from freshly isolated synaptosomes was measured as described previously [31]. To analyze the availability of syntaxin1 to form the ternary SNARE complex, synaptosomes (10 μg total protein) were added to buffer A containing purified proteins (2 μg) and/or detergents at the indicated concentrations. Following a 30 min incubation at 24°C, the reaction was stopped by the addition of SDS sample buffer and was analyzed by SDS-PAGE and Western immunoblotting by using a polyclonal syntaxin1 antibody or a monoclonal synaptobrevin antibody, clone 69.1 (Synaptic Systems). For the crosslinking experiments, synaptosomes or purified syntaxin1/Munc18 were incubated with 1 mM disuccinimidyl suberate (Pierce) for 30 min at 24°C and were analyzed by Western immunoblotting with anti-syntaxin1 (Sigma) and anti-Munc18 (BD Biosciences) antibodies or Coomassie staining. Note that the Sigma anti-syntaxin1 antibody, clone HPC-1, recognizes the syntaxin molecule in Western immunoblotting and can bind free syntaxin in solution, but is not capable of interaction when syntaxin is associated with Munc18. Synaptic membranes were prepared from synaptosomes hypoosmotically shocked in 20 mM HEPES (pH 7.2). Synaptic membranes were pelleted by centrifugation for 20 min, 10,000 × g, 4°C and were resuspended in 20 mM HEPES (pH 7.2), 100 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP (buffer B). SNARE assembly reactions were initiated by the addition of synaptic membranes (20 μg protein) to buffer B containing 1 μM brain-purified SNAP-25 in the absence or presence of 5 μM melittin (Sigma), 0.1 U bovine pancreas phospholipase A2 (Sigma), 1 U bee venom phospholipase A2 (ICN), or 200 μM arachidonic acid. Following a 20 min incubation at 37°C, the ternary SNARE complex formation was triggered by the addition of 1% Triton X-100, which efficiently releases synaptobrevin from vesicles. The reactions were terminated after an additional 10 min by the addition of an SDS-containing sample buffer, and reaction mixtures were divided into two equal aliquots, with one aliquot being boiled at 100°C for 3 min. The ternary SNARE complex assembly was followed by Western immunoblotting with a monoclonal synaptobrevin antibody, clone 69.1 (Synaptic Systems). All experiments were performed at least three times with identical results.

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