Stable SNARE Complex Prior to Evoked Synaptic Vesicle Fusion Revealed by Fluorescence Resonance Energy Transfer*

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Although it is clear that soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex plays an essential role in synaptic vesicle fusion, the dynamics of SNARE assembly during vesicle fusion remain to be determined. In this report, we employ fluorescence resonance energy transfer technique to study the formation of SNARE complexes. Donor/acceptor pair variants of green fluorescent protein (GFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP) are fused with the N termini of SNAP-25 and synaptobrevin, respectively. In vitro assembly of SNARE core complex in the presence of syntaxin shows strong fluorescence resonance energy transfer (FRET) between the CFP-SNAP-25 and YFP-synaptobrevin. Under the same conditions, CFP fused to the C terminus of SNAP-25, and YFP-synaptobrevin have no FRET. Adenovirus-mediated gene transfer is used to express the fusion proteins in PC12 cells and cultured rat cerebellar granule cells. Strong FRET is associated with neurite membranes and vesicular structures in PC12 cells co-expressing CFP-SNAP-25 and YFP-synaptobrevin. In cultured rat cerebellar granule cells, FRET between CFP-SNAP-25 and YFP-synaptobrevin is mostly associated with sites presumed to be synaptic junctions. Neurosecretion in PC12 cells initiated by KCl depolarization leads to an increase in the extent of FRET. These results demonstrate that significant amounts of stable SNARE complex exist prior to evoked synaptic vesicle fusion and that the assembly of SNARE complex occurs during vesicle docking/priming stage. Moreover, it demonstrates that FRET can be used as an effective tool for investigating dynamic SNARE interactions during synaptic vesicle fusion.

The SNARE† core complex involved in synaptic vesicle fusion consists of target SNARE syntaxin and SNAP-25 and vesicle SNARE synaptobrevin/VAMP (1–3). It has been shown by in vitro binding studies that the SNAREs interact directly and form a tight ternary complex (4–9). The recently solved crystal structure of the SNARE core complex and additional biophysical studies of the core complex demonstrate that the N- and C-terminal helix domains of SNAP-25 participate in the formation of a parallel coiled-coil structure with syntaxin and synaptobrevin (10–13). Limited proteolysis and in vitro binding assay suggest that the two SNAP-25 α-helix domains may act independently and contribute equally to form the SNARE complex with syntaxin and synaptobrevin (10). Upon association with additional proteins, e.g. synaptotagmin, NSF, and α-SNAP, the complex undergoes further conformational changes and disassembly (14).

Although the importance of SNARE complex in neurosecretion and the basic structure of the SNARE core complex have been well studied and established, the molecular mechanisms underlying its involvement in the membrane fusion process are less clear. Contrary to the earlier belief that SNARE assembly attaches vesicles to plasma membrane and the subsequent disassembly of the complex in the presence of NSF and α-SNAP drives membrane fusion, recent findings suggest that the formation of SNARE core complex and membrane fusion is tightly linked and the assembly of SNARE complex may provide the energy needed for membrane fusion (15–17). By using a reconstituted liposome system, the formation of SNARE complex is able to drive membrane fusion (18, 19). Nevertheless, the timing and dynamics of SNARE complex assembly prior to vesicle fusion remain to be determined. By using antibodies specific for SNAP-25 peptides, Xu et al. (20) have presented evidence in chromaffin cells, suggesting that SNARE complex exists in two dynamically distinctive “loose” and “tight” states that both contribute to calcium-dependent exocytosis. In addition, studies using neurotoxins also suggest the presence of stable SNARE complex prior to fusion. Therefore, the assembly of SNARE complex may be a rate-limiting step during neurotransmission.

In the present study, we have used fluorescence resonance energy transfer (FRET) to dissect the dynamics of SNARE assembly in PC12 cells and cultured cerebellar neurons. FRET is a process in which energy from a fluorescent donor molecule is transferred to a fluorescent acceptor without the involvement of a proton. One result is that the fluorescence emission of the acceptor is enhanced by the excitation of the donor molecule. The efficiency of energy transfer is dependent on the distance between the donor and acceptor. In this study, we have used FRET to study the assembly of SNARE complex and synaptic vesicle fusion.

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‡ The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; m.o.i., multiplicity of infection; GFP, green fluorescence protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; C-SN, CFP fused to the N terminus of SNAP-25; SN-C, CFP fused to the C terminus of SNAP-25; Y-SB, YFP fused to the N terminus of synaptobrevin; FRET, fluorescence resonance energy transfer; NSF, N-ethylmaleimide-sensitive factor; PMA, phorbol 12-myristate 13-acetate; NGF, nerve growth factor; ROI, region of interest; GST, glutathione S-transferase; PBS, phosphate-buffered saline.
SNARE Complex Revealed by FRET

N-calcmodulin structure and function, (21), Bcl-2-Bax interaction (22), and synaptic activity in the synaptic spine (23). By using a pair of green fluorescent protein (GFP) variants CF and YFP, we measured FRET between SNAP-25 fused with CF and synaptobrevin fused with YFP under both in vitro and in vivo conditions. Our results suggest that an intermediate and a stable form of SNARE complex are present prior to fusion or during vesicle docking stage.

EXPERIMENTAL PROCEDURES

Mutagenesis and Preparation of GFP Fusion Proteins—A mouse cDNA encoding synaptobrevin-2 was purchased from the I.M.A.G.E. Consortium. cDNA encoding mouse SNAP-25b was a generous gift from Dr. Michael C. Wilson (Scripps Research Institute, San Diego, CA) (24). cDNA encoding rat syntaxin 1A was a generous gift from Dr. Richard Scheller (Howard Hughes Medical Institute, Stanford, CA). pECFP-N1, pEYFP-C1, and pEYFP-C1 were from CLONTECH. A BglII site was introduced at the ATG start codon of synaptobrevin cDNA by site-directed mutagenesis, and the resulting BglII-EcoRI fragment containing the coding sequence of synaptobrevin was ligated into the same sites of pEYFP-C1. Similarly, a KpnI site and an EcoRI site were introduced into the SNAP-25 cDNA at its start or stop site, respectively. A KpnI-ApdI and an EcoRI fragment encoding SNAP-25 were ligated into the corresponding sites of pECFP-N1 and pECP-C1, respectively. Recombinant adenoviruses were prepared using the pAdEasy system as described by He et al. (25). ECFP-SNAP-25 (C-SN), SNAP-25-ECFP (SN-C), and EYFP-synaptobrevin (Y-SB) were cloned into pAdShuttle-CMV. Recombination with pAdEasy-1 backbone plasmid was performed in Escherichia coli strain B75183. Recombinant viruses were obtained by transfection of HEK293 cells, and the viruses were amplified and purified to $\approx 10^{12}$ particles/ml by ultracentrifugation on a CsCl gradient.

Cell Culture and Viral Infection—PC12 cells were plated in 35-mm tissue culture dishes coated with 50 $\mu$g/ml poly-D-lysine, and the cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 5% bovine calf serum. NFG (Life Technologies, Inc.) was added to 50 ng/ml final concentration to induce differentiation. Cerebellar granule cells from 5- to 8-day-old Harlan Sprague-Dawley rats were cultured as described previously (26). Briefly, cells were dissociated from the cerebellum by mechanical chopping and treatment with 0.5% trypsin and 1 mM EDTA for 5 min at 37 °C. The cells were dissociated by trituration and plated onto poly-D-lysine (50 $\mu$g/ml, Sigma)-coated dishes. The cells were cultured in minimum essential medium containing 10% fetal bovine serum and 25 mM KCl. To inhibit the growth of non-neuronal cells, cytosine arabinoside was added 24 h later to 10 $\mu$M. The granule cells were cultured for 4–5 days, and recombinant adenoviruses were added directly to the neuron cultures at an m.o.i. of 200–250. The infected cells were maintained for 48 h before use. All of the cells were maintained in a humidified 5% CO$_2$ incubator with 5% CO$_2$.

Immunoprecipitation and Immunoblot Analysis—For immunoprecipitation, the cells were washed once with 50 mM Na$_2$HPO$_4$, pH 7.4, 100 mM NaCl, and PBS (lysed) with the same buffer containing 1% Nonidet P-40. Immunoprecipitation was performed using a monoclonal antibody against syntaxin (clone HPC-1, Sigma) and protein G-plus agarose (Oncogene Science). The immunocomplex was pelleted by centrifugation at 1,000 $\times$ g for 2 min and washed 3 times with 50 mM Na$_2$HPO$_4$, pH 7.4, 100 mM NaCl, and 0.5% Nonidet P-40. The immunoprecipitated proteins were heated to 80 °C for 3 min in 1% SDS, 10 mM EDTA, 10 mM diithiothreitol, 15% glycerol, 20 mM Tris-HCl pH 6.8, and 0.01% bromphenol blue. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane for immunoblot analysis. The following primary antibodies were used: the HPC-1 monoclonal anti-syntaxin (1:1,000), a goat anti-SNAP-25 antibody (1:10,000) (27), and a goat anti-synaptobrevin (1:5,000) raised against a GST-synaptobrevin fusion protein. Alkaline phosphatase-conjugated secondary anti-mouse and anti-goat antibodies from Sigma were used.

In Vitro SNARE Core Complex Formation and Spectrofluorometer Assay—COS-7 cells were infected with recombinant adenoviruses to express C-SN, SN-C, and Y-SB. The cells were lysed with binding buffer containing 20 mM Hepes, pH 7.4, 100 mM KCl, 2 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. GST-Syntaxin bound to glutathione-agarose beads were incubated with C-SN/Y-SB or SN-C/Y-SB for 3 h at room temperature. Beads were then washed three times with the binding buffer, and the bound SNARE complexes were eluted with 5 mM reduced glutathione in binding buffer. The eluted SNARE complexes were immediately measured for FRET using a spectrofluorometer (SLM 8100, SLM-Aminco, Rochester, NY). The samples were excited at 425 nm, and emission spectra were collected from 450 to 550 nm. The final emission spectra were corrected for background, smoothed, and normalized.

Microscopy, Image Analysis, and FRET Calculation—PC12 cells or primary cerebellar neurons were infected with adenoviruses expressing ECFP and YFP fusion proteins. For detection of ECFP, cells were viewed with an inverted fluorescence microscope (Leica DMIL) under a filter set with an excitation filter of 440/21 nm, a dichroic beam splitter of 455, and an emission filter of 480/30 nm. YFP expressing cells were viewed with the same filter set with an excitation filter of 500/25 nm, a dichroic beam splitter of 525 nm, and an emission filter of 545/55 nm. The filters for FRET were an excitation filter of 440/21 nm, a dichroic beam splitter of 455, and an emission filter of 535/26 nm. Images were captured with a cooled CCD camera (Quantix 57, Photometrics, Tucson, AZ) controlled by IPLab 3.5 (Scanalytics, Fairfax, VA) and analyzed with the IPLab software.

FRET was quantified with the three-filter set system to normalize the FRET intensity for ECFP and YFP concentration in each region of interest (ROI) (28). ROIs were selected manually. For an ROI, intensity ($I$) from the three filter sets was obtained after background subtraction. Then FRET was calculated as follows: $FRET = I_{ECFP} - I_{YFP}$. Then FRET was calculated as follows: $FRET = I_{ECFP} - I_{YFP}$. Then FRETN was calculated as follows: $FRET = I_{ECFP} - I_{YFP}$. Where 55% is the percentage of ECFP contribution to FRET intensity, 15% is the percentage of YFP contribution to FRET intensity. In some cases, the images were adjusted pixel-by-pixel using a reference channel of ROI.

RESULTS

FRET between CFP-SNAP-25 and YFP-Synaptobrevin in Vitro—To express effectively the recombinant proteins, the CFP-SNAP-25 (C-SN) and YFP-synaptobrevin (Y-SB) were cloned into an adenovirus vector as described by He et al. (25). A control fusion protein SNAP-25-CFP (SN-C) in which the C terminus of SNAP-25 was fused with CFP was also prepared. The fusion with SNAP-25 or synaptobrevin did not affect the spectrum properties of CFP and YFP (Fig. 1A). To determine whether the fusion proteins still retained the function in SNARE complex assembly, Y-SB was co-expressed with C-SN or SN-C in cultured rat cerebellar granule cells. The cells were lysed with nonionic detergent, and the SNARE complexes were immunoprecipitated using an anti-syntaxin antibody. As shown in Fig. 2, the fusion proteins were co-immunoprecipitated with the SNARE complex containing endogenous SNAP-25 and synaptobrevin. This result suggests that the fusion proteins are able to participate in the formation of SNARE complex.

The assembly of SNARE core complex was first examined by FRET in vitro. Y-SB, C-SN, and SN-C were overexpressed in COS-7 cells by recombinant adenovirus infection. Purified GST-syntaxin was incubated with Triton X-100-solubilized membrane preparations containing similar amounts of Y-SB and C-SN/SN-C. The Y-SB-GST-syntaxin-C-SN or Y-SB-GST-synaptobrevin-SN-C ternary complexes were eluted with excess glutathione and measured for fluorescence absorption at excitation of 425 nm. There are two advantages of using the crude COS-7 preparations instead of purified proteins expressed in bacteria. It allows the correct folding and post-translational modifications of the proteins, and the presence of other cellular proteins further reduced nonspecific absorption by GST-agarose. As shown in Fig. 3A, no FRET between SN-C and Y-SB was observed. However, a significant amount of FRET was detected between C-SN and Y-SB, which exhibited an increased absorption peak at 530 nm (Fig. 3A). For control experiments, an equal aliquot of COS-7 lysate containing C-SN was incubated with the same amount of GST-syntaxin and unlabeled synaptobrevin, and the resulting ternary complex was eluted for fluorescence measurement, which showed a significantly higher absorption at 480 nm in the absence of fluorescence acceptor (data not shown). Assuming that the affinity of Y-SB for syntaxin and SNAP-25 was the same as the...
FRET in PC12 Cells and Cultured Neurons Expressing CFP-SNARE Complex Revealed by FRET

FIG. 1. Spectrum properties of C-SN, SN-C, and Y-SB in solution and in PC12 cells. A, excitation and emission spectra of the fusion proteins. The fusion proteins were expressed in COS-7 cells by adenoviral vectors for 24 h, and the cells were lysed with PBS containing 1% Triton X-100. The cell lysate was measured using a spectrophuorometer. Solid lines indicate the excitation spectra, and dashed lines indicate the emission spectra. B, fluorescence images of PC12 cells expressing the fusion proteins. Differentiated PC12 cells were infected with recombinant adenoviruses at 10 m.o.i. for 48 h. Images were acquired under FRET and CFP/YFP filters. Notice that the C-SN and SN-C were localized to the plasma membranes of the cells bodies and neurites, whereas Y-SB had a punctate localization. Bar, 30 μm.

FIG. 2. Co-immunoprecipitation of C-SN, SN-C, and Y-SB with SNARE complexes from rat cerebellar neuron cultures. A, immunoblot analysis of total cell lysates from cerebellar neurons infected with recombinant adenoviruses expressing 1) C-SN and Y-SB or 2) SN-C and Y-SB. B, immunoprecipitation using anti-syntaxin antibodies. The immunoprecipitated proteins were blotted against goat anti-SNAP-25, goat anti-synaptobrevin, and monoclonal anti-syntaxin antibodies. Lane 1, cells expressing C-SN and Y-SB; lane 2, cells expressing SN-C and Y-SB; and lane 3, cells expressing C-SN and Y-SB, control sample without anti-syntaxin added during immunoprecipitation. Molecular mass markers are 73, 47, 33, 28, and 20 kDa (arrowheads).

unlabeled synaptobrevin, the FRET efficiency between C-SN and Y-SB was estimated to be ~44%. These results are consistent with the crystal structure of SNARE core complex model, in which the two α-helices of SNAP-25 form a parallel 4-α-helical bundle with syntaxin and synaptobrevin (10–19).

FRET in PC12 Cells and Cultured Neurons Expressing CFP-SNAP-25 and YFP-Synaptobrevin—To study SNARE interaction in vitro, PC12 cells and rat cerebellar granule neurons were infected with recombinant adenoviruses expressing the fusion proteins. It had been shown that infection of PC12 cells with recombinant adenovirus at a m.o.i. of up to 100 had no significant effect on endogenous protein expression and norepinephrine release (29). At an m.o.i. of 10, ~10–15% cells were infected and expressed various levels of fusion proteins after 24 h. As expected, C-SN and SN-C were localized to the plasma membranes of cell bodies and neuritic processes of differentiated PC12 cells, whereas Y-SB was mostly associated with intracellular membranes and often with punctate structures clustered in the cells as well as neurites (Fig. 1B). Digital images were acquired under three sets of filters, a CFP set, a YFP set, and a FRET set. To establish background fluorescence, PC12 cells expressing only one fusion protein were examined. There was no crossover between CFP and YFP when viewed under the CFP or YFP filters. When the cells were examined under the FRET filter set, YFP alone yielded less than 15–20% fluorescence and CFP yielded ~50–56% (Fig. 1B). Since many factors may affect the accuracy of FRET measurement, we adopted the method used by Gordon et al. (28), which uses several values for each pair of fusion proteins to correct for background and level of protein expression. As shown in Fig. 3 and Table I, FRET was observed on the plasma membranes and neuritic varicosities of PC12 cells expressing Y-SB and C-SN. However, FRET was not observed in locations including the Golgi-like intracellular membranes and some aggregates in the neurites (Fig. 3B). No significant FRET was observed with the fusion protein pair Y-SB and SN-C (Fig. 3B), which did not show FRET under in vitro conditions (Fig. 3A).

Cultured cerebellar granule cells from 5- to 8-day-old rats undergo synaptogenesis starting at the end of the 1st week when the expression of synaptic proteins is rapidly increased (30). At this stage, the cells are responsible for depolarization and release glutamate as the major neurotransmitters (31, 32). At 6 days in vitro, the endogenous synaptobrevin began to shift its localization from cell bodies and general neuronal processes to a more restricted localization at synaptic junctions (data not shown). SNAP-25, however, was more uniformly distributed in the processes, and little was found in the cell bodies. The Y-SB and SN-C fusion proteins, when co-expressed by adenovirus infection, showed similar localization as their endogenous counterparts, suggesting that the fusion proteins were correctly targeted to the subcellular sites. The virally infected neurons often showed strong fluorescence signals in their cell bodies, probably indicating the transport of newly synthesized recombinant proteins through Golgi apparatus. Three types of neuronal subcellular locations were selected for FRET measurement as follows: the plasma membranes of the cell bodies, the punctate structures presumably to be the synaptic sites, and the large varicosities presumably to be the constitutively secreting vesicles transporting newly synthesized proteins. As
The extent of FRET was also observed, which was likely due to the variance of different membrane fusion events during neurosecretion (3). These results suggested that a tighter cis-SNARE complex may be formed following membrane fusion and that FRET may be used as an effective tool for studying the dynamic interactions between individual SNAREs during synaptic transmission. FRET was also measured in PC12 cells pretreated with 1 mM PMA, a phorbol ester that activates protein kinase C and enhances neurosecretion in PC12 cells. Since several SNAREs are substrates for protein kinase C (33), it is possible that protein kinase C enhances neurosecretion by phosphorylation of SNAREs. No significant changes in FRET were detected in PMA-treated cells (data not shown), suggesting that PMA treatment did not cause major structural reorganization of the SNARE core complexes.

**DISCUSSION**

Recent biochemical and biophysical studies from a number of laboratories have provided much needed insights into the assembly and disassembly of the SNARE core complex and its proposed role in vesicle fusion (11–13, 17, 19). What remains to be delineated is the temporal dynamics of SNARE complex assembly and disassembly in relation to the vesicle fusion process. Such knowledge is essential to the understanding of the function and regulation of SNARE complex during vesicle trafficking. FRET, using fusion proteins of GFP variants, provides a powerful tool for investigating such mechanisms. The CFP (donor)-YFP (acceptor) pair has been used in a number of FRET experiments (21, 23). The critical Förster radius for CFP-YFP is about 50 Å, suggesting that any significant FRET would indicate actual physical interaction between two proteins. Since we have demonstrated that FRET between C-SN and Y-SB can be effectively monitored in vivo, similar experiments with other fusion protein pairs such as syntaxin-synaptobrevin or syntaxin-SNAP-25 can be performed. Furthermore, the technique provides a useful tool for dissecting SNARE complex assembly/disassembly in real time during vesicle docking, fusion, and recycling.

For the FRET technology to work effectively, the fusion proteins must be able to be correctly targeted and participate in SNARE assembly/disassembly. Many studies using GFP fusion proteins have shown high fidelity of subcellular targeting of the fusion proteins, and the same findings with N-terminally and C-terminally fused GFP-synaptobrevin have been reported (34, 35). One concern is that the overexpressed recombinant proteins may be mis-targeted or “spilled over” to other compartments. If Y-SB was mis-targeted to the plasma membranes, it could form cis-SNARE complexes with SNAP-25 and syntaxin as those found in the in vitro binding experiments. Although such a possibility cannot be ruled out, several lines of evidence strongly suggest that the FRET observed with Y-SB and C-SN in vivo was from trans-SNARE complexes. First, only cells moderately expressing the fusion proteins were used for FRET measurement, and similar results were obtained in PC12 cells that had been passed for at least two generations following adenovirus infection, when the expression of the recombinant proteins were comparable to the endogenous protein levels. Second, glycerol gradient ultracentrifugation was able to separate dense core vesicles from the plasma membranes in PC12 cells (36), which showed that >95% Y-SB was associated with the vesicle fractions, and >90% of SNAP-25 was associated with the plasma membranes (data not shown). Third, the Y-SB and C-SN/SN-C were correctly targeted in cultured cerebellar granule cells. Fourth, we have shown that a GFP-synaptobrevin fusion protein was exclusively targeted to neuromuscular junctions when expressed in spinal motor neurons via adenovirus-mediated gene transfer (35).
The evoked synaptic vesicle fusion is a tightly regulated process that is triggered by calcium influx (3, 37). It is not clear if SNARE complex assembly occurs during the vesicle docking/priming stages or immediately preceding the fusion. Studies by Chen et al. (17) suggest that the assembly of SNARE complex is immediately followed by membrane fusion; thus, the two

### Table I

| Protein pair                        | PC12 cells | Granule cells |
|-------------------------------------|------------|---------------|
| SN-C + Y-SB                         | 0.0000245 ± 0.0000375 | 0.0000219 ± 0.000022 |
| C-SN + Y-SB                         | 0.000256 ± 0.000089 | 0.000192 ± 0.000084 |
| C-SN + Y-SB (KCl-treated)           | 0.0030704 ± 0.00015 | ND |

*For PC12 cells, plasma membranes and neuritic varicosities were measured for FRET (n = 18).

*For cerebellar granule cells, putative synaptic junctions were measured (n = 21).

*PC12 cells were treated with 56 mM KCl for 5 min before FRET measurement (n = 17, p < 0.05 by Student’s t test).

*ND, not done.

### Fig. 4. FRET in cultured rat cerebellar neurons expressing C-SN and Y-SB.

Rat cerebellar granule cells were cultured for 5 days before infection with adenoviruses at 200 m.o.i. Forty eight hours post-infection, the cells were examined under CFP, YFP, or FRET filter sets and measured for FRET as described under “Experimental Procedures.” Arrowheads indicate FRET-positive sites presumed to be synaptic junctions, and the arrow indicates the axonally transported varicosities and aggregates in the cell body where no FRET was detected. * indicates cell bodies. Bar, 50 μm.

### Fig. 5. FRET in differentiated PC12 cells stimulated with high KCl.

PC12 cells stably transfected with Y-SB were grown on poly-D-lysine-coated coverslips and infected with C-SN adenoviruses. The cells were treated with 50 ng/ml NGF for 4 days before KCl was added to 56 mM. The cells were incubated for 5 min at 37 °C and fixed with 3.7% formaldehyde in PBS. FRET was measured as described under “Experimental Procedures.” A–D, control cells; E—H, KCl-treated cells. Fluorescence images viewed under CFP filters (A and E), YFP filters (B and F), and FRET filters (C and G). D and H, FRET signals after pixel-by-pixel adjustment and subtraction of nonspecific contributions from CFP and YFP as described under “Experimental Procedures.” Arrowheads indicate FRET positive sites. Bar, 15 μm.
events are practically linked as one step. The findings in our current study suggest that stable SNARE complexes that are fusion-incompetent are assembled. Alternatively, an intermediate form of SNARE complexes may exist during vesicle docking and undergo further conformational changes upon calcium influx. Although it is difficult to estimate the actual amount of SNARE complex based on the FRET values, the strong FRET observed between C-SN and Y-SB indicates the existence of significant numbers of SNARE complexes. Although spontaneous transmitter release by PC12 cells and cerebellar granule cells occurs at the basal level, such large numbers of SNARE complexes favor the notion that assembled yet fusion-incompetent SNARE complexes are present during the vesicle docking/priming stage. The existence of an intermediate form of SNARE complexes is supported by electrophysiological studies in chromaffin cells. Based on the secretion kinetics of chromaffin cells in response to stimulation, it was proposed that a loose form of SNARE complexes exists prior to vesicle fusion, and these complexes are required for the subsequent fusion (20). According to the model, a fusion-competent vesicle can exist in two states that are controlled by a loose or a tight SNARE complex. Another study using neurotoxins also points to the presence of stable SNARE complexes in unstimulated cells (38). Although FRET can be used for measuring distance between proteins, we have not attempted to do so because the conformational property of C-SN and Y-SB has yet to be characterized. Therefore, we could not conclude whether or not the FRET was from a loose or tight SNARE complex. Nevertheless, because the R0 of CFP/YFP is 50 Å, the distance between C-SN and Y-SB would be likely less than 50 Å.

The finding that FRET increases following vesicle fusion supports the proposal that a transition from trans- to cis-SNARE complexes occurs as a result of vesicle fusion (17, 20). Another possibility is that the net amount of SNARE complexes is increased due to membrane fusion under the sustained depolarization conditions. Perhaps both the higher number and tighter SNARE complexes have contributed to the increased FRET observed in KCl-treated cells. This can only be resolved when we can quantify the amount SNARE complex in cells.

In summary, the present study using FRET provides direct evidence of the presence of stable SNARE complexes prior to evoked synaptic vesicle fusion. More importantly, this technique can be applied to investigate the dynamics of SNARE assembly and disassembly in real time during neurotransmitter release in vivo, which would allow us to dissect the molecular event involved in synaptic transmission.

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REFERENCES

1. Bennett, M. K., and Scheller, R. H. (1994) *Annu. Rev. Biochem.* 63, 63–100
2. Jahn, R., and Hanson, P. I. (1998) *Nature* 393, 14–15
3. Sudhof, T. C. (1995) *Nature* 375, 645–653
4. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Gromov, S., Tempst, P., and Rothman, J. E. (1995) *Nature* 376, 318–324
5. Bennett, M. K., Garcia-Arraras, J. E., Elfferich, L. A., Peterson, K., Fleming, A. M., Hanukz, C. D., and Scheller, R. H. (1993) *Cell* 74, 863–873
6. Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K., and Scheller, R. H. (1994) *Neuron* 13, 353–361
7. Chapman, R. K., An, S., Barton, N., and Jahn, R. (1994) *J. Biol. Chem.* 269, 27427–27432
8. McMahon, H. T., and Sudhof, T. C. (1995) *J. Biol. Chem.* 270, 2213–2217
9. Schiavo, G., Stenbeck, G., Lasser, J. E., and Solnier, T. H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 997–1001
10. Fasshauer, D., Eliaason, W. K., Brumer, A. T., and Jahn, R. (1998) *Biochemistry* 37, 10354–10362
11. Fernandez, I., Ubach, J., Duhlubova, I., Zhang, X., Sudhof, T. C., and Rizz, J. (1998) *Cell* 94, 841–849
12. Poirier, M. A., Xiao, W., Macosko, J. C., Chan, C., Shin, Y. K., and Bennett, M. K. (1998) *Nat. Struct. Biol.* 5, 765–769
13. Sutton, R. B., Fasshauer, D., Jahn, R., and Brumer, A. T. (1998) *Nature* 395, 347–353
14. Davis, A. F., Bai, J., Fasshauer, D., Wolowicz, M. J., Lewis, J. L., and Chapman, E. R. (1999) *Nature* 399, 517–519
15. Banerjee, A., Kowalchuk, J. A., Dasgupta, B. R., and Martin, T. F. J. (1996) *J. Biol. Chem.* 271, 20227–20230
16. Littleton, J. T., Serrano, T. L., Rubin, G. M., Ganetzky, B., and Chapman, E. R. (1996) *Nature* 381, 577–580
17. Chen, Y. A., Scales, S. J., Patel, S. M., Dong, Y. C., and Scheller, R. H. (1999) *Cell* 97, 165–174
18. Weber, T., Zenzelmann, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Solnier, T. H., and Rothman, J. E. (1999) *Cell* 92, 759–772
19. Parlati, F., Weber, T., McNew, J. A., Westermann, B., Solnier, T. H., and Rothman, J. E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 12565–12570
20. Xu, T., Rammer, M., Margittai, M., Artalejo, A. R., Neher, E., and Jahn, R. (1999) *Cell* 99, 713–722
21. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) *Nature* 388, 882–887
22. Mahajan, N. P., Linder, K., Berry, G., Gordon, G. W., Heim, B., and Herman, B. (1998) *Nat. Biotechnol.* 16, 547–552
23. Vanderklish, P. W., Krischel, I. A., Holst, B. H., Gally, J. A., Crossin, K. L., and Edelman, G. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 2253–2258
24. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1998) *J. Cell Biol.* 109, 3039–3052
25. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2509–2514
26. Gallo, V., Ciotti, M. T., Coletti, A., Aloisi, F., and Levi, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 7919–7923
27. Lane, S. R., and Liu, Y. (1997) *J. Neurochem.* 69, 1864–1869
28. Gordon, G. W., Berry, G., Liang, X. H., Levine, B., and Herman, B. (1998) *Biophys. J.* 74, 2702–2713
29. Zhou, Q., Xiao, J., and Liu, Y. (2000) *J. Neurosci. Res.* 61, 321–328
30. Sanders, J. D., Yang, Y., and Liu, Y. (1998) *J. Neurosci. Res.* 53, 670–676
31. Marini, A. M., and Paul, S. M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 6555–6559
32. Yang, Y., Xia, Z., and Liu, Y. (2000) *J. Biol. Chem.* 275, 29482–29487
33. Chen, Y. A., Duvvuri, V., Schulman, H., and Scheller, R. H. (1999) *J. Biol. Chem.* 274, 26469–26476
34. Miesenbock, G., De Angelis, D. A., and Rothman, J. E. (1998) *Nature* 394, 192–195
35. Jacob, J. M., Zhou, Q., and Liu, Y. (2000) *J. Neurosci. Res.* 61, 61–66
36. Grote, E., Hao, J. C., Bennett, M. K., and Kelly, R. B. (1995) *Cell* 81, 581–589
37. Jahn, R., and Sudhof, T. C. (1999) *Annu. Rev. Biochem.* 68, 863–811
38. Hua, S. Y., and Charlton, M. P. (1999) *Nat. Neurosci.* 2, 1078–1083
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