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Complexin-1 Enhances the On-Rate of Vesicle Docking via Simultaneous SNARE and Membrane Interactions

Jiajie Diao,[†] Daniel J. Cipriano,[†] Minglei Zhao, Yunxiang Zhang, Sachi Shah, Mark S. Padolina, Richard A. Pfuetzner, and Axel T. Brunger*

Departments of Molecular and Cellular Physiology, Neurology and Neurological Sciences, Structural Biology, and Photon Science and Howard Hughes Medical Institute, Stanford University, Stanford, California 94305, United States

Supporting Information

ABSTRACT: In synaptic terminals, complexin is thought to have inhibitory and activating roles for spontaneous "mini" release and evoked synchronized neurotransmitter release, respectively. We used single vesicle−vesicle microscopy imaging to study the effect of complexin-1 on the on-rate of docking between vesicles that mimic synaptic vesicles and the plasma membrane. We found that complexin-1 enhances the on-rate of docking of synaptic vesicle mimics containing full-length synaptobrevin-2 and full-length synaptotagmin-1 to plasma membrane-mimicking vesicles containing full-length syntaxin-1A and SNAP-25A. This effect requires the C-terminal domain of complexin-1, which binds to the membrane, the presence of PS in the membrane, and the core region of complexin-1, which binds to the SNARE complex.

Ca^{2+}-triggered, synchronized fusion of synaptic vesicles to the presynaptic plasma membrane underlies interneuronal communication. Proteins including neuronal soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), the Ca^{2+} sensor synaptotagmin-1, SM proteins, and complexin are critical for this process.[1] Complexin is a small soluble protein (134 residues) that is mainly found in the presynaptic terminal. It binds to the SNARE complex and has both activating and inhibiting functions for fast synchronous release and spontaneous "mini" release.[2,3] Moreover, overexpression of complexin in PC12[4] and chromaffin[5] cells or expression as a fusion protein to synaptobrevin[6] substantially diminished neurotransmitter release, suggesting an inhibitory role of complexin. In contrast, knockout of complexin isoforms from hippocampal neurons selectively impaired the synchronous component of exocytosis[7] indicating a stimulatory role of complexins in late fusion steps.

The N-terminus (residues 1−27) of complexin-1 is critical for fast synchronized release, whereas the accessory α-helix (residues 27−48) plays a role in suppressing spontaneous release (Figure 1A).[2] A central region of complexin (residues 48−70) binds to the groove between the synaptobrevin and syntaxin α-helices in the core part of the neuronal SNARE complex, which itself is a tight bundle of four α-helices;[8] this central region appears to be critical for all functions of complexin. The C-terminal region (residues 70−134) has a role in synaptic vesicle priming,[3] but the underlying molecular mechanism is unclear.

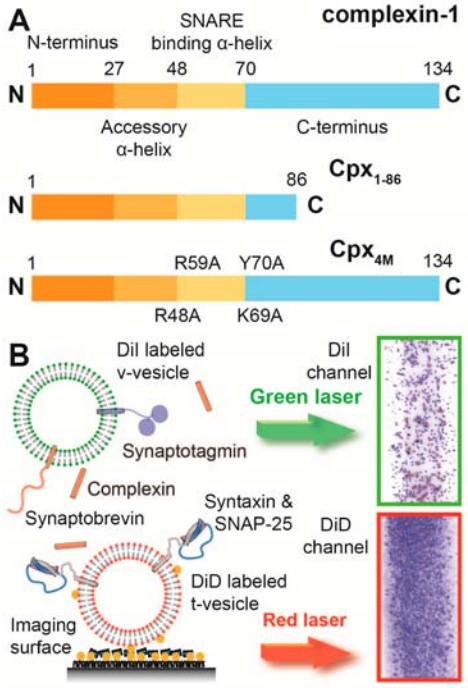

Figure 1. Single vesicle−vesicle docking assay. (A) Primary sequence domain diagrams of complexin-1 (Cpx), Cpx_{4M}, and Cpx_{1-86} with functional annotations. (B) Schematic of our single vesicle−vesicle assay for measuring the docking probability between v- and t-vesicles. A saturated layer of DiD labeled t-vesicles (reconstituted with syntaxin-1A and SNAP-25A) was created by immobilization on the imaging surface through biotin−neutravidin tethers. The saturation and homogeneity of the layer of immobilized t-SNARE vesicles was assessed by (red) laser illumination at 633 nm. Free DiI labeled v-vesicles (reconstituted with full-length synaptobrevin-2 and synaptotagmin-1) were injected into the system in the presence or absence of complexin-1 for a defined incubation time period (25 s unless noted otherwise). Green laser illumination at 532 nm imaged the v-vesicles that were docked to immobilized t-vesicles. The mean diameter of the vesicles is 45 nm as determined by inspection of cryo-EM images of the vesicles (Figure S1). This setup is similar to that of ref 17.

In vitro biophysical studies revealed stimulatory effects of the central region and C-terminal regions of complexin in proteoliposome lipid-mixing experiments[8−10] and inhibitory

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effects of full-length complexin in cell-based fusion, proteoliposome lipid-mixing assays, and synaptotagmin-binding competition experiments.[6,11,12] However, the cell-based fusion assays examined relatively slow fusion events (minute time scale), and the lipid mixing experiments examined lipid mixing, rather than content mixing, the latter correlating with neurotransmitter release. Remarkably, we found that complexin dramatically increases the number of fast (msec) Ca^{2+} triggered fusion events between synaptic vesicle and plasma membrane mimics using a single-vesicle content mixing assay.[13] At the lowest Ca^{2+} concentration that we tested (250 μM), the fusion probability increased from background levels to a substantial burst, in agreement with in vivo studies of synchronous release in neurons.[2]

A "clamping" model of complexin has been proposed, in which complexin stabilizes the t-SNARE complex in an inhibitory conformation that blocks full complex formation with synaptobrevin, until a Ca^{2+} signal arrives,[11,14,15] although the molecular mechanism of the release of the block remains unclear.

Synaptotagmin-1, a synaptic vesicle membrane-anchored Ca^{2+} sensor, plays an essential role for fast synchronous neurotransmitter release.[1] The absence of full-length synaptotagmin-1 in many previous in vitro studies (or, instead, the use of the soluble C2AB domain of synaptotagmin-1) may thus explain the differences between physiological observations and these particular experiments.[16] Here we studied the effect of complexin-1 on the on-rate of docking between vesicles that mimic synaptic vesicles, containing both full-length synaptotagmin-1 and synaptobrevin-2, and vesicles that mimic the plasma membrane, containing both syntaxin-1A and SNAP-25A. We found that complexin-1 enhanced the on-rate of docking. However, this enhancement was critically dependent on the presence of the membrane-binding C-terminal domain of complexin, in agreement with recent in vivo data.[3] In the absence of the C-terminal binding domain, the on-rate of docking was reduced in the presence of complexin. We note that both properties of complexin also depend on the interaction between the central region of complexin and the SNARE complex and on the presence of PS in the membrane.

We immobilized DiD labeled "t-vesicles" (proteoliposomes with reconstituted full-length syntaxin-1A and SNAP-25A) on a polyethylene glycol (PEG) coated imaging surface (Figure 1B). We subsequently injected a solution of DiI labeled "v-vesicles" (proteoliposomes with reconstituted full-length synaptobrevin-2 and synaptotagmin-1) in the presence or absence of 10 μM complexin-1 for a defined incubation time period (see online Supporting Information, SI, for details). The v-vesicles mimic synaptic vesicles, while the t-vesicles mimic the plasma membrane. At the end of the incubation period (25 s, unless mentioned otherwise), unbound v-vesicles and complexin-1 molecules were removed by buffer exchange (SI and Figure S2). Using green laser illumination, we then counted the average number of DiI-labeled v-vesicles per imaging area (50×100 μm^2) that remained bound to t-vesicles. Since our protocol produces a homogeneous and saturated surface-layer of immobilized t-vesicles, the number of fluorescent spots arising from DiI labeled v-vesicles is proportional to the probability that a v-vesicle docks to a surface-tethered t-vesicle during the incubation period,[18] and it is proportional to the duration of the incubation period since the off-rate is rather slow. By design, this experiment measures a non-equilibrium property that depends on the kinetics of the interaction between v- and t-vesicles. Below saturating conditions, the docking probability within the incubation time period is approximately related to the on-rate of docking between free v-vesicles and immobilized t-vesicles. The number of vesicles that dock to a saturated surface within a defined time period has been measured in previous single-particle experiments.[19,20] Figure S3 illustrates the kinetic character of our measurement by using two different incubation time periods (see further discussion below).

As previously noted, we included full-length synaptotagmin-1, in contrast to previous liposome-based studies that examined the effect of complexin in the presence of SNAREs only. Remarkably, in our experiments complexin-1 significantly increased the docking probability by ~60% rather than reducing it (Figure 2A). We next tested if the enhancement

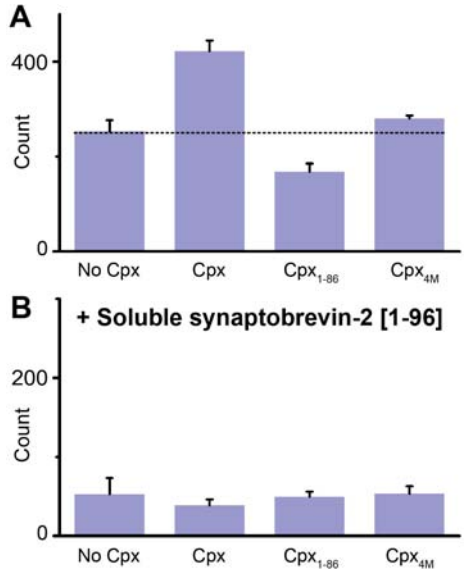

Figure 2. The C-terminus of complexin-1 is critical for enhancing the docking on-rate between v- and t-vesicles in the presence of full-length neuronal SNAREs and synaptotagmin-1. (A) The number of docked v-vesicles for complexin-1 as well as its mutants, using the protocol described in Figure S2 and SI with a 25 s incubation period. (B) As a control, we preincubated the system with 20 μM of the soluble fragment of synaptobrevin-2, residues 1–96, blocking syntaxin-1A/SNAP-25A binary complex and preventing *trans*-SNARE complex formation between t- and v-vesicles. Error bars are SEM from 10 random imaging locations in the same sample channel.

in docking probability by complexin-1 depends on the interaction with the SNARE complex. We employed the "4M" mutant (R48A, R59A, K69A, and Y70A) of complexin-1, Cpx_{4M}, that significantly weakens the interaction with the SNARE complex.[2] The v-vesicle docking on-rate in the presence of the Cpx_{4M} mutant was statistically identical to the case without complexin-1 (Figure 2A). Thus, the enhancement of docking by complexin-1 in the presence of both SNAREs and synaptotagmin-1 depends on this particular interaction with the neuronal SNARE complex.

As a further control, the SNARE dependence of the effects for wild-type complexin and its mutants was tested by preincubating the immobilized v-vesicles with a large excess (20 μM) of the soluble synaptobrevin-2 fragment (residues 1–96) (Figure 2B). In all cases, the pre-incubation of t-vesicles with the synaptobrevin fragment diminished docking of v-vesicles in the absence or presence of complexin as well as its mutants. This result can be explained by the sequestration of all

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t-SNARE complexes by the soluble synaptobrevin fragment, preventing *trans*-SNARE complex formation and, hence, vesicle docking.

Since docked v-vesicles remain associated with the immobilized t-vesicles during the short imaging period,[18] the observed docking probability in our experiments should be proportional to the on-rate of the association reaction. Since this probability would be independent of the duration of the incubation period, one would expect that the docking probability ratios between different conditions to be independent of the incubation period as well. Indeed, for a shorter incubation period, the ratio between two particular conditions is close to that of the longer period (Figure S3). Moreover, as shown in Figure S4A, the relative docking probability pattern was also independent of the v-vesicle concentration. As an additional control, the docking probability ratios are approximately independent of the v-vesicle concentration (Figure S4A). Moreover, the majority of docked v-vesicles are involved in single v-/t-vesicle pairs as assessed by fluorescence intensity profiles (Figure S4B).

We next tested if the enhancement of the docking probability by complexin-1 depends on its C-terminus. Surprisingly, the C-terminally truncated construct of complexin-1, Cpx_{1-86}, reduced the docking on-rate (Figure 2A). Similarly, in the absence of full-length synaptotagmin-1, Cpx_{1-86}, also reduced the docking probability (Figure 3). Moreover, using a liposome

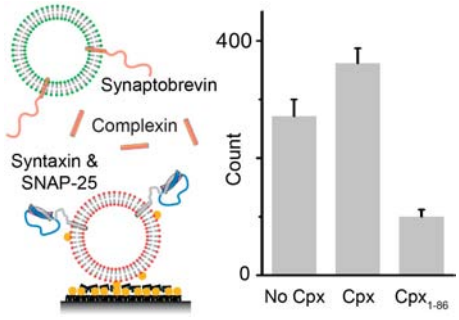

Figure 3. Effect of complexin-1 on the docking on-rate in the absence of synaptotagmin-1. The diagram on the left side shows the experimental setup (identical to the setup shown in Figure 1B, except that v-vesicles only contain synaptobrevin). The bar graph on the right side shows the number of v-vesicles that are docked to immobilized t-vesicles within a 25 s incubation period in the absence and presence of wild-type complexin-1 or presence of the C-terminally truncated mutant Cpx_{1-86}. Error bars are SEM from 15 random imaging locations in the same sample channel.

flotation assay, we found that the C-terminus is critical for binding of complexin-1 to synthetic membranes with a lipid composition similar to that of synaptic vesicles (Figure 4). Finally, the presence of phosphatidylserine (PS) in the v-vesicle membrane is essential for the docking enhancement by complexin (Figure 5). This result is consistent with a previous finding that PS is a binding partner to complexin.[21]

Previous studies based on an ensemble *in vitro* lipid mixing assay proposed a potential fusion promoting role of the C-terminus of complexin-1,[9,10] which contrasts a recent *in vivo* study suggesting that C-terminus is important for vesicle docking, but not fusion.[3] Here, we report a critical role of the complexin C-terminus for enhancing the on-rate of docking between vesicles that mimic synaptic vesicle and the plasma membrane, consistent with the *in vivo* results. How can one

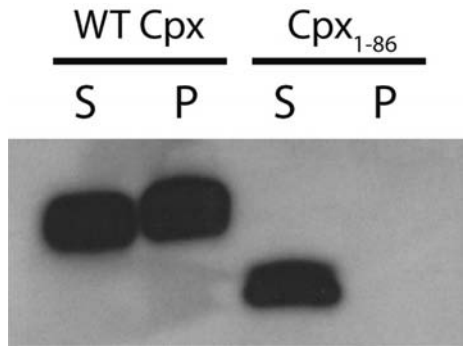

Figure 4. The C-terminus of complexin-1 is essential for membrane binding. Wild-type complexin-1 or the C-terminally truncated complexin mutant Cpx_{1-86} was incubated with protein free vesicles with a lipid composition similar to that of v-vesicles, as described in the SI, Experimental Methods. After centrifugation, membranes were pelleted, and the supernatants (S) and membrane-containing pellet (P) fractions were analyzed using SDS-PAGE followed by Western blotting with an anti-Cpx-1 antibody. Wild-type complexin-1 (Cpx) was associated with the membrane fraction, whereas the C-terminally truncated complexin mutant Cpx_{1-86} was not detectable.

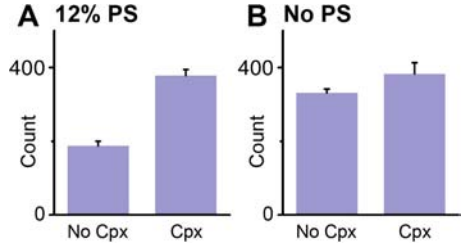

Figure 5. The presence of the anionic phospholipid PS in the v-vesicle membrane is important for complexin's function. Experiments were performed in the presence of neuronal SNAREs and synaptotagmin-1 as described in Figure 1. (A) The exact same lipid composition for t- and v-vesicles were used as in previous experiments (SI, Materials and Methods). (B) Identical conditions as in A were used except without PS in the v-vesicle lipid composition. In the absence of PS, complexin did not enhance the docking on-rate. The corresponding intensity distributions of the observed fluorescent spots are shown in Figure S5, illustrating that mostly single v-/t-vesicle pairs are observed under the conditions of this experiment.

resolve this apparent contradiction between previous *in vitro* bulk lipid-mixing experiments and our single-vesicle results? Fluorescence correlation spectroscopy (FCS) lipid-mixing experiments revealed that the rate-limiting step of an ensemble *in vitro* lipid mixing assay is the docking step itself rather than the fusion reaction.[22] Thus, a factor that promotes vesicle docking would also enhance subsequent lipid mixing and fusion events, rather than affecting the fusion kinetics itself.[17,22] Single-vesicle assays are capable of discriminating between effects related to docking, hemifusion, and complete fusion, so they are not subject to the limitations of certain bulk lipid-mixing experiments.

Our single-vesicle results suggest that the C-terminus of complexin plays a key role in enhancing the docking on-rate of synaptic vesicles. Most recently, a study from Rizo's group showed that the C-terminus of complexin is important for resisting synaptotagmin replacement.[23]

A large body of work has focused on the SNARE-interacting part of complexin-1, the accessory helix, and the N-terminal region for roles in synchronizing fast release and suppressing

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spontaneous release.[11,14,15] Together with previous studies,[3,9,10,21,24] an important functional role of C-terminal membrane-binding region of complexin has been uncovered and warrants further study to decipher the underlying molecular mechanism.

At variance with many previous *in vitro* studies, we included both full-length neuronal SNAREs and synaptotagmin-1 in order to provide better mimics of both synaptic vesicles and the plasma membrane. Compared to the soluble C2AB fragment of synaptotagmin-1, membrane-anchoring of full-length synaptotagmin-1 works in a different manner for efficient Ca^{2+}-triggering.[25] We thus recommend that future studies of Ca^{2+}-triggered fusion should always, at minimum, include both full-length synaptotagmin-1 and complexin-1, in addition to neuronal SNAREs, as was already done in recent studies.[13,26−28]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

brunger@stanford.edu

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

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