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Controlling synaptotagmin activity by electrostatic screening

Yongsoo Park[1], **Javier M. Hernandez**[1], **Geert van den Bogaart**[1], **Saheeb Ahmed**[1], **Matthew Holt**[1], **Dietmar Riedel**[2], and **Reinhard Jahn**[1,*]

[1]Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany

[2]Facility for Electron Microscopy, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany

Summary

Exocytosis of neurosecretory vesicles is mediated bythe SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins syntaxin-1, synaptobrevin, and SNAP-25, with synaptotagmin functioning as the major Ca^{2+}-sensor for triggering membrane fusion. Here we show that bovine chromaffin granules readily fuse with large unilamellar liposomes in a SNARE-dependent manner. Fusion is enhanced by Ca^{2+} but only if the target liposomes contain $PI(4,5)P_2$ and if polyphosphate anions such as nucleotides or pyrophosphate are present. Ca^{2+}-dependent enhancement is mediated by endogenous synaptotagmin-1. Polyphosphates operate by an electrostatic mechanism that reverses an inactivating *cis*-association of synaptotagmin-1 with its own membrane whereas *trans*-binding is not affected. Hence, balancing *trans*- and *cis*-membrane interactions of synaptotagmin may be a crucial element in the pathway of Ca^{2+}-dependent exocytosis.

Neurons communicate via exocytotic release of neurotransmitters. In the resting state, neurotransmitters are stored in synaptic vesicles or secretory granules. Upon depolarization, voltage-gated calcium channels open, resulting in an influx of Ca^{2+} that triggers the fusion of the membrane of the storage vesicles with the plasma membrane, thus releasing the neurotransmitter into the extracellular space[1].

In addition to synaptic vesicles, many neurons contain a second class of secretory vesicles with an electron-dense core and larger diameter, referred to large dense-core vesicles (LDCVs)[2]. They correspond to secretory vesicles of neuroendocrine cells such as

*To whom correspondence should be addressed: Reinhard Jahn, Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, 37077 Göttingen, Germany, phone: x49-551-201-1635, fax: x49-551-201-1639, rjahn@gwdg.de.

Author Contributions

J.M.H. assisted in the generation of SNARE-containing LUVs and performed the light scattering experiments. G.v.d.B. provided labeled-proteins and assisted in the fluorescence anisotropy experiments. S.A. and M.H. provided purified synaptic vesicles. D.R. performed the electron microscopy. Y.P. and R.J. designed the study and wrote the paper. Experiments were mainly conducted by Y.P. All authors discussed the results and commented on the manuscript.

Competing financial interests

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chromaffin cells of the adrenal medulla. LDCVs store proteins and peptides in addition to classical neurotransmitters[2,3]. In neurons, LDCVs also undergo exocytosis in response to Ca^{2+}, but release is differentially regulated from that of synaptic vesicles, requiring bursts of action potentials[4]. However, the release machinery, as far as it is known, utilizes the same proteins as those operating during exocytosis of synaptic vesicles including SNAREs and synaptotagmins. Due to easy access with microelectrodes, exocytosis of LDCVs in chromaffin cells has served as a model for studying the mechanism of SNARE-mediated exocytosis with electrophysiological approaches[5].

Vesicle docking, activation of the fusion machinery (priming), Ca^{2+}-dependent triggering, and subsequent membrane merger are carried out by evolutionarily conserved protein complexes functioning in all neurons and neuroendocrine cells. Of these, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins are currently considered the catalysts of the fusion reaction[6–9]. SNAREs mediating synaptic exocytosis include syntaxin-1 and SNAP-25 localized at the plasma membrane and synaptobrevin located at the vesicle membrane[6,10,11]. SNAREs are small membrane-anchored proteins that possess one or two conserved stretches of 60–70 amino acids arranged in heptad repeats, termed SNARE motifs[6]. Release is triggered by an influx of Ca^{2+} from the extracellular space in response to depolarization. Upon triggering, the SNARE motifs form a helical complex that bridges the membranes (“trans”-complex). The complex assembles in the N-terminal to the C-terminal direction towards the membrane anchors, thus pulling the membranes together and initiating fusion as the SNARE complex relax into the “cis”-configuration[12,13]. Triggering is mediated by synaptotagmins, transmembrane proteins of synaptic vesicles and chromaffin granules containing two Ca^{2+}-binding C2-domains, denoted C2A and C2B, that bind 3 and 2 Ca^{2+}, respectively[14,15]. Synaptotagmin interacts both with SNAREs and with acidic lipids which are widely considered essential for its function. However, how exactly synaptotagmin accelerates fusion is unclear. We have recently shown that interactions with vesicular acidic lipids seem to inactivate synaptotagmin. Incorporation of synaptotagmin into liposomes containing 20% phosphatidylserine (PS) results in binding of synaptotagmin to its own membrane (*cis*-binding), preventing *trans*-binding to the target membranes containing acidic phospholipids and/or SNARE proteins[16,17]. Native vesicles contain ~15% acidic phospholipids[18] including PS and phosphatidylinositol (PI). It is not known how such inactivating *cis*-association of synaptotagmin is prevented *in vivo*[16,19,20].

Here, we have investigated the role of *cis*- and *trans*-binding of synaptotagmin on Ca^{2+}-dependent fusion *in vitro*. To prevent problems that may arise from the differences between artificial and biological membranes, we used purified bovine chromaffin granules as one of the fusion partners. Our data show that chromaffin granules fuse with liposomes carrying syntaxin-1A and SNAP-25A in a SNARE-dependent manner. Ca^{2+} increases the rate of fusion several fold but only if $PI(4,5)P_2$ was present in the target membrane and, surprisingly, ATP was included in the buffer. Intriguingly, no ATP hydrolysis was required, and the stimulatory effect was attributed to an electrostatic effect that prevents the inactivating *cis*-binding of synaptotagmin C2 domains to the vesicle membrane, thereby

allowing endogenous synaptotagmin to interact in *trans* with the lipids and/or SNAREs in the target membrane.

Results

Characterization of purified chromaffin granules

To reconstitute chromaffin granule (CG) fusion *in vitro*, we purified CGs from bovine adrenal medulla using a modified protocol based on continuous sucrose density gradient centrifugation as the final purification step (Supplementary Fig. 1 in the Supplementary Information). Purity of CGs was confirmed by western blotting showing that the final fraction was depleted from markers of other organelles (Fig. 1a). VAMP-4, a SNARE found on immature but not on mature secretory granules[21], was also removed during purification (Supplementary Fig. 1c) showing that our protocol yields mature CGs of high purity.

Cryo-electron microscopyof purified CGs revealed a heterogeneous size distribution (Fig. 1b) with an average diameter of 167.7 nm. Size heterogeneity of CGs in chromaffin cells was also observed in previous studies[22], ranging from 100 nm up to 500 nm, but the average diameter (356 nm)[23] is larger than that found in our study. This is probably due to the inclusion of immature CGs that are known to be larger. CGs become smaller and condensed through removal of water and “shedding” of membrane during the maturation process[24,25]. Our data show that CGs have high purity and mature CGs are mainly collected.

Fusion of CGs with proteoliposomes requires SNARE proteins

We investigated whether purified CGs are able to fuse with liposomes containing SNAP-25A and syntaxin-1A. Proteoliposomes containing NBD- and rhodamine-labeled phospholipids were reconstituted with the stabilized acceptor complex known as the ΔN complex: a preformed complex of syntaxin-1A (lacking the N-terminal Habc domain) and SNAP-25A containing the C-terminal fragment of synaptobrevin (residues 49–96)[26]. Liposomes contained 45% phosphatidylcholine, 15% phosphatidylethanolamine, 10% PS, 25% cholesterol, 4% PI, and 1% PI(4,5)P_2. Fusion was monitored by a lipid-mixing assay in which fluorescence resonance energy transfer (FRET) between the two fluorophore-labeled lipids is reduced because of lipid dilution as a result of fusion with unlabeled lipids, leading to de-quenching of the donor fluorophore[27].

Robust fusion was observed when CGs and proteoliposomes containing the SNARE acceptor complex were mixed (Fig. 1c and Supplementary Fig. 2a). Membrane fusion was SNARE-specific as shown by competitive inhibition with soluble synaptobrevin ($Syb_{1–96}$) or a soluble complex of SyxH3 (H3 domain of syntaxin1A)/SNAP-25A or by incubation with the light chain of tetanus neurotoxin (TeNT), a protease selectively cleaving synaptobrevin (Fig. 1c, Supplementary Fig. 2a,b). Furthermore, endogenous synaptobrevin in the CG membrane was capable of SNARE complex formation as shown by SDS-PAGE (Fig. 1d). Lysophosphatidylcholine (LPC), which destabilizes the negative curvature of stalk-type fusion intermediates[28] by inducing positive curvature, inhibited CG fusion in a dose-dependent manner (Fig. 1e and Supplementary Fig. 2c). Similar results were obtained

when content-mixing instead of lipid-mixing was monitored (Supplementary Fig. 2d,e), indicating that SNARE-dependent fusion of CGs is complete and not arrested at hemifusion.

Ca^{2+} enhances fusion in the presence of ATP

Similar to synaptic vesicles, exocytosis of CGs is triggered by a rise in intracellular Ca^{2+}. We therefore investigated whether addition of Ca^{2+} influenced fusion between purified CGs and SNARE-containing proteoliposomes. A slight but substantial inhibitory effect was observed at Ca^{2+} concentrations above 300 μM (Fig. 2a, Supplementary Fig. 3a,d). In a screen for metabolites that may affect fusion we made the surprising observation that in the presence of ATP, Ca^{2+} did not inhibit but rather dramatically enhanced fusion (Fig. 2a and Supplementary Fig. 3d). Similarly, fusion of synaptic vesicles purified from rat brain was also increased by Ca^{2+} in the presence of ATP whereas it was slightly inhibited in the absence of ATP (Fig. 2b), in agreement with earlier experiments[16,19,20,29]. No such enhancement was seen when $MgCl_2$ was used instead of $CaCl_2$ (Supplementary Fig. 3b,c). As before, fusion was completely blocked when $Syb_{1–96}$ was added as a competitive inhibitor, confirming that Ca^{2+}-enhanced fusion is mediated via SNARE-proteins. Enhancement was observed regardless of whether ATP and Ca^{2+} were added simultaneously or sequentially (Supplementary Fig. 4a). Furthermore, the same rates were observed under all conditions when K-glutamate was replaced with K-gluconate or with KCl or with NaCl (data not shown). Very similar results were obtained when the full-length syntaxin-1A and SNAP-25A binary acceptor complex (2:1 complex) was used, although as expected[26] the rates were lower (Supplementary Fig. 4b).

To confirm that SNARE zippering occurs during fusion, we took advantage of the fact that the C-terminal synaptobrevin fragment stabilizing the acceptor complex in the ΔN complex is displaced by binding of full-length synaptobrevin from vesicle membranes, allowing for monitoring SNARE complex assembly by fluorescence anisotropy[26]. When liposomes containing the ΔN complex with Alexa Fluor 488-labeled $Syb_{49–96}$ were incubated with CGs, we observed a decrease of fluorescence anisotropy indicating SNARE complex zippering. Co-treatment of ATP and Ca^{2+} increased peptide displacement whereas ATP or Ca^{2+} alone had no effect (Fig. 2c and Supplementary Fig. 3e), showing that Ca^{2+}-dependent enhancement of vesicle fusion is associated with an increase in the number of the assembled SNARE complexes.

Next we investigated whether ATP-hydrolysis is required, which would suggest the involvement of an ATPase in the CG membrane. However, this turned out not to be the case as Ca^{2+}-dependent enhancement of CG fusion (Fig. 2d–2f). Synaptic vesicle fusion was also observed when ATP was replaced with the non-hydrolyzable analog ATP-γ-S (Supplementary Fig. 5c). Furthermore, ATP could also be replaced by GTP. Intriguingly, the electrostatic effect of ATP appears to be primarily dependent on the number of negative charges since ADP and AMP were also able to enhance Ca^{2+}-dependent fusion albeit with reduced efficacy (Fig. 2e,f). Finally, we tested pyrophosphate which was as efficient as ADP (Fig. 2f), suggesting that the pyrophosphate moiety of the nucleotides rather than the bases is critical for Ca^{2+}-dependent vesicle fusion.

Ca^{2+}- enhancement depends on synaptotagmin and $PI(4,5)P_2$

In chromaffin cells, members of the synaptotagmin familymediate fast exocytosis in response to Ca^{2+}. Indeed, our results suggest that the Ca^{2+}-dependent enhancement of fusion observed in our experiments is brought about by endogenous synaptotagmin in the CG membrane, which binds to negatively charged phospholipids by electrostatic interactions[30], particularly in the presence of phosphoinositides[31]. Two approaches were used to test this. First, a monoclonal antibody specific for the cytoplasmic domain of synaptotagmin-1[32] was added to the reaction, resulting in a partial inhibition of Ca^{2+}-dependent enhancement while having no effect on the basal fusion rate (Fig. 3a). Second, addition of increasing concentration of a soluble cytoplasmic fragment of synaptotagmin-1 (C2AB domain) as a competitive inhibitor resulted in a progressive inhibition of Ca^{2+}-dependent enhancement, again leaving basal fusion rates unaffected (Fig. 3b). Together, these findings suggest that the Ca^{2+} effect on fusion is mediated by endogenous synaptotagmin-1 although we cannot exclude the presence of other Ca^{2+} sensors (such as synaptotagmin 7 contributing to the effect). To further verify the involvement of synaptotagmin-1, we examined whether acceleration of fusion is dependent on the presence of $PI(4,5)P_2$ in the target membrane. In chromaffin cells, plasma membrane levels of $PI(4,5)P_2$ regulate priming[33] by controlling the size of the releasable CG pool[34]. $PI(4,5)P_2$ is also important for Ca^{2+}-dependent vesicle fusion by enhancing Ca^{2+} affinity of synaptotagmin-1[35] and heavily accumulates at sites of docked vesicles[36]. Intriguingly, the basal fusion rate was lower in the absence of $PI(4,5)P_2$ (Fig. 3c) and no Ca^{2+}-dependent enhancement was observed (Fig. 3c). To gain more insight into the dependence of the Ca^{2+} effect on $PI(4,5)P_2$, we performed titrations at different $PI(4,5)P_2$ concentrations in the target membrane (Fig. 3d). With increasing $PI(4,5)P_2$ concentrations, the Ca^{2+} dose-response curve was shifted to lower Ca^{2+} concentrations. These data suggest that higher local concentration of $PI(4,5)P_2$ enhances Ca^{2+} sensitivity by lowering EC_{50}, in agreement with studies carried out with purified synaptotagmin[35]. Next, we exchanged $PI(4,5)P_2$ with PI3P, a phosphoinositide species specific for endosomal and autophagosomal membranes[37,38]. Ca^{2+}-dependent enhancement was still observed with PI3P, although it was less dramatic than in the presence of $PI(4,5)P_2$ (Fig. 3e and Supplementary Fig. 4c). Enhancement was also observed in the absence of $PI(4,5)P_2$ if the concentration of the acidic phospholipid PS with -1 net charge was increased to 40% (Supplementary Fig. 4d). Similar results for the dependence on $PI(4,5)P_2$ were obtained if synaptic vesicles instead of chromaffin granules were used (Fig. 3f and Supplementary Fig. 5a).

ATP prevents *cis*-binding of synaptotagmin by charge shielding

What is the mechanism by which polyphosphate anions enhance Ca^{2+}/synaptotagmin-mediated fusion of CGs and synaptic vesicles? Considering the data above we hypothesized that polyphosphates such of ATP and related compounds may activate endogenous synaptotagmin in the membrane of CGs and synaptic vesicles. Previous studies have suggested that the C2-domains of membrane-anchored synaptotagmin may interact with its resident membrane (*cis*-binding), which may interfere, or even prevent, the binding to the target membrane[16,17,29]. *Trans*-binding of synaptotagmin, however, is believed to be required for synaptotagmin to drive Ca^{2+}-dependent exocytosis. Since we were unable to

directly measure association of endogenous synaptotagmin, we monitored binding of an exogenously added C2AB fragment of synaptotagmin-1, labeled with Alexa Fluor 488, to CG or synaptic vesicle membranes using fluorescence anisotropy (Fig. 4). Addition of Ca^{2+} increased fluorescence anisotropy indicating that the C2AB domains of synaptotagmin-1 bind to CG membranes in a Ca^{2+}-dependent manner (Fig. 4a–4c). ATP or GTP, when added after Ca^{2+}, decreased the anisotropy signal, suggesting dissociation of the C2AB fragment from the membrane, with AMP being less effective, thus mirroring the effects on fusion enhancement. Very similar observations were made when purified synaptic vesicles instead of chromaffin granules were used (Fig. 4d). Increase of the free Ca^{2+}-concentration up to values above 1 mM did not overcome the ability of ATP to prevent binding (Fig. 4c,d).

Taken together, the results suggest that synaptotagmin binds to its own membrane (*cis*-binding) in response to Ca^{2+}, which abolishes *trans*-interactions, in agreement with our previous observations using artificial vesicles[17]. To analyze the effect of ATP on the Ca^{2+}-dependent binding of synaptotagmin to acidic membranes in more detail, we carried out binding experiments using $PI(4,5)P_2$-containing liposomes with the same composition to those used in the fusion assays. Addition of Ca^{2+} resulted in binding of C2AB domain that was not reversed by ATP (Fig. 4e,f). *Cis*-binding of synaptotagmin is prevented by polyphosphate anions at physiological concentrations whereas it appears that *trans*-interactions with $PI(4,5)P_2$-containing target membranes are not inhibited.

Ca^{2+}-dependent binding of the C2-domains to membranes is strongly enhanced by $PI(4,5)P_2$, but also depends on the concentration of acidic membrane lipids such as PS or PI. We therefore speculated that the inhibitory effect of ATP and paralogs may only be effective at moderate concentrations of such acidic lipids. To test this, we measured Ca^{2+}-dependent binding of synaptotagmin to liposomes with increasing PS concentrations in the presence and absence of ATP (Fig. 5). As described previously[31], Ca^{2+}-dependent binding shifted to lower Ca^{2+} concentrations with increasing PS concentrations. Intriguingly, ATP reversed binding at 10% and 15% PS but not at 20% PS. As a reference, synaptic vesicle membranes contain about 15% acidic phospholipids[18]. Indeed, the Ca^{2+} dose response curve for C2AB binding to CGs (Fig. 4c) and synaptic vesicle (Fig. 4d) resembles that of liposomes containing 15% PS (Fig. 5b), indicating that 15% PS mimics the electrostatic environment of native vesicle membranes.

Finally, we tested whether *cis*-inactivation, its prevention by ATP, and *trans*-acceleration of fusion can be reproduced using liposomes reconstituted with synaptobrevin and synaptotagmin instead of purified CGs and SVs as donor vesicles. Specifically, we asked whether synaptotagmin was capable of enhancing SNARE-mediated fusion in a Ca^{2+}-dependent manner. As shown in Fig. 5, this was indeed the case. Ca^{2+}-dependent acceleration of fusion correlated with the ability of ATP to prevent *cis*-binding (Fig. 5b, right), but the enhancement was not observed when the membrane of the synaptotagmin/synaptobrevin-incorporating vesicle contained more than 20% PS (Fig. 5a, right). This agrees with a recent report showing that Ca^{2+}-dependent enhancement of fusion depends on the amount of PS in synaptotagmin-1-containing liposomes[39]. Intriguingly, Ca^{2+}-dependent fusion of liposomes was less efficient than fusion of native vesicles.

Discussion

Exocytosis of neurosecretory vesicles is mediated by SNAREs and triggered by Ca^{2+}-bound synaptotagmin. However, the mechanism by which synaptotagmin enhances fusion is still unclear. *In vitro* reconstitution of SNARE-dependent membrane fusion in the presence of synaptotagmin has yielded conflicting results, with the effects of Ca^{2+} ranging from slight inhibition to enhancement under widely varying conditions[16,29,39–41]. We have previously shown that membrane-anchored synaptotagmin binds to its own membrane (*cis*-binding) when acidic phospholipids are present, preventing *trans*-interactions with the target membrane[16,17]. Here we have shown that such *cis*-binding of synaptotagmin occurs in native vesicles including chromaffin granules and synaptic vesicles but that such *cis*-binding is prevented by polyphosphate anions including ATP at physiological concentrations. ATP and analogous compounds probably operate by charge screening, i.e. by directly competing with the acidic membrane lipids in chelating Ca^{2+} and disrupting *cis*-binding of synaptotagmin (see Fig. 6 for model). The native vesicle membrane which contains ~15% acidic phospholipids has a Ca^{2+} affinity with EC_{50} of 233 ± 29 μM for the binding of C2AB domain (Fig. 4c) and ATP on its own chelates Ca^{2+} with a similar affinity (EC_{50}: 230 μM, data not shown, see also[42]), thereby inhibiting C2AB binding to the vesicle membrane. Intriguingly, screening is only effective if the concentration of acidic phospholipids does not exceed 15% and if no $PI(4,5)P_2$ is present in the membrane. $PI(4,5)P_2$-containing target membrane has a much higher Ca^{2+} affinity for C2AB binding than that of the vesicle (EC_{50} : 56 μM), and synaptotagmin can then act in “trans”, resulting in a major Ca^{2+}-dependent enhancement of SNARE-dependent fusion.

Our data shed new light on the mechanism by which the Ca^{2+}-sensor synaptotagmin may operate between the vesicle and the plasma membrane. It is well established that both C2-domains display highly cooperative Ca^{2+}-dependent binding to membranes containing acidic phospholipids[15,30], with an increasing concentration of acidic phospholipids in the membrane resulting in higher affinities[31]. Furthermore, synaptotagmin contains a basic patch in the C2B domain that binds to $PI(4,5)P_2$ in an at least partially Ca^{2+}-independent manner[43,44] and enhances Ca^{2+} sensitivity of exocytosis[45]. Our data now suggest that there may be a delicate balance between “*cis*” and “*trans*” binding of synaptotagmin: while the target membrane containing high concentrations of $PI(4,5)P_2$ allows for strong Ca^{2+}-dependent (and partially Ca^{2+}-independent) binding of synaptotagmin, the concentration of acidic phospholipids in the vesicle membrane appears to be adjusted to regulate *cis*-binding of synaptotagmin.

We do not yet know whether polyphosphate-dependent screening of inactivating *cis*-binding plays a role in the regulation of Ca^{2+}-dependent exocytosis under physiological conditions. In permeabilized neuroendocrine cells, triggering of exocytosis by Ca^{2+} depends on the presence of ATP[46]. Moreover, we have shown previously that in a cell-free preparation composed of inverted lawns of plasma membrane containing docked secretory vesicles, addition of Ca^{2+} elicits exocytosis but only if ATP is present[47]. Obviously, ATP-dependence may simply reflect the involvement of ATP-utilizing enzymes such as NSF or PI(4) kinase. However, it is conceivable that the strict dependence on the presence of ATP

even during the Ca^{2+}-triggering phase may also be attributable to the prevention of inactivating *cis*-binding of synaptotagmin to the vesicle membrane.

How do these data contribute to the understanding of the still debated mechanism by which Ca^{2+}-binding to synaptotagmin accelerates the rate of exocytosis by more than five orders of magnitude? Many models have been suggested for the action of synaptotagmin in the fusion pathway such as unblocking or activating arrested *trans*-SNARE complexes, generation of localized membrane curvature in the plasma membrane, or perturbation of the hydrophilic/hydrophobic boundary of the membranes at the contact site. While we cannot yet exclude any of these mechanisms, it has recently been suggested that synaptotagmin may act as a distance regulator that pulls the vesicle and the plasma membrane a bit closer upon Ca^{2+} triggering, thus triggering SNARE assembly and fusion[48]. It is conceivable that synaptotagmin first binds in *trans* to the target membrane, but that *trans*-binding is then followed by *cis*-binding to the vesicle membrane involving the C2-domains, shortening the distance between the membranes. Such a two-step mechanism may allow for regulation of *cis* binding, e.g. by fine-tuning the concentration of acidic phospholipids and/or phosphorylated variants of phosphatidylinositol in the vesicle membrane at the contact site.

Online Methods

Materials

Calcein, ATP, ADP, AMP, GTP, ATP-γ-S, and pyrophosphate were purchased from Sigma (St Louis, MO). L-α-lysophosphatidylcholine (LPC) and other lipids were from Avanti (Alabaster, AL). Antibodies against synaptobrevin, synaptophysin, synaptotagmin-1 (monoclonal antibodies 41.1 and 604.1), and VAMP-4 were from Synaptic Systems (Göttingen, Germany). Antibodies against SDHA, EEA-1, Calnexin, LAMP-1, Rpt-4, Catalase, and Na^+/K^+-ATPase were from Abcam (Cambridge, MA).

Purification of chromaffin granules (CGs) and synaptic vesicles (SVs)

CGs were purified according to Smith and Winkler[49], with several modifications. Fresh bovine adrenal glands were obtained from a local slaughterhouse. After trimming away the cortex and fat, the medullae were minced with a scissor in 300 mM sucrose buffer (300 mM sucrose, 20 mM HEPES, pH 7.4 adjusted with KOH) and then homogenized using a cooled glass-teflon homogeniser at 1,000 rpm (H, homogenate). PMSF (200 μM) was added to prevent protein degradation. All subsequent steps were carried out at 0°–4°C. After centrifugation at 1,000 g for 15 min at 4°C, the pellet containing nuclei and cell debris (P1) was discarded. The supernatant (S1) was further centrifuged (12,000 g, 15 min, 4°C), followed by the additional cycle of resuspension and centrifugation for washing step. The resulting pellet (P2, crude CG fraction) was resuspended in 300 mM sucrose buffer and loaded on top of a continuous sucrose gradient (from 300 mM to 2.0 M) to remove other contaminants including mitochondria. CGs were collected from the pellet after centrifugation at 27,000 rpm for 60 min in a Beckman SW 41 Ti rotor and resuspended with the buffer (120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES. KOH, pH 7.4). The fraction directly on top of the pellet was removed and the pellet was only resuspended in order to purify mature CGs.

Synaptic vesicles from rat brain were purified according to a new method that will be described in detail elsewhere. Briefly, rat brains were homogenized in homogenization buffer supplemented with protease inhibitors, using a glass-Teflon homogenizer with 10 strokes at 900 rpm. The homogenate was centrifuged for 10 min at 1,000 g and the resulting supernatant was further centrifuged for 15 min at 15,000 g. The supernatant S2 was stored on ice for later use. The synaptosome pellet was lysed by adding ice-cold water and 3 strokes at 2,000 rpm were applied. Protease inhibitors and HEPES were added to the lysate immediately. The lysate was centrifuged for 15 min at 17,000 g and the supernatant LS1 was combined with the S2. The mixture of LS1/S2 was centrifuged for 25 min at 48,000 g. The resulting supernatant CS1 was overlaid onto a 0.7 M sucrose cushion and centrifuged for 1 h at 133,000 g. The pellet was resuspended in column buffer (100 mM Tris-HCl, 100 mM KCl, pH7.4) and loaded onto a Sephacryl S-1000 size exclusion chromatography column (100×1 cm).

Protein purification

All SNARE constructs were based on rat sequences and were cloned in the pET28a vector. TeNT light chain (both wildtype and the inactive E234A mutant[50]), the SNARE proteins including soluble form of synaptobrevin lacking the transmembrane domain ($Syb_{1–96}$), and C2AB domain of synaptotagmin-1 (aa 97–421) were expressed in E. coli and purified by Ni^{2+}-NTA affinity chromatography followed by ion exchange chromatography with Mono S column on an Äkta system (GE Healthcare, Piscataway, NJ). The stabilized Q-SNARE complex, referred to as ΔN complex, containing syntaxin-1A (183–288), SNAP-25A (no cysteine, cysteines were replaced by alanines) and the C-terminal synaptobrevin fragment (49–96)) was purified, as described previously[16]. The 2:1 binary Q-SNARE complex containing syntaxin-1A (1–288) and SNAP-25A (no cysteine) was expressed using co-transformation[51]. The ΔN complex, the syntaxin-1A/SNAP-25A 2:1 binary complex, SNAP-25A (no cysteine), and syntaxin-1A (aa 1–288, 183–288, and 183–262 (SyxH3)) were purified by Ni^{2+}-NTA affinity chromatography followed by ion-exchange chromatography on an Mono Q column (GE Healthcare, Piscataway, NJ) in the presence of 50 mM n-octyl-β-D-glucoside.

For anisotropy measurements, point mutated C2AB (S342C)[35] and $Syb_{49–96}$ (T79C) in ΔN complex were labeled with Alexa Fluor 488 C5 maleimide.

Preparation of proteoliposomes

Unless indicated otherwise, the lipid composition of proteoliposomes (molar ratios) consists of 45% PC (L-α-phosphatidylcholine), 15% PE (L-α-phosphatidylethanolamine), 10% PS (Lα-phosphatidylserine), 25% Chol (cholesterol), and 5% PI (L-α-phosphatidylinositol). $PI(4,5)P_2$ or PI3P, at the indicated concentrations, were replacing PI. For FRET-based dequenching assays, 1.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD-DOPE) and 1.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (Rhodamine-DOPE) were used as a donor and an acceptor dye, respectively. Synaptobrevin- and synaptotagmin-1-containing liposomes consist of 50% PC, 20% PE, 20% PS, and 10% cholesterol. When 15% PS or 10% PS was used, PC contents were adjusted accordingly.

As described before[52], liposomes were extruded using polycarbonate membranes of pore size 100 nm (Avanti Polar lipids) to give uniformly distributed large unilamellar vesicles (LUVs) in the diameter range of 100 nm as confirmed by field-flow-fractionation coupled to multi angle laser light scattering (FFF-MALLS, Wyatt Technology Corporation, Santa Barbara, CA, USA).

Incorporation of the proteins into liposomes was achieved by OG (n-octyl-β-D-glucoside)-mediated reconstitution. Proteoliposomes containing the stabilized acceptor complex (ΔN complex) or the syntaxin-1A/SNAP-25A binary complex in 2:1 stoichiometry (2:1 complex) were prepared by detergent-assisted insertion of proteins as described previously[16,52]. ΔN complex in 50 mM OG (n-octyl-β-D-glucoside) was mixed with LUVs (lipid to protein ratio of 500:1 (n/n)). In case of 2:1 complex incorporation with LUVs, lipid to protein ratio was 200:1. For content-mixing assays, 50 mM calcein (495/515 nm) was encapsulated in proteoliposomes according to Kendall and MacDonald[53]. Lipids were dissolved in diethyl ether (1.5 ml) and resuspended with 0.5 ml of 50 mM Calcein ($2Na^+$-$calcein^{2-}$) in 20 mM HEPES/KOH pH 7.4, 75 mM KCl, and 1 mM DTT. Content mixing was specific to SNARE proteins incorporated in liposomes and leakage, determined by quenching calcein leaked into the medium by addition of Co^{2+}, was only 4–5% of total calcein (for details see[48]).

Fusion reaction

CG fusion reactions were performed at 37 °C. For each reaction, 50 μg of CGs and 10μl of proteoliposomes were mixed in 1 ml of buffer containing 120 mM K- glutamate, 20 mM K-acetate, 20 mM HEPES-KOH (pH 7.4), and 5 mM $MgCl_2$. Unless indicated otherwise, acceptor liposomes contained the stabilized Q-SNARE complex, termed ΔN complex[26]. The 2:1 (syntaxin-1A/SNAP-25A) Q-SNARE complex was also tested for SNARE- and Ca^{2+}-dependent fusion (Supplementary Fig. 4b). For Ca^{2+}-dependent fusion, 5 mM $2Na^+$-ATP was added. ATP should be made freshly for experiments, because ATP is easily destroyed by freezing and thawing. Fluorescence dequenching signal was measured using Fluorolog and Fluoromax (Horiba Jobin Yvon) with wavelengths of 460 nm (slit width of 1 nm) for excitation and 538 nm (slit width of 3 nm) for emission. Fluorescence values were normalized as the percentage value of the maximum donor fluorescence induced by 0.1% Triton X-100 detergent treatment at the end of experiments. No addition represents basal fusion without any treatment or Ca^{2+}. Quantification of vesicle fusion data of lipid-mixing and content-mixing assay were presented as the percentage by normalizing basal fusion after 20 min of reaction time.

Cryoelectron Microscopy

Samples were bound in a VitrobotT Mark IV (FEI Company) to a glow discharged carbon foil covered grid. The suspension was blotted 2x for 1 sec at blott force 2 and vitrified at 24°C, 97% humidity. The samples were evaluated with a CM 120 transmission electron microscope and pictures were taken with a TemCam 224A slow scan CCD camera (TVIPS, Gauting, Germany).

Fluorescence anisotropy measurements

Anisotropy measurements were carried out in a Fluorolog 3 spectrometer in T-configuration equipped for polarization (Model FL322, Jobin Yvon). All experiments were performed at 37 °C in 1 ml of the buffer containing 120 mM K-glutamate, 20 mM K-acetate, 20 mM HEPES-KOH (pH 7.4), and 5 mM $MgCl_2$. $2Na^+$-ATP and $CaCl_2$ were treated as indicated. Alexa Fluor 488-labeled proteins were excited at 488 nm (slit width of 8 nm) and emission was measured at 520 nm (slit width of 10 nm). To monitor SNARE assembly, 200 μg CGs were incubated with 1% $PI(4,5)P_2$-containing liposomes which incorporate ΔN complex ($Syb_{49–96}$ labeled with Alexa Fluor 488 at T79C). To monitor the binding of C2AB domain, 30 nM C2AB (S342C, Alexa Fluor 488-labeled) was incubated with 30 μg CGs or protein-free liposomes consisting 20%, 15%, or 10% PS.

Ca^{2+} calibration

ATP contains negatively charged oxygen atoms which bind to Mg^{2+}, Ca^{2+}, or Sr^{2+}, thereby chelating divalent cations[42]. Ca^{2+} concentrations were calibrated with Fluo-5N, a low-affinity Ca^{2+} indicator with a K_d of 90 μM, and experiment data was correlated with simulation that calculates the free Ca^{2+} concentrations (http://maxchelator.stanford.edu).

Statistical analysis

All quantitative data are mean ± SD from ≥ 3 independent experiments. Dose-response curves were fitted using four parameter logistic equations (4PL) to calculate EC_{50} (SigmaPlot).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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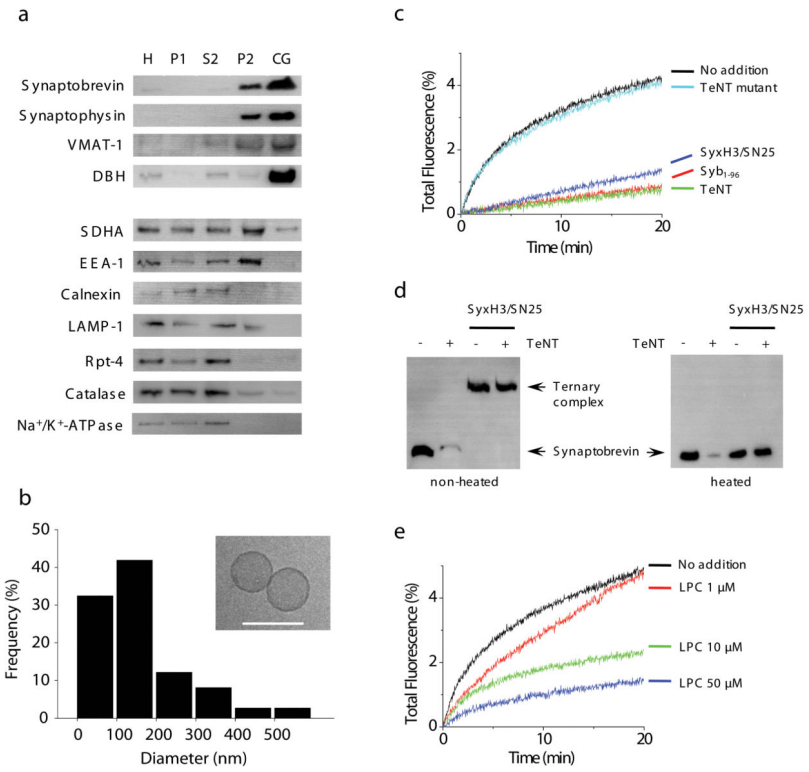

Figure 1.

Purified chromaffin granules (CGs) fuse with large unilamellar liposomes (LUVs) in a SNARE-dependent manner. (**a**) Purified CGs are highly enriched in CG-specific proteins whereas contaminating membranes are removed during purification. Antibodies for the following organelle-specific marker proteins were used: synaptobrevin, synaptophysin, vesicular monoamine transporter-1 (VMAT-1), dopamine-beta-hydroxylase (DBH) for the membrane of CGs, succinate dehydrogenase complex subunit A (SDHA, mitochondria), calnexin (endoplasmic reticulum), early endosome-associated protein 1 (EEA-1, early endosomes), lysosomal-associated membrane protein 1 (LAMP-1, lysosomes), Rpt-4 (proteosomes), catalase (peroxisomes), and Na^+/K^+-ATPase (plasma membrane)(see Online Methods for details). See Supplementary Fig. 1 for fractionation scheme and complete blots (**b**) Size distribution of CGs determined by cryo-electron microscopy (n = 74). The average diameter was calculated to be 167.7 ± 14.3 nm (inset figure, scale bar indicates 200 nm). (**c**) Fusion (lipid mixing) of purified CGs with large unilamellar liposomes (LUVs) containing a stabilized SNARE acceptor complex, measured by a fluorescence dequenching assay. Preincubation of LUVs with soluble synaptobrevin ($Syb_{1–96}$, 2 μM), and of CGs with syntaxin-1A (183–262)/SNAP-25A (SyxH3/SN25, 1 μM) for 30 min at 37°C completely blocked lipid mixing. Similarly, lipid mixing was inhibited by preincubation of CGs with TeNT light chain (200 nM, 30 min at 37°C), but not with an inactive light chain mutant. (**d**) Incubation of CGs with excess SyxH3/SN25 resulted in the formation of ternary SNARE complexes with endogenous synaptobrevin, which does not dissociate in SDS (left) unless heated (right). Only complexed synaptobrevin is resistant to cleavage by TeNT light chain (immunoblots probed for synaptobrevin). (**e**) Lysophosphatidylcholine (LPC) inhibited lipid mixing of CGs in a dose-dependent manner.

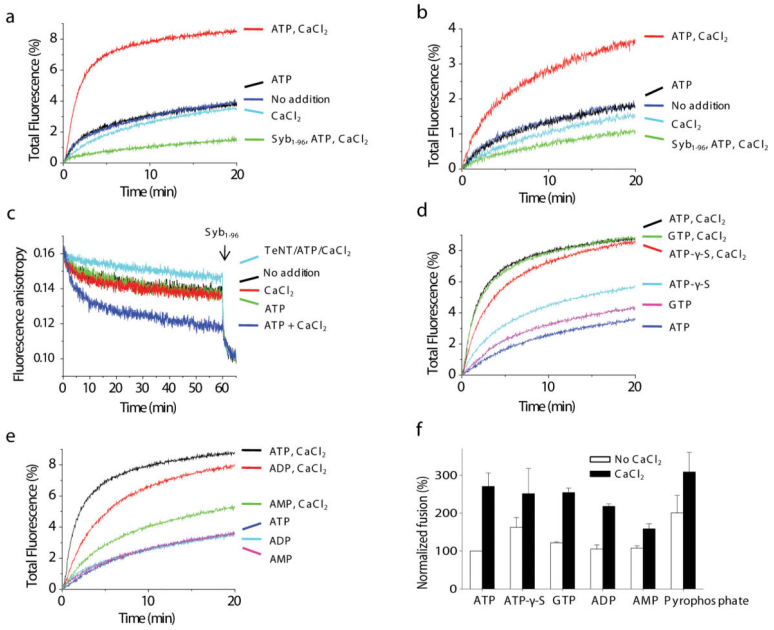

Figure 2.

Polyphosphates are required for Ca^{2+}-dependent vesicle fusion (measured by a lipid mixing assay). (**a**) Ca^{2+} enhances CG fusion with LUVs containing the stabilized acceptor complex only if ATP (5 mM) is present. CGs, Ca^{2+}, and ATP were added at the onset of the fusion reaction (84 μM free Ca^{2+} and 5 mM ATP was used here and in all subsequent experiments unless indicated otherwise). Preincubation with soluble synaptobrevin ($Syb_{1–96}$) abolished Ca^{2+}-induced CG fusion. (**b**). Fusion of purified rat brain synaptic vesicles was also enhanced by Ca^{2+} but again only in the presence of ATP. (**c**) Ca^{2+}-dependent enhancement of fusion is associated with increased SNARE assembly, measured by the release of the stabilizing $Syb_{49–96}$ fragment, which is labeled with Alexa Fluor 488 (decrease in fluorescence anisotropy). Preincubation of CGs with TeNT light chain blocked SNARE assembly. At the end of each reaction, 1 μM unlabeled $Syb_{1–96}$ was added to displace all remaining $Syb_{49–96}$. (**d**) ATP hydrolysis is not required for Ca^{2+}-dependent enhancement of CG fusion. No change was observable if ATP was replaced with ATP-γ-S. Furthermore, GTP was as effective as ATP. (**e**) Ca^{2+}-dependent enhancement of CG fusion was also observed in the presence of 5 mM ADP an AMP, respectively, but with lower efficacy. (**f**) Electrostatic effect of polyphosphates for Ca^{2+}-dependent CG fusion. 5 mM ATP was replaced with ATP-γ-S, GTP, ADP, AMP, or pyrophosphate. Data (20 min time points) were normalized as the percentage of fusion in the presence of ATP. All quantitative data are mean ± SD from ≥ 3 independent experiments.

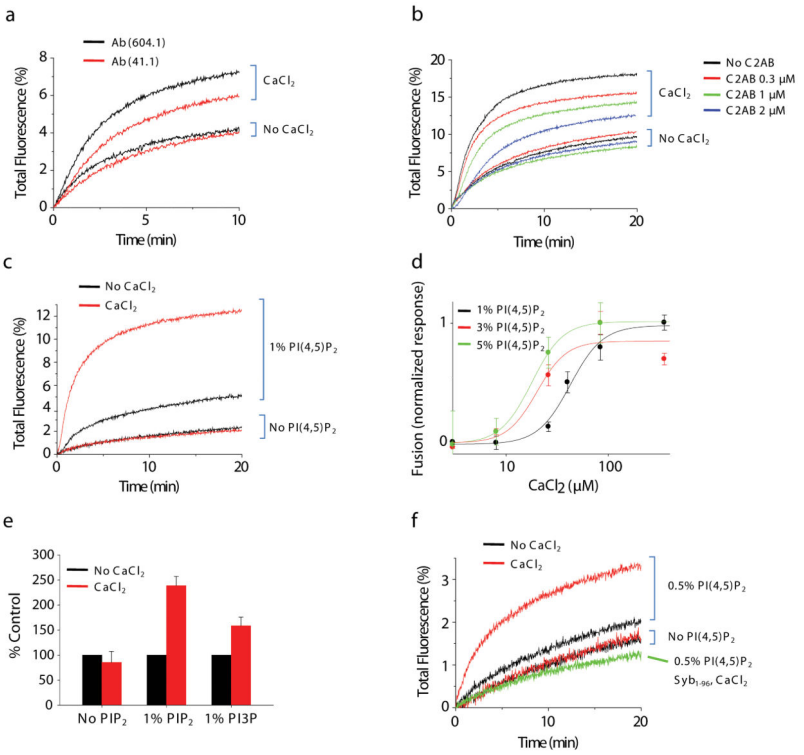

Figure 3.

Ca^{2+}-dependent enhancement of vesicle fusion is mediated by endogenous synaptotagmin-1 and dependent on $PI(4,5)P_2$ in the target membrane. (**a**) A monoclonal antibody specific for the cytoplasmic domain of synaptotagmin-1 (Ab 41.1) reduced Ca^{2+}-dependent enhancement of CG fusion whereas no effect was observed on the basal fusion rate (10 μM of antibody for 10 min at 37°C). No effect was seen when an antibody specific for the intravesicular domain of synaptotagmin-1 (Ab 604.1) was used as control. (**b**) Addition of recombinant C2AB domain of synaptotagmin-1 blocked Ca^{2+}-dependent enhancement of CG fusion in a dose-dependent manner. Again, no effect on basal fusion was observed. (**c**) $PI(4,5)P_2$ is required for Ca^{2+}-dependent enhancement of CG fusion. Omission of $PI(4,5)P_2$ from the LUV membrane reduced basal fusion and abolished Ca^{2+}-dependent enhancement of fusion. (**d**) Higher $PI(4,5)P_2$ concentrations increase Ca^{2+} sensitivity for CG fusion by lowering EC_{50}. Dose-dependence of Ca^{2+}-dependent enhancement of fusion (apparent EC_{50} values for Ca^{2+} in parentheses) at $PI(4,5)P_2$ concentrations of 1% (40.1 ± 2 μM), 3% (20 ± 8 μM), and 5% (17.8 ± 8 μM) $PI(4,5)P_2$ in the LUV membranes. Basal fusion level was subtracted and maximum fusion was normalized to 1. All quantitative data are mean ± SD from ≥ 3 independent experiments. (**e**) PI3P in the target LUVs also enhanced Ca^{2+}-dependent vesicle fusion of CG, but the effect was weaker than with $PI(4,5)P_2$. (**f**) Purified synaptic vesicles from rat brain also require $PI(4,5)P_2$ in the target LUVs for Ca^{2+}-dependent enhancement of fusion.

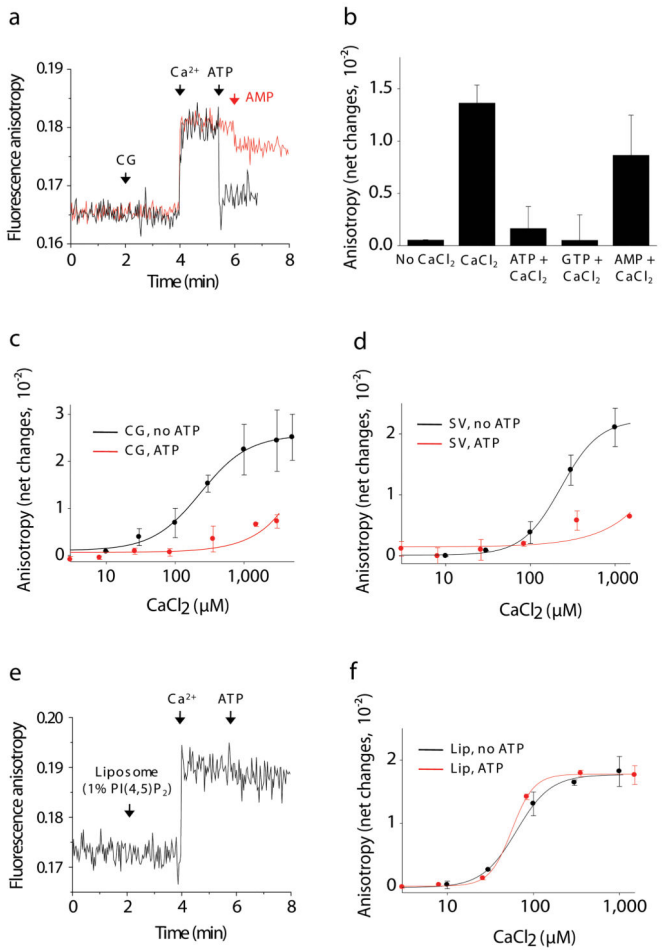

Figure 4.

ATP prevents *cis*-association of synaptotagmin-1 to the vesicle membrane. (**a**,**b**) C2AB binding to CG membranes was monitored by fluorescence anisotropy. CGs were incubated with a purified fragment of synaptotagmin-1 encompassing both C2 domains ($Syt_{97–421}$, Alexa Fluor 488-labeled at S342C). Binding resulted in an increase of anisotropy due to a reduced mobility of the fluorophores[35]. Binding was dependent on the presence of Ca^{2+} (300 μM) and reverted by the addition of 5 mM ATP (note that ATP addition reduces the free Ca^{2+} concentration to 84 μM due to chelation, see Online Methods for details). AMP is less efficient whereas GTP is as effective as ATP. (**c**,**d**) Ca^{2+}-dependence of C2AB binding to purified CGs (**c**) and SVs (**d**) in the presence and absence of 5 mM ATP. ATP completely dissociated C2AB domain from CG or synaptic vesicle membranes at all Ca^{2+} concentrations (note that the Ca^{2+}-concentrations are corrected to represent free Ca^{2+} concentration, see Online Methods for details). (**e**,**f**) The presence of 1% $PI(4,5)P_2$ in liposomes abolished ATP- dependent interference with Ca^{2+}-dependent binding of C2AB to the membrane of liposomes. All quantitative data are mean ± SD from ≥ 3 independent experiments.

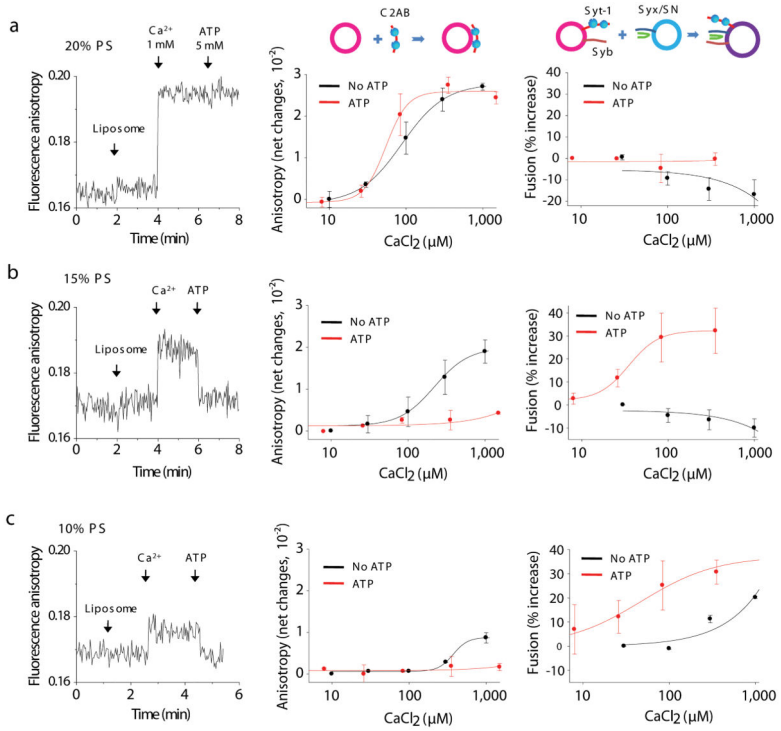

Figure 5.

ATP-dependent screening of *cis*-binding of synaptotagmin-1 depends on the concentration of acidic phospholipids. Using LUVs with decreasing concentrations of phosphatidylserine (PS), we monitored Ca^{2+}-dependent binding of C2AB domain by fluorescence anisotropy (left and middle panels). In parallel, we used LUVs of the same composition reconstituted with synaptobrevin and synaptotagmin-1 in fusion experiments, using standard acceptor LUVs containing the Q-SNARE complex and 1% $PI(4,5)P_2$ (right panels). ATP was able to prevent C2AB binding to liposomes containing 10% (**c**) and 15% (**b**), but not 20% PS (**a**), regardless of whether it was added simultaneously (middle panels) or after the addition of Ca^{2+} (left traces, note that we used 1 mM Ca^{2+} to exclude that the ATP-effect is due to partial chelation of free Ca^{2+} upon addition). Note that Ca^{2+}-dependent enhancement of fusion correlates with the ability of ATP to prevent *cis*-binding of synaptotagmin. All quantitative data are mean ± SD from ≥ 3 independent experiments.

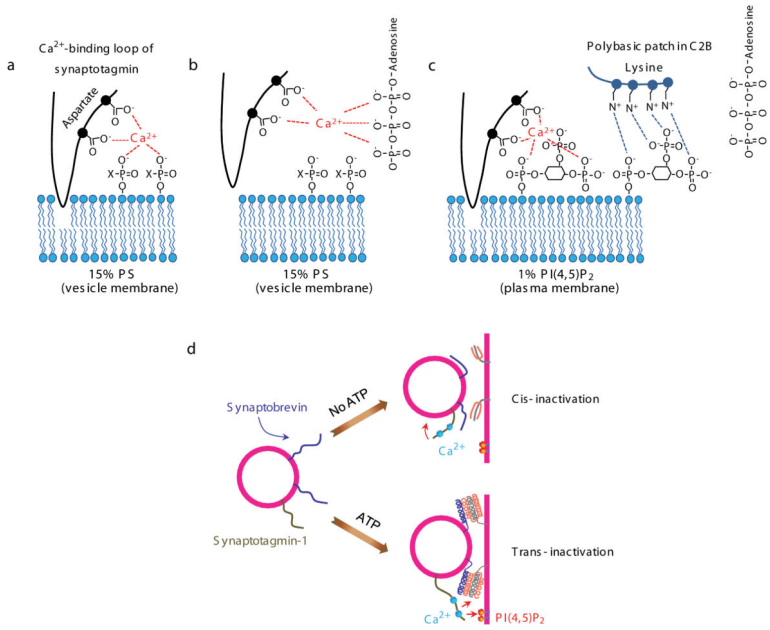

Figure 6.

Overview over the effect of polyphosphates on Ca^{2+}-dependent binding of synaptotagmin to membranes containing acidic phospholipids. (**a**) In the absence of ATP, synaptotagmin-1 binds to the vesicle membrane in a Ca^{2+}-dependent manner with EC_{50} of 233 μM Ca^{2+}. Aspartate residues of the C2-domains (black balls) provide partial coordination for Ca^{2+}-ions, with a more complete coordination sphere being contributed by acidic membrane lipids. (**b**) ATP, which chelates Ca^{2+} with a K_d of 230 μM[42], competes with Ca^{2+}-dependent membrane binding of synaptotagmin by shielding the coordination site of acidic phospholipids. (**c**) In the presence of $PI(4,5)P_2$, Ca^{2+}-binding between the membrane and the C2 domains is enhanced with an EC_{50} of 56 μM (Fig. 4f), probably involving a polybasic $_{2+}$ patch within the C2B domain that is known to bind to $PI(4,5)P_2$ on its own. The high Ca affinity of C2AB binding to $PI(4,5)P_2$-containing target membranes cannot be competed for by ATP. (**d**) In the absence of ATP, Ca^{2+} inactivates synaptotagmin-1 via *cis*-association (binding to vesicle membrane). ATP selectively abolishes *cis*-binding of synaptotagmin-1, thus leaving the C2AB domains active to interact with the plasma membrane containing $PI(4,5)P_2$, as required for Ca^{2+}-triggering of exocytosis.