THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 23, pp. 15845–15852, June 9, 2006

Phosphatidylinositol Phosphates as Co-activators of Ca^{2+} Binding to C_2 Domains of Synaptotagmin 1*[S]

Received for publication, January 30, 2006, and in revised form, March 16, 2006 Published, JBC Papers in Press, April 4, 2006, DOI 10.1074/jbc.M600888200

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Ca^{2+}-dependent phospholipid binding to the C_2A and C_2B domains of synaptotagmin 1 is thought to trigger fast neurotransmitter release, but only Ca^{2+} binding to the C_2B domain is essential for release. To investigate the underlying mechanism, we have compared the role of basic residues in Ca^{2+}/phospholipid binding and in release. Mutations in a polybasic sequence on the side of the C_2B domain β-sandwich or in a basic residue in a top Ca^{2+}-binding loop of the C_2A domain (R233) cause comparable decreases in the apparent Ca^{2+} affinity of synaptotagmin 1 and the Ca^{2+} sensitivity of release, whereas mutation of the residue homologous to Arg^{233} in the C_2B domain (Lys^{366}) has no effect. Phosphatidylinositol polyphosphates co-activate Ca^{2+}-dependent and -independent phospholipid binding to synaptotagmin 1, but the effects of these mutations on release only correlate with their effects on the Ca^{2+}-dependent component. These results reveal clear distinctions in the Ca^{2+}-dependent phospholipid binding modes of the synaptotagmin 1 C_2 domains that may underlie their functional asymmetry and suggest that phosphatidylinositol polyphosphates may serve as physiological modulators of Ca^{2+} affinity of synaptotagmin 1 *in vivo*.

The synaptic vesicle protein synaptotagmin 1 acts as a major Ca^{2+} sensor in neurotransmitter release at excitatory and inhibitory synapses (1, 2). This function can be attributed to Ca^{2+} binding to the two C_2 domains of synaptotagmin 1 (referred to as the C_2A and C_2B domain; Ref. 3). The C_2A and C_2B domains bind three and two Ca^{2+} ions, respectively, through loops located at the tips of similar β-sandwich structures (4–7). Both C_2 domains bind to negatively charged phospholipids, including phosphoinositides, as a function of Ca^{2+}, and exhibit comparable apparent Ca^{2+} affinities (7–9). Furthermore, in the absence of Ca^{2+}, the C_2B domain, but not the C_2A domain, of synaptotagmin 1 avidly binds to inositolpolyphosphates (such as inositol 1,3,4,5-tetrakisphosphate) and to phosphoinositides (such as phosphatidylinositol 4,5-bisphosphate (PIP_2)[2]) via a polybasic sequence that is located in a β-strand on the side of the domain (10, 11). Moreover, the C_2 domains interact Ca^{2+}-dependently and -independently with individual SNARE proteins such as syntaxin1 and SNAP-25 and with SNARE complexes (12–17). Finally, the synaptotagmin C_2 domains engage in additional interactions *in vitro*, including binding of the clathrin adaptor protein complex AP-2 (18–20) and Ca^{2+} channels (21–23).

Although the biochemical properties of synaptotagmin 1 have been studied in detail, the functional importance of individual properties has remained unclear. Ca^{2+}-dependent phospholipid binding by synaptotagmin 1 *in vitro* correlates with its functional role in Ca^{2+} triggering of release *in vivo*, as demonstrated with both loss-of-function and gain-of-function mutations (1, 24). This correlation suggests that Ca^{2+}-dependent phospholipid binding represents a crucial step in synaptotagmin 1 function. However, mutational studies revealed that although both C_2 domains of synaptotagmin 1 are involved in Ca^{2+}-triggered release, Ca^{2+} binding to the C_2A domain only boosts release, whereas Ca^{2+} binding to the C_2B domain is essential for synchronous release (1, 25–29). Thus, it is puzzling that the two C_2 domains of synaptotagmin 1 appear to exhibit similar Ca^{2+}-dependent phospholipid binding properties *in vitro* but a striking functional asymmetry *in vivo*. The differential requirements of the C_2A *versus* C_2B domain for the Ca^{2+} triggering of release could potentially arise from the unique ability of the C_2B domain (but not the C_2A domain) to bind to phosphoinositides in a Ca^{2+}-independent manner (10, 11). Indeed, consistent with this idea, microinjection of soluble inositol polyphosphates into nerve terminals potently inhibits release (30). Two observations, however, argue against this interpretation. First, the C_2B domain acts in release by binding Ca^{2+} (26, 29), making it difficult to imagine that a Ca^{2+}-independent activity of the C_2B domain mediates its essential role. Second and more importantly, mutations in the phosphatidylinositol phosphate-binding site in the C_2B domain of synaptotagmin 1 (the polybasic sequence on the side of the domain) abolish phosphatidylinositol phosphate binding (10, 11) but impair synaptotagmin 1 function only moderately (31, 32). Thus the physiological significance of Ca^{2+}-independent binding of phosphatidylinositol phosphates to the polybasic sequences remains unclear.

To address this conundrum, we have now tested whether the polybasic sequence of the C_2B domain may participate in Ca^{2+}-dependent phospholipid binding and whether such an activity correlates with the *in vivo* function of synaptotagmin 1. We did not examine this activity previously because the location of the polybasic sequence in the atomic structure of the C_2B domain, on the side, away from the Ca^{2+}-binding sequences, made a participation in Ca^{2+}-dependent activities appear

* This work was supported by the Brown Foundation and National Institutes of Health Grants NS50655 (to C. R.) and NS40944 (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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[2] The abbreviations used are: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SFV, Semliki Forest virus; EPSC, excitatory postsynaptic current; mEPSC, miniature EPSC; NBD, 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; GST, glutathione *S*-transferase; MES, 4-morpholineethanesulfonic acid; HSQC, heteronuclear single quantum correlation.

highly unlikely. Surprisingly, we find that mutation of the polybasic sequence in the C_2B domain (the KAKA mutation) impairs Ca^{2+}-dependent phospholipid binding to a similar extent as mutation of the positively charged Arg^{233} in the C_2A domain. We also show that under approximately physiological conditions, binding of phosphoinositides to synaptotagmin 1 is entirely Ca^{2+}-dependent, although at low ionic strength or in the absence of Mg^{2+}, Ca^{2+}-independent binding of phosphoinositides occurs. Moreover, we demonstrate that the effects of the R233Q and KAKA mutations on the Ca^{2+} sensitivity of release correlate with their impairment of the Ca^{2+}-dependent component of phospholipid binding but not with their effects on the Ca^{2+}-independent component. Finally, we show that the K366Q substitution in the C_2B domain (that corresponds to the R233Q mutation in the C_2A domain) has no effect on phospholipid binding and release. These results show that the polybasic region contributes to Ca^{2+}-dependent phospholipid binding to synaptotagmin 1, reinforcing the notion that this is a major function of synaptotagmin 1 during fast Ca^{2+}-triggered release and revealing an overt asymmetry in the contributions of the two C_2 domains to this activity. In addition, the enhancement of Ca^{2+}-induced phospholipid binding by phosphoinositides suggests that phosphoinositides may modulate the apparent Ca^{2+} affinity of synaptotagmin 1 during release.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Viral Preparation—Mutations were introduced into rat cDNA encoding synaptotagmin1 using point mutation PCR or QuikChange™ mutagenesis technique as described previously (52). Mutated synaptotagmin 1 inserts were subsequently subcloned into the pSFV1 vector (Invitrogen). Semliki Forest virus (SFV) production was carried out as described previously (40). Briefly, linearized SFV plasmids were transcribed *in vitro*, and the resulting RNA were transfected into BHK21 cells by electroporation. 24 h later, cell culture media containing inactive virus were collected and frozen in aliquots. For one experiment, one frozen aliquot was thawed and activated by α-chymotrypsin for 30 min.

Neuronal Culture and Viral Infection—Autaptic cultures of neonatal synaptotagmin-deficient mice hippocampal neurons were prepared as described previously (53). Briefly, islands of substrate (polylysine/collagen) were applied with a stamp containing regularly spaced squares (200 × 200 μm). Astrocytes (5,000/cm^2) were preplated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. As soon as astrocytes reached confluence, 5-fluoro-2′-deoxyuridine was added (10 μM) to inhibit overgrowth of the astrocytes. Before neurons were plated at a density of 500/cm^2, the medium was replaced with serum-free medium (Neurobasal medium A supplemented with B27). Islands containing single neurons were examined after 10–15 days growth in culture. Successfully transfected neurons were detected by development of eGFP-based fluorescence, resulting from internal ribosome entry site (IRES) driven expression of the green fluorescent protein. We compared expression levels of WT and synaptotagmin 1^{KAKA} overexpression levels by analyses of transfection efficiency and using Western blot analyses. Transfection efficiency after 15 h was 70–80% in both wild-type synaptotagmin as well as synaptotagmin 1^{KAKA}-transfected cultures. Neurons with viral infection of 15–20 h resulted in the highest rescue efficiency. Western blot analysis was performed from high density cultures from neurons (200,000 neurons/20 cm^2/15 days *in vitro*) that were transfected 15 h prior to harvesting of the proteins. Equal amounts of proteins (10 μg) were used for SDS-PAGE and blotted onto nitrocellulose membrane. The viral expressed synaptotagmin 1 was probed with a monoclonal mouse anti-synaptotagmin 1 antibody (1:5,000; clone 41.1, Synaptic Systems, Göttingen, Germany) and the endogenous expressed synaptobrevin 2 with a polyclonal rabbit anti-synaptobrevin 2 antibody (1:10000, Synaptic Systems, Göttingen, Germany). Blots were developed using horseradish peroxidase-conjugated secondary antibody (1:10,000, Amersham Biosciences) together with the ECL™ Western blotting detection reagent. Semiquantitative estimates for synaptotagmin 1 overexpression were obtained by normalizing the densitometric Western blot signals against the densitometric signal from endogenous synaptobrevin 2. The degree of synaptotagmin overexpression for synaptotagmin wild-type and synaptoatagmin 1^{KAKA} was comparable in both groups (approximately 4–6-fold, three independent experiments). Patch-pipette solutions contained (in mM): 120 KCl, 20 HEPES, 1 EGTA, 4.6 $MgCl_2$, 4 K_2-ATP, 15 creatine phosphate, 50 units ml^{-1} phosphocreatine kinase (300 mosM, pH 7.3). The extracellular saline solution contained (in mM) 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$, and 4 $MgCl_2$ except otherwise noted (305 mosM, pH 7.3). All chemicals, except for 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)-quinoxaline (NBQX) and tetrodotoxin (Tocris, Bristol, UK), were purchased from Sigma. All solutions were applied using a fast flow system at room temperature (53).

Electrophysiology—Cells were whole cell voltage-clamped at −70 mV with an Axopatch 200B amplifier (Axon Instruments) under the control of Clampex 8.0 (Axon Instruments) program. Excitatory postsynaptic currents were evoked by somatic depolarization to 0 mV for 2 ms. The readily releasable pool was determined by integrating the transient inward current component evoked by 4-s application of external solution added with 500 mM sucrose. Miniature excitatory postsynaptic currents (mEPSCs) were measured in 300 nM tetrodotoxin. For the determination of apparent Ca^{2+} sensitivity of EPSC amplitudes, EPSCs were recorded in Ca^{2+} concentrations varying from 0.5 to 12 mM in presence of 1 mM Mg^{2+} and were subsequently normalized to EPSC recorded intermittently at standard conditions (4 mM Ca^{2+}, 4 mM Mg^{2+}). Data were low pass-filtered at 1 or 5 kHz and stored at either 10 or 20 kHz. The series resistance was compensated to 70–90%. Only cells with series resistances below 15 MOhm were analyzed. Data were analyzed with software Axograph 4.6 and Kaleidagraph 3.0 running on a Mac OS X system. Statistical significance was tested using student's *t* test; *** and ** in Figs. 4 and 6 indicate *t* test values of $p < 0.001$ and $p < 0.01$, respectively. All values are presented as the mean ± S.E.

Recombinant Proteins—Site-directed mutagenesis to produce expression vectors for K326A,K327A-C_2B were performed by standard PCR techniques using custom-designed primers and the QuikChange™ site-directed mutagenesis kit (Stratagene). The ^{15}N-labeled wild-type and K326A,K327A mutant C_2B domains were expressed in bacteria and purified as described (34).

Centrifugation Phospholipid Binding Assay—Lipid mixture of synaptic vesicles (41% phosphatidylcholine (PC), 32% phosphatidylethanolamine (PE), 12% phosphatidylserine (PS), 5% phosphatidylinositol (PI), and 10% cholesterol; Ref. 36) with or without additional phosphatidylinositol 4-phosphate (PIP) and PIP_2 was dried as a thin layer under a stream of nitrogen gas. HEPES buffer (50 mM HEPES, pH 6.8, 100 mM NaCl, and 4 mM EGTA) containing 0.5 M sucrose was added to the dried lipid layer, vortexed for 20 min, and sonicated for 5 min in a bath sonicator (model G112SP1G; Laboratory Supply Co. Inc.). After liposome formation, 4 volumes of HEPES buffer without sucrose were added and centrifuged to separate heavy liposomes from free phospholipids (100,000 × *g* for 30 min). Heavy liposomes were washed once and repelleted (20,800 × *g* for 10 min). Recombinant wild-type or mutant forms of GST-synaptotagmin 1-C_2A/B proteins (10 μg) were mixed with 100 μg of liposomes in the presence of different concentrations of free Ca^{2+} calculated with EqCal software (Biosoft, Ferguson, MI). After

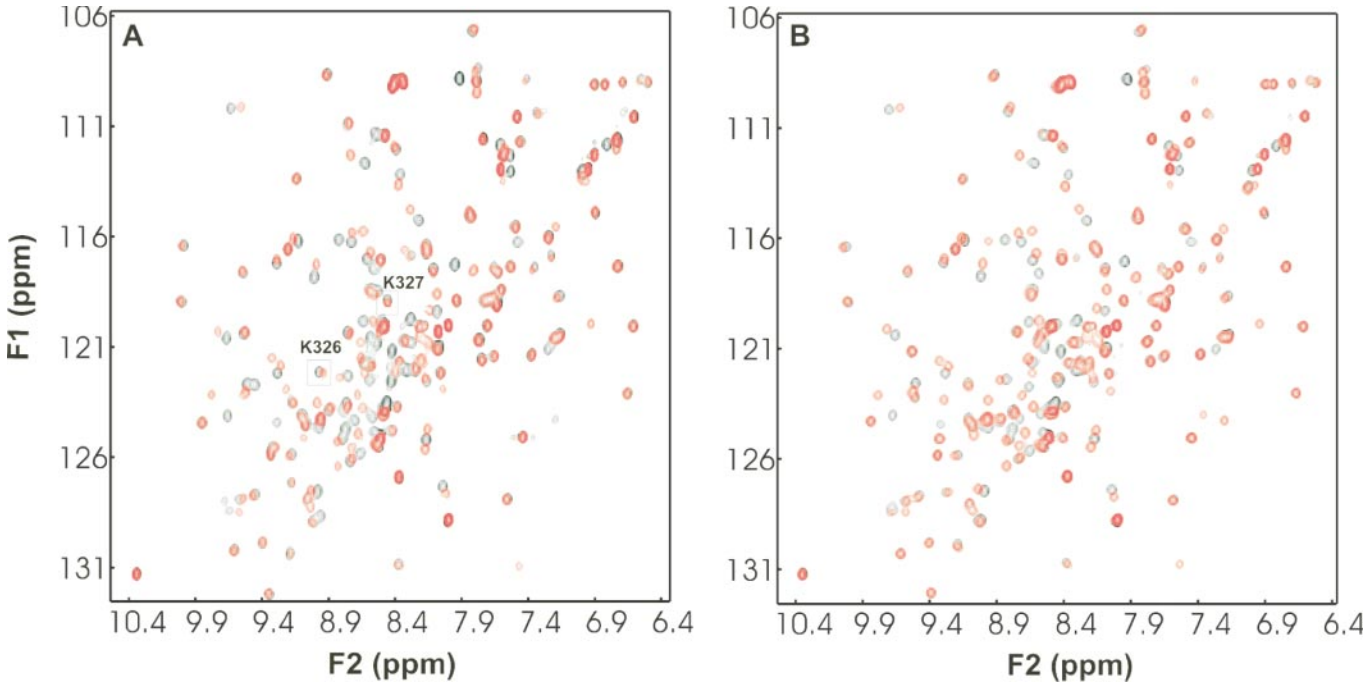

FIGURE 1. **The KAKA mutation does not affect the proper folding of the C_2B domain.** The diagrams show ^1H-^{15}N HSQC spectra of the wild-type (*A*) and KAKA mutant (*B*) synaptotagmin 1 C_2B domain in 1 mM EDTA (*black contours*) or 20 mM Ca^{2+} (*red contours*). The cross-peaks corresponding to the two mutated residues (Lys^{326} and Lys^{327}) in the spectrum of the wild-type C_2B domain are labeled.

10-min incubation of total 1-ml reaction mixture on an Eppendorf thermal mixer at 30 °C and 800 rpm, liposomes were re-isolated by centrifugation (20,800 × *g* for 10 min) and washed three times with 1 ml of the corresponding buffers. Chloroform:methanol (1:2, v/v) solution was added into the pelleted liposomes to denature protein and dissolve lipids. After centrifugation (20,800 × *g* for 15 min), the protein precipitate was resuspended in 30 μl of 2× SDS sample buffer and analyzed by SDS-PAGE and Coomassie Blue staining (7).

NMR Spectroscopy—NMR spectra were acquired on a Varian INOVA500 spectrometer at 27 °C in 50 mM MES, pH 6.3, 0.15 M NaCl, and 2 mM dithiothreitol, with samples containing 0.1 mM wild-type or K326A,K327A mutant C_2B domain, and 1 mM EDTA or 20 mM Ca^{2+}. ^1H-^{15}N heteronuclear single quantum correlation (HSQC) spectra were acquired using a sensitivity-enhanced pulse sequence (54) with total acquisition times of 1–2 h.

RESULTS

Neutralization of the Polybasic Region in the C_2B Domain of Synaptotagmin 1 by the K326A,K327A Substitution (the "KAKA Substitution") Does Not Cause Substantial Structural Changes—To examine the structural effects of the KAKA substitution, we purified recombinant mutant and wild-type C_2B domains that were uniformly ^{15}N-labeled and acquired ^1H-^{15}N HSQC spectra in the absence and presence of Ca^{2+} (Fig. 1). The spectra and the Ca^{2+}-induced cross-peak shifts observed for the KAKA mutant of the C_2B domain were very similar to those observed for the wild-type C_2B domain, demonstrating that the synaptotagmin 1^{KAKA} mutation does not induce major structural perturbations in the C_2B domain or impair its ability to bind Ca^{2+}.

The Polybasic Region of the Synaptotagmin 1 C_2B Domain Participates in Ca^{2+}-dependent Phospholipid Binding—The polybasic region of the synaptotagmin 1 C_2B domain is located in the fourth β-strand on a side of the C_2B domain, quite distant from the top loop sequences that are thought to mediate Ca^{2+}-dependent phospholipid binding in C_2 domains. Consistent with this spatial separation, the polybasic region was found to bind to phosphatidylinositides in a Ca^{2+}-independent. but not Ca^{2+}-dependent. manner (11, 33). However, both synaptotagmin 1 C_2 domains (and other C_2 domains) are known to bind in a Ca^{2+}-dependent manner to all negatively charged phospholipids, including phosphatidylinositides (9). To explore the relation between Ca^{2+}-dependent and -independent binding of phosphoinositides to the C_2B domain, we systematically examined the role of the polybasic region. In these studies, we employed recombinant fragments containing both C_2 domains (the C_2AB domain fragment) instead of isolated C_2 domains because the effects of mutations on the biochemical properties of the individual C_2 domains may not reflect their effects on the normally present double C_2 domain configuration (*e.g.* Ref. 25). We produced recombinant C_2 domain proteins under conditions that minimize bacterial non-proteinaceous contaminations bound to the polybasic region (34) and compared the properties of the wild-type C_2AB domain fragment with those of mutant C_2AB domains containing either the KAKA substitution in the C_2B domain or the R233Q substitution in the C_2A domain. The latter mutation was used because it decreases the apparent Ca^{2+} affinity of the C_2AB domain fragment and thus serves as a control for changes in apparent Ca^{2+} affinity (1).

We performed Ca^{2+} titrations to compare the effects of the R233Q and KAKA mutations on the Ca^{2+} dependence of phospholipid binding in the presence of increasing concentrations of PIP and PIP_2. In addition, we also studied the effects of Mg^{2+} on these properties because Mg^{2+} may bind to PIP and PIP_2. In these experiments, we employed a centrifugation assay that yields more reliable results than standard GST pulldown assays (7, 35). The phospholipid vesicles used in these experiments contained a mixture of PC, PS, PE, PI, and cholesterol that corresponds to the reported phospholipid composition of synaptic vesicle membranes (36, 37). Small, incremental amounts of PIP and PIP_2 were added to mimic those that are likely to be present *in vivo*, based on the estimation that phosphoinositides constitute 5% of the synaptic membrane lipids and that these phosphoinositides are composed of: 80–90%

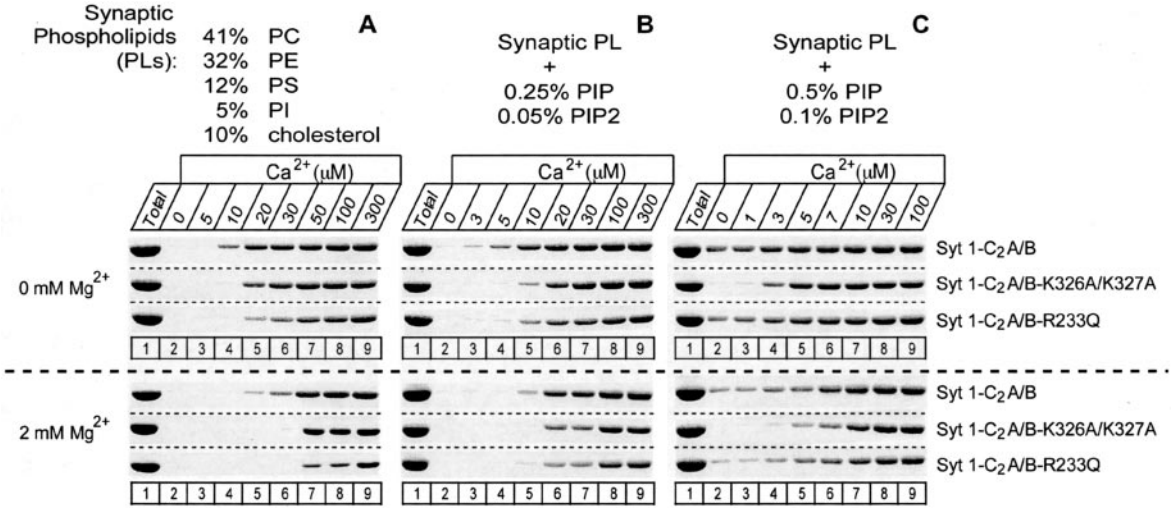

FIGURE 2. **The electrostatics of WT, synaptotagmin 1^{KAKA} (*Syt 1*) and synaptotagmin 1^{R233Q} to phospholipid.** GST fusions of the wild-type, K326A,K327A mutant, or R233Q mutant synaptotagmin 1-C_2A/B fragments were incubated with liposomes whose compositions are indicated on the top of each panel. *A–C*, Ca^{2+} dependence of phospholipid binding as an additional function of PIP/PIP_2 concentrations. Free Ca^{2+} concentrations in the presence or absence of 2 mM free Mg^{2+} were calculated with EqCal software. Data shown are representative of experiments performed multiple times (see supplemental Fig. 1).

PI, 5–10% PIP, 2–5% PIP_2, and less than 0.5% of other phosphoinositides (38, 39).[3]

In the absence of PIP and PIP_2, phospholipid binding to the wild-type and mutant synaptotagmin 1 C_2AB domain fragments required Ca^{2+}. At physiological ionic strength, the KAKA mutation markedly decreased the apparent Ca^{2+} affinity of all phospholipid binding similar to the R233Q mutation (Fig. 2 and supplemental Fig. 1). This unexpected result shows that the polybasic region of the C_2B domain, although quite distant from the Ca^{2+}-binding loops, contributes to Ca^{2+}-dependent phospholipid binding by the C_2B domain even in the absence phosphatidylinositol polyphosphates. The addition of increasing amounts of PIP and PIP_2 dramatically increased the apparent Ca^{2+} affinity of the wildtype and mutant C_2AB fragments (Fig. 2, *B* and *C*). Binding remained largely Ca^{2+}-dependent, although we observed partially Ca^{2+}-independent binding at the higher PIP and PIP_2 concentration. Consistent with previous reports (11), this binding was abolished by the KAKA mutation but not by the R233Q mutation (Fig. 2, *B* and *C*). This result supports the notion that the synaptotagmin C_2 domains interact with phospholipids in a Ca^{2+}-independent manner via the polybasic region of the C_2B domain if the concentration of negative charges on the phospholipid surfaces is increased by addition of phosphoinositides. However, these binding measurements were carried out in the absence of Mg^{2+}, which is universally present in the cytosol. Addition of Mg^{2+} decreased the apparent Ca^{2+} affinity of the wild-type and mutant C_2AB fragments and impaired Ca^{2+}-independent binding even of wild-type C_2 domains to membranes containing the higher PIP/PIP_2 concentrations (Fig. 2), suggesting that the Ca^{2+}-independent binding may not be physiological.

To further analyze the contribution of electrostatic interactions to phospholipid binding by synaptotagmin 1, we then examined the effects of changes in ionic strength (Fig. 3 and supplemental Fig. 2). As expected, increasing the ionic strength decreased phospholipid binding observed in the absence and presence of Ca^{2+}. Consistent with the central role of electrostatic forces in shaping phospholipid binding by synaptotagmin 1, both types of phospholipid interactions became more resistant to NaCl with increasing concentrations of PIP/PIP_2 and less resistant in the presence of Mg^{2+}. Similarly consistent with the role of electrostatic interactions, decreasing the ionic strength caused even the wild-type C_2AB fragment to bind to any negatively charged phospholipid in the absence of Ca^{2+} as long as Mg^{2+} was also lacking (Fig. 3). Binding was weakened but not abolished by the KAKA mutation (Fig. 3). The effects of the R233Q and KAKA mutations at different ionic strengths (Fig. 3) paralleled those observed in the Ca^{2+} titrations (Fig. 2). These observations show that the ability of synaptotagmin 1 to interact with membranes in the absence of Ca^{2+} does not specifically depend on polyphosphoinositides but is a function of the density of negative charges on the phospholipid surface and of the ionic strength. Furthermore, these observations demonstrate that although the polybasic region enhances binding to phosphoinositides, it is not actually required for this interaction.

Mutations in the Polybasic Region of the Synaptotagmin 1 C_2B Domain Decrease the Neurotransmitter Release Probability—We next examined the functional relevance of the polybasic region of the C_2B domain of synaptotagmin 1 physiologically. Primary autaptic cultures of synaptotagmin 1-deficient hippocampal neurons were infected with Semliki forest viruses that express wild-type or mutant synaptotagmin 1 (40). Wild-type, KAKA mutant, and R233Q mutant synaptotagmin 1 were compared side-by-side. A good initial test for efficiency of synaptic function is the EPSC amplitudes. The mean EPSC amplitude of the synaptotagmin 1^{KAKA} mutant was reduced ~50% (WT: 1.05 ± 0.13 nA, $n = 40$; synaptotagmin 1^{KAKA}: 0.46 ± 0.05 nA, $n = 40$; Fig. 4*A*), similar to earlier results (31, 32). The reduced EPSC amplitude observed for the synaptotagmin 1^{KAKA} mutant, however, could just as well be the result of a decrease in the readily releasable vesicle pool. To test this, we applied hypertonic sucrose solution to the WT or synaptotagmin 1^{KAKA} transfected neurons and quantified the transient component of the synaptic response corresponding to the pool of fusion competent vesicles (RRP) (41). We observed no significant differences in the RRP size between neurons rescued with wild-type or KAKA mutant synaptotagmin 1 (Fig. 4*C*). Based on the observation of reduced synaptic output and an unchanged pool of readily releasable vesicles, the computed probability that an individual fusion-competent vesicle fuses following an action potential (the vesicular release probability, P_{vr}) was reduced from 7.1 ± 0.6% ($n = 34$) for wild-type synaptotagmin 1 to 4.1 ± 0.6% ($n = 34$) ($p < 0.01$) for the synaptotagmin 1^{KAKA} mutant (Fig. 4*D*).

[3] P. DeCamilli, personal communication.

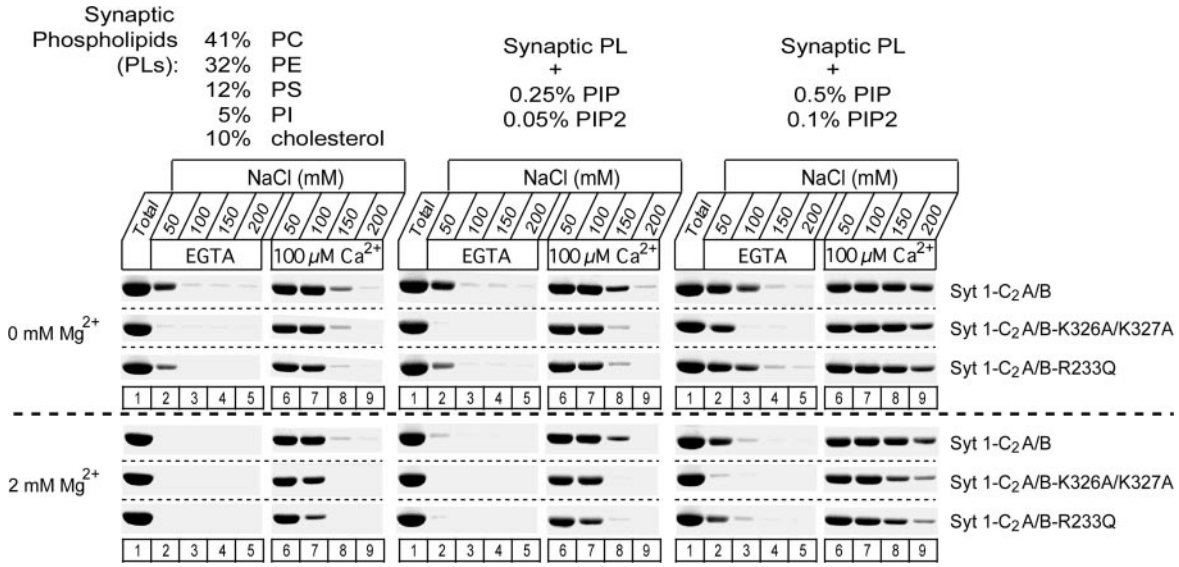

FIGURE 3. **Ca^{2+}-dependent and –independent phospholipid binding properties of WT, synaptotagmin 1^{KAKA} and synaptotagmin 1^{R233Q} as a function of ionic strength.** GST fusions of the wild-type, K326A,K327A mutant, or R233Q mutant synaptotagmin 1 (*Syt 1*)-C_2A/B fragments were incubated with liposomes whose compositions are indicated on the *bottom* of each panel in the presence of the different NaCl concentrations shown. 100 μM Ca^{2+} concentrations in the presence or absence of 2 mM free Mg^{2+} were calculated with EqCal software. Liposomes were centrifuged and washed, and bound proteins were analyzed by SDS-PAGE and Coomasie Blue staining. Data shown are representative of experiments performed multiple times (see supplemental Fig. 2).

Synaptotagmin 1 critically determines the time course of neurotransmitter release (42–44). The Ca^{2+}-independent interaction of the polybasic C_2B site with PIP_2 has been hypothesized to contribute to the fast action of synaptotagmin 1 by prepositioning synaptotagmin 1 at the target membrane (11). Although such Ca^{2+}-independent interactions would occur at a physiological ionic strength and Mg^{2+} concentration only in the presence of very high concentrations of PIP_2 (Figs. 2 and 3; Ref. 11), we tested this hypothesis by studying the effect of the KAKA mutation on the time course of release. We integrated the EPSCs observed in synapses expressing wild-type or KAKA mutant synaptotagmin 1 and fitted the responses with a double exponential equation (45). We found no significant changes in the time constants of the fast and slow components (WT: τ_{fast} = 8.0 ± 0.6 ms, τ_{slow} = 252 ± 27 ms, n = 17; synaptotagmin 1^{KAKA}: τ_{fast} = 10.1 ± 1.0 ms, τ_{slow} = 236 ± 33 ms, n = 12; Fig. 4*B*). The reduction in EPSC amplitude for the synaptotagmin 1^{KAKA} mutant was entirely the result of a 50% decrease in the amplitude of the fast component (wild-type: Q_{fast} = 11.3 ± 1.4 pC, Q_{slow} = 2.7 ± 0.4 pC, n = 17; synaptotagmin 1^{KAKA}: Q_{fast} = 6.2 ± 0.8 pC, Q_{slow} = 2.5 ± 0.5 pC, n = 12; Fig. 4*B*). While these results suggest a specific role for synaptotagmin 1 in enabling fast release, they also undermine the exclusive role played by the polybasic patch of the C_2B domain in positioning synaptotagmin 1 to speed up fast release.

Ca^{2+}-independent binding of synaptotagmin 1 may influence spontaneous release activity and, indeed, mutating the polybasic region of the C_2B domain of synaptotagmin 1 in drosophila led to a reduction in miniature excitatory junctional potential frequency (31). Our analysis of spontaneous release activity, however, revealed no significant difference in mEPSC frequency between wild-type and KAKA mutant synaptotagmin 1 (wild-type: 2.4 ± 0.4 Hz, n = 16; synaptotagmin 1^{KAKA}: 1.9 ± 0.5 Hz, n = 12). Furthermore, the mEPSC amplitudes were not different between WT and synaptotagmin 1^{KAKA} mutants (WT: 21.0 ± 1.2 pA, n = 16; synaptotagmin 1^{KAKA}: 19.9 ± 1.4 pA, n = 12), indicating that the reduced synaptic output is neither due to changes in neurotransmitter content in the vesicle nor the sensitivity of the postsynaptic AMPA receptors.

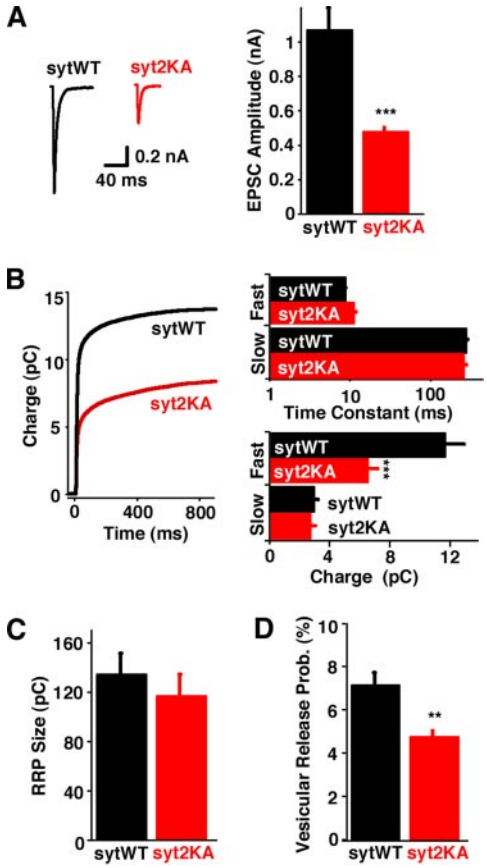

FIGURE 4. **Synaptotagmin (*syt*) 1^{KAKA} mutation reduces vesicular release probability in excitatory neurons.** *A*, *left side*, typical synaptic responses from excitatory hippocampal murine synaptotagmin 1 (synaptotagmin) knock-out neurons rescued either with wild-type synaptotagmin 1 WT or the C_2B polybasic mutant synaptotagmin 1^{KAKA}. *Right side*, mean EPSC amplitudes of WT and synaptotagmin 1^{KAKA} rescued neurons. *B*, *left side*, exemplary integrated EPSC charge responses from WT and synaptotagmin 1^{KAKA} rescued neurons. *Right side*, analysis of time constants and amplitudes after two-component exponential fitting. *C*, mean readily releasable pool RRP sizes of the WT (n = 34) and synaptotagmin 1^{KAKA} (n = 34). *D*, mean vesicular release probability of the WT (n = 34) or synaptotagmin 1^{KAKA} (n = 34).

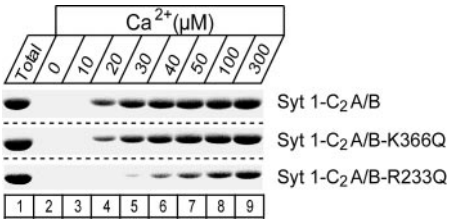

FIGURE 5. **Comparison of the effects of the R233Q and K366Q substitutions on the apparent Ca^{2+} affinity of the synaptotagmin (*Syt 1*) C_2 domains.** GST fusions of the wild-type, K366Q mutant, or R233Q mutant synaptotagmin 1-C_2A/B fragments were incubated with liposomes reconstituted with synaptic phospholipid composition (41% PC, 32% PE, 12% PS, 5% PI, and 10% cholesterol) in the presence of both 2 mM Mg^{2+} and free Ca^{2+} at the concentrations shown, clamped by Ca^{2+}/Mg^{2+}/EGTA buffers. Liposomes were centrifuged and washed, and bound proteins were analyzed by SDS-PAGE and Coomassie Blue staining. Data shown are representative of experiments performed multiple times.

Comparison of the Effects of Positively Charged Residues in the Ca^{2+}-binding Loops Versus the Polybasic Region—The experiments described above suggest that, surprisingly, the polybasic region in the C_2B domain contributes to Ca^{2+}-dependent phospholipid binding. This is an unexpected finding not only because of the physical distance of the polybasic region from the Ca^{2+}-binding sites but also because we previously defined a similar role for a positively charged residue in the Ca^{2+}-binding loops of the C_2A domain (Arg^{233}). These observations, together with the fact that the C_2A domain does not contain the polybasic region, raise the intriguing possibility that the C_2A and C_2B domains interact with phospholipids in a distinct manner. To test this hypothesis, we investigated the role of Lys^{366}, a lysine residue in the C_2B domain that precisely corresponds to the Arg^{233} residue in the C_2A domain. However, we found that the K366Q substitution had no significant effect on the apparent Ca^{2+} affinity of the C_2AB domain fragment (Fig. 5). This result further emphasizes the asymmetry in the Ca^{2+}-dependent phospholipid binding modes of the C_2A and C_2B domains. This was validated in a systematic analysis of the R233Q, K366Q, and KAKA mutations on release in the same experiments, where we failed to detect a significant effect of the K366Q mutation on release. Rescue experiments revealed that during repetitive stimulation at 10 Hz, neurons expressing either KAKA or R233Q mutant synaptotagmin 1 exhibited strong initial facilitation, whereas neurons expressing wild-type synaptotagmin 1 or the K366Q mutant synaptotagmin 1 exhibited almost identical moderate depression (Fig. 6, *A* and *B*). This result is consistent with a similar decrease in release probability in neurons expressing KAKA or R233Q mutant synaptotagmin 1 but not in neurons expressing K366Q mutant synaptotagmin 1 (Fig. 6*C*). Finally, we compared the apparent Ca^{2+} sensitivity of release (Fig. 6*D*). As expected, the synaptotagmin 1^{R233Q} mutant led to a nearly 2-fold right shift of the dose-response curve, consistent with previous data (1). The synaptotagmin 1^{KAKA} mutant showed a similar shift in apparent Ca^{2+} sensitivity (see also Ref. 32), whereas the synaptotagmin 1^{K366Q} mutant exhibited no shift and was practically identical to wild-type synaptotagmin 1 (Fig. 6*D*).

DISCUSSION

Synaptotagmin 1 acts as a major Ca^{2+} sensor in neurotransmitter release via Ca^{2+} binding to its two C_2 domains (1). The two C_2 domains exhibit similar overall structures and properties but differ dramatically in their function: Ca^{2+} binding to the C_2A domain serves a regulatory role in release, whereas Ca^{2+} binding to the C_2B domain is essential for release (1, 31, 46). In the present study, we have expanded on previous studies indicating that only the C_2B but not the C_2A domain may interact with phosphatidylinositol polyphosphates in a Ca^{2+}-independent manner (10, 47) and used a combination of biophysical, biochemical,

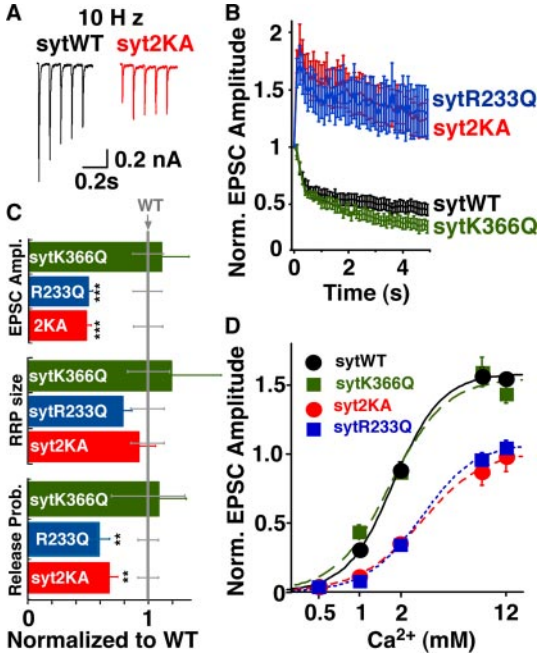

FIGURE 6. **Synaptic properties of the three synaptotagmin (*syt*) 1 basic residue mutants (synaptotagmin 1^{R233Q}, synaptotagmin 1^{K366Q}, and synaptotagmin 1^{KAKA}) and WT.** *A*, *left side*, raw traces of the initial five consecutive EPSCs of WT and synaptotagmin 1^{KAKA} (*syt2KA*) mutant evoked at 10 Hz. *B*, plot of normalized EPSC amplitudes during trains of action potentials applied at 10 Hz (synaptotagmin 1^{R233Q}, $n = 35$; synaptotagmin 1^{KAKA}, $n = 35$; WT, $n = 36$; and synaptotagmin 1^{K366Q}, $n = 8$). *C*, EPSC amplitudes, RRP sizes, and vesicular release probabilities of the three mutants normalized to WT (WT, $n = 34$; synaptotagmin 1^{R233Q}, $n = 33$; synaptotagmin 1^{KAKA}, $n = 34$; separate experiment: WT, $n = 9$; synaptotagmin 1^{K366Q}, $n = 8$). *D*, mean normalized EPSC amplitude as a function of external Ca^{2+} concentration (WT, $n = 9$–15; synaptotagmin 1^{R233Q}, $n = 9$; synaptotagmin 1^{K366Q}, $n = 8$–12; synaptotagmin 1^{KAKA}, $n = 9$–13).

and electrophysiological techniques to investigate the role of this binding *in vitro* and *in vivo*. Our data suggest three conclusions: 1) phospholipid binding to synaptotagmin 1 C_2 domains is governed by a dynamic energetic equilibrium that is governed by similar forces in both C_2 domains and can be Ca^{2+}-dependent or -independent, depending on the ionic strength of the medium. 2) Although the C_2A and C_2B domains of synaptotagmin 1 similarly interact with negatively charged phospholipids, there is a clear asymmetry in the sequences involved in binding. 3) Phosphoinositides are co-activators of Ca^{2+} binding to synaptotagmin 1 and of Ca^{2+} triggering of release by synaptotagmin 1, but phosphoinositides are unlikely to bind to synaptotagmin 1 in a Ca^{2+}-independent physiologically relevant interaction.

A Dynamic Energetic Equilibrium Governs Phospholipid Binding to Synaptotagmin 1 C_2 Domains—We show that the synaptotagmin C_2AB fragment interacts with negatively charged phospholipids in a Ca^{2+}-dependent or a -independent manner depending on the ionic strength and the presence of Mg^{2+}. Ca^{2+}-independent binding can occur even in the absence of phosphoinositides, provided that the ionic strength is low and Mg^{2+} is absent. The much higher efficiency of Mg^{2+} compared with Na^+ in inhibiting binding likely arises because Mg^{2+} has a higher charge and binds tightly to the lipid phosphate groups, thus screening their charge. On the other hand, lipid head groups with a higher charge density are more efficient in promoting synaptotagmin 1 binding, likely due to the smaller entropy loss associated with immobilization of lipid diffusion upon binding (*e.g.* comparing binding to one lipid molecule bearing three phosphate groups with binding to three lipid molecules bearing a single phosphate group each). Our data thus demonstrate that, at physiological ionic strength, the C_2AB fragment is suspended in a precarious balance where basic and hydrophobic residues are predisposed to interact with the lipids

but are not quite sufficient to induce binding. Ca^{2+} shifts the equilibrium toward binding by acting as an electrostatic switch that converts repulsive interactions (between the phospholipids and negatively charged residues on the top loops of the C_2 domains) into energetically favorable interactions (by sandwiching Ca^{2+} between the two). Any change that increases the electrostatic attraction between the C_2AB fragment and the membrane surface, such as a decrease in Mg^{2+} concentration or an increase in the membrane negative charge density due to the presence of phosphoinositides, facilitates binding and thus decreases the amount of Ca^{2+} required to shift this dynamic energetic equilibrium.

Differences in the Phospholipid Binding Mechanism between the C_2A and C_2B Domains—We found that the KAKA mutation in the C_2B domain phenocopies the R233Q substitution in the C_2A domain both in terms of phospholipid binding and in terms of electrophysiological phenotype. For both mutations, the apparent Ca^{2+} affinity of the double C_2 domain fragment in the presence of negatively charged phospholipids is decreased, and the Ca^{2+} concentration dependence of release is shifted to higher Ca^{2+} concentrations. However, the K366Q mutation, which in the C_2B domain corresponds to the R233Q mutation in the C_2A domain, had no effect on the apparent Ca^{2+} affinity of synaptotagmin 1 or on neurotransmitter release. These results show that both C_2 domains contribute to the overall apparent Ca^{2+} affinity of the double C_2 domain fragment but bind to phospholipids via different positively charged residues. For both C_2 domains, the Ca^{2+}-binding loops are critical, as mutation of the Ca^{2+}-binding sites abolishes Ca^{2+}-dependent phospholipid binding. However, in the C_2A domain, residue Arg^{233} is in a Ca^{2+}-binding loop and makes a substantial energetic contribution to the interaction with phospholipids, whereas in the C_2B domain the equivalent positioned residue Lys^{366} does not. In contrast, in the C_2B domain the polybasic region is critical, whereas the C_2A domain does not have an equivalent sequence. These findings reveal a clear asymmetry in the mode of Ca^{2+}-dependent phospholipid binding of the two C_2 domains. This asymmetry could be explained by a model whereby the two C_2 domains bind to a single membrane in different orientations, although the observation that the C_2B domain (but not the C_2A domain) binds simultaneously to two membranes provides an alternative explanation for this asymmetry (55). Regardless of which of these two possibilities is correct, it is possible that the distinct Ca^{2+}-dependent phospholipid binding properties of the C_2B domain are crucial for the role of synaptotagmin 1 in triggering fusion pore opening.

Phosphoinositides are Co-activators of Ca^{2+} Binding to Synaptotagmin 1—Our results show that phosphoinositides enhance the apparent Ca^{2+} affinity of synaptotagmin 1 and that this enhancement is diminished when the polybasic region of the C_2B domain is mutated. However, phosphatidylinositols enhance Ca^{2+}-dependent and -independent binding to the synaptotagmin 1 C_2B domains even upon mutation of the polybasic region, which hence does not constitute a specific receptor site for phosphoinositides but only contributes to the overall binding via its high charge density. The synaptotagmin 1 C_2 domains therefore do not constitute true phosphatidylinositol phosphate receptors like pleckstrin homology domains or Phox homology domains (48, 49). Our data suggest that the local concentration of phosphoinositides in the nerve terminal determines the apparent Ca^{2+} affinity of synaptotagmin 1 and that changes in this concentration can modulate the efficiency of Ca^{2+} triggering of release. Our data also provide a potential explanation for the inhibitory effect of inositolpolyphosphates on release. As these bind to the polybasic region, they block Ca^{2+}-dependent phospholipid binding, including phosphatidylinositol phosphate binding to the polybasic region, and thereby inhibit C_2B domain function. Our observation that the KAKA mutation induces a major change in apparent Ca^{2+} affinity of the C_2 domains contradicts a previous study that failed to detect an effect of this mutation on the apparent Ca^{2+} affinity of synaptotagmin 1 (17). This discrepancy is probably due to the fact that the previous study employed the GST pulldown assay for measuring phospholipid binding (8), an assay that does not properly monitor phospholipid binding to the C_2B domain (7).

Viewed together, our electrophysiological results extend earlier results obtained in the *Drosophila* neuromuscular junction (31) and autapses from mouse hippocampal neurons (32) showing that mutations in the polybasic region impair but do not abolish synaptotagmin 1 function. They contrast, however, with overexpression experiments in PC12 cells that suggested an essential functional role for the polybasic region of the C_2B domain, based on the observation that wild-type synaptotagmin 1 inhibits exocytosis, while the KAKA mutation abolishes such inhibition (50). In addition, the polybasic region had been implicated in multiple interactions of the C_2B domain, including synaptotagmin oligomerization, that were abolished by the KAKA mutation (19, 51). Hence, these interactions are unlikely to be physiologically relevant based on the moderate phenotype caused by the KAKA mutation. Overall, it is striking that the effects on release of the KAKA mutation and other substitutions in basic sites of the synaptotagmin 1 C_2 domains (Fig. 6) correlate perfectly with their effects on Ca^{2+}-dependent phospholipid binding (Figs. 2 and 5), further validating the notion that Ca^{2+}-dependent phospholipid binding is a central component of synaptotagmin 1 function. On the other hand, the poor correlation between the impairment caused by these mutations in Ca^{2+}-independent phospholipid binding and their effects on release suggest that this activity is not critical or relevant. It should be noted, however, that this correlation does not exclude a potential role for SNARE binding by synaptotagmin 1 in triggering release, which could place synaptotagmin 1 into the right position at the site of fusion induced by SNARE complex assembly (reviewed in Ref. 56). Such positioning could allow Ca^{2+} binding to synaptotagmin to stimulate fusion pore expansion by causing a change in phospholipids. Future experiments will have to address this central question.

Acknowlegments—We thank Dr. Ralf Nehring for help with the synaptotagmin mutagenesis and Ina Herfort, Hui Deng, and Dirk Reuter for the preparation of astrocyte culture and SFV virus production. We acknowledge Dr. Pietro De Camilli for providing us with unpublished information regarding synaptic PIP and PIP_2 concentrations.

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