Regulated secretion is a fundamental process underlying the function of many cell types. In particular, acrosomal exocytosis in mammalian sperm is essential for egg fertilization. Regulated secretion requires SNARE proteins and, in neurons, also synaptotagmin I and complexin. Recent reports suggest that complexin imposes a fusion block that is released by Ca$^{2+}$ and synaptotagmin I. However, no direct evidence for this model in secreting cells has been provided and whether this complexin/synaptotagmin interplay functions in other types of secretion is unknown. In this report, we show that the C2B domain of synaptotagmin VI and an anti-complexin antibody blocked the formation of trans SNARE complexes in permeabilized human sperm, and that this effect was reversed by adding complexin. In contrast, an excess of complexin stopped exocytosis at a later step, when SNAREs were assembled in loose trans complexes. Interestingly, this blockage was released by the addition of the C2B domain of synaptotagmin VI C2B domain in the presence of Ca$^{2+}$. We have previously demonstrated that the activity of this domain is regulated by protein kinase C-mediated phosphorylation. Here, we show that a phosphomimetic mutation in the polybasic region of the C2B domain strongly affects its Ca$^{2+}$ binding properties. Importantly, this mutation completely abrogates its ability to rescue the complexin block. Our results show that the functional interplay between complexin and synaptotagmin has a central role in a physiological secretion event, and that this interplay can be modulated by phosphorylation of the C2B domain.

All types of intracellular membrane fusions, including regulated exocytosis (i.e. the stimulus-triggered fusion of secretory vesicles with the plasma membrane), require proteins from the SNARE$^3$ family (1). SNAREs are small membrane proteins that form stable hetero-oligomeric complexes consisting of a bundle of four parallel helices. Assembly of trans SNARE complexes between SNAREs from two opposing membranes is a key event for membrane fusion. SNAREs residing on the same membrane can form cis complexes that must be disassembled to render free SNAREs competent for membrane fusion. The exocytotic SNAREs involved in neurotransmission are syntaxin1A and SNAP25 in the plasma membrane, and synaptobrevin 2 (also called VAMP2) in secretory vesicles. These proteins are the targets of botulinum and tetanus toxins, a set of highly specific zinc-dependent endoproteases (2). Only when not assembled in tight complexes are SNAREs susceptible to cleavage, making these toxins excellent tools for the diagnosis of SNARE assembly status.

Additional proteins that are key for Ca$^{2+}$-triggered neurotransmitter release include complexins and synaptotagmins (3). Complexins are small cytosolic proteins that bind with high affinity to SNARE complexes (4), forming an antiparallel α helix that inserts into a groove between the synaptobrevin and syntaxin helices (5). Experiments where complexins were deleted or overexpressed have suggested that they play both activating and inhibitory roles, possibly to achieve synchronization of the fast component of neurotransmitter release (5–9). Synaptotagmins can interact with several combinations of SNARE proteins including ternary complexes (3). These proteins are Ca$^{2+}$ sensors coupling cell stimulation to exocytosis (3). Synaptotagmins are transmembrane proteins with a cytoplasmic region that contains two conserved C2 domains capable of binding Ca$^{2+}$ and negatively charged phospholipids (10–12). The synaptotagmin I C2A (membrane proximal) and C2B (membrane distal) domains have similar structure (10, 13), but only the C2B domain is able to bind simultaneously to two membranes (14) and Ca$^{2+}$ binding to the C2B domain is more critical for neurotransmitter release than Ca$^{2+}$ binding to the C2A domain (15–17).

A recent report using purified proteins has showed that the cytoplasmic domain of synaptotagmin I can displace complexin from SNARE complexes in a Ca$^{2+}$-dependent manner in the presence of membranes (9). An interplay between complexin and synaptotagmin I in neurotransmitter release has been also
Complexin/Synaptotagmin Interplay in Exocytosis

Inferred from the observation that their deletions produce a selective inhibition of the fast synchronous component of release (6, 18). A similar effect was observed inducing a local excess of complexin by overexpressing a synaptobrevin/complexin fusion protein in cultured cortical neurons (9). Moreover, complexin was found to block SNARE-induced liposome fusion and intercellular fusion caused by flipped SNAREs, and synaptotagmin I released this block in a Ca\(^{2+}\)-dependent manner (19, 20). However, no direct evidence has been provided that synaptotagmin can rescue the blockade in exocytosis imposed by an excess of complexin in cells. Therefore, we decided to test the interplay of synaptotagmin with complexin in the acrosomal exocytosis, a particularly well suited model to study molecular interactions during secretion (21). Mammalian spermatozoa are highly differentiated cells specialized in performing a limited number of functions with high efficiency (22). Sperm can move very fast by means of a flagellum, exocytose the content of their acrosomal granule, and fertilize eggs. Therefore, from the point of view of intracellular trafficking, sperm are specialized in a single membrane fusion event: the exocytosis of the acrosomal granule (AE). Secretion is initiated when a sustained intracellular Ca\(^{2+}\) increase is induced by activation of sperm receptors that recognize specific glycoproteins in the zona pellucida of the egg (23). AE depends on Rab3A (24) and neurotoxin-sensitive members of the SNARE family (25). According to our present working model, AE proceeds through a sequential set of events initiated when Ca\(^{2+}\) triggers the activation of Rab3A, which in turn leads the NSF/α-SNAP-mediated disassembly of cis SNARE complexes (26). SNAREs then re-associate in loose trans complexes until an efflux of intra-acrosomal Ca\(^{2+}\) promotes synaptotagmin VI- and SNARE-dependent membrane fusion (26).

In this report, we show that in permeabilized human sperm, an experimental model with naturally occurring membranes containing all factors involved in physiological secretion, the functional interplay between complexin and synaptotagmin has a central role in exocytosis. This interplay can be modulated by phosphorylation or by a phosphomimetic mutation in the polybasic region of the synaptotagmin C2B domain.

**EXPERIMENTAL PROCEDURES**

*Reagents—Recombinant streptolysin O was obtained from Dr. Bhakdi (University of Mainz, Mainz, Germany). Human tubal fluid media was from Irvine Scientific, Santa Ana, CA. O-Nitrophenyl EGTA-AM (NP-EGTA-AM), Hoechst 33258, and an Alexa Fluor 488-conjugated goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Anti-complexin I/II (rabbit polyclonal, purified IgG) was from Synaptic Systems (Göttingen, Germany). A horseradish peroxidase-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc. Protein kinase C (PKC) βII was from Calbiochem. Glutathione-Sepharose was from GE Healthcare and nickel-nitriotriacetic acid-agarose from Qiagen GmbH (Hilden, Germany). All other chemicals were reagent of analytical grade and were purchased from Sigma or ICN Biochemicals, Inc.*

*Recombinant Proteins—Plasmids encoding the C2A (residues 226–358) and C2B (residues 361–511) domains of rat synaptotagmin VI fused to GST were kindly provided by Dr. T. Sudhof (University of Texas Southwestern Medical Center, Dallas, TX). The C2A-(T284A and T284E) and C2B-T418A,T419A and T418E,T419E) domain mutants were obtained as described (27). Recombinant synaptotagmins were expressed in BL21(DE3) cells (Stratagene, La Jolla, CA) and purified by affinity chromatography on glutathione-Sepharose beads as described (28). Synaptotagmin VI domains were further purified by gel filtration on Superdex S75 in 20 mM Hepes, pH 7.0, 1 mM dithiothreitol, and 150 mM NaCl. The cDNAs encoding human complexin I and II fused to GST in a pGEX-4T vector were kindly provided by Drs. J. Rothman (Department of Physiology and Cellular Biophysics, Columbia University, New York) and B. Davletov (MRC Laboratory of Molecular Biology, Cambridge, UK), respectively. An expression plasmid pGEX-2T containing the cDNA-encoding human Rab3A was generously provided by Dr. P. Stahl (Washington University, St. Louis, MO). DNAs encoding Rab3A and complexins were transformed into Escherichia coli BL21 (Stratagene), expression was induced overnight at 22 °C with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and recombinant proteins were purified on glutathione-Sepharose following standard procedures. Rab3A was always used prenylated and loaded with GTPγS (26). The GST moiety was removed from complexins and synaptotagmin domains by cleavage with thrombin (0.25 mg/ml of resin) in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2.5 mM CaCl\(_2\) during 3 h at 25 °C. The light chains of botulinum neurotoxin B (BoNT/B) and tetanus toxin fused to His\(_6\) (pQE3 plasmid, Qiagen) were generously provided by Dr. T. Binz (Medizinische Hochschule Hannover, Hannover, Germany). DNA encoding His\(_6\)-tetanus toxin was transformed into E. coli XL-1Blue (Stratagene) and protein expression was induced overnight at 20 °C with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside. A plasmid construct encoding BoNT/B was transformed into E. coli M15pRep4 (Qiagen) and protein expression induced 4 h at 30 °C with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Purification of His\(_6\)-tagged recombinant proteins was accomplished according to The QiAexpressionist (www.qiagen.com).

To generate uniformly \(^{15}\)N-labeled wild type and mutant synaptotagmin VI C2B domains for NMR studies, bacteria were grown in M9 minimal medium supplemented with \(^{15}\)NH\(_4\)Cl (CIL, Andover, MA) as the sole nitrogen source. Proteins were purified as before.

In phosphorylation experiments, 20 μM synaptotagmin VI domains were incubated in 20 mM Hepes-K, pH 7.4, 2 mM dithiothreitol, 1 mM ATP, 5 mM MgCl\(_2\), and 5 mM NaF, containing 0.6 units/ml of PKCβII under activating (140 μM phosphatidylyserine, 325 nM phorbol 12-myristate 13-acetate, 100 μM CaCl\(_2\)) conditions for 30 min at 37 °C. After incubation, the mixtures were filtered through Sephadex G-25 spin columns equilibrated with sucrose buffer to eliminate phorbol 12-myristate 13-acetate and small molecules.

**Acrosomal Exocytosis in Permeabilized Sperm**—Human semen samples were obtained from normal healthy donors. Highly motile sperm were recovered following a swim-up separation in human tubal fluid supplemented with 0.5% bovine serum albumin for 1 h at 37 °C in an atmosphere of 5% CO\(_2\), 95% air. Concentration was adjusted to 5–10 × 10^6/ml, and
incubation proceeded for at least 2 h. Permeabilization was accomplished as described (26). Sperm were resuspended (7 × 10^6/ml) in ice-cold sucrose buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-K, pH 7) containing 2 mM dithiothreitol and divided in 30–50-μl aliquots. Each aliquot was treated as described in the figure legends. The different reagents were added successively without washing. Care was taken to maintain the same incubation times for all tubes in the same experiment. All incubations were carried out at 37 °C. Negative (no stimulation) and positive (10 μM free Ca^{2+}) controls were included in all experiments. At the end of the assay, 10 μl of each sample was spotted on slides and fixed/permeabilized in ice-cold methanol. Acrosomal status was evaluated by staining with FITC-coupled *Pisum sativum* agglutinin (PSA-FITC). Briefly, spermatozoa that have preserved an intact acrosome show strong label with the fluorescent lectin at the acrosomal region. Cells that have undergone acrosomal exocytosis show no labeling at the acrosomal region or labeling limited to the posterior edge of the granule (ecuatorial staining). At least 200 cells were scored for each condition. For each experiment, acrosomal exocytosis indexes were calculated by subtracting the number of reacted spermatozoa in the negative control (range of 12–28%) from all values, and expressing the resulting values as a percentage of the acrosome reaction observed in the positive control (range of 30–48%). The average difference between positive and negative controls was 14% (experiments where the difference was less than 10% were discarded).

**Phospholipid Binding**—FRET experiments were performed on a PerkinElmer Life Sciences LS50-B spectrofluorimeter excited at 280 nm and recording the emission spectra from 400 to 600 nm. The experiments were performed at room temperature in 20 mM Tris-Cl, pH 7.2, 100 mM NaCl, with 0.01 mg/ml phospholipid vesicles containing 10% dansyl-phosphatidyethanolamine, 25% phosphatidylserine, and 65% phosphatidylcholine, and 1 μM protein, and the corresponding additions of 1 mM EDTA or 1 mM CaCl_2. A fluorescence spectra acquired under identical conditions but in the absence of protein was subtracted from the corresponding spectra acquired in the presence of protein (10).

**NMR Spectroscopy**—All NMR experiments were carried out at 27 °C on Varian INOVA500 or INOVA600 spectrometers with samples containing ~100 μM C2B domains dissolved in 20 mM Hepes, pH 7.0, and 150 mM NaCl, with 0.01 mg/ml phospholipid vesicles containing 10% dansyl-phosphatidyethanolamine, 25% phosphatidylserine, and 65% phosphatidylcholine, and 1 μM protein, and the corresponding additions of 1 mM EDTA or 1 mM CaCl_2. A fluorescence spectra acquired under identical conditions but in the absence of protein was subtracted from the corresponding spectra acquired in the presence of protein (10).

**INDIRECT IMMUNOFLOURESCENCE**—Twenty μg/ml (134 nm) of an anti-complexin I/II antibody was added to the suspension of streptolysin O-permeabilized sperm and incubated for 15 min at 37 °C before spotting on round coverslips. After fixation in 2% paraformaldehyde, sperm were washed once in PBS containing 0.4% polyvinylpyrrolidone (40,000 average M, PBS/PVP). The cells were then incubated with 0.1% Triton X-100 in PBS for 10 min at room temperature. After washing twice with PBS/PVP, sperm were incubated in 50 mM glycine-PBS overnight at 4 °C. Nonspecific staining was blocked with 5% bovine fetal serum in PBS/PVP for 1 h at room temperature. An Alexa Fluor 488-labeled anti-rabbit IgG (1 h at 20 °C, 6.7 μg/ml in 0.5% bovine fetal serum, PBS/PVP) was used as secondary antibody. Coverslips were washed (3 times) with PBS/PVP between incubations. Finally, cells were incubated 1 min in cold methanol and stained with Hoechst 33258 (20 min at 20 °C, 1 μg/ml in PBS) followed by PSA-FITC (30 min at 20 °C, 50 μg/ml in PBS) and washed with distilled water 20 min at 4 °C. Coverslips were mounted in Gelvatol, and examined with an Eclipse TE3000 Nikon microscope equipped with a Plan Apo ×60/1.40 oil objective and a Hamamatsu Orca 100 camera operated with Metamorph 6.1 software (Universal Imaging Corp.). Background was subtracted and brightness/contrast were adjusted to render an all or nothing labeling pattern using Corel Draw version 12 (Corel, Ottawa, Ontario, Canada).

**SDS-PAGE and Immunoblot Analysis**—Sperm were washed in PBS and proteins extracted in cold 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and a protease inhibitor mixture (P2714, Sigma). After sonication for 15 s (3 times with 10-s intervals) and extraction for 30 min at 4 °C, the sperm extracts were clarified by centrifugation at 14,000 × g for 20 min and used immediately or stored at −20 °C. Proteins were separated on 12.5% polyacrylamide slab gels (31) and transferred to 0.22-μm nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany). Nonspecific reactivity was blocked by incubation for 1 h at room temperature with 5% nonfat dry milk dissolved in washing buffer (PBS, pH 7.6, 0.2% Tween 20). Blots were incubated with the anti-complexin antibody (0.2 μg/ml in blocking solution) for 60 min at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit-IgG dissolved in blocking buffer was used as secondary antibody (0.25 μg/ml, 45 min at room temperature). Excess first and second antibodies were removed by washing 5 times for 5 min in blocking solution. Detection was accomplished with an enhanced chemiluminescence system (ECL, Amersham Biosciences) and subsequent exposure to Kodak X-AR film (Eastman Kodak) for 5–30 s.

**Statistical Analysis**—Data were evaluated using one-way analysis of variance. The Tukey-Kramer post hoc test was used for pairwise comparisons. Differences were considered significant at the p < 0.05 level.

**RESULTS**

**Complexin Regulates Acrosomal Exocytosis**—We hypothesized that complexin may be part of the membrane fusion machinery involved in AE. Complexin was detected in human sperm extracts by Western blot (Fig. 1A). Although the antibody does not distinguish between isoforms I and II, the electrophoretic mobility of sperm complexin suggests that II is the predominant one. The protein localized to the acrosomal region (Fig. 1B), as it has been shown in sperm of other species (32, 33), consistent with a role in the exocytosis of this granule. When introduced into streptolysin O-permeabilized sperm, nanomolar concentrations of a specific anti-complexin I/II antibody prevented the AE stimulated by 10 μM Ca^{2+} in a dose-response fashion, suggesting that endogenous complexin is necessary for secretion (Fig. 1C). The effect of the antibody was specific, because it was abolished by preincubation with purified complexin (Fig. 1D). Interestingly, addition of low concen-
Complexin/Synaptotagmin Interplay in Exocytosis

FIGURE 1. Complexin participates in acrosomal exocytosis. A, a postnuclear membrane pellet from rat brain (1 μg of protein, brain) and human sperm (10⁶ cells, sperm) extracts, and recombinant complexins I (cpx I) and II (cpx II) (1 ng each) were resolved in 12.5% Tricine gels, transferred to nitrocellulose membranes, and probed with an anti-complexin I/II antibody. M, standards (×10⁻⁵) are indicated on the left. B, sperm were fixed on coverslips and labeled with the anti-complexin antibody (anti-cpx), a lectin that recognizes the intra-acrosomal content (PSA-FITC), and with a DNA-staining probe (Hoechst 333258). Shown are epifluorescence micrographs of typically stained cells. Notice that complexin was mostly localized to the acrosomal region. Bar = 5 μm. C, permeabilized spermatozoa were treated at 37 °C for 10 min with increasing concentrations of the anti-complexin antibody. AE was initiated by adding 0.5 mM CaCl₂ (10⁻³ M free Ca²⁺) and the incubation continued for an additional 15 min. D, permeabilized spermatozoa were treated at 37°C for 10 min with 34 nM anti-complexin antibody premixed with 68 nM GST-complexin II (anti-cpx + cpx, black bar) and AE was initiated with Ca²⁺ (Ca²⁺⁻) as in C. Controls include (gray bars): background AE in the absence of any stimulation (control); AE stimulated by 10 μM free Ca²⁺ (Ca²⁺⁺); treatment with 34 nM antibody (anti-cpx). E, permeabilized spermatozoa were treated at 37°C for 10 min with increasing concentrations of purified complexin II fused to GST (gray circles) or without GST (black circles), and with complexin I without GST (gray triangles); AE was subsequently initiated as in C. F, permeabilized spermatozoa were treated with 200 nM complexin II (cpx II) or 67 nM anti-complexin antibody (anti-cpx) and AE stimulated with 300 nM Rab3A (black bars). Controls include (gray bars): background AE in the absence of any stimulation (control); AE stimulated with 10 μM free Ca²⁺ (Ca²⁺⁺) or with 300 nM Rab3A (Rab3A). Sperm in C–F were fixed and AE was measured. The percentage of reacted sperm was normalized as described under "Experimental Procedures." Data represent mean ± S.E. of at least three independent experiments.

could rescue the complexin block. For these experiments, subinhibitory concentrations (100 nM) of both domains were added to assays containing inhibitory concentrations (200 nM) of complexin II. Interestingly, the C2B domain efficiently restored exocytosis. In contrast, the C2A domain was ineffective (Fig. 2, top). Rab3A-triggered exocytosis was used to assess whether the effect of the synaptotagmin VI domains on complexin inhibition was Ca²⁺ dependent. As shown in Fig. 2, middle, no rescue was observed in the absence of Ca²⁺ with the C2B or C2A domains. If Ca²⁺ plus Rab3A were used to activate exocytosis, the C2B (but not the C2A) domain relieved the complexin block (Fig. 2, bottom), indicating that the lack of effect in the presence of Rab3A alone was not due to a misbalance caused by an excess of the small GTPase. These observations indicate that the C2B domain of synaptotagmin VI is sufficient to compete the inhibitory effect of complexin in AE. Moreover, rescue is only observed in the presence of cytosolic Ca²⁺.

Complexin II and the Synaptotagmin VI C2B Domain Arrest Fusion at Different Stages of the Fusion Process—We took advantage of the reversibility of the complexin block by displacement with the C2B domain of synaptotagmin VI to investigate the point in the exocytotic cascade where complexin exerts its function. In resting sperm, SNAREs are assembled in cis complexes resistant to neurotoxins (26).

trations of recombinant complexin II, either as a fusion protein with GST or without the GST moiety, inhibited AE. Complexin I was as efficient as complexin II in blocking exocytosis (Fig. 1E). Pre-activated Rab3A can trigger AE in permeabilized sperm in the absence of Ca²⁺ (34), an effect that was also inhibited by an anti-complexin antibody and by recombinant complexin II, suggesting a role for this protein downstream of the small GTPase activation (Fig. 1F). These results indicate that complexin is present in human sperm and that a precise amount of this protein is required for AE.

The C2B Domain of Synaptotagmin VI Rescues the Complexin Block—We then set conditions to test whether synaptotagmin could modulate the effect of complexin in AE. In sperm, the C2A and C2B domains of synaptotagmin VI affect exocytosis (27). Therefore, we tested whether the separated domains

Complexin may bind to these complexes and inhibit exocytosis by interfering with their disassembly during the early steps of AE. To test this possibility, sperm were incubated with tetanus toxin in the presence of complexin and then activated with Ca²⁺. If complexin prevents disassembly, SNAREs will remain engaged in cis complexes and synaptobrevin will be protected from cleavage. At the end of the incubation, TPEN, a zinc chelator, was added to inactivate the toxin and the C2B domain to investigate the point in the exocytotic cascade where complexin exerts its function. In resting sperm, SNAREs are assembled in cis complexes resistant to neurotoxins (26).
If addition of complexin does not inhibit SNARE disassembly, it should block exocytosis at a later step. To assess the sensitivity of synaptobrevin to toxins, sperm were activated by adding 10 μM free Ca\(^{2+}\) (Ca\(^{2+}\), top) or 300 nM Rab3A (Rab3A, middle) or with the combination of both stimuli (Rab3A + Ca\(^{2+}\), bottom) and the incubation continued for an additional 15 min. Controls include (gray bars): background AE in the absence of any stimulation (control); AE stimulated with 10 μM free Ca\(^{2+}\) (Ca\(^{2+}\)), or with 300 nM GST-Rab3A (Rab3A), or with a combination of both stimuli (Rab3A + Ca\(^{2+}\)), effect of C2A (C2A), C2B (C2B), or complexin II (cpx) alone on the AE. Sperm were fixed and AE was measured. The percentage of reacted sperm was normalized as described under "Experimental Procedures." Data represent mean ± S.E. of at least three independent experiments.

In a previous report (27), we have shown that an excess of complexin blocks exocytosis when SNAREs are assembled in loose trans complexes. This inhibition can be relieved by addition of recombinant complexin. At a later stage, an excess of recombinant complexin also prevents the stabilization of loose complexes; this effect can be competed by adding recombinant complexin. At a later stage, an excess of complexin blocks exocytosis when SNAREs are assembled in loose trans complexes. This inhibition can be relieved by adding recombinant synaptotagmin VI C2B domain.

Complexin II Facilitates the Assembly of Tetanus Toxin-resistant SNARE Complexes—The experiments depicted in Fig. 3 suggest that complexin may stabilize the formation of loose complexin II to rescue, we observed that the process stopped at a stage in which synaptobrevin was sensitive to tetanus toxin and BoNT/B. These observations suggest that synaptobrevin was not stably engaged in loose trans complexes under these conditions but either remained as a monomer or cycled between monomers and SNARE complexes.
Complexin/Synaptotagmin Interplay in Exocytosis

FIGURE 4. Complexin II facilitates the assembly of tetanus toxin-resistant SNAREs complexes. A, permeabilized spermatozoa were loaded with 10 μM NP-EGTA-AM (NP) for 15 min at 37 °C to chelate intra-acrosomal Ca$$^{2+}$$, AE was then initiated by adding 10 μM free Ca$$^{2+}$$ (Ca$$^{2+}$$). After a further 10-min incubation to allow exocytosis to proceed up to the intra-acrosomal Ca$$^{2+}$$-sensitive step, sperm were treated for 10 min with 250 nm complexin II (cpx), 500 nm synaptotagmin VI C2B domain (C2B), or 67 nm anti-complexin antibody (anti-cpx). All these procedures were carried out in the dark. UV photolysis of the chelator was induced at the end of the incubation period (hv) and the samples were incubated for 5 min to promote exocytosis (black bars). Controls include (gray bars): background AE in the absence of any stimulation (control); AE stimulated by 10 μM free Ca$$^{2+}$$; inhibitory effect of NP-EGTA-AM in the dark and the recovery upon illumination; inhibitory effect of the proteins when added before Ca$$^{2+}$$ stimulation. B, permeabilized sperm were incubated with 34 nm anti-complexin antibody (anti-cpx) for 10 min. AE was initiated by adding 10 μM free Ca$$^{2+}$$. After 10 min, different concentrations of complexin I (gray triangles) or complexin II (black circles) were added to the tubes and the incubation proceeded for 10 more min. C, in these experiments, intra-acrosomal calcium was depleted as in A. Samples were then incubated with 34 nm (anti-cpx) or 20 nm (anti-cpx) anti-complexin antibody for 10 min and then AE was stimulated with 10 μM free Ca$$^{2+}$$ (Ca$$^{2+}$$) for 10 min. At this stage, samples were treated with 100 nm tetanus toxin (TeTx) for 10 min to assess SNARE sensitivity. To relieve the inhibitory effect of 34 nm anti-complexin, 200 nm complexin II was added and the incubation proceeded for 10 min (complexin was not added to samples treated with 20 nm anti-complexin). All these procedures were carried out in the dark. UV photolysis of the chelator was then induced (hv) and the samples were incubated for 5 min to promote exocytosis (black bars). Controls include (gray bars): background AE in the absence of any stimulation (control); AE stimulated by 10 μM free Ca$$^{2+}$$; inhibitory effect of NP-EGTA-AM in the dark and the recovery upon illumination; tetanus toxin resistance when AE was initiated in the absence of complexin; recovery from 34 nm anti-complexin blockade with complexin; partial inhibition when AE was initiated in the presence of 20 nm anti-complexin. Sperm were fixed and AE was measured. The percentage of reacted sperm was normalized as described under “Experimental Procedures.” Data represent mean ± S.E. of at least three independent experiments.
has been implicated in Ca\(^{2+}\)-dependent (35) and -independent (36, 37) functions of synaptotagmin. When two threonines present in the middle of the polybasic region are mutated to alanine (to eliminate phosphorylation; TA mutant) or to glutamic acid (to mimic phosphorylation; TE mutant), the properties of this protein are strongly affected: the effect of the TA mutant on AE cannot be modulated by phosphorylation and the TE mutant does not affect exocytosis (27). To assess whether these mutations alter the Ca\(^{2+}\) and phospholipid binding properties of the C2B domain, wild type, TA, and TE mutant proteins were purified as GST fusion proteins and the GST moiety was removed. Ca\(^{2+}\)-dependent binding of these domains to liposomes was studied by a FRET assay involving energy transfer between the protein tryptophans and dansyl groups incorporated into lipids (10). The wild type synaptotagmin VI C2B domain showed a prominent FRET signal increase upon addition of Ca\(^{2+}\) (Fig. 5A, left), indicating that, as expected, the protein binds to liposomes in a Ca\(^{2+}\)-dependent manner. A similar result was observed with the TA mutant (Fig. 5A, center). In contrast, the signal with the TE mutant was not affected by Ca\(^{2+}\), indicating that this mutant does not interact with lipids in a Ca\(^{2+}\)-dependent manner (Fig. 5A, right).

To examine the intrinsic Ca\(^{2+}\)-binding properties of the wild type and mutant synaptotagmin VI C2B domains, we used HSQC experiments, which provide a powerful tool to monitor Ca\(^{2+}\) binding to proteins (12). The \(^{1}H\)\(^{15}N\) HSQC spectra of the Ca\(^{2+}\)-free wild type and mutant C2B domains exhibited well dispersed cross-peak patterns indicating that they are folded (Fig. 5B, black contours). Note that both the TA and TE mutations induced significant changes in the spectra that likely arise from structural perturbations, because the side chain of one of the mutated threonines forms a hydrogen bond with the backbone. Importantly, Ca\(^{2+}\) induced substantial changes in the \(^{1}H\)\(^{15}N\) HSQC spectra of the wild type and TA mutant C2B domains (Fig. 5B, left and center) but not in the TE mutant (Fig. 5B, right). Hence, the TA mutant is still able to bind Ca\(^{2+}\), whereas the TE

The Ca\(^{2+}\) and Phospholipid Binding Activity of the C2B Domain Is Dramatically Affected by a Phosphomimetic Mutation in the Polybasic Region—According to studies with purified proteins, the competition of synaptotagmin I with complexin for SNARE complex binding is Ca\(^{2+}\) and membrane-dependent (9). In a previous report, we have shown that PKC-mediated phosphorylation of the polybasic region of the C2B domain of synaptotagmin VI abolishes its effect on AE (27). This region

C

FIGURE 5. A phosphomimetic mutation in the polybasic region of the C2B domain affects its Ca\(^{2+}\)- and phospholipids binding properties and completely abrogates its ability to rescue the complexin block. A, FRET analysis involving energy transfer between the protein tryptophans and dansyl groups incorporated into liposomes (10) in the presence of 1 mM EDTA (black line) or 1 mM CaCl\(_2\) (red line) of wild type C2B domain of synaptotagmin VI (stgC2B-WT), or mutants with substitutions in Thr-418 and Thr-419 (Thr\(^{3}\)Glu, phosphomimetic mutation, stgC2B-TE). The Ca\(^{2+}\)/H\(^{11001}\). A phosphomimetic mutation in the polybasic region of the C2B domain affects its Ca\(^{2+}\) and phospholipids binding properties and completely abrogates its ability to rescue the complexin block. A, FRET analysis involving energy transfer between the protein tryptophans and dansyl groups incorporated into liposomes (10) in the presence of 1 mM EDTA (black line) or 1 mM CaCl\(_2\) (red line) of wild type C2B domain of synaptotagmin VI (stgC2B-WT), or mutants with substitutions in Thr-418 and Thr-419 (Thr\(^{3}\)Glu, phosphomimetic mutation, stgC2B-TE). B, superpositions of \(^{1}H\)\(^{15}N\) HSQC spectra of \(^{15}N\)-labeled synaptotagmin VI C2B domain in the presence of 1 mM EDTA (black contours) or 1 mM CaCl\(_2\) (red contours) of the wild type protein (stgC2B-WT), TA mutant (stgC2B-TA), and TE mutant (stgC2B-TE). C, permeabilized sperm were incubated at 37 °C for 10 min with 200 nM complexin II (cpx) and 100 nM wild type C2A domain untreated (C2A), previously phosphorylated in vitro with PKC (p-C2A), or with point mutations in Thr-284 (Thr\(^{3}\)Ala, non-phosphorylatable mutant, stgC2B-TA; Thr\(^{3}\)Glu, phosphomimetic mutation, stgC2B-TE). The percentage of reacted sperm was normalized as described under “Experimental Procedures.” Data represent mean ± S.E. of at least three independent experiments.

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JOURNAL OF BIOLOGICAL CHEMISTRY 26341
**Complexin/Synaptotagmin Interplay in Exocytosis**

![Diagram of exocytosis involving complexin and synaptotagmin](image)

**FIGURE 6. Working model for complexin/synaptotagmin interplay during the acrosomal exocytosis.** In resting sperm, SNAREs are assembled in inactive cis complexes and synaptotagmin is phosphorylated (26, 27). Upon activation, Ca$^{2+}$ coming from the extracellular medium triggers SNARE complex disassembly and synaptotagmin dephosphorylation. Free SNAREs are now able to re-assemble in-trans, a process that is facilitated by complexin binding to the partially assembled complexes. This step is inhibited by the synaptotagmin VI C2B domain or by the anti-complexin antibody. Endogenous complexin arrests exocytosis at a step when SNAREs are assembled in tetanus toxin-resistant, BoNT/B-sensitive trans complexes. A local increase in Ca$^{2+}$ coming from the acrosome activates the synaptotagmin-dependent relief of the complexin block and secretion is completed. This stage is inhibited by chelating intracellular calcium influx.

**DISCUSSION**

The molecular bases for the effect of complexin in exocytosis are only partially understood. The recent observation that synaptotagmin I, the best characterized Ca$^{2+}$ sensor in secretion, competes with complexin for SNARE binding (9) may provide a molecular connection between complexin and Ca$^{2+}$ stimulation. Evidence for an interplay between complexin and synaptotagmin I has been reported using purified SNARE-induced cell (20) and liposome (19) fusion. However, no direct evidence for this model using biological membranes engaged in a physiological regulated secretion has been provided. Also, it is unknown whether the complexin/synaptotagmin interplay is a signature of neurotransmitter release or it also functions in other regulated secretions. This is an important issue because complexins have been implicated in pancreatic β cell (38) and mast cell (8) secretion. In support of a crucial and widely spread role for the synaptotagmin/complexin functional interaction, we report here that complexin is required for acrosomal exocytosis in human sperm, that exocytosis is blocked by excess complexin, and that the C2B domain of synaptotagmin VI releases this inhibition. It is interesting to note that the C2A domain does not, in agreement with the observation that this domain cannot displace complexin from SNARE complexes reconstituted in lipid bilayers (39).

In permeabilized sperm, both complexin and the synaptotagmin VI C2B domain are inhibitory and both proteins, under certain conditions, can rescue the blockage imposed by the other. These observations give strong support to the notion of an active interplay between these proteins in acrosomal exocytosis. Moreover, the results with NP-EGTA and the sensitivity to neurotoxins indicate that an excess of C2B domain is inhibitory at an early stage, before the assembly of loose trans SNARE complexes, whereas an excess of complexin blocks at a later stage, when tetanus toxin-resistant complexes have already formed. All these observations can be explained by the competition between complexin and the synaptotagmin C2B domain for binding to SNARE complexes reported using purified proteins reconstituted in lipid bilayers (39). In an initial stage, complexin has an activating role in exocytosis, stabilizing the formation of trans complexes. At this stage, an excess of C2B domain, which displaces complexin from the complex, or the depletion of complexin (by an inhibitory antibody) would render the trans complexes unstable and presumably in equilibrium with monomeric SNAREs, a condition that would be sensitive to neurotoxins. After the formation of the loose trans complexes, the release of Ca$^{2+}$ from the acrosome would trigger the final steps.
of membrane fusion that require the synaptotagmin-dependent displacement of complexin from the SNARE complexes. At this stage, the C2B domain is no longer inhibitory, but can rescue a blockage imposed by an excess of complexin (Fig. 6).

In sperm, complexin arrested exocytosis at a stage where synaptobrevin was resistant to tetanus toxin but sensitive to BoNT/B. Similar neurotoxin sensitivity has been reported in the cell fusion assay using flipped SNAREs (20). In the proteoliposome assay, complexin arrests fusion mostly at the level of hemifusion (19); if this stage is compatible with loose trans complexes is at present unclear (1).

Ca\(^{2+}\) is essential for the complexin/synaptotagmin interplay in fusion reconstitution assays (19, 20). This ion is also necessary for the synaptotagmin I-mediated displacement of complexin from SNARE complexes (9). In sperm, we show the C2B domain can rescue the complexin block only when exocytosis is initiated with Ca\(^{2+}\). Moreover, when the Ca\(^{2+}\) and phospholipids binding properties of the C2B domain are altered by a phosphomimetic mutation or by phosphorylation with PKC, the domain loses the capacity to restore secretion. Therefore, we propose that phosphorylation of synaptotagmin stands as a key regulatory process that modulates its functional interaction with complexin (Fig. 6). This regulatory process may be of outstanding importance for sperm, which after a long trip have a single opportunity to release the acrosomal content at the exact time and place to successfully fertilize an egg.

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