Receptor-mediated Regulation of Tomosyn-Syntaxin 1A Interactions in Bovine Adrenal Chromaffin Cells

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Tomosyn, a soluble R-SNARE protein identified as a binding partner of the Q-SNARE syntaxin 1A, is thought to be critical in setting the level of fusion-competent SNARE complexes for neurosecretion. To date, there has been no direct evaluation of the dynamics in which tomosyn transits through tomosyn-SNARE complexes or of the extent to which tomosyn-SNARE complexes are regulated by secretory demand. Here, we employed biochemical and optical approaches to characterize the dynamic properties of tomosyn-syntaxin 1A complexes in live adrenal chromaffin cells. We demonstrate that secretagogue stimulation results in the rapid translocation of tomosyn from the cytosol to plasma membrane regions and that this translocation is associated with an increase in the tomosyn-syntaxin 1A interaction, including increased cycling of tomosyn into tomosyn-SNARE complexes. The secretagogue-induced interaction was strongly reduced by pharmacological inhibition of the Rho-associated coiled-coil forming kinase, a result consistent with findings demonstrating secretagogue-induced activation of RhoA. Stimulation of chromaffin cells with lysophosphatidic acid, a nonsecretory stimulus that strongly activates RhoA, resulted in effects on tomosyn similar to that of application of the secretagogue. In PC-12 cells overexpressing tomosyn, secretagogue stimulation in the presence of lysophosphatidic acid resulted in reduced evoked secretory responses, an effect that was eliminated upon inhibition of Rho-associated coiled-coil forming kinase. Moreover, this effect required an intact interaction between tomosyn and syntaxin 1A. Thus, modulation of the tomosyn-syntaxin 1A interaction in response to secretagogue activation is an important mechanism allowing for dynamic regulation of the secretory response.

Regulated neurotransmitter release requires the well orchestrated spatial and temporal actions of many presynaptic proteins (1). Although the primary molecular entities in the release pathway have been identified, the exact mechanics of synaptic vesicle fusion and its precise regulation are still not established. Central to the fusion process is the transient formation of SNARE4 core complexes that include the target membrane SNARE proteins syntaxin 1A and SNAP25 and the vesicle SNARE protein syntaxin (2–4). A SNARE core complex is a highly stable, four–α-helix parallel bundle consisting of one SNARE motif from each of syntaxin 1A and synaptobrevin/VAMP and two SNARE motifs from SNAP25 and SNAP25. Although these proteins alone are sufficient to induce a slow fusion when reconstituted into liposomes (7), additional proteins are necessary to establish the properties that describe fast, Ca2+-dependent neurotransmitter release (8). For example, assembly of SNARE core complexes is subject to temporal and spatial regulation by a variety of protein families, including Rab-GTPases (9–13), Sec/Munc18s (14–16), exocyst tethering complexes (17–20), and Munc13s (21–24). In addition, recent evidence suggests that the temporal and spatial availability of SNAREs for membrane fusion may be subject to precise regulation by the presence of soluble R-SNARE motif-containing proteins, such as amysin (25, 26) and tomosyn (27–29).

Tomosyn was originally identified in neurons as a binding partner of the Q-SNARE, syntaxin 1A (27), and belongs to a larger family of proteins that includes the yeast proteins Sro7p and Sro77p, the Drosophila tumor suppressor lethal giant larvae family, and the mammalian Mgfl family (30–33). Tomosyn homologues also appear in the Fungi and Plantae kingdoms (34).

Structurally, tomosyns are soluble proteins that contain two distinguishable domains. An R-SNARE homology motif near the C terminus defines the primary interaction of tomosyn with the Q-SNARE syntaxin 1A, whereas the remaining N-terminal region contains 7–9 repeating β-transducin-like WD-40 motifs

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4 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; cYFP, citrine mutant of yellow fluorescent protein; NEM, N-ethylmaleimide-sensitive factor; NEM, N-ethylmaleimide; ROCK, Rho-associated coiled-coil-forming kinase; LPA, lysophosphatidic acid; DMPP, 1,1-dimethyl-4-phenylpiperazinum iodide; PSS, physiological saline solution; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced citrine mutant of yellow fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; hGH, human growth hormone; GST, glutathione S-transferase.
that form additional protein-protein interaction sites (27, 35, 36). In the mouse genome, two paralogous genes for tomosyn exist (tomosyn-1 and -2) and lead to the expression of seven tomosyn isoforms (37). Variability between these tomosyn isoforms is clustered within a hypervariable domain that separates the N-terminal WD-40 repeats from the C-terminal SNARE domain. Functional actions of tomosyn family members have been ascribed to their interaction with cognate Q-SNAREs; however, as the lethal giant larvae family and Sro7p and Sro77p proteins do not possess a well defined C-terminal R-SNARE homology domain, interactions between these families and their cognate Q-SNAREs have been proposed to involve alternative interaction motifs (30, 31, 38).

Increasing evidence demonstrates that tomosyn and its homologues are critical regulators in vesicular trafficking and membrane fusion processes. Overexpression of tomosyn in PC-12 and adrenal chromaffin cells negatively regulates neurotransmitter secretion (27, 29), which, in chromaffin cells, results from inhibition in priming of large dense core vesicles and decreased readily releasable pool size (28). Tomosyn has also been shown to exert an important role in polarized exocytosis in yeast and epithelial cells (30, 39) to negatively modulate insulin release from pancreatic β-cells (40), and to interact with syntaxin 4 and SNAP23 and inhibit insulin-induced fusion of GLUT4-containing vesicles in 3T3-L1 adipocytes (41). Recent genetic studies in Caenorhabditis elegans have also clearly established that Tom-1, the ortholog of mammalian tomosyn, exerts an inhibitory role on neurotransmitter secretion by negatively regulating synaptic vesicle priming (42, 43).

The inhibitory effects of tomosyn have been proposed to result from the formation of specific tomosyn-protein complexes that reduce the availability of interacting proteins to perform functional roles in exocytosis. For example, tomosyn has been shown to compete with Munc18 for binding to syntaxin, and, probably of greater significance, tomosyn competes with synaptobrevin for binding to syntaxin/SNAP25 dimers to form tomosyn-SNARE complexes (27). The latter complexes, as shown by their resolved crystal structure, are almost identical to the synaptobrevin/VAMP-containing SNARE complexes (34), including a required action by the ATPase NSF for complex disassembly and reuse of the interacting proteins (29). However, whereas tomosyns can participate in the formation of stable tomosyn-SNARE complexes, the absence of a membrane anchor in all tomosyn family members precludes them from acting as fusogenic synaptobrevin/VAMP analogues. Rather, formation of these nonfusogenic tomosyn-SNARE complexes diminishes the availability and formation of fusion-competent SNARE complexes between membrane-anchored SNAREs, and it is this feature that has been proposed to underlie the negative regulation by tomosyn of exocytotic activity.

The interaction of tomosyn with syntaxin 1A has recently been reported to be differentially regulated by the Rho/Rock kinase (ROCK) (44) and protein kinase A signaling pathways (45). Activation of RhoA and its signaling effector ROCK facilitated syntaxin 1A phosphorylation and formation of tomosyn-SNARE complexes at the palms of growth cones in extending neurites in NG108 neuroblastoma cells and cultured neurons (44). This resulted in the localized inhibition of functional SNARE complex formation in these areas and spatially directed fusion of plasmalemmal precursor vesicles to the leading edge of growth cones. On the other hand, protein kinase A-catalyzed phosphorylation of tomosyn decreased the interaction of tomosyn with syntaxin 1A and thereby up-regulated SNARE complex formation and enhanced neurotransmitter release in cultured superior cervical ganglion neurons (45). Thus, although tomosyn is not essential for neurotransmitter release, its complex regulation suggests that it may play a critical role in integrating multiple receptor-mediated signaling pathways to ultimately achieve a fine modulatory control over the site and extent of secretory responses.

To date, there has been no direct evaluation in living cells of the time course or extent to which the assembly/disassembly of tomosyn-SNARE complexes is regulated by secretory demand for neurotransmitter release. Furthermore, although a Rho signaling pathway has been demonstrated to alter tomosyn-SNARE interactions during neurite development, it remains unknown whether this signaling pathway operates to direct tomosyn-SNARE complex assembly during regulated neurotransmitter release. In this paper, we evaluated the spatiotemporal dynamics and regulation of the tomosyn-syntaxin 1A interaction during stimulated secretion in neuroendocrine chromaffin cells. We show that activation of nicotinic acetylcholine receptors, as occurs normally during neurally evoked secretory responses, as well as treatment with lysophosphatidic acid (LPA), activates Rho-GTPase and increases tomosyn-syntaxin 1A complex formation at the plasma membrane in chromaffin cells. These effects of secretagogue stimulation and LPA treatment were inhibited by Y27632, a specific inhibitor of the Rho-GTP effector ROCK. We also show using dynamic FRET measurements between CFP-tomosyn and yFyp-syntaxin 1A that the formation of these complexes is strongly augmented under conditions where NSF action is inhibited, suggesting that a rapid and dynamic cycling of tomosyn-syntaxin 1A interactions occurs in vivo. Finally, we present functional data to demonstrate that LPA activation of the RhoA/ROCK pathway during evoked secretion enhances tomosyn-mediated inhibition of secretion.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Expression Constructs—pEGFP-C1 and monomeric mutants (A206K) of pECFP-C1 and pEcYFP-C1 (citrine) vectors containing the LoxP sequence were used as recipient vectors for subcloning using the Cre-recombinase-mediated Creator system (Clontech). Rat syntaxin 1A, m-tomosyn, SNAP25, and Munc18-1 were merged to the C terminus of the EGF, ECFP, and EcYFP. Effector binding mutants of syntaxin 1A (I209A and I233A), a soluble mutant of SNAP25 (SNAP25c/a, C-80, 88, 90, and 92-A), as well as a carboxyl-terminal deletion of tomosyn-(1–1067) (tomosyn ΔCT, glutamate at residue 1068 changed to stop codon) were constructed using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene). The sequence fidelity of all constructs was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core). RhoA activation was measured using an enzyme-linked immunosorbent assay-based kit (Cytoskeleton). HEK293 cells stably expressing the rat α1B and human β2B
α,δ voltage-gated calcium channel subunits (HEK293-S3 cells) were a gift from D. Rock (Warner-Lambert Parke Davis, Ann Arbor, MI). All other chemicals were obtained from Sigma unless specifically indicated otherwise.

**Cell Culture and Transfection**—HEK293-S3 cells were plated and cultured in RPMI 1640 with 1% glutamine supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), 0.4 mg/ml hygromycin, and 0.6 mg/ml Geneticin at 37 °C in 95% O2, 5% CO2 for 2 days on coverslips (thickness 1) attached to the bottom of 35-mm culture dishes before transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. One hour prior to transfection, cells were placed into DMEM lacking antibiotics and supplemented with 1% 1-glutamine, 1% nonessential amino acids, and 10% fetal bovine serum. 4–6 h after the transfection, cells were returned to the RPMI 1640 medium. Cells were used for imaging 24–48 h after transfection.

Chromaffin cells were isolated from bovine adrenal glands using divalent metal ion-free rinse, collagenase digestion, and gradient centrifugation as described previously (46). Cells were cultured in 6-well plates in DMEM/F-12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 10 μg/ml gentamicin, and 10 μm cytosine arabinosuromide. Three days following isolation, cells were transfected using biolistic particle bombardment according to the manufacturer’s instructions with plasmid DNA-laden (2 μg/mg of beads) 1-μm diameter gold beads (Bio-Rad). Cells were replated 4–24 h before imaging onto collagen coated glass coverslips.

PC-12 cells were cultured in 10% CO2 in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 1% gentamicin (10 μg/ml). PC-12 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. 30 min before transfection, cells were placed in OptiMem medium; cells were returned to the DMEM 4–6 h following transfection.

**Tomosyn Translocation Assay**—Cells were serum-starved in DMEM/F-12 medium for 4 h prior to treatment with selected receptor agonists or signaling antagonists. Following the serum-starved period, medium was changed to physiological saline (PSS) containing 140 mM NaCl, 5 mM KCl, 2.2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH 7.4 adjusted with NaOH). Treatments included incubation with LPA (10 μM, Biomol), nicotinic acetylcholine receptor agonist DMPP (20 μM), or, for control, PSS alone. Following either a 2- or 10-min period of receptor activation, the medium was rapidly removed, and the cells were rinsed once in ice-cold phosphate-buffered saline. Lysis buffer was then rapidly added, cells were scraped, and lysates were collected and immediately frozen in liquid N2. The relative level of RhoA activation with respect to control conditions was then measured using an enzyme-linked immunosorosorbant assay-based kit according to the manufacturer’s instructions (Cytoskeleton). Each sample was assayed in duplicate, and each condition was repeated on at least three individual cell preparations. The level of RhoA activation was calculated with respect to that of the control condition following subtraction of the assay blank.

**Human Growth Hormone Secretion Assay**—PC-12 cells were plated onto 24-well plates and co-transfected with plasmids coding for human growth hormone (hGH), in addition to full-length tomosyn, tomosynΔC, or a neomycin control. The total concentration of DNA was held equal across all treatments. hGH was used as a reporter for regulated secretion specifically from transfected cells (47). Secretion assays were performed 48–72 h following transfection. 16–20 h before the start of the assay, cells were placed in serum-free medium; where applicable, cells were pretreated with Y27632 (20 μM) 4 h prior to the start of the assay. To test secretion, cells were rinsed for 10 min in a physiological saline solution (145 mM NaCl, 5.6 mM KCl, 15 mM NaHEPES, 0.5 mM MgCl2, 2.2 mM CaCl2, 5.6 mM glucose, 0.5 mM sodium ascorbate, 2 mg/ml fatty acid-free bovine serum albumin, pH 7.3) in the presence or absence of Y27632. Cells
were then stimulated to secrete by a 6-min treatment with 70 mM KCl (same saline solution but with equimolar substitution of KCl for NaCl). Where applicable, 10 μM LPA and/or (20 μM) Y27632 were added to the stimulus solution. The saline solution containing the secreted hGH was collected, and cells were lysed to determine the percentage of total hGH content secreted. hGH content was measured using an hGH enzyme-linked immunosorbent assay kit (Roche Applied Science). Each experiment was performed with quadruplicate replicates for each treatment.

**GST-Syntaxin 1A- Tomosyn Binding in Vitro and Immunoblotting**—Soluble syntaxin 1A (residues 1–264) was expressed in Escherichia coli as a gluthathione S-transferase (GST) fusion protein. GST was expressed in a similar manner. Both proteins were purified by glutathione-Sepharose (Sigma) binding and extensive washing. The bound GST or GST-syntaxin 1A were then incubated for 16 h at 4°C with lysates prepared from PC-12 cells expressing EGFP-tomosyn or EGFP-tomosyn ∆CT. The lysis buffer contained 100 mM KCl, 20 mM HEPES-KOH, 2 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol with protease inhibitor mixture (Roche Applied Science; PI Complete). The beads were then collected by centrifugation and washed four times with phosphate-buffered saline, and bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-GFP antibody (Stressgen).

**Fluorescence Photobleaching in Subcellular Localization of EGFP-tagged Proteins**—Chromaffin cells were plated onto collagen-coated glass-bottomed 35-mm culture dishes, cultured for 24 h prior to use, and imaged in PSS. Localization of expressed EGFP-tagged soluble proteins to plasma membrane regions was determined following a spatially directed laser-induced (488-nm line of the argon laser) photobleach of EGFP fluorescence in a rectangular region of the cytosol of live cells using a Zeiss LSM 510 confocal microscope. A targeting factor for distribution of EGFP-tagged proteins to the plasma membrane region was determined from images of the cell taken pre- and post-photobleach of the photobleach. Initially, averaged fluorescence intensities of the whole cell (I_{total}) and its cytosolic (I_{cyt}) and membrane (I_{mem}) regions in pre- and postbleach images were measured along with the corresponding total (A_{total}), membrane (A_{mem}), and cytosolic (A_{cyt}) areas. The value of membrane targeting of a protein was defined as the fraction of membrane-bound EGFP-tagged protein in total fluorescence of the cell before the photobleach. For each photobleachable, relative membrane fluorescence parameter M = (I_{post}/I_{pre})_{tot} was determined. Assuming that total intensity in a membrane region consists of contributions from that of membrane-bound and near membrane cytosol fluorescence (I_{mem} = I_{trueMem} + I_{cyt}A_{mem}), and that I_{trueMem} does not change with photobleach, the targeting factor value was calculated as follows: targeting factor = I_{trueMem}/I_{total} where I_{trueMem} = [(I_{pre}A_{mem}M - I_{post}A_{cyt})/A_{mem}]/(1 - M).

**Confocal and Conventional Fluorescence Microscopy of ECFP- and EcYFP-tagged Proteins and Imaging of FRET**—Chromaffin cells were cultured for 36 h after transfection and then plated and cultured for 4–24 h on collagen-coated glass coverslips prior to fluorescence imaging. For fluorescence imaging of fixed cells, the cells were fixed in 4% paraformaldehyde in PSS for 20 min, rinsed with PSS, and quenched with 50 mM NH_4Cl-PSS for 12 min. After rinsing with PSS, cells were submerged in 75% glycerol-PSS and subjected to fluorescence imaging on a Zeiss LSM 510 META confocal microscope. Imaging and morphological analysis of subcellular localization of expressed fluorophore-tagged proteins were primarily performed on the Zeiss confocal microscope. Imaging of FRET was carried out using acceptor photobleach (i.e. donor dequenching) methodology on the Zeiss confocal microscope and by detection and analysis of sensitized EcYFP emission on a conventional Olympus fluorescence microscope. FRET imaging with the Zeiss LSM 510 META relied on pixel-by-pixel linear unmixing calculations (Meta software) performed prior to and following photobleach of the acceptor. This software utilizes control emission spectra of the fluorophores to completely decompose or digitally separate the contribution from the ECFP- and EcYFP-tagged signals in the mixed emission spectrum recorded from cotransfected cells. Relative FRET efficiency by acceptor photobleach was calculated as (1 – (ECFP I_{prebleach}/ECFP I_{postbleach})) × 100%. Sensitized emission FRET was detected using a Olympus microscope coupled to a Tilt-Photonics Polychrome IV xenon lamp-based monochrometer (Tilt-Photonics, Grafelfing, Germany), a polychroic mirror that allowed detection of multiple fluorophores (436–500 nm; Chroma Technology Corp., Brattleboro, VT), a Planapo X60 water immersion objective (1.2 numerical aperture), a multispec microimager (Optical Insights, Sante Fe, NM) containing a dichroic splitter (505dcxrx) and emission filters (D465/30 and HQ535/30), and a cooled digital CCD camera (TILL-IMAGO QE). Images corresponding to three excitation/emission wavelength settings (excitation/emission, 436/465, 436/535, and 500/535) were collected. Quantification of sensitized emission FRET efficiency from shade- and background-corrected images was determined according to a method termed FRET stoichiometry (48). Analysis of the acquired cell images determined three parameters for each pixel of an image. The first parameter is the apparent FRET efficiency, EA, which is the product of the FRET efficiency of the specific bimolecular interaction, termed characteristic FRET efficiency (E_c), and the fraction of acceptor in complex with the donor. The second parameter is ED, which is the apparent donor efficiency and is the product of E_c and the fraction of donor in complex. The third parameter is the acceptor-donor molar ratio (Ratio). Ratio indicates the molar fraction of acceptor to donor in each pixel of the cell image. Because EA and ED are proportional to the fraction of acceptor and donor in complex, respectively, they can be used to measure time- or agonist-dependent changes in the fraction of acceptor and donor in complex. Full details concerning controls performed for validation of FRET signals, acquisition parameters of background and shade images, methods used for determination of proportionality constants required for complete separation of ECFP and EcYFP and FRET signals, and the specific calculations performed to quantify FRET efficiency values are given in our recent report (49).

**Data Analysis and Statistics**—Data were analyzed, and statistical analysis was performed using IGOR PRO (Wavemetrics Inc.) and GraphPad Instat (GraphPad Software Inc., San Diego, CA) software. Population data were expressed as means ± S.E.,
and statistical significance was determined using Student’s unpaired t tests or for multiple comparisons using analysis of variance with Dunnett’s post hoc test on normally distributed data. In the case of nonparametric data, a Mann-Whitney U test was performed using the original calculated values. Significant differences were defined by p < 0.05 and indicated by an asterisk.

RESULTS

Expression and Targeting of Fluorescently Tagged Tomosyn, Syntaxin 1A, and Munc18-1 in HEK293 Cells—HEK-293 cells were initially used to demonstrate expression and targeting of the recombinant fluoroprotein-tagged tomosyn, syntaxin 1A, and Munc18-1 proteins and to test for specific interactions between these proteins in an in vivo situation. The HEK-293 cell line does not demonstrate endogenous expression of these proteins (50) and, therefore, provides a clean background upon which to test interactions. Fig. 1A shows confocal fluorescence images that compare the subcellular distribution of cYFP-tomosyn, cYFP-syntaxin 1A, and CFP-Munc18-1 when individually expressed. Although both the cYFP-tomosyn and CFP-Munc18-1 showed a diffuse distribution in the cytosol typical of soluble proteins, syntaxin 1A was observed to accumulate primarily in perinuclear membrane regions and in some cells in a number of highly localized sites in the cytosol characterized by intense fluorescence. By comparison, coexpression of cYFP-syntaxin 1A with CFP-Munc18-1 resulted in efficient targeting of cYFP-syntaxin 1A to the plasma membrane region (Fig. 1B), and cells were remarkably devoid of perinuclear fluorescence. The coexpression of CFP-tomosyn with cYFP-syntaxin 1A did not result in a similar dramatic targeting consequence, since strong perinuclear cYFP-syntaxin 1A labeling was retained with little enrichment of fluorescence at the plasma membrane. Thus, tomosyn and Munc18-1 are functionally distinct with regard to their ability to facilitate syntaxin 1A targeting to the plasma membrane in HEK-293 cells.

We next determined whether overexpressed CFP-tomosyn directly interacted with syntaxin 1A in this cell line. For this purpose, we used FRET between the CFP/cYFP pair to visualize and quantify direct CFP-tomosyn/cYFP-syntaxin 1A interactions. FRET measurements were performed by acceptor photobleach on fixed cells using an LSM Zeiss 510 META confocal microscope, which was optimal for acquiring images in a single optical plane coincident with FRET and analysis of its subcellular compartmentation. Fig. 2A compares images acquired prior to and following a localized photobleach of the cYFP acceptor from a representative HEK-293 cell. A marked increase in CFP fluorescence occurred following photobleach that was restricted to the bleached perinuclear membrane region, a result indicative of FRET between CFP-tomosyn and cYFP-syntaxin 1A. The increased CFP signal following photobleach was readily apparent upon comparison of spectrally pseudocolored images of this cell region prior and following photobleach (shown below cell images). Averaged relative FRET efficiency values for photobleached and nonphotobleached cell regions are presented in Fig. 2C and clearly establish that a CFP-tomosyn/cYFP-syntaxin 1A interaction occurred in HEK-293 cells. Thus, the compartmentalization of CFP-tomosyn observed in the presence of syntaxin 1A coexpression is reflective of a colocalization of these proteins that results from a direct protein-protein interaction. As we have previously reported, analysis of nonphotobleached regions of CFP-expressing cells also demonstrates a slight increase in CFP signal of ~9% following the laser photobleach protocol, which we define as background (49). The cause of this small and consistent change remains undetermined and has not been subtracted from the relatively large FRET values determined for photobleached regions of the cells.

Since Munc18-1 and tomosyn both interact with syntaxin 1A, we next determined whether overexpression of Munc18-1 altered the interaction between syntaxin 1A and tomosyn. As shown in Fig. 2B, coexpression of tomosyn, syntaxin 1A, and Munc18-1 resulted in targeting of syntaxin 1A to plasma membrane regions, indicative of Munc18-1 facilitation of syntaxin 1A targeting. Of specific importance, with these coexpression conditions, CFP-tomosyn retained significant FRET with cYFP-syntaxin 1A, although it was now present spatially at plasma membrane regions (Fig. 2B), versus the perinuclear/ER and Golgi localization that was observed in the absence of Munc18-1 coexpression. In addition, the averaged relative FRET efficiency was reduced slightly but significantly from that measured in the absence of Munc18-1 coexpression (Fig. 2C). These results are complementary to prior reports for the HEK-293 cell line in establishing that Munc18-1 is important for the distribution of overexpressed syntaxin 1A to the plasma membrane region (50–52). The present FRET results demonstrate that, although tomosyn is capable of interacting with syntaxin
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In multiple subcellular compartments, it is likely to preferentially associate with syntaxin 1A at the plasma membrane in neurons where Munc18-1, syntaxin 1A, and tomosyn are endogenously expressed.

In additional experiments, we tested whether the tomosyn-syntaxin 1A heterodimeric complex at the plasma membrane in HEK-293 cells was capable of progression to a SNARE core complex by the incorporation of a cognate t-SNARE, SNAP25. For these experiments, we used a SNAP25 mutant that contained EGFP fused to the N terminus of a SNAP25 mutant in which the four normally palmitoylated cysteine residues that direct SNAP25 membrane targeting were mutated to alanine (EGFP-S25 c/a). We have previously demonstrated that this SNAP25 mutant becomes membrane-localized only if it is capable of pairing its SNARE motifs with that of syntaxin 1A (50) or, presumably, a syntaxin 1A-tomosyn complex. The subcellular distribution of EGFP-S25 c/a was determined by calculating a targeting factor that compares EGFP fluorescence at the plasma membrane region relative to that in the cytosol from confocal images taken prior to and following a laser-induced photobleach (488-nm line of argon laser) of the cytosolic fluorescence using the Zeiss LSM confocal microscope. Fig. 2D shows that EGFP-S25 c/a expressed alone in HEK-293 cells shows little labeling of the plasma membrane but that its coexpression with syntaxin 1A resulted in a substantial increase in targeting. Under these coexpression conditions, targeting of syntaxin 1A to the plasma membrane occurred in the absence of Munc18-1. Notably, the syntaxin 1A induced increase in EGFP-S25 c/a targeting was completely inhibited with the added expression of Munc18-1, probably as a result of stabilizing syntaxin 1A in an inactive SNARE complex forming conformation. Coexpression of EGFP-S25 c/a with syntaxin 1A and tomosyn also resulted in significant targeting of EGFP-S25 c/a to the plasma membrane, and this targeting was unaffected by the additional expression of Munc18-1.

These data, taken together with the prior FRET results (Fig. 2C), indicate that tomosyn competes with Munc18-1 for binding to plasma membrane-localized syntaxin 1A in vivo and permits formation of SNARE complexes.

Location and Properties of Tomosyn Interaction with Syntaxin 1A in Adrenal Chromaffin Cells—To establish the components that normally regulate tomosyn-syntaxin 1A interactions and to relate changes in their interaction to functional consequences within the exocytotic pathway, the properties of the interaction were further investigated in bovine adrenal chromaffin cells, where these proteins are endogenously present and demonstrated to be functionally important (26, 28). In these cells, exogenously expressed CFP-tomosyn and cYFP-syntaxin 1A localized to the plasma membrane region without exogenous coexpression of Munc18-1 (Fig. 3A). To establish that the colocalized CFP-tomosyn and cYFP-syntaxin 1A at the
plasma membrane region were indicative of a direct bimolecular interaction, we examined the properties of colocalization using both acceptor photobleach and sensitized emission FRET approaches. Sensitized emission FRET was performed on live chromaffin cells using a conventional fluorescence microscope and was beneficial for examining FRET under low protein overexpression conditions. Both FRET approaches demonstrated that CFP-tomosyn bound directly to cYFP-syntaxin 1A at the cell periphery (Fig. 3, A and D). Relative FRET efficiency at the plasma membrane region by acceptor photobleach averaged 42.9 ± 3.6% (n = 5), which was >5-fold over that of background, as calculated from the nonphotobleached region of the same cells (Fig. 3B). The averaged apparent FRET efficiency (EA, ED) values determined by sensitized emission (14 ± 2.9%) were lower than those calculated by acceptor photobleach FRET. The values differ as a result of the specific analysis schemes applied, particularly since the expression levels of the fusion protein constructs, which define the degree of FRET interaction, were not accounted for in the analysis of acceptor photobleach FRET. Indeed, a plot of molar ratio (cYFP/CFP) against corresponding apparent efficiency of donor in complex ED for each imaged pixel of a single cell as determined by sensitized emission illustrates the sensitivity of FRET efficiency to the relative expression of acceptor and donor proteins (Fig. 3E). Therefore, all subsequent comparisons between FRET values by sensitized emission have included only those FRET values where the molar ratio fell within 0.9–1.1 Ratio range.

**Requirement of SNARE/SNARE Interaction for CFP-Tomosyn/cYFP-Syntaxin 1A FRET**—The R-SNARE homology domain near the C terminus of tomosyn is believed to be primarily responsible for the interaction of tomosyn with the Q-SNARE motif of syntaxin 1A. Therefore, to define the extent to which the in vivo interaction of tomosyn with syntaxin 1A is dependent upon formation of an R-SNARE-Q-SNARE complex, we measured, using FRET, the effect of specific Q-SNARE mutations of syntaxin 1A on the interaction. Two site-directed mutants of cYFP-syntaxin 1A were tested: cYFP-syntaxin 1A (I209A) and cYFP-syntaxin 1A (I233A). The I209A mutation occurs within the SNARE motif of syntaxin 1A, and it has been shown to strongly reduce the binding of syntaxin 1A to its co-
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native t-SNARE, SNAP25 (53). By comparison, the I233A mutation lies within the N-terminal domain of the SNARE motif that normally occupies the central cavity of Munc18 during syntaxin 1A interaction with Munc18. Correspondingly, the syntaxin 1A (I233A) mutant has been shown to reduce syntaxin 1A/Munc18 binding in vitro without reduction in syntaxin 1A/SNAP25 binding (54). As shown in Fig. 3, B and F, expression of cYFP-syntaxin 1A (I209A) with CFP-tomosyn resulted in a significant reduction in FRET (by ~50%) with respect to control. These results are consistent with the R-SNARE/Q-SNARE interaction being critically important to the tomosyn-syntaxin 1A interaction. The retention of measurable binding may be representative of the lowered affinity of interaction with the mutant syntaxin 1A or may result from interacting sites beyond the SNARE motifs that may also be important for the tomosyn-syntaxin 1A interaction in vivo. By comparison, FRET between CFP-tomosyn and cYFP-syntaxin 1A (I233A) measured by sensitized emission was similar to that of control (Fig. 3E). Evaluation of the syntaxin 1A (I233A)-tomosyn interaction was limited to sensitized emission as a result of low levels of exogenous expression of the cYFP-syntaxin 1A (I233A) protein. A central interaction of tomosyn with syntaxin 1A is, therefore, similar to that of SNAP25 with syntaxin 1A, which is mediated by SNARE/SNARE motif interactions.

Tomosyn and Munc18-1 have also been reported to interact with structurally distinct conformations of syntaxin 1A. In addition, these differences in binding interactions are proposed to reflect the important functional differences these proteins exert on the exocytotic pathway. To determine if tomosyn and Munc18-1 compete for syntaxin 1A within chromaffin cells, we analyzed the effect of Munc18-1 coexpression on CFP-tomosyn/cYFP-syntaxin 1A FRET. The results showed that Munc18-1 overexpression reduced CFP-tomosyn binding to cYFP-syntaxin 1A, as evidenced by an ~40% reduction in apparent FRET efficiency (Fig. 3B and F). In complementary experiments, overexpression of tomosyn was found to reduce the apparent FRET efficiency between coexpressed CFP-Munc18-1 and cYFP-syntaxin 1A (Fig. 3C and G), as would be expected for a competitive binding reaction.

Tomosyn Association with a Membrane Fraction Is Enhanced by a Secretory Stimulus and Activation of Rho-GTPase—Interaction of syntaxin 1A with Munc18-1 is essential for regulated exocytosis, whereas its interaction with tomosyn is believed to negatively impact exocytotic activity through the formation of fusion-incompetent SNARE core complexes. Therefore, a key question is whether the level of tomosyn-SNARE complexes is subject to dynamic regulation, as would be required to fine tune secretory responses. As an initial test for dynamic regulation, we determined if stimulation of chromaffin cells for secretion by activation of acetylcholine receptors with DMPP induced changes in the association of endogenous tomosyn with the plasma membrane. Fig. 4 shows that the amount of membrane-localized tomosyn significantly increased upon treatment of the chromaffin cells with DMPP (30 min, 10 μM), a stimulus that normally evokes a strong secretory response. In these experiments, tomosyn was immunoprecipitated from a membrane fraction prepared from control or DMPP-treated chromaffin cells. In each case, cells were subjected to N-ethylmaleimide (NEM; 100 μM, 10 min) treatment prior to lysis to inhibit NSF-mediated disassembly of formed tomosyn-SNARE complexes (100 μM NEM was also included in the lysis buffer). Immunoprecipitation was required in these experiments, since the level of tomosyn associated with the membrane fraction under control and secretagogue stimulation conditions represented a small fraction of total tomosyn within the chromaffin cell lysate.

We next sought to establish the signaling mechanism that mediates the increase in tomosyn association with the membrane in response to a secretory stimulus in chromaffin cells. Previous studies have established that activation of Rho-GTPase/ROCK signaling pathways can enhance formation of tomosyn-SNARE complexes in cultured embryonic neurons. Therefore, we investigated whether DMPP stimulation of chromaffin cells may act via the Rho-GTPase signaling pathway to alter tomosyn association with the plasma membrane. As an initial experiment, we determined whether LPA, a well known receptor-mediated activator of Rho-GTPase (RhoA-GTP) in chromaffin cells treated with LPA (10 μM; 2 min or 10 min) or DMPP (20 μM; 2 min or 10 min) relative to control (C, nonstimulated, 10 min) conditions. Data shown are the average of four (DMPP) or three (LPA) separate experiments, with each sample measured in duplicate.

![FIGURE 4. Agonist activation of nicotinic acetylcholine receptor and application of LPA induce translocation of endogenous tomosyn to a membrane fraction in chromaffin cells and activate endogenous RhoA. A, averaged level of endogenous tomosyn immunoreactivity in a membrane fraction of cultured chromaffin cells incubated with DMPP or LPA relative to that in control (C, nonstimulated) conditions. Translocation induced by receptor agonists was specifically blocked by co-incubation with the Rho kinase inhibitor. Serum-starved chromaffin cells were treated with DMPP (30 min, 20 μM) or LPA (10 min, 10 μM) with subsequent NEM treatment (10 min, 100 μM). The Rho kinase inhibitor (Y27632, 20 μM), when included, was added to the serum-starved medium 4 h prior to stimulation and maintained throughout stimulation. Following treatment, cells were lysed, membrane fractions were extracted by ultracentrifugation, and tomosyn was immunoprecipitated. Samples were then analyzed by SDS-PAGE and immunoblotting. The number of individual determinations comprising the average is shown above the bars. The panel above the plot shows a representative immunoblot. B, level of activated RhoA (RhoA-GTP) in chromaffin cells treated with LPA (10 μM; 2 min or 10 min) or DMPP (20 μM; 2 min or 10 min) relative to control (C, nonstimulated, 10 min) conditions. Data shown are the average of four (DMPP) or three (LPA) separate experiments, with each sample measured in duplicate.]
(20 μM) also activated Rho-GTPase, although the level of activation was substantially less than that observed to LPA stimulation (Fig. 4B). Of particular importance, preincubation of the cells with Y27632 (4 h, 20 μM), a specific ROCK inhibitor, completely eliminated both the DMPP- and LPA-mediated increase in tomosyn within the membrane fraction (Fig. 4A).

These data suggest that enhancement of tomosyn association with the membrane in response to DMPP stimulation of chromaffin cells occurred via activation of the Rho-GTPase/ROCK signaling pathway.

Photobleaching of Freely Diffusible EGFP Fluorescence Reports DMPP- and LPA-induced Targeting of EGFP-Tomosyn to the Cell Periphery in Live Chromaffin Cells—Previous studies established that DMPP and LPA treatment of adrenal chromaffin cells promoted tomosyn association with a membrane particulate fraction prepared by ultracentrifugation of cell lysates. Our expectation was that in response to these stimulation conditions, tomosyn translocated and localized to the plasma membrane prior to cell lysis through direct interactions with syntaxin 1A. However, cell lysis disrupts subcellular integrity, thereby precluding direct assignment of tomosyn in a particulate fraction to the plasma membrane. In addition, syntaxin 1A may form protein complexes following cell lysis, which compromises interpretations on physiological relevance of immunoprecipitated tomosyn-syntaxin 1A complexes. To overcome these problems, a fluorescence photobleach approach was used to visualize and quantify the subcellular distribution of overexpressed EGFP-tomosyn in confocal images of living chromaffin cells that were exposed to DMPP or LPA stimulation. As a soluble protein, the contribution of cytosolic EGFP-tomosyn to the fluorescent image of the cell was selectively removed by application of brief laser photobleaching within a discrete area in the cell’s cytoplasm. The remaining EGFP fluorescence reflected EGFP-tomosyn that was diffusively restricted, presumably through its specific interaction with proteins or lipids associated with membrane-delimited organelles or the plasma membrane. As shown in the images of Fig. 5A and the averaged targeting factor data (Fig. 5B), this approach was able to detect EGFP-tomosyn that was specifically localized to the plasma membrane region in response to a series of specific treatments. The results showed that expression of EGFP-tomosyn alone demonstrated little specific EGFP fluorescence at the membrane region, whereas, by comparison, a strong EGFP-tomosyn signal was observed at the plasma membrane region when it was coexpressed with exogenous syntaxin 1A. The difference in plasma membrane-localized fluorescence between these conditions indicated that it is the interaction of EGFP-tomosyn with syntaxin 1A that brings EGFP-tomosyn to the plasma membrane. Most importantly, the results demonstrated that application of DMPP or LPA led to a significant increase in EGFP-tomosyn membrane targeting over control. Furthermore, the LPA-induced increase was inhibited by preincubation of the cells with the Rho kinase inhibitor, Y27632 (Fig. 5B).

Kinetics of Receptor-mediated Change in Tomosyn-Syntaxin 1A Interaction and Involvement of Rho/ROCK Signaling Pathways—Our next goal was to use the developed sensitized emission FRET assay between CFP-tomosyn and CyFP-syntaxin 1A together with time lapse fluorescent imaging to define the kinetics of the receptor-mediated targeting of tomosyn to the plasma membrane and to establish that this targeting resulted in a direct interaction of tomosyn with syntaxin 1A. For these FRET experiments, we optimized our ability to detect changes in the fraction of cYFP-syntaxin 1A in complex with CFP-tomosyn (proportional to ΔEA) by transfection with a DNA ratio of cYFP-syntaxin 1A to CFP-tomosyn that resulted in a low cYFP/CFP molar ratio of protein expression (cYFP/ CFP ratio ~0.3). As shown in Fig. 6A, application of 20 μM LPA to the CFP-tomosyn/cYFP-syntaxin 1A-expressing chromaffin cells resulted in a rapid step increase from the base-line FRET EA value that was sustained throughout the application period. The subsequent addition of NEM, while continuing LPA treatment, resulted in a marked further time-dependent increase in EA from base line. As shown in Fig. 6B, preincubation of chromaffin cells for 4 h with the Rho kinase inhibitor Y27632 (10 μM) eliminated the effect of LPA to increase an interaction between CFP-syntaxin 1A and cYFP-tomosyn. This was evidenced by no significant change in the EA values from base line over the time frame of the recordings. These data are consistent with LPA-mediated activation of the Rho-ROCK pathway in chromaffin cells promoting a steady-state increase in tomosyn-syntaxin 1A SNARe complexes. In additional experiments, FRET measurements were also used to examine the effect of DMPP stimulation on the kinetics of direct interaction of CFP-tomosyn/cyFP-syntaxin 1A in transfected chromaffin cells. As shown in Fig. 6C, DMPP stimulation resulted in an increased interaction between these proteins similar to that observed with LPA stimulation, and the interaction was further increased upon application of NEM. Preincubation with the Rho kinase inhibitor abolished the DMPP-induced increase in...
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FRET (Fig. 6D). The substantial increase in FRET from baseline by the addition of NEM occurred only in LPA- or DMPP-stimulated cells (Fig. 6E), thereby indicating that it probably resulted from an inhibition of NSF-mediated disassembly of tomosyn-SNARE complexes and that LPA and DMPP strongly enhance cycling of tomosyn-SNARE complexes.

Stimulus-induced Secretion Is Modulated by Rho-mediated Regulation of Tomosyn—In a final series of experiments, we investigated whether there was a functional link between Rho/ROCK signaling pathways and tomosyn upon regulation of evoked secretion. For these experiments, we compared the effects of tomosyn with that of a tomosyn mutant that lacks the R-SNARE motif (tomosyn ΔCT) on secretion from PC-12 cells. Our rationale was that if Rho/ROCK pathways augment the inhibitory action of tomosyn on secretion by promoting a direct tomosyn-syntaxin interaction, then these effects should be mitigated by a tomosyn mutant (tomosyn ΔCT) that lacks the syntaxin 1A-interacting SNARE motif. The lack of a direct high affinity interaction between the tomosyn ΔCT construct and syntaxin 1A was confirmed using GST-syntaxin 1A pull-down experiments (Fig. 7A). By comparison, considerable interaction was observed between GST-syntaxin 1A and wild-type tomosyn. For the secretion experiments, we used PC-12 cells cotransfected with the regulated secretory pathway reporter human growth hormone (hGH) to report on effects of the exogenously expressed tomosyn constructs on regulated secretion only from transfected cells. As shown in Fig. 7B, transfection with tomosyn or tomosyn ΔCT inhibited elevated K⁺-induced secretion by ~25% with respect to control transfected (neomycin plasmid) PC-12 cells. Moreover, LPA, applied at the onset of the elevated K⁺ stimulus, increased the extent of secretory inhibition in tomosyn- but not tomosyn ΔCT-expressing cells, with respect to control cells. Importantly, the effect of LPA to increase secretory inhibition in tomosyn-transfected cells was reversed by preincubation of the cells with the Rho kinase inhibitor (Y27632). Preincubation with the Rho kinase inhibitor had no effect on the secretory inhibition mediated by tomosyn ΔCT. The differences in the effect of LPA treatment upon secretion between tomosyn and tomosyn ΔCT were unlikely to have resulted from differences in relative levels of expression of these constructs, since no significant difference in levels of expression of CFP-tagged variants of the constructs was observed from measurements of CFP intensity (Fig. 7, C and D). Taken together, these data demonstrate that LPA exposure augments tomosyn inhibition of stimulus-induced secretion from PC-12 cells and that this probably occurs as a result of tomosyn interaction with syntaxin 1A (i.e. the ability of LPA to augment inhibition of secretion was absent in the tomosyn ΔCT mutant-expressing cells).

DISCUSSION

Recent studies suggest that tomosyn exerts regulatory control over specific presynaptic pathways, including neurite extension and neurotransmitter release (27, 29, 44). Furthermore, the activity of tomosyn in these pathways has been reported to be fine tuned by the phosphorylation state of tomo-
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FIGURE 7. Effect of tomosyn translocation induced by LPA treatment on evoked hGH secretion from PC-12 cells. A, GST-syntaxin pull-down assay of EGFP-fused tomosyn (top) or EGFP-tomosyn ΔCT (bottom). Lysates from PC-12 cells expressing EGFP-tomosyn or EGFP-tomosyn ΔCT were bound to GST- or GST-syntaxin 1A-containing beads. Bound proteins were analyzed by SDS-PAGE and immunoblotted with anti-EGFP antibody. B, PC-12 cells were transfected with control empty plasmid (C; encodes only neomycin resistance), tomosyn (T), or a tomosyn construct (ΔCT) in which the SNARE motif at the C terminus was deleted. 2 days after transfection, secretion was induced by application of elevated K+ saline alone (left set of bars), with co-application of LPA (10 μM; middle set of bars) or in cells preincubated with Y27632 (4 h) prior to co-application of elevated K+ saline and LPA. In each case, the stimulus period lasted for 10 min, after which the medium was collected for analysis of secreted hGH, and cell lysates were prepared for determination of total cell hGH content. For each experiment, hGH secretion was calculated as a percentage of the total cell content using quadruplicate samples and then normalized to the control condition of each treatment. C, averaged ECFP fluorescent intensity in tomosyn- and tomosyn ΔCT-transfected PC-12 cells (± S.E.; n = 184 and 207 cells for tomosyn and tomosyn ΔCT, respectively). Cells were imaged 48 h post-transfection, and images of at least 50 random fields of cells were taken for each condition. D, frequency distribution of spatially averaged ECFP intensity values for tomosyn (top) and tomosyn ΔCT-transfected cells (bottom).

syn as well as that of its immediate effectors (e.g. syntaxin 1A) (44, 45). We hypothesized that physiological regulation of these key tomosyn-protein interactions during neurosecretion might occur rapidly in response to receptor-mediated signals of secretory demand. In this study, we examined the spatiotemporal dynamics of the interaction of tomosyn with the t-SNARE syntaxin 1A, both in the nonsecretory HEK293 cell line and in neurosecretory bovine adrenal chromaffin cells. Our findings establish that the tomosyn-syntaxin interaction is up-regulated in response to secretory demand and that under these conditions, there is a substantial increase in the rate of tomosyn-SNARE complex cycling (i.e. formation and dissolution). Moreover, modulation of the tomosyn-syntaxin 1A interaction by secretagogue stimulation provides for dynamic regulation of the secretory response.

Our characterization of the tomosyn-syntaxin 1A interaction in HEK293 cells illustrates an important difference between Munc18-1-syntaxin 1A and tomosyn-syntaxin 1A heterodimer complexes. In HEK293 cells, targeting of syntaxin 1A to plasma membrane regions is dramatically facilitated by co-expression and direct interaction with Munc18-1 (50–52). Although facilitating, the Munc18-1-syntaxin 1A interaction is not essential for syntaxin 1A targeting, as has been identified in several recent studies (55–57). Indeed, SNAP25, as well as other syntaxin 1A-interacting proteins, may enhance syntaxin 1A membrane localization (50). Here, we have extended those findings to show that, unlike the Munc18-1-syntaxin 1A interaction, the interaction of syntaxin 1A with tomosyn does not promote the targeting of either syntaxin 1A or tomosyn to plasma membrane regions. Rather, coexpression of tomosyn and syntaxin 1A results in localization of these proteins to perinuclear compartments, whereas coexpression of these proteins with Munc18-1 results in the targeting of syntaxin 1A and tomosyn to the plasma membrane region. We propose a model whereby an initial interaction of Munc18-1 with syntaxin 1A facilitates syntaxin 1A targeting to the plasma membrane; once syntaxin 1A localizes to the plasma membrane, tomosyn can effectively compete with Munc18-1 to interact with syntaxin 1A. Indeed, our FRET data indicate that the tomosyn-syntaxin 1A interaction is reduced upon overexpression of Munc18-1 and, likewise, that the Munc18-1-

suggested atrophy of microvilli by light and electron microscopy.

FIGURE 10. Regulation of tomosyn localization following exposure to secretogogue in PC-12 cells. A, To visualize the subcellular localization of tomosyn in control and Y27632 preincubated PC-12 cells (4 h), cells were transfected with EGFP-tomosyn or EGFP-tomosyn ΔCT and treated for 10 min with elevated K+ saline alone (top set of bars) or in the presence of Y27632 (4 h) prior to application of elevated K+ saline (middle set of bars). Bound proteins were analyzed by SDS-PAGE and immunoblotted with anti-EGFP antibody. B, hGH secretion was calculated as a percentage of the total cell content using quadruplicate samples and then normalized to the control condition of each treatment. C, averaged ECFP fluorescent intensity in tomosyn- and tomosyn ΔCT-transfected PC-12 cells (± S.E.; n = 184 and 207 cells for tomosyn and tomosyn ΔCT, respectively). Cells were imaged 48 h post-transfection, and images of at least 50 random fields of cells were taken for each condition. D, frequency distribution of spatially averaged ECFP intensity values for tomosyn (top) and tomosyn ΔCT-transfected cells (bottom).
time lapse imaging experiments of FRET between CFP-tomosyn and cYFP-syntaxin 1A. The FRET signal was a relevant reporter of tomosyn-syntaxin 1A interactions in vivo, since control experiments demonstrated predictable changes in FRET efficiency with site-directed mutations within the syntaxin 1A SNARE motif. Interestingly, the DMPP-induced interaction between tomosyn and syntaxin 1A was markedly increased by the addition of the NSF inhibitor, NEM. Tomosyn-syntaxin 1A interactions were not, however, increased by treatment with NEM in non-receptor-activated chromaffin cells. Since tomosyn-SNARE (i.e. tomosyn-syntaxin 1A-SNAP25) complexes, but not the heterodimeric tomosyn-syntaxin 1A complexes, are sensitive to NSF inhibition by NEM (29), these data demonstrate that secretagogue receptor activation results in a rapid and substantial increase in the cycling of tomosyn-containing ternary SNARE complexes. In addition, using membrane localization of soluble EGFP-S25 c/a as a reporter of a syntaxin 1A-SNAP25 interaction, we show that tomosyn can compete with Munc18-1 to promote the formation of EGFP-S25 c/a-containing SNARE complexes.

Importantly, we determined that secretagogue-induced interaction between tomosyn and syntaxin 1A occurs downstream of the Rho-GTPase/ROCK signaling pathway. First, we demonstrated that DMPP stimulation results in activation of the Rho signaling pathway in chromaffin cells. Second, treatment of chromaffin cells with LPA, a nonsecretory stimulus that also activates the Rho pathway, was sufficient to recapitulate the effects of DMPP both on translocation of tomosyn to the plasma membrane region and on the increased interaction between tomosyn and syntaxin 1A. Notably, although DMPP stimulation induced only a modest increase in RhoA activation relative to that induced by LPA, it resulted in a substantially larger change in CFP-tomosyn/cYFP-syntaxin 1A FRET. Both the DMPP- and LPA-induced increases in FRET were mitigated by Y27632. The differences in tomosyn-syntaxin 1A association probably result from the diversity in the extent and type of signaling pathways activated by these different stimuli and suggest that additional signaling pathways may be activated by DMPP that facilitate the RhoA/ROCK pathway. Taken together, the data are consistent with a model whereby the level of tomosyn-syntaxin 1A interaction rapidly responds to activation of receptors mechanistically linked to RhoA and is probably mediated by signaling via RhoA/ROCK phosphorylation of syntaxin 1A. Although certain of the experiments necessitated the overexpression of a fluorophore-tagged tomosyn, the effects seen in these overexpression studies were corroborated in a nonoverexpression system, by biochemical assays establishing that endogenous tomosyn also translocated to membrane fractions in response to both DMPP and LPA and that this translocation was similarly blocked by Y27632.

Although a Rho signaling pathway was previously demonstrated to alter tomosyn-SNARE interactions during neurite development in NG108 cells and cultured hippocampal neurons (44), it was unknown whether the Rho signaling pathway also modulated tomosyn-SNARE complex assembly during regulated neurotransmitter release. Members of the Rho GTPase family are primary regulators of actin cytoskeleton organization, although they regulate a wide range of cellular processes, including exocytosis (58–60). A potential role for GTPases in regulated exocytosis in chromaffin cells was initially suggested from in vitro observations indicating their presence on purified chromaffin granule membranes (61–63). Several distinct GTPases were found, including heterotrimeric Go protein, ARF6, Rab3 and, importantly, RhoA. Most of the RhoA in chromaffin cells is associated with secretory granules, and selective activation of the trimeric G-protein Go1, on chromaffin granules was found to inhibit priming steps of exocytosis (64, 65). This inhibition was significantly reduced in cells treated with Clostridium botulinum C3 exoenzyme, which selectively inactivates the small G-protein Rho (66, 67). Activation of Go1 thus induces RhoA activation, which acts through an effector pathway to decrease vesicle priming. Although phosphatidylinositol 4-kinase has been proposed as a RhoA effector, no direct link of it to vesicle priming has been demonstrated, and other mediators, such as ROCK, have not been tested. Our data indicate that tomosyn must be considered as a target for agonist activation of the Rho/ROCK signaling pathway in chromaffin cells and that this pathway may contribute to the previously reported effect of RhoA-GTP to inhibit vesicle priming.

A central and important question that remains is whether the increased cycling of tomosyn-SNARE complexes exerts a positive or negative role on the maintenance of exocytotic secretion. Our present results indicate that the increase in tomosyn-SNARE cycling in response to receptor activation functions as a negative feedback system that ultimately limits the extent or sites of secretion. Moreover, comparison of the wild-type and ΔCT tomosyn suggests that the Rho-mediated augmentation of the inhibitory effects of tomosyn on secretion is mediated primarily via the tomosyn-syntaxin 1A interaction. It should be noted that overexpression of the tomosyn ΔCT mutant resulted in secretory inhibition only slightly less than that of full-length tomosyn. Since tomosyn ΔCT does not bind syntaxin, these data suggest that the tomosyn SNARE motif, although not critical for the inhibitory effect of tomosyn, might be involved in activity-dependent regulation of the inhibition imposed by tomosyn. Interactions of the tomosyn R-SNARE with syntaxin 1A could designate spatial information, whereas the N-terminal WD40 domains or the hypervariable linker region in tomosyn could carry out the actual inhibitory function on exocytotic activity. Interestingly, a prior report (26) demonstrated that overexpression of a tomosyn mutant with reduced syntaxin 1A interaction still resulted in secretory inhibition.

The participation of the N-terminal motifs and the hypervariable domain in tomosyn must be considered along with the R-SNARE motif as a regulator of the protein interactions of tomosyn or as a scaffold for additional protein interactions important for the inhibitory function of tomosyn. The tomosyn N terminus contains 7–9 repeating β-transducin-like WD-40 motifs that comprise ~90% of the protein sequence and represent the region of highest conservation among tomosyn homologues. Moreover, tomosyn also contains a hypervariable region that separates the N-terminal WD-40 repeats and the C-terminal SNARE domain. Serine phosphorylation by protein kinase A within this region has recently been reported to exert negative regulatory action upon tomosyn-syntaxin 1A interaction (45). In addition, tomosyn homologues, such as the lethal
giant larval family and yeast Sro7p and Sro77p and the mammalian Lgl1 (Mlgl), which do not possess well defined SNARE motifs (34), still interact with SNARE proteins. Importantly, deletion of the yeast proteins Sro7p and Sro77p results in a strong cold-sensitive block in exocytosis (30).

In summary, these present studies are among the first to illustrate, in real time and in live cells, a physiological and dynamic modulation of the localization and interaction of tomosyn with syntaxin 1A in response to secretagogue activation. Moreover, they demonstrate the functional relevance of this modulation in enhancing the inhibitory effect of tomosyn on secretion. Thus, the functions of proteins that regulate the secretory exocytotic pathway may themselves be regulated by secretory demand. Further work remains to elucidate the structural roles of tomosyn’s N-terminal and hypervariable linker domains in mediating tomosyn’s inhibitory effects on neurosecretion.

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