Gβγ Interferes with Ca$^{2+}$-Dependent Binding of Synaptotagmin to the Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor (SNARE) Complex

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ABSTRACT

Presynaptic inhibitory G protein-coupled receptors (GPCRs) can decrease neurotransmission by inducing interaction of Gβγ with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. We have shown that this action of Gβγ requires the carboxyl terminus of the 25-kDa synaptosome-associated protein (SNAP25) and is downstream of the well known inhibition of Ca$^{2+}$ entry through voltage-gated calcium channels. We propose a mechanism in which Gβγ and synaptotagmin compete for binding to the SNARE complex. Here, we characterized the Gβγ interaction sites on syntaxin1A and SNAP25 and demonstrated an overlap of the Gβγ- and synaptotagmin I-binding regions on each member of the SNARE complex. Synaptotagmin competes in a Ca$^{2+}$-sensitive manner with binding of Gβγ to SNAP25, syntaxin1A, and the assembled SNARE complex. We predict, based on these findings, that at high intracellular Ca$^{2+}$ concentrations, Ca$^{2+}$-synaptotagmin I can displace Gβγ binding and the Gβγ-dependent inhibition of exocytosis can be blocked. We tested this hypothesis in giant synapses of the lamprey spinal cord, where 5-HT works via Gβγ to inhibit neurotransmission (Blackmer et al., 2001). We showed that increased presynaptic Ca$^{2+}$ suppresses the 5-HT- and Gβγ-dependent inhibition of exocytosis. We suggest that this effect may be due to Ca$^{2+}$-dependent competition between Gβγ and synaptotagmin I for SNARE binding. This type of dynamic regulation may represent a novel mechanism for modifying transmitter release in a graded manner based on the history of action potentials that increase intracellular Ca$^{2+}$ concentrations and of inhibitory signals through G$\text{G}_{\beta\gamma}$-coupled GPCRs.

Modification of neurotransmitter release by presynaptic G protein-coupled receptors (GPCRs) is ubiquitous and vital to nervous system function. Gβγ inhibits synchronous exocytosis directly at the SNARE complex, the heart of the fusion machinery (Silinsky, 1984; Man-Son-Hing et al., 1989; Blackmer et al., 2001, 2005; Takahashi et al., 2001; Chen et al., 2005; Gerachshenko et al., 2005) and consequently modifies the quantal size of release by causing transient fusion of vesicles in both lamprey giant synapses and mammalian chromaffin cells (Chen et al., 2005; Photovala et al., 2006). Gβγ proteins may also inhibit exocytosis through inhibition of voltage-gated calcium channels, blunting the influx of Ca$^{2+}$ required for exocytosis (Herlitze et al., 1996; Ikeda, 1996).

Synaptotagmin I is believed to mediate the requirement for Ca$^{2+}$ in the initiation of exocytosis. Before the fusion event, vesicles are primed by formation of a stable protein structure, the ternary SNARE complex, comprising the plasma membrane t-SNAREs SNAP25 and syntaxin and the vesicular SNARE VAMP2 (Fig. 1A). Synaptotagmin, as the putative Ca$^{2+}$ sensor, is thought to act as the trigger by which Ca$^{2+}$ influx effects the fusion of primed vesicles (Bai et al., 2004; Maximov and Sudhof, 2005; Wang et al., 2006).

Given its central role in triggering exocytosis, interactions between synaptotagmins and the SNARE complex have been studied extensively. Upon Ca$^{2+}$ binding to its C2 domains (Fig. 1A) during regulated exocytosis, synaptotagmin interacts with phospholipids and the SNARE complex (Perin et

ABBREVIATIONS: GPCR, G protein-coupled receptor; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; t-SNARE, target membrane-associated snare; SNAP25, 25-kDa synaptosome-associated protein; DTT, dithiothreitol; OG, N-octyl-β-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; GST, glutathione transferase; MIANS, 2-(4′-maleimidyl)anilino) naphthalene-6-sulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; BoNT/A, botulinum toxin A; EPSC, excitatory postsynaptic current; 5-HT, 5-hydroxytryptamine.
al., 1990; Schiavo et al., 1997). This interaction occurs through the H3 SNARE domain of syntaxin1A and the C terminus of SNAP25 (Bennett et al., 1992; Chapman et al., 1995; Gerona et al., 2000; Bai et al., 2004). Although the mechanism by which the SNARE complex initiates fusion is under debate (Rizo et al., 2006), interactions with synaptotagmin may cause stabilized ternary SNAREs to zip up, uniting the vesicular and plasma membranes (Margittai et al., 1999; Sorensen et al., 2006). Other evidence suggests that ternary SNARE complex formation is a concerted process with more than one transition state during initial and sustained dilation of a protein fusion pore (Han and Jackson, 2006).

Despite the controversy regarding SNARE complex-mediated membrane fusion, widespread consensus holds that synaptotagmin is the Ca\(^{2+}\) sensor for stimulated exocytosis and may provide the cue for full fusion versus kiss-and-run fusion. Mutagenesis studies, for example, indicate that fusion is inhibited in the presence of synaptotagmin mutants with diminished SNARE interactions (Bai et al., 2004). Furthermore, overexpression of different isoforms of synaptotagmin in PC-12 cells leads to different fusion pore opening durations, suggesting that the interaction of synaptotagmin with the SNARE complex and the membrane affects the nature of fusion pore formation and dilation (Wang et al., 2001). G\(\beta\gamma\) signaling is another mediator of fusion pore dynamics (Wang et al., 2004). We now identify G\(\beta\gamma\) binding sites on the ternary SNARE complex. In addition, we identify a role for Ca\(^{2+}\) and demonstrate competition between G\(\beta\gamma\) and synaptotagmin for binding to SNARE complexes. We propose that modulation of exocytosis is subject to convergent and complementary pathways directed to alteration of synaptotagmin/SNARE complex interactions in which presynaptic Ca\(^{2+}\) both stimulates fusion and modifies the impact of G\(\beta\gamma\) coupled inhibitory GPCRs on exocytosis.

**Fig. 1.** G\(\beta\gamma\) binds individual SNARE proteins and their ternary complex. A, the SNAREs in the primed state and their relationship to synaptotagmin. We hypothesize that G\(\beta\gamma\) counteracts synaptotagmin by interacting with the C-terminal region of the SNARE complex. B, G\(\beta\gamma\) interacts with SNAP25 and syntaxin1A in a GST pulldown assay (top). Equimolar amounts of GST (lanes 1), GST-SNAP25 (lanes 2–4), or GST-syntaxin1A (lanes 5–7) were incubated with the noted concentrations of G\(\beta\gamma\). Precipitated G\(\beta\gamma\) was detected with an anti-pan G\(\beta\gamma\) antibody. Purified G\(\beta\gamma\) was labeled with MIANS and various concentrations of SNAP25 and syntaxin1A and then resuspended in lysis buffer (50 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT)). G\(\beta\gamma\) was induced with 0.1 mM isopropyl \(\beta\)-thiogalactoside for 16 h at room temperature. Bacterial cultures were pelleted, washed with 1x phosphate-buffered saline, and then resuspend in lysis buffer (50 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT)). Cells were lysed with a sonic dismembrator at 4°C. GST-SNAP25

**Materials and Methods**

**Plasmids.** The open reading frames for the SNARE component proteins were subcloned into the pGEX6p1 vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for expression in bacteria. Individual truncation mutants were generated via a PCR-based strategy.

**SNARE Protein Purification.** Recombinant bacterially expressed glutathione transferase (GST) fusion proteins were expressed in *Escherichia coli* strain BL21(DE3). Protein expression was induced with 0.1 mM isopropyl \(\beta\)-thiogalactoside for 16 h at room temperature. Bacterial cultures were pelleted, washed with 1x phosphate-buffered saline, and then resuspend in lysis buffer (50 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT)). Cells were lysed with a sonic dismembrator at 4°C. GST-SNAP25
and GST-VAMP2 were purified from cleared lysates by affinity chromatography on glutathione-agarose (GE Healthcare), following the manufacturer’s instructions. While the proteins were bound to the column, the buffer was exchanged to 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% n-octyl β-D-glucopyranoside (OG), and 2.0 mM dithiothreitol. In some cases, the proteins were eluted by cleaving from GST with PreScission protease (GE Healthcare) for 4 h at 4°C and then dialyzed extensively against 20 mM HEPES, pH 7.0, 80 mM KCl, 20 mM NaCl, and 0.05% OG. GST-syntaxin1A (lacking the transmembrane domain) was purified from the sonicated bacterial supernatant by affinity chromatography on glutathione-agarose (GE Healthcare) in 10 mM HEPES, pH 7.4, 0.05% OG, and 2 mM DTT. Protein concentrations were determined with a Bradford assay kit (Pierce, Rockford, IL), and purity was verified by SDS-PAGE analysis.

**Binary and Ternary SNARE Complex Reassembly.** An equimolar ratio (3 μM) of GST-syntaxin1A on glutathione-agarose and SNAP25 were incubated overnight at 4°C in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% OG, and 2.0 mM dithiothreitol. The binary t-SNARE complex was washed three times with phosphate-buffered saline and eluted from the column by removing GST with PreScission protease (GE Healthcare) for 4 h at 4°C. Equimolar protein-protein interaction was confirmed by SDS-PAGE/Coomassie staining analysis. To reassemble ternary SNARE complex, an equimolar ratio (3 μM) of GST-syntaxin1A on glutathione-agarose, VAMP2, and SNAP25 was incubated overnight at 4°C in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% OG, and 2.0 mM DTT. The SNARE proteins were washed and eluted from the column by removing GST. SDS-and heat-stable SNARE complex formation was verified by SDS/-PAGE analysis with or without a 15-min incubation at 100°C.

**Gβγ Purification and Labeling.** Gβγ1,2 was purified from bovine retina as described previously (Mazzoni et al., 1991). Recombinant Gβγ1,2 was expressed in Sf9 cells and purified via a His6 tag on Gγ1 using nickel-nitrilotriacetic acid affinity chromatography (Sigma-Aldrich, St. Louis, MO). Fluorescence labeling of Gβγ1,2 and binding assays were conducted as described previously (Phillips and Cerione, 1991). In brief, purified Gβγ1,2 was dialyzed into labeling buffer (20 mM HEPES, pH 7.4, 5 mM MgCl2, 150 mM NaCl, and 10% glycerol), then mixed with 2(4’-maleimidylalanine)naphthalene-6-sulfonic acid (MIANS) in a 5-fold molar excess. The reaction proceeded for 3 h at 4°C before quenching with 5 mM 2-mercaptoethanol. The MIANS-Gβγ1,2 complex was separated from unreacted MIANS using a PD-10 desalting column (GE Healthcare). MIANS-Gβγ1,2 was stored in aliquots at −80°C.

**Fluorescence Binding and Competition Assay.** All fluorescence measurements were performed in a fluorescence spectrophotometer (Cary Eclipse; Varian, Inc., Palo Alto, CA) at room temperature. In general, MIANS-Gβγ1,2 was diluted into 0.5 ml of assay buffer (20 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM DTT, 0.1 M NaCl, and 1 mM EDTA) to a final concentration of 20 nM. The MIANS fluorescence was monitored with excitation at 322 nm and emission at 417 nm. All proteins purified as GST fusion proteins were cleaved from GST with PreScission protease (GE Healthcare) before analysis. The fluorescent changes caused by the addition of SNARE complexes were monitored continuously. Note that the amplitude of the fluorescence increase is not a measure of the affinity of the complex; rather, it reflects the specificity on site fluorescently labeled Gβγ of interaction with each protein. There was no nonspecific binding of the free probe to the SNARE proteins and MIANS-Gβγ was resistant to photobleaching under experimental conditions (data not shown). For Gβγ-syntaxotagmin competition assays, various concentrations of syntaxotagmin with SNARE proteins were added to labeled Gβγ1,2 with the noted Ca2+ concentrations, and fluorescence changes were monitored. The EC50 concentrations were determined by sigmoidal curve fitting using Prism software (GraphPad Software, San Diego, CA).

**GST Pull-Down Assay.** GST-SNAP25, GST-VAMP2, and GST-syntaxin1A (5 μg of each protein) were incubated with various amounts of Gβγ1,2 proteins for 1 h at 4°C and washed three times with assay buffer (20 mM HEPES, pH 7.0, 80 mM KCl, 20 mM NaCl, and 0.1% OG). The complex was eluted with 20 μl of SDS sample buffer followed by separation via SDS-PAGE. Precipitated Gβγ was detected using Western blotting with a rabbit anti-pan Gβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Electrophysiology and Microinjection.** Lamprey spinal cord preparations (Petromyzon marinus) were performed as described previously (Wickelgren, 1977). Whole-cell patch-clamp recordings were achieved with a modified blind technique (Blanton et al., 1989) using pipettes (5−10 MO) containing solution of the following composition: 102.5 mM MeSO4, 1 mM NaCl, 1 mM MgCl2, 5 mM HEPES, and 0.05 mM EGTA, brought to a pH of 7.4 with KOH. All paired recordings to measure the effect of protein injection in this study were made at a minimum of 5 min after injection and less than 100 μm from the presynaptic injection site. Recombinant Gβγ1,2 protein was stored at −20°C in a solution containing 100 mM MeSO4, 5 mM HEPES, and 0.05 mM EGTA at pH 7.4. Concentrations ranged from 3 to 5 mg/ml. For microinjection into the presynaptic terminal, 50% (by volume) of 1 M KMeSO4 was added to the Gβγ1,2-containing solution. Protein was pressure microinjected through presynaptic microelectrodes using Picospitzer II (General Valve, Fairfield, NJ).

**Imaging.** Fluorescence images were recorded with a modified confocal microscope (MRC600; Bio-Rad Laboratories, Hercules, CA) run with custom software available at http://alford.bios.uiuc.edu. Reticulospinal axons were labeled retrogradely with a dextran amine-sensitive dye Flu-o-4 (high affinity; Invitrogen, Carlsbad, CA). The dye was applied using a suction pipette fitted to the cut end, immediately after the end of the spinal cord was cut. The tissue was then incubated overnight for the dye to be transported throughout the axons. Images were collected at high speed by scanning a laser over a single line at 500 Hz. Imaging data were analyzed using NIH ImageJ software (http://rsb.info.nih.gov/ij/) on a Macintosh computer. ImageJ was used to calculate the brightness value (range of possible values, 10 bit) for each pixel in a field of view. For each individual axon of interest, the brightness values were measured, and after background subtraction, images were normalized to the baseline level of fluorescence to give (F + 1/P) values.

**Calibration of the Ca2+ Sensitive Dyes.** The dye sensitivity to Ca2+ was determined using the same optical path (confocal microscope) that was used to measure Ca2+ transients in the tissue. Dyes (5 μM) were prepared in blends of two Ca2+ buffer standards (10 mM KEGTA, 100 mM KCl, and 30 mM MOPS) and (10 mM CaEGTA, 100 mM KCl, and 30 mM MOPS) both at pH 7.2 (Invitrogen) to make an 11-point standard curve. The absolute Ca2+ values of the mix were corrected for the low temperatures used in this study (10°C). The dye buffer mix was placed between cover slips cooled from below with a liquid cooling system to 10°C and imaged from above with the 40X water immersion lens.

Western blots are representative of at least three independent experiments. Data points are presented as mean ± S.E.M. (*, p < 0.01; **, p < 0.001, two-tailed Student's t test).

**Results**

**Gβγ1 Interacted with Individual SNARE Proteins and Ternary SNARE Complex.** To understand the relationship between Gβγ and the SNARE complex in the modulation of exocytosis, we characterized sites of interaction by two complementary in vitro approaches, GST pulldown assays and fluorescent binding assays. In GST pulldowns, both components of the t-SNARE (syntaxin1A and SNAP25) interacted with Gβγ1 individually. In addition, the vesicular SNARE component, VAMP2, was found to interact directly with Gβγ (Fig. 1B). To demonstrate that the GST pulldown assay provides specific binding, we performed pulldown assays with Gα subunit and heterotrimeric G protein that contains Gβγ. The Gβγ subunit, but not the Gα subunit or the heterotr-
meric G proteins, interacts with SNARE proteins, suggesting that G protein interaction with SNARE occurs upon inhibitory G protein-coupled receptor activation that induces Gα and Gβγ dissociation (Supplementary Figure S1).

To quantify the relative contributions to Gβγ binding by the individual SNARE components, we employed a sensitive and quantitative fluorescence assay for measuring protein-protein interactions (Phillips and Cerione, 1991). Gβγγ1γ was labeled with the environmentally sensitive fluorescent probe MIANS. This probe serves to report interaction with a binding partner because it undergoes enhanced fluorescence when the local environment becomes more hydrophobic. In these assays, labeled Gβγ (final concentration, 20 nM) was used, whereas interactions with different concentrations of individual SNARE proteins or complexes were assessed through fluorescence intensity changes. Therefore, apparent affinities were estimated by determining concentrations of binding proteins at which MIANS fluorescence reached 50% of maximum (EC50).

This assay was used to quantify the interaction of Gβγ with the ternary SNARE complex and its constituent proteins (Fig. 1, C and D). The ternary SNARE complex indeed caused a dose-dependent increase in the fluorescence of MIANS-Gβγγ1γ, consistent with our previous findings that Gβγγ1γ and the SNARE complex interact in vitro (Blackmer et al., 2001, 2005) (Fig. 1C and EC50 = 0.27 μM). Likewise, we tested the apparent Gβγ-binding affinities of the individual SNARE components (Fig. 1D). Each of the three components caused a fluorescence increase, confirming direct interactions. The relative affinities followed the order syntaxin1A (EC50 = 0.33 μM), VAMP2 (0.94 μM), and SNAP25 (1.07 μM).

Gβγ Interacted with the Syntaxin1a H3 Motif and the C Terminus of SNAP25. Syntaxin1A has two major regions, the H3 SNARE domain and the modulatory Habc region (Fig. 2A). Truncation mutants representing the two major syntaxin1A regions were generated (Fig. 2A), and EC50 values for binding to 20 nM MIANS-labeled Gβγγ1γ were determined. The syntaxin1A H3 domain showed a right-shifted binding curve (EC50 = 0.89 μM; Fig. 2B) relative to the entire molecule (EC50 = 0.33 μM). Syntaxin1A (1–190) did not induce a significant Gβγ fluorescence change. These results, which were confirmed by GST pulldown assays (data not shown), indicate that Gβγ binds to the syntaxin1A H3 SNARE domain. However, because Gβγ bound to the H3 domain with an affinity lower than that for intact syntaxin1A, we cannot rule out a masked minor contribution of the Habc domain or the possibility that the isolated domain does not fold optimally (Jarvis et al., 2002).

Recent evidence suggests that nine amino acids from the C terminus of SNAP25 are crucial for interaction with Gβγγ1γ as well (Blackmer et al., 2005; Gerachshenko et al., 2005). Botulinum toxin A (BoNT/A) treatment, which removes nine C-terminal residues of SNAP25 (SNAP259) (Fig. 2C), diminishes Gβγγ1γ-mediated inhibition of exocytosis (Blackmer et al., 2005; Gerachshenko et al., 2005). In addition, a ternary SNARE complex comprising VAMP2, syntaxin1A, and SNAP259 shows reduced Gβγ binding compared with the wild-type SNARE complex (Gerachshenko et al., 2005). Here, we quantified the direct interaction of Gβγγ1γ with the C terminus of SNAP25. A truncation mutant of SNAP25 that mimics the BoNT/A cleavage product (SNAP259) was analyzed for its binding to Gβγ. Relative to full-length SNAP25, SNAP259 showed significantly decreased affinity for Gβγ (Fig. 2D).

In presynaptic terminals of neurons in situ and in cultured PC-12 and chromaffin cells, BoNT/E, which cleaves 26 amino acids from the C terminus of SNAP25 (Fig. 2C), inhibits Ca2+-dependent exocytosis (Ferrer-Montiel et al., 1998; Chen et al., 2001), consistent with an observed loss of interaction between synaptotagmin and SNAP25 (Chen et al., 1999; Gerona et al., 2000). We investigated whether a further trun-

![Fig. 2.](https://molpharm.aspetjournals.org/article/S0014429517300321/FIG2.jpg)
ated SNAP25 (SNAP25Δ26) also weakens binding to Gβγ. Fluorescent binding assays demonstrated that deletion of 26 amino acids caused a great loss of Gβγ binding (Fig. 2D). These data indicate that 26 C-terminal amino acids of SNAP25 are also critical for Gβγ binding.

Gβγ and Synaptotagmin Competed in Binary and Ternary SNARE Complexes in a Ca²⁺-Dependent Manner. It is noteworthy that the Gβγ-binding domains of the SNARE complex, the syntaxin1A C3 domain and the C terminus of SNAP25, are also known to constitute functionally important Ca²⁺-dependent synaptotagmin binding sites (Chapman et al., 1995; Gerona et al., 2000). The overlapping binding sites suggests that there might be competition between Gβγ and synaptotagmin on the complexed SNAREs. To test whether Gβγ competes with synaptotagmin for binding to SNARE proteins in complex, we conducted competition assays in vitro. We used a mutant of synaptotagmin I (K326A, K327A) that exhibits wild-type SNARE binding affinity but holds a reduced propensity to oligomerize and to form insoluble aggregates in response to high concentrations of Ca²⁺ (Bai et al., 2004). In fluorescent binding assays, synaptotagmin does not interact significantly with MIANS-Gβγ (data not shown). We therefore tested competition between Gβγ and synaptotagmin for binding to the binary t-SNARE complex, comprising syntaxin1A and SNAP25. The interaction between Gβγ and binary t-SNARE complex in the absence of synaptotagmin was normalized to 100%. In 50 μM Ca²⁺, the interaction between MIANS-Gβγ and 1 μM binary t-SNARE complex was almost completely inhibited by 250 nM synaptotagmin I (Fig. 3A). Increasing synaptotagmin concentrations competed more effectively with Gβγ, resulting in reduced fluorescence changes (Fig. 3B), further indicating that Gβγ and synaptotagmin compete for SNARE binding.

Synchronous vesicle fusion is initiated by Ca²⁺, but additional Ca²⁺ influx may modify large dense core vesicle fusion properties and possibly alter the quantal size of synaptic neurotransmitter release (Elhamdani et al., 2001, 2006; Harata et al., 2006). Because this modulation may reflect the Ca²⁺ sensitivity of synaptotagmin (Morimoto et al., 1995), we tested whether competition with Gβγ is also Ca²⁺ sensitive. We found that the competition between synaptotagmin and Gβγ for binary t-SNARE complex binding is dependent on intracellular Ca²⁺ concentrations. In buffer lacking Ca²⁺, synaptotagmin caused a slight but statistically significant inhibition of the ability of t-SNAREs to increase the fluorescence of MIANS-Gβγ (Fig. 3C), possibly because of a limited Ca²⁺-independent interaction between synaptotagmin and t-SNAREs (Bennett et al., 1992; Sollner et al., 1993). However, upon addition of physiologically relevant concentrations of Ca²⁺, we observed an increased inhibition of Gβγ binding by synaptotagmin, with maximum effectiveness at 50 μM Ca²⁺ (Fig. 3C).
Interactions of both Gβγ and synaptotagmin with the ternary SNARE complex modify the dilation of the vesicle fusion pore (Bai et al., 2004; Photowala et al., 2006). Thus, we tested the hypothesis that Gβγ competes with synaptotagmin for binding to the ternary SNARE complex. We first measured the Gβγ/SNARE interaction by determining the change in fluorescence of 20 nM MIANS-Gβγ upon addition of the ternary SNARE complex. Addition of 1 μM SNARE complex induced an increase of fluorescence intensity (Fig. 3D). This increase was reduced by inclusion of 250 nM synaptotagmin, indicating that Gβγ and synaptotagmin compete for binding to the ternary SNARE complex. In addition, competition by synaptotagmin and the resulting reduced fluorescence changes were dependent on the concentration of synaptotagmin (Fig. 3E) and the concentration of Ca2⁺ (Fig. 3F). Together, these data suggest that indeed Gβγ and synaptotagmin compete for binding to the ternary SNARE complex.

Gβγ and Synaptotagmin Competed for Binding to the Individual t-SNAREs (Syntaxin 1A and SNAP25) but Not the Vesicular SNARE (VAMP2). The H3 domain of syntaxin1A and the C terminus of SNAP25 provide interaction regions for Gβγ and synaptotagmin. We saw significantly more efficient competition between Gβγ and synaptotagmin in the context of binary t-SNAREs than ternary SNARE complexes. Therefore, we measured the individual contributions of SNARE components to competition between Gβγ and synaptotagmin.

First, to determine whether Gβγ and synaptotagmin compete for binding to syntaxin1A or SNAP25, the interaction of 20 nM MIANS-Gβγ with syntaxin1A and SNAP25 was monitored with or without preincubation of the SNARE proteins with 20 nM synaptotagmin. The interactions between Gβγ and SNAP25 proteins were unaffected by Ca2⁺ in the absence of synaptotagmin (data not shown). In the absence of Ca2⁺, we observed a slight, statistically insignificant decrease in Gβγ binding to both syntaxin1A (Fig. 4A) and SNAP25 (Fig. 4B) upon preincubation of the SNARE proteins with 20 nM synaptotagmin. However, in 100 μM Ca2⁺, the ability of synaptotagmin to compete for binding to SNAREs was greatly enhanced. Syntaxin1A binding to Gβγ was reduced by 48% (Fig. 4A), and binding of SNAP25 to Gβγ was reduced by 78% (Fig. 4B). Thus, Gβγ competes with Ca2⁺-synaptotagmin for binding to both syntaxin1A and SNAP25.

We observed a direct interaction between Gβγ and VAMP2 (Fig. 1, B and D). An earlier study suggested that synaptotagmin interacts with VAMP2 (Fukuda, 2002), leading to the possibility that this protein forms an additional site of competition between Gβγ and synaptotagmin. However, the existence of a direct synaptotagmin/VAMP2 interaction remains controversial (Chapman et al., 1995). Consequently, we monitored the Gβγ interaction with VAMP2 with or without preincubation of VAMP2 with synaptotagmin in the presence or absence of Ca2⁺. No alteration in Gβγ binding to VAMP2 by synaptotagmin was observed, suggesting that Gβγ and synaptotagmin do not share a binding region on VAMP2 (Fig. 4C). This result may explain why competition at the ternary SNARE complex seems to favor Gβγ binding compared with competition at the binary t-SNARE, in the ternary SNARE complex, Gβγ binding is enhanced by an additional binding site within VAMP2 that is unaffected by synaptotagmin I. Thus, its ability to compete with Gβγ in the ternary SNARE complex is decreased.

The C Terminus of SNAP25 in the Ternary SNARE Complex Was Important for Gβγ-Synaptotagmin Competition. We have shown that BoNT/A, which specifically removes the C-terminal nine amino acids from SNAP25, greatly reduced the inhibitory effect of Gβγ on regulated exocytosis (Blackmer et al., 2001; Gerachshenko et al., 2005). We propose that this reduction can be explained if removal of these residues diminishes Gβγ competition with synaptotagmin for binding to the t-SNAREs and the ternary SNARE complex in vivo. Therefore, we examined the contribution of the C terminus of SNAP25 in the competition between Gβγ and synaptotagmin within the ternary SNARE complex. It is noteworthy that whereas BoNT/A cleavage of SNAP25 reduced binding of synaptotagmin and Gβγ individually, significant interactions were still observed (Fig. 2D). Thus, it was important to quantify the interactions using the fluores-

![Fig. 4. Gβγ and synaptotagmin compete for binding to SNAP25 and syntaxin1A but not to VAMP2. A, Gβγ competes with synaptotagmin for binding to syntaxin1A. Competition assays were performed as described for Fig. 3. The relative change in fluorescence of 20 nM MIANS-Gβγ in 100 μM Ca²⁺ upon addition of 0.5 μM syntaxin1A was normalized to 100%. Addition of 20 nM synaptotagmin in the absence of Ca²⁺ induced a slight decrease in the Gβγ-syntaxin1A interaction, but inclusion of both 20 nM synaptotagmin and 100 μM Ca²⁺ reduced binding by approximately 50%. B, Gβγ competes with synaptotagmin for binding to SNAP25. Synaptotagmin (20 nM) caused a slight decrease in the interaction of 20 nM MIANS-Gβγ with 2 μM SNAP25 in the absence of Ca²⁺, whereas inclusion of both 20 nM synaptotagmin and 100 μM Ca²⁺ reduced binding by approximately 80%. C, Gβγ and synaptotagmin do not compete for binding to VAMP2. The change in MIANS-Gβγ fluorescence upon addition of 2 μM VAMP2, normalized to 100%, was not reduced significantly in the presence of 20 nM synaptotagmin, with or without 100 μM Ca²⁺. Data are presented as mean ± S.E.M. (n = 3).](https://molpharm.aspetjournals.org/doi/abs/10.1124/mol.106.092585)
cent binding assay to determine whether this cleavage shifted the Gβγ/synaptotagmin competition toward synaptotagmin binding.

To test the relative contributions of the C terminus of SNAP25 in binding Gβγ and synaptotagmin, we quantified binding to ternary SNARE complexes comprising syntaxin1A, VAMP2, and SNAP25Δ9. Competition by synaptotagmin for Gβγ binding to SNARE complex that contains SNAP25Δ9 was increased compared with the wild-type SNARE complex at 50 μM Ca2+ (Fig. 5A). This finding is consistent with a model in which the nine C-terminal amino acids of SNAP25 represent an important determinant mediating exclusion of synaptotagmin binding by Gβγ.

We then determined the concentration dependence of the synaptotagmin competition. The strength of synaptotagmin competition in SNARE complexes formed with SNAP25Δ9 increased dramatically compared with the wild type SNARE complex (Fig. 5B). These results suggest that the C-terminal nine amino acids of SNAP25 are more critical for Gβγ than for synaptotagmin binding. These data support the dramatic loss of inhibitory effect of Gβγ in the BoNTA-treated synapse (Gerachshenko et al., 2005).

The 5-HT Mediated Regulation of Exocytosis Was Diminished with High [Ca2+] at the Lamprey Giant Synapse. What role does Gβγ-synaptotagmin I competition for SNARE complexes play at synapses? At lamprey giant synapses, we have shown that 5-HT inhibits transmitter release through direct Gβγ/SNARE complex interactions. 5-HT mediated Gβγ does not inhibit voltage-gated Ca2+ channels in this preparation but acts directly on the ternary SNARE complex to inhibit exocytosis (Blackmer et al., 2001; Takahashi et al., 2001; Gerachshenko et al., 2005). From our in vitro study, we hypothesized that Gβγ-mediated inhibition requires competition between Gβγ and Ca2+-dependent synaptotagmin binding on the SNARE complex. These findings suggested that the effect of GPCR-mediated activation of

Fig. 5. The C-terminal nine amino acids of SNAP25 comprise a critical region for competition between Gβγ and synaptotagmin. Gβγ competition with synaptotagmin to bind ternary SNARE complex was made up of SNAP25 (black bar) or SNAP25Δ9 (gray bar). Gβγ and SNARE complex binding was normalized to 100% fluorescence in the presence of 1 mM Ca2+ and in the absence of synaptotagmin. Competition by synaptotagmin for Gβγ binding to SNARE containing SNAP25Δ9 was increased compared with the wild-type SNARE complex. A, competition for binding to SNAP25Δ9 is more sensitive to [Ca2+] than SNAP25Δ9. B, synaptotagmin I competes in a dose-dependent manner with Gβγ for binding to SNARE, more potently with SNAP25Δ9. Data are represented as mean ± S.E.M. (n = 3).

Fig. 6. Raising extracellular [Ca2+] increases intracellular [Ca2+] transients in a presynaptic terminal. A, lamprey giant axons labeled retrogradely with a Ca2+-sensitive dye (Fluo-4 dextran high affinity) and imaged with confocal microscopy. To quantify rapid action potential-induced Ca2+ transients, high-speed line scanning of Ca2+ entry (500 Hz) was repeated at the edge of the axon marked with the arrowheads. B–F, raising extracellular [Ca2+] enhances action-potential-induced Ca2+ influx. B, a single action potential in the axon evoked at the time point marked by the arrowhead caused a localized Ca2+ transient in the axon. The line scan is pseudocolored with the lookup table shown below A. The pixel values were measured and, after background substraction, images were normalized to the baseline level of fluorescence [ΔF(F + 1)]. C, increasing the extracellular Ca2+ concentration from 2.6 to 50 mM markedly enhanced the amplitude of the Ca2+ transient. D, during wash-in of 50 mM Ca2+, the resting fluorescence was monitored at 30-s intervals. A small increase in the resting fluorescence was observed. Fluorescence is recorded to the same scale as E. E, the Ca2+ transients were measured between the white horizontal lines in B and C to quantify Ca2+ entry. Estimates of recorded Ca2+ transient amplitudes were made as described under Materials and Methods. The calculated Ca2+ concentration scale for this estimate is shown to the right. F, the relative effect of raising extracellular Ca2+ concentration on the amplitude of the axonal Ca2+ transient is recorded. Note that high extracellular Ca2+ concentrations have a proportionately smaller effect on the amplitudes of the recorded intracellular transient.
Gβγ would be mitigated by raising the synaptic terminal Ca$^{2+}$ concentration.

We tested this possibility using paired recordings between giant axons and postsynaptic neurons in lamprey synapse. To increase action potential-evoked intracellular Ca$^{2+}$ concentrations, we raised extracellular Ca$^{2+}$ concentrations (Fig. 6, A–C). We first quantified the consequent effect on evoked presynaptic Ca$^{2+}$ entry. Axons were filled with a Ca$^{2+}$-sensitive dye (Fluo-4 dextran high affinity) and imaged with confocal microscopy. Action potential-evoked presynaptic fluorescence transients were imaged under increasing extracellular Ca$^{2+}$ (2.6, 10, and 50 mM; Fig. 6, B and C, and data not shown). The relationship between extracellular Ca$^{2+}$ concentrations and the fluorescence transient was alinear (Fig. 6E). Therefore, to calculate the relationship between extracellular Ca$^{2+}$ and the intracellular Ca$^{2+}$ transient amplitude, we determined the peak intracellular Ca$^{2+}$ during the transients. The affinity of the dye (1.2 µM) was obtained from fluorescence curves that showed a 120-fold increase in fluorescence between 0 µM Ca$^{2+}$ and saturation (41 µM) at a physiological temperature. Using these data and the resting [Ca$^{2+}$] in lamprey axons (approximately 100 nM), we estimated the peak transient intracellular Ca$^{2+}$ (Fig. 6E). Extracellular Ca$^{2+}$ (50 mM) slightly increased basal presynaptic intracellular Ca$^{2+}$ (Fig. 6D). However, the evoked fluorescence transient, and therefore its peak Ca$^{2+}$ concentration, was markedly increased (Fig. 6E). Note that absolute Ca$^{2+}$ concentrations calculated in this way underestimate the concentration at the vesicle fusion site because we cannot image with sufficient resolution to resolve the close spatial relationship between Ca$^{2+}$ channels and the release machinery (Adler et al., 1991). Consequently, we used these data to calculate the relative amplitude of the Ca$^{2+}$ transient at extracellular concentrations of 10 and 50 mM (Fig. 6F) normalized to control (2.6 mM).

We then tested the effect of increased Ca$^{2+}$ on 5-HT-mediated presynaptic inhibition. At a control extracellular Ca$^{2+}$ concentration (2.6 mM), 5-HT (600 nM) reduced the EPSC to 55.9 ± 7.3% of control (n = 5; P < 0.05). The EPSC fully recovered upon 5-HT removal (Fig. 7A, top). Extracellular Ca$^{2+}$ (50 mM) increased the EPSC amplitude to 129.7 ± 4.7% of control (n = 5; P < 0.01), but 600 nM 5-HT caused no inhibition (Fig. 7A, bottom; EPSC was 97.1 ± 8.1% of the EPSC modified by 50 mM Ca$^{2+}$; n = 5 pairs). At 50 mM extracellular Ca$^{2+}$, even a saturating dose of 5-HT (10 µM) resulted in only a slight reduction in the EPSC response was 71.3 ± 0.9% of the control response modified by high Ca$^{2+}$ (n = 3, P > 0.05), compared with a reduction to 20% of control in normal Ca$^{2+}$ (Takahashi et al., 2001) (Fig. 7, B and C). We also confirmed that 5-HT does not change Ca$^{2+}$ influx in lamprey synapses (Blackmer et al., 2001; Takahashi et al., 2001) treated with 50 mM extracellular Ca$^{2+}$ concentration (Supplementary Figure S2). Thus, increased intracellular Ca$^{2+}$ transients attenuated GPCR mediated presynaptic inhibition.

High Extracellular Ca$^{2+}$ Attenuated the Presynaptic Inhibitory Effect of Gβγ. We have shown that the reduction of the EPSC upon activation of 5-HT receptors is caused by liberated Gβγ (Blackmer et al., 2001; Gerachshenko et al., 2005). Here, we examined the effects of manipulating intracellular Ca$^{2+}$ on EPSC inhibition by free Gβγ introduced through direct injection of purified Gβγ protein into presynaptic axons. We compared Gβγ-mediated inhibition of exocytosis at a control (2.6 mM) and a raised Ca$^{2+}$ concentration (10 mM); the latter increased the EPSC amplitude (Fig. 8A) to 122 ± 2% of control (n = 3). In four more paired recordings, we probed the effect of raising Ca$^{2+}$ concentrations on inhibition by presynaptic Gβγ. In control Ca$^{2+}$ (2.6 mM), Gβγ injection significantly reduced the EPSC, as described previously (Blackmer et al., 2001), to 37 ± 12% (Fig. 8, B and C). In the same pairs, the Ca$^{2+}$ concentration was raised to 10 mM and Gβγ inhibition was reversed (10 mM; Fig. 8, B and C; EPSC was 100 ± 9% of control). Thus raised Ca$^{2+}$ transient amplitudes significantly attenuated the inhibitory effect of presynaptic Gβγ. The effects of both 5-HT and presynaptic Gβγ injection are attenuated by enhancing the amplitude of the action potential evoked presynaptic Ca$^{2+}$ transient.

![Figure 7](image-url)
Discussion

In primed vesicles, Ca\(^{2+}\) effects exocytosis in part by increasing synaptotagmin’s affinity for the primed SNARE complex (Perin et al., 1990; Schiavo et al., 1997; Yoshihara and Littleton, 2002; Bai et al., 2004; Wang et al., 2006). G\(\beta\gamma\) liberated by inhibitory G\(_{i/o}\)-coupled GPCRs inhibits neurotransmitter release by at least two mechanisms: inhibition of presynaptic Ca\(^{2+}\) entry at many synapses by modulating voltage-gated calcium channels (Herlitze et al., 1996; Ikeda, 1996), as well as interaction with the ternary SNARE complex late in membrane fusion (Herlitze et al., 1996; Ikeda, 1996), as well as interaction with the ternary SNARE complex late in membrane fusion (Blackmer et al., 1996; Ikeda, 1996), as well as interaction with the ternary SNARE complex late in membrane fusion (Blackmer et al., 2005; Gerachshenko et al., 2005). Here we show that G\(\beta\gamma\) interacts with SNARE proteins and that this binding is not directly modified by calcium. G\(\beta\gamma\) interacts with VAMP2 at a site different from synaptotagmin’s interaction. Perhaps the G\(\beta\gamma/VAMP2\) interaction strengthens G\(\beta\gamma\) binding to the SNARE complex, whereas occlusion of synaptotagmin I binding occurs through the t-SNARE components (syntaxin1A and SNAP25) in the ternary SNARE complex. In addition, G\(\beta\gamma\) binds directly to the t-SNAREs SNAP25 and syntaxin1A at sites similar to those used by synaptotagmin to interact with these proteins in a Ca\(^{2+}\)-dependent manner. This sets up a potent competition with the Ca\(^{2+}\) sensor to inhibit regulated exocytosis. This differential Ca\(^{2+}\) requirement can lead to a Ca\(^{2+}\)-sensitivity of the G\(\beta\gamma\) inhibition of SNARE/synaptotagmin complex formation: at low and moderate Ca\(^{2+}\) concentrations, the presence of inhibitory signals generates G\(\beta\gamma\) that competes with synaptotagmin for binding to SNARE, leading to inhibition of exocytosis. At high Ca\(^{2+}\) concentrations, the affinity of synaptotagmin for SNARE increases, whereas the G\(\beta\gamma/SNARE\) affinity remains steady, and thus high synaptic activity overrides the inhibition by G\(\beta\gamma\).

At the synapse, after C-terminal truncation of SNAP-25 with BoNT/A, exocytosis recovers in high Ca\(^{2+}\) in many preparations, including the lamprey (Gerachshenko et al., 2005), presumably because high Ca\(^{2+}\) enhances the residual synaptotagmin/SNARE interaction that remains after BoNT/A treatment. However, the affinity of G\(\beta\gamma\) for the modified SNARE complex is much lower, and G\(\beta\gamma\) therefore competes less well with synaptotagmin for the same site, particularly at raised intracellular Ca\(^{2+}\). These findings led to a testable hypothesis: at the synapse, larger evoked Ca\(^{2+}\) transients should prevent G\(\beta\gamma\)-mediated presynaptic inhibition induced by 5-HT. Accordingly, in the lamprey giant synapse, although 5-HT powerfully inhibits exocytosis through G\(\beta\gamma\) interaction with the SNARE complex (Silinsky, 1984; Man-Son-Hing et al., 1989; Blackmer et al., 2001, 2005; Takahashi et al., 2001; Chen et al., 2005; Gerachshenko et al., 2005), we observed that enhancing the Ca\(^{2+}\) transient reversed this inhibition (Figs. 6 and 7). These data are consistent with the biochemical results showing that inhibition of synaptotagmin/SNARE complex interaction by G\(\beta\gamma\) is sensitive to Ca\(^{2+}\). Although Ca\(^{2+}\) could have other effects in the synapse, the fact that synaptotagmin is thought to be the major Ca\(^{2+}\) receptor for exocytosis (Bai et al., 2004; Maximov and Sudhof, 2005; Wang et al., 2006) suggests that G\(\beta\gamma\) may work physiologically by competing with Ca\(^{2+}\)-synaptotagmin for SNARE, leading to inhibition of exocytosis. G\(\beta\gamma\) also has many other targets, and enhancing intracellular Ca\(^{2+}\) could modify the effects of these interactions on exocytosis. The most obvious other G\(\beta\gamma\) effector is the voltage-gated Ca\(^{2+}\) channel, which has been shown by many investigators to be inhibited by G\(\beta\gamma\). However, G\(\beta\gamma\) has no effect on Ca\(^{2+}\) entry even at 50 mM extracellular Ca\(^{2+}\), because 5-HT does not modify evoked Ca\(^{2+}\) entry (Supplementary Figure S2).

This Ca\(^{2+}\)-dependent regulation may complete a physiological feedback loop, because G\(\beta\gamma\) may control both Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (Herlitze et al., 1996; Ikeda, 1996) and access of the Ca\(^{2+}\) effector synaptotagmin to its site on the SNARE. Our data suggest that this competition is elegantly controlled by overlap of the binding sites of G\(\beta\gamma\) and synaptotagmin at the SNARE proteins SNAP25 and syntaxin. In a further level of regulation, during repetitive stimulation, buildup of intracellular Ca\(^{2+}\) concentration may reverse G\(\beta\gamma\) inhibition. Thus, depending on presynaptic G\(_{i/o}\)-coupled GPCR activation and Ca\(^{2+}\) influx in...
response to synaptic activity, a range of efficiencies of exocytosis potentially exists. Thus, we predict that a complex and dynamic regulation of vesicle fusion properties will occur. Finally, at low levels of inhibitory GPCR signaling, Gβγ causes kiss-and-run exocytosis in lamprey synapses (Photowala et al., 2006) and adrenal chromaffin cells (data not shown) (Chen et al., 2005). In hippocampal neurons, sustained stimulus trains forcing high presynaptic Ca2+ concentrations change fusion from a state dominated by kiss-and-run to one favoring full fusion (Harata et al., 2006). However, the exact Ca2+ concentrations required to reverse Gβγ-mediated presynaptic inhibition are likely to exceed those obtained by residual Ca2+ during short bursts of stimuli, in which saturating doses of 5-HT remains effective (Takahashi et al., 2001). Thus, we speculate that the dynamic relationship between inhibitory GPCRs and presynaptic Ca2+ constantly modifies the nature of the vesicle fusion event itself.

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References

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Supplemental Figure Legend

Figure S1. Gβγ but not Gα or heterotrimeric G protein interacts with the SNARE proteins.

(A) 5 µg of GST-SNARE (syntaxin1A, SNAP25 or VAMP2) was incubated with noted concentration of Gβ1γ2, Gαt and heterotrimeric G protein (Gt). Precipitated G proteins were detected with coomassie staining. Gβγ interacts with syntaxin, SNAP25 and VAMP2 and shows different affinity. Gβγ and syntaxin have the highest-affinity interaction (note that VAMP2 has background band at the same molecular weight as Gβ). However, Gαt and heterotrimeric G proteins were not pulled down. (B) 5 µg of GST-SNAREs interacted with 14 µg of Ga and 28 µg of heterotrimeric G protein and these proteins were not precipitated at these concentrations.

Figure S2. 5-HT dose not change presynaptic Ca2+ entry.

*Lamprey giant* axons labeled retrogradely with a Ca2+-sensitive dye (Fluo-4 dextran high affinity) and imaged with confocal microscopy. To quantify rapid action potential-induced Ca2+ transients, high-speed line scanning of Ca2+ entry (500 Hz) was repeated at the edge of the axon. Line scan images of calcium transients by a given action potential were measured before (black trace) and after 5-HT (3 µM) application (grey trace) in
50mM extracellular calcium. The mean change in calcium transient amplitude was to 98±2% of control on addition of 5-HT (n=5).
Figure S1.

A

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G protein (µg)

GST-syntxin

Gα

Gβ

GST-SNAP25

Gβ

GST-VAMP

Gβ

B

GST-syntaxin

Gα

Gαβγ

GST-SNAP25

Gα

Gαβγ

GST-VAMP

Gα

Gβ
Figure S2.