Phosphomimetic Mutation of Ser-187 of SNAP-25 Increases both Syntaxin Binding and Highly Ca\textsuperscript{2+}-sensitive Exocytosis

Yan Yang,¹ Tim J. Craig,⁴ Xiaohui Chen,¹,² Leonora F. Ciufo,⁴ Masami Takahashi,⁵ Alan Morgan,⁴ and Kevin D. Gillis¹,²,³

¹Dalton Cardiovascular Research Center, ²Department of Biological Engineering, ³Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO 65211
⁴The Physiological Laboratory, School of Biomedical Sciences, University of Liverpool, UK
⁵Department of Biochemistry, Kitasato University School of Medicine, Kanagawa, Japan

The phosphorylation targets that mediate the enhancement of exocytosis by PKC are unknown. PKC phosphorylates the SNARE protein SNAP-25 at Ser-187. We expressed mutants of SNAP-25 using the Semliki Forest Virus system in bovine adrenal chromaffin cells and then directly measured the Ca\textsuperscript{2+} dependence of exocytosis using photorelease of caged Ca\textsuperscript{2+} together with patch-clamp capacitance measurements. A flash of UV light used to elevate [Ca\textsuperscript{2+}], to several μM and release the highly Ca\textsuperscript{2+}-sensitive pool (HCS) of vesicles was followed by a train of depolarizing pulses to elicit exocytosis from the less Ca\textsuperscript{2+}-sensitive readily releasable pool (RRP) of vesicles. Carbon fiber amperometry confirmed that the amount and kinetics of catecholamine release from individual granules were similar for the two phases of exocytosis. Mimicking PKC phosphorylation with expression of the S187E SNAP-25 mutant resulted in an approximately threefold increase in the HCS, whereas the response to depolarization increased only 1.5-fold. The phosphomimetic S187D mutation resulted in an ~1.5-fold increase in the HCS but a 30% smaller response to depolarization. In vitro binding assays with recombinant SNARE proteins were performed to examine shifts in protein–protein binding that may promote the highly Ca\textsuperscript{2+}-sensitive state. The S187E mutant exhibited increased binding to syntaxin but decreased Ca\textsuperscript{2+}-independent binding to synaptotagmin I. Mimicking phosphorylation of the putative PKA phosphorylation site of SNAP-25 with the T138E mutation decreased binding to both syntaxin and synaptotagmin I in vitro. Expressing the T138E/S187E double mutant in chromaffin cells demonstrated that enhancing the size of the HCS correlates with an increase in SNAP-25 binding to syntaxin in vitro, but not with Ca\textsuperscript{2+}-independent binding of SNAP-25 to synaptotagmin I. Our results support the hypothesis that exocytosis triggered by lower Ca\textsuperscript{2+} concentrations (from the HCS) occurs by different molecular mechanisms than exocytosis triggered by higher Ca\textsuperscript{2+} levels.

INTRODUCTION

Activation of PKC potently enhances Ca\textsuperscript{2+}-triggered exocytosis from neurons and excitable endocrine cells (e.g., Gillis et al., 1996; Smith et al., 1998; Stevens and Sullivan, 1998; Wu and Wu, 2001; Zhu et al., 2002; Wan et al., 2004; Yang and Gillis, 2004), but the identity of the phosphorylation targets that mediate these effects are unknown. The SNARE proteins syntaxin, SNAP-25, and synaptobrevin/VAMP are targets of clostridial neurotoxins and play an essential role in exocytosis (Sollner et al., 1993; Pellizzari et al., 1999; for review see Jahn and Sudhof, 1999). Phosphorylation of cellular SNAP-25 has been shown to occur in response to phorbol ester application and physiological stimuli in various cell types, including PC12 cells (Shimazaki et al., 1996; Kataoka et al., 2000), hippocampal organotypic cultures (Genoud et al., 1999), pancreatic β cells (Gonelle-Gispert et al., 2002), and adrenal chromaffin cells (Nagy et al., 2002). Early studies using botulinum neurotoxin cleavage suggested the likely PKC phosphorylation site as Ser-187 (Shimazaki et al., 1996), and this was subsequently confirmed using a phospho-Ser-187–specific antibody (Kataoka et al., 2000). One report claims that PKC can also phosphorylate SNAP-25 on Thr-138 (Hepp et al., 2002), which has been characterized as an in vivo and in vitro PKA phosphorylation site (Risinger and Bennett, 1999; Nagy et al., 2004).

A role of Ser-187 phosphorylation in regulating exocytosis has been questioned because Ser-187 phosphorylation does not always correlate with effects of PKC-activating agents that modulate exocytosis (Iwasaki et al., 2000; Gonelle-Gispert et al., 2002), and Ser-187 mutations do not produce large effects on exocytosis (Gonelle-Gispert et al., 2002; Nagy et al., 2002; Finley et al., 2003). A powerful approach to answer this question is the use of photorelease of caged Ca\textsuperscript{2+} together with patch-clamp capacitance measurements to directly study the modulation of Ca\textsuperscript{2+}-triggered exocytosis. A study of

Abbreviations used in this paper: GST, glutathione S-transferase; HCS, highly Ca\textsuperscript{2+}-sensitive pool; RRP, readily releasable pool; SFV, Semliki Forest virus; SRP, slowly releasable pool.
this type by Nagy et al. (2002) using chromaffin cells reveals that expressing the putative phosphomimetic Ser-187-Glu/Asp SNAP-25 mutants increases the phase of exocytosis that occurs seconds after photoelevation of [Ca^{2+}], to \( \approx 20 \mu M \). They interpreted this result as indicating that the refilling rate of the readily releasable pool (RRP) of granules is enhanced by phosphomimetic Ser-187 mutations. In contrast, unphosphorylatable Ser-187-Ala/Cys mutants inhibit the rate of refilling (Nagy et al., 2002). This suggests that one effect of PKC activation, namely the increased rate of releasable pool refilling, occurs via phosphorylation of Ser-187 of SNAP-25. On the other hand, expression studies with Thr-138 mutants suggest that tonic phosphorylation of SNAP-25 on Thr-138 by PKA regulates the size of the RRP and the slowly releasable pool (SRP) in chromaffin cells, with little effect on the rate of refilling (Nagy et al., 2004).

Most studies using photorelease of caged Ca^{2+} have concentrated on [Ca^{2+}], levels greater than \( \approx 10 \mu M \). Recently, a previously overlooked small burst of exocytosis has been observed at [Ca^{2+}], levels of several \( \mu M \) in chromaffin cells (Yang et al., 2002) and pancreatic β cells (Wan et al., 2004; Yang and Gillis, 2004). Exocytosis with similar kinetics and Ca^{2+} sensitivity has also been observed in photoreceptors (Thoreson et al., 2004) and gonadotropes (Zhu et al., 2002). This kinetic phase of exocytosis has been interpreted as release from a highly Ca^{2+}-sensitive pool (HCSP) of vesicles that coexists with the RRP and SRP in chromaffin cells and may play an important role in regulating exocytosis that occurs asynchronously with action potentials. The HCSP is even more sensitive to activation of PKC than the RRP and SRP because the HCSP is enhanced in size approximately sixfold by phorbol esters, an effect that can be blocked with a selective PKC inhibitor (Yang et al., 2002). Here we report that phosphomimetic mutations of Ser-187 preferentially increase the size of the HCSP.

The molecular mechanisms whereby phosphorylation of Ser-187 or Thr-138 affect exocytosis are unclear. Indeed, the only published data on this issue is that treatment of PC12 cell extracts with purified PKC and ATPγS reduces the amount of syntaxin communoprecipitating with SNAP-25 (Shimazaki et al., 1996). This observation suggests that phosphorylation of Ser-187 reduces the affinity of SNAP-25 for syntaxin, although such a mechanism is not easy to reconcile with the stimulatory effects of phorbol esters on exocytosis. Furthermore, stoichiometric phosphorylation of Thr-138 has no obvious effect on the binding of recombinant SNAP-25 to SNAREs or synaptotagmin (Risinger and Bennett, 1999). Here we present evidence that the increase in size of the HCSP by phosphomimetic mutations of Ser-187 of SNAP-25 is correlated with an increase in binding of recombinant SNAP-25 with syntaxin measured in vitro, but not with Ca^{2+}-independent binding of SNAP-25 to synaptotagmin I.

Materials and Methods

Preparation of Bovine Chromaffin Cells

Cell preparation was modified from previously published protocols (Ashery et al., 1999; Smith, 1999) and is therefore described in some detail below. Bovine adrenal glands from a local slaughterhouse were kept in a Ca^{2+}- and Mg^{2+}-free isolation buffer and transported to the laboratory at room temperature. Fat was trimmed from the glands using scissors under a sterile hood. Blood was washed out of the glands by gently injecting isolation buffer into the portal vein and then allowing the solution to run out while gently massaging the glands. This was repeated several times until the liquid running out was clear. Collagenase type P (Roche Diagnostics) was dissolved in isolation buffer at a concentration of 1 mg/ml, injected into the portal vein, and then the glands were placed in a beaker in a 37°C shaking water bath for 20–25 min. The glands were opened by cutting around the circumference of the cortex with scissors, and the medulla was peeled away from the cortex and placed into a culture dish containing isolation buffer. The medullae were then minced to small pieces with a scalpel. The medulla-containing suspension was filtered through a nylon mesh (70 μm; Fisher Scientific). The filtered liquid was collected in a 50-ml tube and centrifuged at 1,000 rpm for 10 min at room temperature. The supernatant was aspirated and discarded, and then the pellet was resuspended in isolation buffer. In another tube, a 10-fold concentrated isolation buffer solution was mixed with percoll at a ratio of 1:9 to make a solution with physiological tonicity and a pH of 7.2. The percoll solution was combined with the cell solution at a 1:1 ratio and this mixture was centrifuged at 15,000 rpm for 30 min at 15°C. This resulting solution contains several distinct layers: cell debris in the top layer, chromaffin cells in the middle layer, and red blood cells in the bottom layer. The middle layer was gently transferred to a centrifuge tube using a pipette. Isolation buffer was added again to fill the tube and the suspension was centrifuged at 1,000 rpm for 5 min. The supernatant was aspirated and discarded and the pellet resuspended in isolation buffer. Chromaffin cell culture media was added at a 1:3 ratio to the cell suspension to begin the adaptation of cells to the Ca^{2+}-containing media. The suspension was centrifuged again at 1,000 rpm for 5 min and the supernatant was discarded. After resuspending the pellet in chromaffin cell media, the cells were counted using a hemacytometer. The solution was then diluted with media to give a density of 6 \( \times 10^3 \) cells/ml. The cell suspension was then plated into six-well culture plates, containing 25-mm diameter round glass coverslips that had been previously coated with 0.0025% poly-l-lysine. Cells were plated at a density of 6 \( \times 10^3 \) cells per coverslip for experiments using virus infection and at a density of 10^4 cells per coverslip for patch-clamp experiments. The cells were housed in a humidified incubator at 37°C with 5% CO₂ and used 1–3 d after preparation.

Generation of Semliki Forest Virus (SFV)

We used both a DNA-based Semliki Forest virus (SFV) vector (DiGioioso and Bremner, 1998) and an RNA-based SFV vector (Invitrogen) (Ashery et al., 1999; Duncan et al., 1999) to produce SFV. Both methods expressed a construct with GFP fused to the nucleus of SNAP-25A. An oligonucleotide cassette was introduced into the Xmal site of the viral vector of either pSFV1 or pSCA1 as described previously (Wei et al., 2000) to generate singular restriction sites. Site-directed mutations of SNAP-25 were generated using the Quickchange mutagenesis kit (Stratagen). The sequence of all constructs was verified by DNA sequencing. For the DNA-based method, the pSCA1 and pSCAHelper vectors were gifts from R. Bremner (University of Toronto, Toronto, Canada) (DiGioioso and Bremner, 1998). We inserted GFP-SNAP-25 into the restriction site of pSCA1 to create the pSCA1-GFP-SNAP-25 plasmid.
HEK 295 cells were transfected with both pSCAI-GFP-SNAP-25 and pSCAHelper to produce virus as follows. 2.5 μg of each DNA plasmid, 150 μl serum-free IMDM medium, and 30 μl Superfect transfection reagent (Invitrogen) were combined. After allowing 10 min for complexes to form, 1 ml of regular IMDM media was added. The media from the HEK cells (grown to 80% confluence) was removed and the cells were washed gently with warm PBS. Transfection solution was then added and cells were incubated at 37°C for 2–3 h. The transfection solution was then removed and fresh HEK complete media supplemented with 0.1 mM sodium butyrate was added to the cells. After 5–6 h incubation, the SFV-containing media was collected and either used immediately or stored at −70°C. The RNA-based method to produce SFV followed the protocol described by Ashery et al. (1999). We obtained pSFV1 constructs for SNAP-25 mutants S187E, S187C, and SNAP-25 wt as a gift from J. Sorensen and E. Neher (MPI for Biophysical Chemistry, Goettingen, Germany). We found no apparent difference in the response of chromaffin cells infected with virus prepared according to the RNA or DNA methods, therefore we combined the data.

Infection of Chromaffin Cells
Chromaffin cells were infected the day after isolation. Thawed SFV-containing media was diluted 1:1 with Dulbecco’s modified Eagle’s medium (DMEM) without FBS. α-Chymotrypsin (0.2 mg/ml in HBS solution) was added and the solution was allowed to incubate for 50 min at room temperature to activate the virus. Then aprotinin (0.6 mg/ml in HBS solution) was added and allowed to incubate for an additional 5 min to inactivate α-chymotrypsin. Media was removed from the chromaffin cells and replaced with the activated SFV solution. After allowing 12 h for infection, the virus-containing media was replaced with fresh chromaffin cell media. Cells were used for experiments 1–2 d after infection. Cells expressing GFP-SNAP-25 were identified by fluorescence microscopy using 480-nm excitation and used for patch-clamp experiments.

Solutions and Reagents
Chromaffin cell isolation buffer consisted of (in mM): 145 NaCl, 2.8 KCl, 0.85 NaH2PO4, 2.15 Na2HPO4, 10 glucose, 10 HEPES-NaOH, pH 7.2. The extracellular solution for patch-clamp experiments consisted of 145 NaCl, 2.8 KCl, 1 MgCl2, 10 CaCl2, 10 glucose, 10 HEPES-NaOH, pH 7.2. For Ca2+ calibration experiments, the bath solution contained 1 mM CaCl2. The base pipette solution contained 120 N-methylglucamine, 120 t-glutamic acid, 8 NaCl, and 40 HEPES titrated to pH 7.2 with N-methylglucamine. In caged Ca2+ experiments, 0.5–1 mM NP-EGTA (Ellis-Davies and Kaplan, 1994), 80% loaded with CaCl2, together with 2 mM Na2-ATP, and 1 mM MgCl2 were added to the pipette solution. To measure [Ca2+]i over a wide dynamic range, a mixture of the high-affinity indicator bisfura-2 (0.1 mM, K+ salt, Molecular Probes) and the lower-affinity indicator fura-2ff (0.1 mM, K+ salt, Teflabs) were added to the pipette solution (Voets, 2000). Basal ([Ca2+]i) was set to ~600 nM.

Chromaffin cell culture media consisted of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 4,500 mg/liter glucose, enriched with 10% (vol/vol) FBS (Invitrogen) and 1% penicillin/streptomycin. HBS solution for dissolving α-chymotrypsin and aprotinin consisted of (in mM) 140 NaCl, 5 KCl, 10 MgCl2, 20 CaCl2, 10 HEPES-NaOH, pH 7.2. All chemicals were from Sigma-Aldrich unless otherwise indicated.

Electrophysiology and Photometry
Patch electrodes were pulled from Kimaq glass capillaries, coated with dental wax, and fire polished. The pipette resistance ranged from 2 to 4 MΩ when filled with the intracellular solutions. All electrophysiological experiments were conducted using the standard whole-cell configuration. The series resistance ranged from 5 to 10 MΩ and the membrane conductance was 600 pS or lower. The pipette holding potential was −70 mV in all experiments. Capacitance measurements were performed using the “sine + dc” software lock-in amplifier method implemented in PULSE software (Pusch and Neher, 1988; Gillis, 2000). The assumed reversal potential was 0 mV and the sinusoid had an amplitude of 25 mV and a frequency of 1.3 kHz.

The capacitance increase immediately following flash photolysis of caged Ca2+ was fitted by an exponential, or by an exponential plus a line, and the amplitude of the exponential fit was interpreted as the size of the HCSP. The capacitance increase following the initial exponential burst is believed to reflect release from the RRP. Release from the RRP was accelerated 300 ms after the flash by applying 10 depolarizing pulses, 30 ms in duration to +20 mV, to elicit Ca2+ influx. The total increase in capacitance between the initial exponential burst and the end of the 10th depolarization was defined as ∆Cm, an index of the size of the RRP (see Fig. 2 A).

Values indicated are mean ± SEM. Statistical comparisons were performed using Student’s paired t test.

A monochromator (Polychrome 4, TILL Photonics) coupled to the epifluorescence port of an Olympus IX-50 microscope with a fiber-optic cable excited the Ca2+ indicators at 340 and 365 nm. A two-port condenser (TILL Photonics) combined the monochromator excitation path with that of a flash lamp (TILL Photonics). A 4× 1.15 NA water immersion lens (U-APO; Olympus) focused the excitation light and collected fluorescent light. The fluorescent light (535 ± 25 nm) was measured using a photodiode mounted in a viewer (TILL Photonics). To reduce the variance between datasets, data recordings from both control and experimental cells were performed in the same day.

Ca2+ Measurements
[Ca2+]i was measured by dual-excitation wavelength spectrally-rimetry using an equimolar combination of the indicators fura-2FF and bisfura-2. For calibration, eight solutions were prepared with free [Ca2+], 0, 0.31, 1.2, 3.6, 7.2, 28, and 127 μM and 10 mM by using the Ca2+ buffers EGTA (10 mM, Kd of 150 nM at pH 7.2), N-(2-hydroxyethyl)-ethylendiaminetetraacetic acid (HEDTA, 10 mM, Kd of 3.6 μM), or 1,5-diaminopropan-2-ol-tetraacetic acid (DPTA, 30 mM, Kd of 81 μM) (Martell and Smith, 1974–1989). Whole-cell recordings were made using each of the calibration solutions in the pipette, and the ratio of fluorescence emission (535 ± 25 nm) for 340- and 365-nm excitation was recorded after waiting 2 min for the pipette solution to diffuse into the cell. The resulting [Ca2+]i versus ratio data were then fit by the equation of Voets (2000) to yield estimates of the five free calibration parameters. We did not apply any correction for the fluorescence perturbation introduced by photolysis of cage (Zucker, 1992) because we used low cage concentrations and light intensities to achieve the low μM [Ca2+]i, used in this study.

Carbon Fiber Amperometry
Carbon-fiber electrodes were purchased from ALA Scientific Inc. and were positioned to just touch the surface of the cell using a micromanipulator (PCS-5000, Burleigh). The amperometric current was measured using a patch-clamp amplifier (EPC-9, HEKA) at a holding potential of +700 mV. The signal was filtered at 2.9 kHz and sampled at 17.5 ksamples/s. After acquisition, samples were averaged (“decimated”) in groups of 12 to give a final temporal resolution of ~0.7 ms per point. Analysis of amperometric spikes was performed using software described by Segura et al. (2000).

Recombinant Protein Molecular Biology
A fragment of synaptotagmin I encoding the cytoplasmic domain was PCR-amplified from rat brain cDNA (CLONTECH Laboratories Inc.) and cloned into the pET41a vector (Novagen) to create pET41α-Syt1(96–331). This plasmid encodes residues 96–331.
fused at the N terminus to glutathione-S-transferase (GST) and a His$_6$ tag. The pGEK-Syx1A construct encoding residues 4–266 of syntaxin 1A fused at the N terminus to GST was a gift from R.H. Scheller (Stanford University, Stanford, CA) and has been previously described (Risinger and Bennett, 1999). The pQE9-SNAP-25 construct encoding His$_6$-tagged full-length SNAP-25 was a gift from S.W. Whiteheart (University of Kentucky, Lexington, KY) and has been previously described (Matveeva and Whiteheart, 1999). Mutant SNAP-25 constructs were produced by PCR-based mutagenesis of this plasmid. Mutations at Thr-138 were identified by removal of a SmaI restriction site. Mutations at Ser-187 were identified by creation of a new CiaI site. Combined double mutants were created by subcloning fragments from single mutants. All constructs were verified by automated sequencing.

Protein Biochemistry

Recombinant proteins were produced by transformation of constructs into M15 Escherichia coli, induction of expression with IPTG, and purification by FPLC. Fractions containing recombinant protein were pooled, concentrated, and stored in aliquots at −80°C for subsequent use. Binding assays were performed using a previously described microtitre plate-based method (Craig et al., 2004). The primary (bait) protein used was either 200 ng GST-syntaxin 1A or 200 ng GST-synaptotagmin I. The secondary (prey) SNAP-25 protein was detected by monoclonal anti-RGS-His$_6$ antibody (QIAGEN) for assays using GST-syntaxin 1A, or anti-SNAP-25 mAb for assays using GST-synaptotagmin I. The RGS-His$_6$ antibody could not be used for assays involving synaptotagmin, as the GST-synaptotagmin I construct also contained a His$_6$ tag. Syntaxin/SNAP-25 heterodimers were assembled by overnight incubation of equimolar amounts of GST-syntaxin and His$_6$-SNAP-25 at 4°C. This resulted in the majority of SNAP-25 forming a higher molecular weight complex with syntaxin, as determined by gel filtration chromatography using a G75 FPLC column (GE Healthcare). In vitro phosphorylation assays were performed using purified catalytically active kinases (Calbiochem) at 30°C in MES buffer (50 mM MES, pH 6.9, 10 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT) and initiated with the addition of 2 μCi [γ-32P]ATP and unlabeled ATP to a final concentration of 100 μM, as previously described (Evans et al., 2001). Phosphorylation at Ser-187 was assessed using the previously characterized phosphospecific antibody Pi-SNAP-25 (Kataoka et al., 2000).

RESULTS

The Ser187Glu Mutation Mimics PKC Phosphorylation

Phosphorylation of SNAP-25 on Ser-187 has been documented in several cell types and mutation of this residue to a putative phosphomimetic glutamate has been shown to affect pool refilling in chromaffin cells (Shimazaki et al., 1996; Genoud et al., 1999; Kataoka et al., 2000; Gonelle-Gispert et al., 2002; Nagy et al., 2002). However, glutamate/aspartate mutations do not always result in phosphomimetic proteins, and there are no data on whether the presumed phosphomimetic SNAP-25 constructs behave similarly to PKC-phosphorylated wild-type SNAP-25 in protein interaction assays. To study the biochemical effects of phosphorylation of SNAP-25 on Ser-187, we performed in vitro kinase assays. Wild-type recombinant SNAP-25 was incubated with the catalytic subunit of PKA or a catalytic fragment of PKC at 30°C for 3 h, in the presence of 100 μM ATP. Phosphorylation at this residue was detected using a phosphospecific antibody (Pi-SNAP-25), which has been shown in previous studies to be specific for SNAP-25 phosphorylated on Ser-187 (Kataoka et al., 2000). Fig. 1 A clearly demonstrates that phosphorylation of SNAP-25 on Ser-187 can be achieved with PKC. In contrast, PKA is much less effective as a Ser-187 kinase, producing similar levels of Pi-SNAP-25 immunoreactivity to control (mock) reactions. In spite of the apparent strength of signal obtained with this phosphospecific antibody from PKC-phosphorylated SNAP-25, kinase assays using [32P]-ATP show very low stoichiometry of 32P incorporation (unpublished data), consistent with previous reports (Risinger and Bennett, 1999; Kataoka et al., 2000). It has been suggested that this may be due to the relatively unstructured nature of SNAP-25 in isolation, and that interaction with syntaxin might be required to achieve stoichiometric phosphorylation (Kataoka et al., 2000). To address this, we prepared stable heterodimeric complexes of recombinant syntaxin and SNAP-25 and used these for in vitro phosphorylation reactions. We find no increase in Ser-187

Figure 1. Phosphorylation of purified recombinant SNAP-25 on Ser-187. Recombinant SNAP-25 was phosphorylated using either PKC or PKA with 100 μM ATP. Kinase reactions were stopped with an equal volume of Laemmli buffer, boiled, and resolved on SDSPAGE. Phosphorylation on Ser-187 was assessed by immunoblotting with a phosphospecific antibody for pS187 of SNAP-25. (A) Comparison of PKA and PKC as Ser-187 kinases. (B) Comparison of monomeric SNAP-25 and heterodimeric syntaxin/SNAP-25 complexes as PKC substrates. (C) Effect of mutations at Ser-187 on Pi-SNAP-25 immunoreactivity.
phosphorylation and mutation of this residue on syntaxin binding. As an alternative test of the putative phosphomimetic nature of Ser-187 modifications, we determined the Pi-SNAP-25 immunoreactivity of wild-type and mutant constructs (Fig. 1 C). The increased phosphorylation of wild-type SNAP-25 caused by PKC is eliminated in the S187A construct, which itself exhibits similar immunoreactivity to mock incubations lacking kinase, thus confirming the nonphosphomimetic nature of this alanine substitution mutant. In contrast, the S187E mutant exhibits a level of immunoreactivity comparable to PKC-phosphorylated wild-type SNAP-25 even in the absence of kinase, with no further increase in this signal upon incubation with PKC. The EE mutant, in which both Thr-138 and Ser-187 are mutated to glutamate, is indistinguishable in this assay to the single S187E mutant. As the Pi-SNAP-25 antibody is by definition a phospho-Ser-187–specific SNAP-25 binding protein, these data suggest that substitution of glutamate at this position does indeed create a phosphomimetic mutant protein.

**Phosphomimetic Mutations of Ser-187 Increase the Size of the HCSP but only modestly affect the size of the RRP**

We used SFV to express mutants of SNAP-25A in bovine chromaffin cells. EGFP fused to the N terminus was used to allow fluorescent selection of cells expressing the protein for patch-clamp experiments. It has previously been shown that expression of EGFP-SNAP-25 with SFV results in a 25-fold overexpression compared with native SNAP-25, yet has no apparent effect on Ca\(^{2+}\)-triggered exocytosis (Wei et al., 2000). Caged Ca\(^{2+}\) together with a combination of a high affinity Ca\(^{2+}\) indicator (bisfura2) and a lower affinity indicator (fura2-FF) were loaded into GFP-positive cells through the patch pipette during whole-cell recording. The combination of the two indicators allows ratiometric measurement of [Ca\(^{2+}\)]\(_i\) over a wide concentration range (Voets, 2000).

We and others have previously described a small but rapid burst of exocytosis in response to photoelevation of [Ca\(^{2+}\)], into the low \(\mu\)M range that we ascribe to release from an HCSP, whereas elevation of [Ca\(^{2+}\)] into the tens of \(\mu\)M is required to elicit fast release from the RRP. Fig. 2 depicts a protocol we developed to estimate the size of the HCSP and RRP in a single sweep (Yang and Gillis, 2004). We first use flash photolysis of caged Ca\(^{2+}\) to release the HCSP followed by 10 depolarizing pulses (30 ms in duration to \(+20\) mV) to release most or all of the RRP. In the sample experiment of Fig. 2 A, the HCSP is \(26\) fF, whereas the increase in Cm after 10 depolarizing pulses (\(\Delta C_{10}\)) is \(177\) fF, similar in size to the RRP defined from caged Ca\(^{2+}\) experiments in chromaffin cells (e.g., Voets, 2000). In the following experiments we will use \(\Delta C_{10}\) as an index of the size of the RRP (Yang and Gillis, 2004).

**Figure 2.** Measurement of the HCSP and depolarization-evoked exocytosis in chromaffin cells expressing GFP-SNAP-25 using the Semliki Forrest virus expression system. Whole-cell recordings were made from cells exhibiting GFP fluorescence. Flash photolysis of caged Ca\(^{2+}\) at the time indicated by the arrow leads to a burst of exocytosis (increase in membrane capacitance, \(\Delta C_m\)) from the HCSP. This is followed 300 ms later by a train of 10 depolarizing pulses to trigger Ca\(^{2+}\) influx (\(I_{Ca}\)) in order to accelerate release of chromaffin granules that are less responsive to Ca\(^{2+}\) (the RRP). (A) Sample traces from an individual cell. (B) Averaged responses from 18 cells. Note that, whereas [Ca\(^{2+}\)] is elevated uniformly throughout the cell upon photolysis of caged Ca\(^{2+}\), the near-membrane [Ca\(^{2+}\)], that triggers release from the RRP is likely to be significantly higher than the spatially averaged [Ca\(^{2+}\)], reported by the fluorescent indicators (middle trace) following depolarization-triggered Ca\(^{2+}\) influx (\(I_{Ca}\)).
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GFP-SNAP-25, whereas averaged responses from 18 cells are depicted in Fig. 2 B. These responses are indistinguishable from responses from untransfected cells (Yang et al., 2002) and thus demonstrate that viral expression of GFP-SNAP-25 does not significantly alter the sizes of the HCSP or RRP.

We next performed experiments comparing responses from cells expressing the phosphomimetic mutation S187E with those expressing the putative phosphomimetic mutation S187D. Whereas the HCSP was approximately twofold greater for S187D-expressing cells compared with cells expressing S187A (P < 0.001), the ΔC10 response is similar for the two mutations. However, both of these mutants had ΔC10 values significantly less than cells expressing control GFP-SNAP-25 (P < 0.001 for both mutants; also see Nagy et al., 2002). These results are summarized in Fig. 8.

The HCSP Consist of Granules with Similar Catecholamine Content and Release Kinetics as the RRP

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We next performed experiments comparing responses from cells expressing the phosphomimetic mutation S187E with cells expressing an S187C mutation that precludes phosphorylation. Sample traces are depicted in Fig. 3 A whereas averaged responses are depicted in Fig. 3 B. The average size of the HCSP is 23 fF for S187C, a value indistinguishable from wild type, whereas expression of the S187E mutant results in an approximately threefold larger HCSP (P < 0.001). In contrast, the ΔC10 response was only ~50% larger for cells expressing the S187E mutation. This increase in ΔC10 was also significant (P < 0.001).

We also compared cells expressing the S187A mutation with those expressing the putative phosphomimetic mutation S187D. Whereas the HCSP was approximately twofold greater for S187D-expressing cells compared with cells expressing S187A (P < 0.001), the ΔC10 response is similar for the two mutations. However, both of these mutants had ΔC10 values significantly less than cells expressing control GFP-SNAP-25 (P < 0.001 for both mutants; also see Nagy et al., 2002). These results are summarized in Fig. 8.

The HCSP Consist of Granules with Similar Catecholamine Content and Release Kinetics as the RRP

Capacitance measurements indicate changes in surface area that result from both exocytosis and endocytosis. Declines in capacitance due to endocytosis following stimulus-induced rises are generally quite slow or absent during whole-cell recording if [Ca2+]i is kept below 10 μM (see Figs. 2 A, 3 A, 4 A, 7 A, and Smith and Neher, 1997), therefore, fast changes in Cm likely accurately reflect exocytosis under our recording conditions. To test this hypothesis, we performed a set of experiments with the addition of a carbon fiber electrode to directly record quantal catecholamine release using amperometry. Fig. 4 A depicts a sample response using a similar protocol as Fig. 2, whereas Fig. 4 B depicts the average of 44 traces obtained from 16 cells. In Fig. 4 B we compare the integral of the amperometric trace (Q, red line) with the capacitance response. The scaled Q follows the ΔC trace with a delay due to the time it takes for catecholamine to be released from granules following membrane fusion. Note that the ratio of Q (which scales with granule volume) to ΔC (which scales with granule surface area) is similar for release from the HCSP as from the depolarization-evoked release, suggesting that the granules in the two phases of release have a similar size. Also note that the similarity in time course between Q and ΔC demonstrates that endocytosis does not greatly affect the kinetics of ΔCm under our recording conditions.

We analyzed individual amperometric spikes to see if quantal release differs between the HCSP and the RRP. Fig. 4 C presents cumulative histograms of the charge of individual amperometric spikes resulting from the HCSP phase (dashed line) and the depolarization-evoked response (solid line). Little difference in quantal content (Q) is apparent. Cumulative histograms of the spike duration at half-maximal current (t1/2) are compared in Fig. 4 D. Again, no difference is apparent. Therefore we conclude the phase of exocytosis we attribute to release of the HCSP consists of granules with

Figure 3. Expression of the phosphomimetic SNAP-25 mutation S187E leads to a larger HCSP size than the S187C mutation that precludes phosphorylation by PKC. (A) Sample responses of individual cells expressing either the S187C mutation (black traces) or the S187E mutation (red traces). (B) Averaged responses from cells expressing the S187C mutation (black traces, 35 cells) or S187E mutation (red traces, 40 cells).
similar catecholamine content and release kinetics as depolarization-evoked release from the RRP.

**Phosphomimetic Mutation of SNAP-25 at Ser-187 Increases Binding to Syntaxin but Decreases Ca$$^{2+}$$-independent Binding to Synaptotagmin I in an In Vitro Protein Binding Assay**

Results with the phosphomimetic S187E and S187D mutations suggest that phosphorylation of SNAP-25 on Ser-187 increases the size of the HCSP. As phosphorylation of this residue has been suggested to inhibit syntaxin binding (Shimazaki et al., 1996), we analyzed the effect of Ser-187 mutations on the ability of recombinant SNAP-25 to bind GST-syntaxin. Unexpectedly, the S187E phosphomimetic mutation results in an increased binding affinity for syntaxin (Fig. 5 C). This effect is specific for the phosphomimetic glutamate residue, as substitution with a nonphosphorylatable alanine residue at Ser-187 does not exhibit increased binding (Fig. 5 A). Although we also created the S187C cysteine substitution mutation, we were unable to prepare soluble mutant recombinant protein, thus precluding biochemical analysis of this mutant. Substitution of glutamate at Thr-138, a PKA phosphorylation site (Risinger and Bennett, 1999; Nagy et al., 2004) that has also been suggested to be a PKC target (Hepp et al., 2002), also fails to increase syntaxin binding (Fig. 5 B). It has been suggested that phosphorylation of Thr-138 may occur tonically in chromaffin cells (Nagy et al., 2004), and so we investigated the effect of a double Thr-138/Ser-187 glutamate substitution (“EE” mutant), which would potentially mimic SNAP-25 phosphorylated on both sites. Interestingly, the Ser-187 mutation was dominant in this double mutant, as the EE mutant displayed a virtually identical increase in syntaxin binding to that seen in the single S187E mutant (compare Fig. 5, C and D). We therefore conclude that phosphomimetic mutation of Ser-187 increases syntaxin binding affinity irrespective of the phosphorylation state of Thr-138.

In addition to syntaxin, SNAP-25 has been shown to bind to a variety of other proteins implicated in exocytosis. Amongst these, synaptotagmin is of particular interest, as its interaction with SNAP-25 involves the C-terminal helix that contains Ser-187 and is functionally important in the regulation of exocytosis (Gerona et al., 2000; Zhang et al., 2002; Bai et al., 2004). We therefore assessed the Ca$$^{2+}$$-independent binding of the wild-type and mutant SNAP-25 proteins to a GST fusion protein encoding the cytoplasmic domain of synaptotagmin 1. The S187E mutant exhibits a reduction in synaptotagmin binding (Fig. 6 C), in direct contrast to the increased syntaxin binding exhibited by this mutant. Again, some sequence selectivity was evident, as an alanine substitution at the same position in the S187A mutant has little effect on synaptotagmin binding (Fig. 6 A). The T138E and EE mutants display similarly reduced synaptotagmin binding as the S187E mutation (Fig. 6, C and D). Taken together, this indicates that...
phosphomimetic glutamate mutation at Ser-187 increases syntaxin binding yet decreases synaptotagmin binding, whereas glutamate mutation at Thr-138 inhibits both interactions.

The Stimulatory Effect of the Phosphomimetic SNAP-25 Mutant S187E on Highly Ca\(^{2+}\)-sensitive Exocytosis Is Not due to Decreased Ca\(^{2+}\)-independent Binding of SNAP-25 to Synaptotagmin I

The decreased binding of SNAP-25 to synaptotagmin of the phosphomimetic mutation S187E is of particular interest because the Ca\(^{2+}\)-sensing role of synaptotagmin in exocytosis. It is possible that synaptotagmin I is the low-affinity Ca\(^{2+}\) sensor for exocytosis from the RRP (Sorensen et al., 2003), but that a different, higher affinity Ca\(^{2+}\) sensor mediates release from the HCSP. In this case, decreasing the affinity of SNAP-25 for synaptotagmin I may make granules more likely to be in the highly Ca\(^{2+}\)-sensitive state. To test for this possibility we performed experiments using cells expressing the T138E
mutation, which has a similarly decreased affinity for synaptotagmin I as the S187E mutation (Fig. 6). A typical experiment is depicted in Fig. 7 A whereas the average response is given in Fig. 7 B (black traces). The sizes of the HCSP and the ΔC_{10} response for T138E are compared in Fig. 8. It is apparent that expression of the T138E mutant results in responses very similar to expression of wild-type GFP-SNAP-25, consistent with the view that this residue is phosphorylated by PKA under tonic conditions in chromaffin cells (Nagy et al., 2004). We next compared the response of cells containing T138E with those expressing the double “EE” mutant (Fig. 7). These two mutants exhibit very similar Ca^{2+}-independent binding to synaptotagmin I (Fig. 6), but the EE SNAP-25 mutant exhibits increased binding to syntaxin (Fig. 5). The results depicted in Fig. 7 and summarized in Fig. 8 demonstrate that the double mutant has a phenotype similar to that of S187E, i.e., the HCSP is enhanced several fold compared with wild type whereas the response to depolarization (ΔC_{10}) is enhanced to a lesser extent. Thus the stimulatory effect of the S187E mutation on the HCSP cannot be attributed to a decreased Ca^{2+}-independent binding to synaptotagmin I, but is correlated with increased binding of SNAP-25 with syntaxin.

**DISCUSSION**

Effects of Phosphomimetic Mutations of SNAP-25 on Binding To Syntaxin and Synaptotagmin I Assayed In Vitro

Many lines of evidence suggest that neuronal exocytosis is modulated by PKA and PKC (for reviews see Turner et al., 1999; Evans and Morgan, 2003; Leenders and Sheng, 2005; Morgan et al., 2005). However the precise roles of individual phosphorylation events on specific proteins are still largely unclear. The core complex protein SNAP-25 has been previously reported to be phosphorylated on Ser-187 by PKC (Shimazaki et al., 1996) and at Thr-138 by PKA (Risinger and Bennett, 1999) and PKC (Hepp et al., 2002), and these two phosphorylation events have been suggested to regulate the refilling kinetics (Nagy et al., 2002) and size (Nagy et al., 2004) of releasable vesicle pools, respectively. These studies were based on the phenotypes of chromaffin cells expressing putative phosphomimetic glutamate/aspartate mutants, although no assessment of their phosphomimetic properties has been provided. In the present study, we found that the S187E mutation does indeed create a phosphomimetic protein, as evidenced by enhanced binding of a phospho-Ser-187–specific antibody to the recombinant protein. Furthermore, phosphomimetic mutation of Ser-187 (either singly or in combination with mutation of T138) was found to selectively increase SNAP-25’s binding affinity for syntaxin, but not synaptotagmin. As this enhanced syntaxin binding correlated with increased exocytosis from the HCSP, the simplest interpretation is that these effects are causally linked. This remains speculative, however, because these functional effects may be due to changes in SNAP-25 interactions with other proteins not assayed in this study.

How then might an increase in binding affinity of SNAP-25 for syntaxin explain the various reported stimulatory effects of PKC on exocytosis? Formation of the syntaxin–SNAP-25 heterodimer is the initial, rate-limiting step in SNARE complex assembly. Therefore, in situations where the availability of syntaxin–SNAP-25 dimers are limiting, increased formation of these t-SNARE heterodimers by Ser-187 phosphorylation would facilitate formation of the ternary SNARE complex and hence membrane fusion. This model is consistent with the observed effect of the S187E mutant in increasing
the rate of releasable pool refilling after stimulation (Nagy et al., 2002), and the increase in the size of the HCSP reported here, assuming that t-SNARE heterodimer formation is limiting in these assays.

The only previously published data on how phosphorylation of SNAP-25 on Ser-187 might affect syntaxin binding is the observation that treatment of PC12 cell extracts with purified PKC and ATPγS reduces the amount of syntaxin communoprecipitating with SNAP-25 (Shimazaki et al., 1996). In the complex environment of a cell lysate, it is clearly possible that effects of PKC and ATPγS other than phosphorylation of SNAP-25 could explain the observed reduction in SNARE complex (e.g., effects on NSF). In any case, it is difficult to reconcile the enhancement of exocytosis in various systems with a model where PKC induces a reduction in syntaxin–SNAP-25 heterodimer formation, as this should antagonize membrane fusion.

Interestingly, mimicking PKC phosphorylation with the S187E mutation decreases Ca\(^{2+}\)-independent binding of SNAP-25 with the cytoplasmic domain of synaptotagmin I assayed in vitro (Fig. 6). However, there is no difference in Ca\(^{2+}\)-independent synaptotagmin binding between a Thr-138 phosphomimetic SNAP-25 mutant (T138E) and the T138E/S187E double mutant (EE, Fig. 6), whereas the EE mutant results in a larger HCSP than the T138E mutant when expressed in chromaffin cells (Fig. 7). Therefore the difference in Ca\(^{2+}\)-independent binding to synaptotagmin I does not appear to explain changes in exocytosis caused by S187E expression in chromaffin cells, which, as discussed above, seems most likely to be explained by an increased affinity for syntaxin.

Nevertheless it is possible that Ser-187 participates in Ca\(^{2+}\)-dependent binding of SNAP-25 to synaptotagmin I (Gerona et al., 2000) or in other interactions between SNAP-25 and synaptotagmin isoforms not assayed here.

### Phosphomimetic Mutations of SNAP-25 Partially Mimic the Effects of Protein Kinases on Vesicle Pool Sizes

This work extends previous reports that demonstrate that exocytosis triggered by low levels of [Ca\(^{2+}\)], is regulated differently than exocytosis triggered by higher [Ca\(^{2+}\)], levels (Yang et al., 2002; Wan et al., 2004; Yang and Gillis, 2004). Here we show that expression of the phosphomimetic S187E SNAP-25 mutant has a greater effect on increasing the size of the HCSP compared with the RRP (Fig. 8), and thus results in an increase in the fraction of granules that are in a highly Ca\(^{2+}\)-sensitive state. Activation of PKC with a phorbol ester also increases the size of the HCSP to a greater extent than the RRP (Yang et al., 2002; Zhu et al., 2002; Wan et al., 2004; Yang and Gillis, 2004). On the other hand, PKC activation has additional effects on exocytosis that are not reproduced by expression of S187E such as a greater increase in the size of the RRP and the maintenance of RRP size in response to repeated stimulation (Nagy et al., 2002).

An interesting observation is that the S187E SNAP-25 mutation results in a approximately two to threefold increase in both the HCSP (this work) and the steady-state release that continues for seconds after photoelevation of [Ca\(^{2+}\)] to levels >\approx 15 \mu M (Nagy et al., 2002). This “sustained” phase of release is usually interpreted as reflecting the rate of vesicle recruitment since this refilling process presumably becomes rate limiting following depletion of vesicle pools (for review see Sorensen, 2004). It is somewhat puzzling, then, why neither the RRP nor the SRP increase in size if the refilling step is enhanced with the S187E mutation. A possible explanation for these observations is that the S187E mutation increases the refilling step of the HCSP, rather than the RRP or SRP, and this accounts for both the increase of the “sustained” phase of exocytosis and the increase of the HCSP size. Further experiments are necessary to test this hypothesis.

Finally, whereas our results demonstrate a preferential increase in a highly Ca\(^{2+}\)-sensitive state upon expression of SNAP-25 Ser-187 phosphomimetic mutations, other groups have demonstrated that different mutations of the C terminus of SNAP-25, including truncation of the nine C-terminal amino acid residues to mimic BoNT/A cleavage, greatly decrease the sensitivity of exocytosis to Ca\(^{2+}\) (Gerona et al., 2000; Sorensen et al., 2006). Thus our results provide further evidence that the C terminus of SNAP-25 plays an important role in Ca\(^{2+}\) sensing for exocytosis.

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