PKC Modulation of Transmitter Release by SNAP-25 at Sensory-to-Motor Synapses in Aplysia

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INTRODUCTION

PKC is a Ca²⁺ phospholipid-dependent protein kinase and plays a prominent role in many neuronal processes including the regulation of transmitter release (Hilfiker and Augustine 1999; Waters and Smith 2000). Activation of PKC increases both the size and the rate at which the readily releasable pool (RRP) refills in cultured hippocampal autapses (Stevens and Sullivan 1998) and in chromaffin cells (Smith et al. 1998). In chromaffin cells, PKC enhances exocytosis both by increasing the RRP and by shifting vesicles to a highly Ca²⁺ sensitive state, enabling exocytosis at sites relatively distant from Ca²⁺ channels (Yang et al. 2002).

Aplysia sensory-to-motor neuron synapses show a marked depression at low frequencies that contributes to behavioral habituation (Byrne et al. 1978). Hosomsynaptic depression is reversed by the neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) through activation of a Ca²⁺-independent form of PKC, PKC ApII (Ghirardi et al. 1992; Manseau et al. 2001). Whereas activation of PKC by 5-HT increases transmitter release only at depressed synapses, activation of PKC by phorbol esters increases transmitter release at both naive and depressed synapses (Brah et al. 1990; Ghirardi et al. 1992; Nakhost et al. 2003). However, the molecular targets of PKC at either depressed or naive synapses have not been identified. We previously examined the role of the potential target synaptotagmin I (Syt I) in synaptic modulation of the sensory–motor synapse (Nakhost et al. 2003). However, although PKC phosphorylates Syt I in Aplysia and splicing of Syt I regulates transmitter release, synaptotagmin was not the phosphorylation target for PKC (Nakhost et al. 2003). Here, we focus on another possible substrate, synaptosomal-associated protein of 25 kDa (SNAP-25).

SNAP-25 is essential for evoked synaptic transmission. In SNAP-25-deficient mice, spontaneous release persists, whereas evoked release is abolished (Sørensen et al. 2003; Washbourne et al. 2002). SNAP-25 is involved in the molecular regulation of neurotransmitter release through its association with vehicle-associated membrane protein (VAMP, synaptobrevin) and syntaxin (Südhof 1995). Together these three factors form a stable SNARE complex of proteins (Fernández-Chacon and Südhof 1999), which is essential for evoked neurotransmitter release. In vitro evidence indicates that the SNARE complex can promote fusion between lipid vesicles (Schuette et al. 2004; Weber et al. 1998). Moreover, this complex is likely to provide a framework for a variety of protein interactions involved in assembly and recycling of synaptic vesicles (Brunner 2000).

Although the role of the SNARE complex in synaptic vesicle release is clear, there is less evidence that the physiological modulation of transmitter release arises from posttranslational modification of SNARE proteins. Perhaps the best evidence for this phenomenon is the phosphorylation of SNAP-25 at Ser187 by PKC (Genoud et al. 1999; Shimazaki et al. 1996). The amount of SNAP-25 associated with syntaxin decreases after PKC-induced phosphorylation of SNAP-25 in PC12 cells (Shimazaki et al. 1996) and phosphorylation at this site increases recruitment of dense-core vesicles in chromaffin cells (Nagy et al. 2002). However, SNAP-25 phosphorylation was not important for phorbol 12,13-dibutyrate (PDBu)–mediated increase in transmitter release in CA1 neurons (Finley et al. 2003), calling into question whether SNAP-25 phosphorylation is also important in regulating release of synaptic vesicles.

In the present paper, we used the plastic synapses between mechanoreceptor sensory neurons and siphon motor neurons of Aplysia as a model to investigate whether PKC phosphorylation of SNAP-25 plays a modulatory role in evoked release of synaptic vesicles. Our results suggest an important role for SNAP-25 and phosphorylation of SNAP-25 in multiple aspects...
of the regulation of synaptic vesicle release at sensory–motor neuron synapses.

**METHODS**

*Aplysia californica* (75–200 g) were purchased from Marine Specimens Unlimited (Pacific Palisades, CA) or the Aplysia Resource Facility at the University of Miami (Miami, FL). They were kept in an aquarium for ≥3 days before experimentation. Dissection and isolation of tissues and cultured neurons were as previously described (Manseau et al. 2001).

**Cloning of SNAP-25**

*Aplysia* nervous system cDNA was generated using pleural, pedal, and abdominal ganglia from the animal. These ganglia were dissected and immediately frozen in liquid nitrogen, and then processed using the Qiagen RNEasy Mini kit (Qiagen, Santa Clara, CA) to obtain total RNA. cDNA template was made using Superscript II RT (Life Technologies, Gaithersburg, MD). The cDNA product was subsequently used as a template in PCR to amplify fragments of SNAP-25 using degenerate primers. The following primers were used: SNAP-25 degenerate 5′ primers: F1 [GA(T/C)GAN CA(A/G)GG NGA(A/G)CA], F2 [GA(T/C)ATGA(A/G)GA(A/G)GCNGA], degenerate 3′ primers R1 [CAT(A/G)TC NGA(T/A)GNGC CAT(A/G)T], and R2 [TCN TCC AT(T/C)TC(G/A) T(T/C)TC(G/A) T(T/C)TC(T/C)TC].

The nested degenerate PCR provided a fragment of SNAP-25 that was then extended using rapid amplification of cDNA ends (RACE) to generate the full-length sequence. GST–SNAP-25 was then generated using RT-PCR from total nervous system RNA to insert the full length *Aplysia* SNAP-25 into pGEX-5X-1. This plasmid was then cut with *Bam* HI and *Kpn* I to insert into the plasmid pNEX-3 for expression in *Aplysia* neurons and pGEX5.2 for generation of a GST–SNAP-25 fusion protein. To confirm that this isoform of SNAP-25 was expressed in sensory neurons we recloned SNAP-25 using RT-PCR from total sensory neuron RNA and examined three independent PCR products. All PCR products were identical to the previously isolated *Aplysia* SNAP-25. EGFP fusion proteins were generated by amplifying SNAP-25 with PCR and inserting into the *Kpn* site of pNEX-3–EGFP. The lack of introduced errors was again confirmed with sequencing. Products were sequenced in both strands.

**Generation of an antibody to SNAP-25**

The GST–SNAP 25 fusion protein was used as an immunogen for injection into rabbits. The resultant serum was used without purification.

**Fluorescence microscopy**

Injected sensory neurons were visualized at ×4 with a Nikon fluorescence microscope (Nikon Eclipse E600). The success rate of expression ranged from 50 to 90%. Expression was stable 24 h after injection and the cells remained fluorescent for about 1 wk.

**Cell culture preparation**

Injections of plasmid DNA and physiological paradigms were as previously described (Manseau et al. 2001). Briefly, 4 days after injection of the EGFP-tagged construct into the sensory neuron (SN) and 2 days after pairing with a motor neuron (MN), excitatory postsynaptic potentials (EPSPs) were evoked every 20 s in the motor neuron that was kept at −50 mV. After 40 evoked EPSPs, 5-HT (10 μM final concentration) was added to the bath and 10 additional EPSPs were sampled. In another set of experiments, the phorbol ester phorbol 12,13-dibutyrate (PDBu, 100 nM final concentration) was added after the first EPSP, a second EPSP was evoked 10 min later, followed by 38 evoked EPSPs to observe the rate of depression afterward. In some of the PDBu experiments a PKC inhibitor, bisindolylmaleimide I (Bis) (10 μM final concentration), was added to the bath 10 min before the start of an experiment and remained in the bath throughout. All experiments were performed at room temperature and the initial EPSP amplitude had to be ≥2 mV for an experiment to progress. In a couple of experiments the MN spiked in the first EPSP or after the addition of PDBu. In this case we measured the EPSP at the shoulder of the spike.

**Data acquisition and analysis**

Data were acquired and analyzed digitally using CLAMPEx 9 and a modified version of pCLAMP (Axon Instruments) (Manseau et al. 2001). The effects of the various constructs were always tested in parallel. EPSPs were always normalized to the size of the initial EPSP.

**Changes in synaptic transmission**

The difference in homosynaptic depression was quantified using a nonparametric bootstrap test procedure with 10,000 repetitions and a criterion of *P* < 0.05 (Efron and Tibshirani 1986). The amount of facilitation was calculated as the difference between EPSPs after treatment (averages of EPSPs 41–43) and EPSPs before treatment (averages of EPSPs 38–40), or in the case of PDBu, the difference between EPSP 1 and EPSP 2 measured after PDBu. Significance was calculated by Student’s *t*-test.

**Immunofluorescence**

To identify the localization of SNAP-25 wild-type and SNAP-25 mutants at *Aplysia* sensory–motor synapses, dissociated *Aplysia* SNs were plated on Matek (Ashland, MA) polylysine-coated glass-bottom culture dishes and coinjected with EGFP–SNAP-25 and DsRed–VAMP (as an indicator for synapse localization) or DsRed–VAMP alone. The cultures were subsequently labeled with an *Aplysia*-specific SNAP-25 antibody (Anti SNAP-25). Four days after injection of the EGFP-tagged construct into the SN and 2 days after pairing with an MN, the cells were fixed in 4% paraformaldehyde in a 30% sucrose-buffered PBS solution for 60 min. The cells were then solubilized and permeabilized in a 30% sucrose-buffered PBS containing 0.1% Triton for 10 min. The cultures were rinsed in PBS and free aldehydes were quenched with 50 mM NH₄Cl for 30 min, then incubated in 10% normal goat serum containing 0.5% Triton in PBS for 30 min to block nonspecific binding. After 1-h incubation with primary antibodies (Anti SNAP-25) diluted (1:50) in blocking buffer, cultures were rinsed in PBS and incubated for 1 h in Alexa Fluor 644–conjugated goat anti-rabbit secondary antibodies (1:200) (Molecular Probes, Eugene, OR) diluted in blocking buffer. Cultures were rinsed in PBS and then mounted with Shandon Immu-Mount (Thermo, Pittsburg, PA) mounting media. All steps were performed at room temperature.
Confocal microscopy

The triple-labeled sections were visualized simultaneously with a Zeiss LSM 510 confocal laser microscope (Carl Zeiss, Jena, Germany), equipped with one argon (488 nm) and two He/Ne lasers of 543 and 633 nm.

RESULTS
Cloning of Aplysia SNAP-25

Aplysia SNAP-25 was cloned using degenerate PCR and RACE and confirmed by sequencing three clones isolated from sensory neurons. We found that the PKC phosphorylation site Ser198 in Aplysia (the equivalent of mammalian Ser187) is conserved over evolution. Figure 1A shows the amino acid alignment with other published SNAP-25 sequences. To study the effect of the phosphorylation of Ser198 in SNAP-25, various plasmid constructs encoding EGFP or EGFP-tagged SNAP-25 constructs were generated. We replaced Aplysia Ser198 with the negatively charged glutamate (S198E) or aspartate (S198D), both of which mimic the effect of PKC phosphorylation by introducing a negative charge, but may differ in their orientation based on possible rotamer possibilities, and thus may impose spatial constraints on the three-dimensional structure of SNAP-25. Conversely, we substituted serine with the nonpolar alanine (S198A) or cysteine (S198C), both of which cannot be phosphorylated. Cysteine is suggested to be a better substitute for the nonphosphorylated state because of its longer side chain, but there is a danger of forming inappropriate disulfide bonds. EGFP was coupled to the N-terminus of SNAP-25 as an indicator of expression. N-terminal–coupled EGFP–SNAP-25 was used in a number of functional studies in other systems and is reported to fully functionally replace SNAP-25 (Finley et al. 2002, 2003; Nagy et al. 2002, 2004; Sørensen et al. 2002). All EGFP–SNAP-25 constructs expressed well in SF9 cells and we did not observe any cleavage between the EGFP and SNAP-25 (Fig. 1B).

SNAP and SNAP-25 mutants are both localized to synaptic vesicles

We further examined the subcellular distribution of EGFP–SNAP-25 and EGFP–SNAP-25 mutants and tested whether these constructs localized at synaptic sites. Both wild-type and mutant EGFP–SNAP-25 co-localized with DsRed-tagged Aplysia VAMP at concentrations of VAMP likely to mark pools of synaptic vesicles (Fig. 2A). FP-tagged VAMP was previously used to mark synaptic vesicle pools in many systems (Ahmari et al. 2000; Nonet 1999) and in Aplysia sensory neurons, tagged VAMP co-localizes with antibodies to clustered glutamate at sensory-to-motor neuron synapses (data not shown). However, some of the puncta of tagged VAMP (see wt SNAP-25 in Fig. 2A) do not co-localize. These are most probably small clusters of synaptic vesicles traveling down the neurites (Ahmari et al. 2000). Using an antibody raised to endogenous SNAP-25 (Fig. 2B), we also show that, as expected, endogenous SNAP-25 also localizes to sites where VAMP is concentrated (Fig. 2C, asterisk), although this is less obvious because SNAP-25 immunoreactivity is also present in the motor neuron and the immunolocalization of endogenous protein has more background staining than the fluorescent proteins. These experiments also demonstrate that the levels of overexpressed EGFP–SNAP-25 are much greater than those of the endogenous protein, as when sensory neurons are expressing EGFP–SNAP-25 (or the mutants; data not shown), at laser levels five times lower than in Fig. 2C and more than sufficient
SNAP phosphomimetic mutants decrease the rate of synaptic depression

Sensory neurons injected with various plasmids were paired with motor neurons to obtain functional synapses in isolated cell cultures (see Manseau et al. 2001). Synaptic depression was produced by 40 repeated intracellular stimulations of the sensory cell with an interstimulus interval of 20 s. Mutants mimicking the phosphorylated state (S-E, S-D) slowed down the rates of depression (Fig. 3A). Homosynaptic depression (HSD) in Aplysia is characterized by an initial rapid phase and a subsequent slow phase, during which the response remains fairly stationary, after the major part of HSD has already taken place (Armitage and Siegelbaum 1998; Royer et al. 2000). After seven to ten stimuli, the EPSP amplitude of the control EGFP–SNAP-25 was rapidly reduced to 27% of the initial EPSP amplitude and slowly decreased to 12% at the end of the stimulation period (EPSPs 38–40), whereas when sensory cells expressed the phosphomimetic mutant S-E or S-D, the plateau started at 48 and 40% of the initial EPSP, respectively, and decreased to about 22 and 18% after 40 stimuli. We observed a statistically significant difference ($P < 0.05$) for S-E throughout the stimulation period. For S-D we observed a statistical difference ($P < 0.05$) only through parts of the stimulation period (EPSPs 4–5, 7–8, 20–25, 28–31, and 36–37). In contrast, there was no significant difference on the rate of depression between mutants mimicking the nonphosphorylated state of SNAP-25 (S-A or S-C) and the control (Fig. 3B).

Whereas phosphomimetics of SNAP-25 decreased the rate of depression, SNAP-25 variants that could not be phosphorylated did not. This is not surprising because it is unlikely that PKC is activated by depression itself. To determine whether PKC-mediated phosphorylation of SNAP-25 could also decrease the rate of depression and to determine whether SNAP-25 could be a target for phorbol-ester–mediated increases in synaptic facilitation, the effect of wild-type SNAP-25 and one of the nonphosphorylated state mutants (SNAP-25 S-A) was examined in the presence of the PKC activator PDBu. Given the recent data from mammalian cells, such as palimtylation between the species. (25 kDa), it is within the range that one would expect the SNAP-25 antibody; one major band of about 30 kDa is observed. Although this is slightly higher than the molecular weight of SNAP-25 in vertebrates (25 kDa), it is within the range that one would expect the Aplysia SNAP-25 antibody (Anti-SNAP-25). Asterisk indicates potential synapse where endogenous SNAP-25 overlap with DsRed-VAMP. D: as in C, only here DsRed-VAMP and EGFP-SNAP-25 were injected into Aplysia sensory cells. First part shows a transmitted light image with superimposed fluorescent staining of DsRed-VAMP and EGFP-SNAP-25. High-power views of synaptic regions show the amount of overexpression. EGFP-SNAP-25 (green) and Anti-SNAP-25 (blue). Laser power was 5 times lower than that in C.

![Fig. 2. Localization of SNAP-25 constructs in sensory neurons. Plasmids encoding DsRed-labeled Aplysia vehicle-associated membrane protein (VAMP, synaptobrevin) and EGFP-labeled Aplysia SNAP-25 were coinjected into sensory neurons. Expressing neurons were then paired with motor neurons and visualized 3 days later. High-power views of the synaptic regions are shown. Similar results were seen with staining of SNAP-25 as opposed to EGFP labeling. B: 10 μg of nervous system tissue were blotted with the anti-Aplysia SNAP-25 antibody; one major band of about 30 kDa is observed. Although this is slightly higher than the molecular weight of SNAP-25 in vertebrates (25 kDa), it is within the range that one would expect the Aplysia SNAP-25 antibody (Anti-SNAP-25)].

![Fig. 3. Effects of SNAP-25 Ser198 mutations on the rates of depression. A: significant difference is observed among the phosphomimetic mutants compared with the wild-type (EGFP–SNAP-25) control. EGFP–SNAP-25 S-D (n = 7); EGFP–SNAP-25 S-E (n = 7); EGFP–SNAP-25 (n = 14). B: no significant difference is observed among the nonphosphorylated state mutants compared with the control (n = 14 as in A). EGFP–SNAP-25 S-C (n = 6); EGFP–SNAP-25 S-A (n = 7). Comparison of the rates of depression between the different constructs was quantified by subtracting the mean of the constructs from the reference control. Mean was calculated for every 2 normalized excitatory postsynaptic potential (EPSP) amplitudes, starting at the second stimulation, and was compared with the mean of the control. Difference was determined using a nonparametric bootstrap test procedure (Efron and Tibshirani 1986; Georgopoulos et al. 1988) with 10,000 repetitions and a criterion of $P < 0.05$. Briefly, each individual EPSP within the mean was shuffled randomly and compared with the equally shuffled control.}
that PDBu effects on transmitter release can also be mediated by Munc-13 (Rhee et al. 2002), we first confirmed that in Aplysia neurons the effects of PDBu on synaptic strength are mediated by PKC using a specific PKC inhibitor, Bisindoleamide I (Bis). Bis completely blocked the ability of PDBu to increase transmitter release at naive synapses, confirming earlier results (Braha et al. 1990) that this effect is mediated by PKC (Fig. 4, A and B). Strikingly, the increase in synaptic strength seen after PDBu treatment was also significantly blocked in sensory neurons expressing the nonphosphorylated state mutant of SNAP-25 (P < 0.01), but not when wild-type EGFP–SNAP-25 was expressed (Fig. 4, A and B). The effect of expressing the nonphosphorylated state mutant of SNAP-25 was similar to that of Bis.

Because PDBu initially increased synaptic strength, it is difficult to determine the effect of PDBu on the rates of depression because one cannot directly compare the rates of depression after PDBu to control because they start at different points. Instead, we examined the effect of PDBu on the steady-state amount of depression (i.e., the percentage of the initial EPSP reached after 15–20 stimulations). In the presence of PDBu, there is less synaptic depression in the steady state (Fig. 4A), similar to the effect of overexpressing EGFP–SNAP-25 phosphomimetics. Moreover, although wild-type SNAP-25 does not affect the steady state of depression in the absence of PDBu (Fig. 3) or the increase in synaptic strength mediated by PDBu, wild-type EGFP–SNAP-25 did further decrease the amount of steady-state synaptic depression (Fig. 4A). The effect of PDBu on the steady state of depression is blocked by Bis (P < 0.05) and by EGFP–SNAP-25 S-A, (Fig. 4A). These results are consistent with a model where PKC phosphorylation of SNAP-25 leads to an increase in synaptic strength in naive synapses and to a lower level of steady-state depression after repeated stimulations; both of these effects are mediated mainly through phosphorylation of SNAP-25.

Overexpression of SNAP-25 or phosphomutants does not affect initial synaptic strength

If phosphorylation of SNAP-25 was sufficient to increase synaptic strength, one might expect that neurons expressing phosphomimetics of SNAP-25 would have higher initial synaptic strength and the nonphosphorylated-state SNAP-25 would have lower initial synaptic strength. Although, similar to other studies (Nakhost et al. 2003), we observed large variabilities in initial synaptic strength, there was certainly not a trend for any of the phosphomimetic constructs to increase synaptic strength (P > 0.05) (Fig. 5). One hypothesis proposed by Waters and Smith (2002) argues that the proportion of recycling vesicles in the readily releasable pool differs from synapse to synapse (Waters and Smith 2002; Zhao and Klein 2003) and this may account for the heterogeneity of our data.

Effect of SNAP-25 expression on the 5-HT–induced reversal of depression

We next examined the ability of 5-HT to reverse synaptic depression in paired neurons where the sensory cells were preinjected with various constructs. 5-HT (10 μM, final concentration) was added to induce PKC-dependent facilitation (Ghirardi et al. 1992). Our electrophysiological results show that the reversal of depression was reduced by overexpression of EGFP–SNAP-25 (Fig. 6A). This was a surprising result; EGFP–SNAP-25 was previously reported to functionally replace SNAP-25 in a number of systems, yet here it appeared to act as a dominant negative construct. To determine whether the EGFP attachment at the N-terminal end was responsible for this effect, we separately injected EGFP and SNAP-25 (SNAP-25 + EGFP in Fig. 6). This did not interfere with the reversal of depression, suggesting that the addition of EGFP to the N-terminal converted SNAP-25 to a dominant negative construct. This effect is specific for the reversal of depression because EGFP–SNAP 25 did not affect initial synaptic strength (Fig. 5), PDBu-mediated increases in synaptic strength (Fig. 4A), or the normal rate of synaptic depression (Fig. 3). Interestingly, overexpression of EGFP–SNAP-25 with mutations at the PKC phosphorylation site, with the exception of the Ser-Asp (S198D) mutation, did not interfere with facilitation, suggesting that this amino acid was important for the ability of SNAP-25 to act as a dominant negative (Fig. 6B).

Discussion

Phosphorylation of SNAP-25 slows down the rate of depression in SM synapses

Although the data corroborate that phosphorylation of Ser187 in dense-core vesicle release in chromaffin cells is strong, no previous evidence indicates that this site is important for release of synaptic vesicles. Synaptic vesicles like dense-core vesicles exhibit multiple kinetic components of release, but the rates are about tenfold faster for synaptic vesicles (Mennerick and Matthews 1996; Sakaba and Neher 2001). Phosphomimetics of Aplysia SNAP-25 decreased the rate of depression (Fig. 3). Unlike in chromaffin cells where exocytosis can be measured directly using capacitance and amperometry measurement, we can measure exocytosis only indirectly using the EPSP in the motor cells. Nevertheless, this result is similar to a study in adrenal chromaffin cells where overexpression of phosphomimetic mutants at SNAP-25 Ser187 resulted in enhanced vesicle recruitment (Nagy et al. 2002). In contrast to our results, this study demonstrated that overexpression of nonphosphorylated-state mutants at SNAP-25 Ser187 resulted in inhibition of vesicle pool refilling (Nagy et al. 2002). However, in this study the stimulus used for release led to enhanced phosphorylation of SNAP-25. Because PKC is not activated during 0.05-Hz stimulation and there was no effect of the nonphosphorylated-state EGFP–SNAP-25 on depression, it is unlikely that PKC phosphorylation of SNAP-25 plays a role in depression in Aplysia sensory–motor neuron synapses.

Synaptic depression in Aplysia appears to consist of two separate mechanisms. Klein and colleagues compared the effect of depression on the readily releasable pool (as measured by transmitter release by sucrose) and the effect on overall release. They found that only a fraction of the decrease in synaptic transmission during depression was the result of a decrease in the readily releasable pool (RRP) and the remainder was the result of a decrease in calcium-secretion coupling (Fig. 6; Zhao and Klein 2002). The percentage of depression attributed to the decrease in the RRP is approximately the same as the amount of depression recovered by expressing the
FIG. 4. Rates of depression. A: after the first EPSPs, phorbol 12,13-dibutyrate (PDBu, 100 nM) was applied to induce synaptic facilitation. Interstimulus interval (ISI) between the first and the second EPSP was 10 min. Sensory-to-motor transmission was then depressed by a series of 40 repeated intracellular stimuli (ISI = 20 s). EPSP amplitude was normalized to the initial control value. EGFP (black dotted line; n = 11); EGFP–SNAP-25 (black solid line; n = 12); EGFP–SNAP-25 S-A (gray dotted line; n = 5); EGFP–SNAP-25 + bisinolylmaleimide I (Bis; gray solid line; n = 5). Region between 0 and 150% on the y-axis was expanded for clarity below. We removed one anomalous experiment with an extremely high facilitation (13-fold) from the SNAP-25 group. Comparison of the steady-state level of depression (stimuli 15–20) between the EGFP–SNAP-25 and the EGFP control was quantified by using a nonparametric bootstrap test procedure (see Fig. 2). Bootstrap test showed that EGFP–SNAP-25 was significantly different from EGFP–SNAP-25 + Bis (P < 0.005) throughout the period of steady-state depression. With respect to EGFP–SNAP-25 S-A there was a significant difference throughout the entire stimulation period (P < 0.05), apart from stimuli 18 and 19 (P = 0.0535). Similarly, EGFP alone, compared with EGFP–SNAP-25 + Bis, showed a significant difference (P < 0.005) throughout the steady-state stimulation period and vs. EGFP–SNAP-25 S-A (P < 0.05). B: initial effect of PDBu. A measurement of the fold increase in EPSP amplitude by PDBu between EPSP1 and EPSP2. Unpaired one-tailed t-test with Welch correction showed that EGFP–SNAP-25 was significantly different from both EGFP–SNAP-25 S-A and Bis (P < 0.01) and that PDBu significantly increases the EPSP vs. the wild-type EGFP–SNAP-25, in the absence of PDBu (P < 0.001).
phosphomimetic mutants, suggesting that these mutants act by blocking this decrease in the RRP seen with stimulation.

**Phorbol esters increase transmission at naïve synapses through phosphorylation of SNAP-25**

A striking result from this study is that the increase in transmitter release seen after PDBu treatment is almost completely blocked by expression of the SNAP-25 S-A mutant. This suggests that PDBu increases synaptic strength mainly through PKC phosphorylation of SNAP-25. Finley et al. (2003) examined the effect of replacement of SNAP-25 with phosphomimetic mutants in hippocampal neurons, but saw no effect of S187A mutants on PDBu increases in transmitter release and actually saw an increase in synaptic failures with an S187E

**FIG. 5.** Scatterplot of initial EPSP for all constructs. Despite large variability, none of the mutants significantly changed the initial EPSP. Horizontal lines indicate the average value in each group (ANOVA; P > 0.5). EGFP (n = 24); SNAP-25 + EGFP (n = 11); EGFP–SNAP-25 S-C (n = 8); EGFP–SNAP-25 (n = 14); EGFP–SNAP-25 S-A (n = 8); EGFP–SNAP-25 S-D (n = 7); EGFP–SNAP-25 S-E (n = 7).

**FIG. 6.** Short-term facilitation of depressed synapses is partly blocked by overexpression of EGFP–SNAP-25 and EGFP–SNAP-25 S-D but not by any of the other mutants. A: sensory-to-motor transmission was depressed by a series of 40 repeated intracellular stimuli (ISI = 20 s) and 5-hydroxytryptamine (5-HT, 10 μM) was applied to induce synaptic facilitation, followed by an additional 10 stimuli. Control (n = 35), we pooled cells injected with EGFP alone with the cells where EGFP and SNAP-25 were injected separately: EGFP alone (n = 24); SNAP-25 + EGFP (n = 11). B: amount of facilitation was calculated as the difference between the average of the 3 normalized EPSPs after and before 5-HT application: EPSP 2 (after 5-HT) − EPSP 1 (before 5-HT). Unpaired one-tailed t-test with Welch’s correction showed that EGFP–SNAP-25 was significantly different from control (P < 0.05) (SNAP-25 + EGFP, n = 35 as in A); EGFP–SNAP-25 S-C, n = 6; EGFP–SNAP-25, n = 14; EGFP–SNAP-25 S-A, n = 7; EGFP–SNAP-25 S-D, n = 7; EGFP–SNAP-25 S-E, n = 7.
PKC phosphorylation site. In chromaffin cells the role of PKC residues in the C-terminal portion of SNAP-25 containing the phosphorylation. Most of the above effect was localized to the 180–197 was sufficient to decrease the sensitivity of release to depolarization. Exogenously expressing SNAP-25 into GABAergic neurons negatively regulates neuronal calcium responsive-}

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SNAP-25 was sufficient to increase transmitter release, one would have expected the phosphomimetics to significantly increase initial synaptic strength, although there was no trend to suggest this effect. One possibility is that the phosphomimetic SNAP-25s do not effectively mimic phosphorylated SNAP-25 for this physiological effect. Another possibility is that prolonged expression of SNAP-25 is necessary, but not sufficient, for the phorbol-ester–mediated increases in synaptic strength. Thus additional substrates of PKC, or activation of other phorbol-ester targets such as Munc-13 or ras guanine exchange factors (Dyer et al. 2003) may also be required in addition to SNAP-25 phosphorylation for the increase in transmitter release. Because 5-HT increases synaptic strength at depressed synapses through PKC (Ghirardi et al. 1992; Mansseau et al. 2001), why does 5-HT not increase the synaptic strength of naïve synapses through PKC activation, especially because phorbol esters can? Many explanations have been proposed for this conundrum. One possibility is that the isoforms of PKC activated by phorbol esters and 5-HT may be distinct (Zhao et al. 2006) and phosphorylation of SNAP-25 may be isoform specific. Another possibility is that the initial increase in synaptic strength caused by activation of PKA may make irrelevant the modifications induced by 5-HT. Indeed, some data suggest that prolonged applications of 5-HT do result in PKC-mediated increases in synaptic strength at naïve synapses (Jin et al. 2005). PKA-mediated phosphorylation of SNAP-25 at a separate site may also increase synaptic strength (Hepp et al. 2002; Nagy et al. 2004).

Does SNAP-25 modulation of calcium dynamics play a role in depression?

During HSD the depression of transmitter release occurs downstream of calcium influx (Armitage and Siegelbaum 1998) and probably affects calcium-secretion coupling. A recent study in hippocampal GABAergic synapses shows that SNAP-25 negatively regulates neuronal calcium responsiveness to depolarization (Verderio et al. 2004). These neurons naturally lack SNAP-25, but contain the related SNAP-23. Exogenously expressing SNAP-25 into GABAergic neurons was sufficient to decrease the sensitivity of release to depolarization. Most of the above effect was localized to the 180–197 residues in the C-terminal portion of SNAP-25 containing the PKC phosphorylation site. In chromaffin cells the role of PKC is to increase the proportion of vesicles that are highly sensitive to calcium (Yang et al. 2002). These results together suggest a model where SNAP-25 naturally inhibits a step in calcium secretion coupling and that PKC activation can reverse this step.

In our experiments, overexpression of EGFP-coupled SNAP-25 acted as a dominant negative for the 5-HT–mediated reversal of depression, but not for initial synaptic strength or PDBu-mediated increases in synaptic strength. The specificity of the dominant negative effect suggests a distinct role of SNAP-25 in regulating depression independent of its role in transmitter release and vesicle availability. Thus although somewhat speculative, one model that is consistent with our data posits that during depression SNAP-25 acts to inhibit release at a postpriming step and thus blocking calcium-secretion coupling. This inhibition by SNAP-25 is sensitive to the amino acid at the phosphorylation site and thus many of the mutants do not compete with endogenous SNAP-25 for this interaction. The fact that SNAP-25 cannot be released through PKC phosphorylation. This may be attributable either to phosphorylation of SNAP-25 by PKC or to PKC phosphorylation of an interacting partner.

How could SNAP-25 mediate this inhibition? The carboxy-terminal region is also required for Ca\(^{2+}\)--dependent interaction of the calcium sensor Syt I and SNAP-25 during Ca\(^{2+}\)--triggered exocytosis (Gerona et al. 2000; Zhang et al. 2002). The interaction of Syt I with the SNARE complex is a leading candidate for the calcium-sensitive step regulating exocytosis (Bai et al. 2004; Tucker et al. 2004) and thus regulation of this interaction by phosphorylation could regulate calcium-secretion coupling. In previous work we showed that Syt I binding to SNAP-25 increases about two- to fourfold in the presence of calcium (Nakhost et al. 2004). However, we did not observe any differences in Syt I–SNAP-25 interactions with any of the mutations in the phosphorylation site (data not shown; see Nakhost et al. 2004). It should be noted though that these experiments investigated only the low-affinity interaction of Syt I with SNAP-25 compared with the high-affinity interaction of Syt I with the entire SNARE complex because SNAP-25 is largely unstructured as a monomer but acquires \(\alpha\)-helicity in binary and ternary SNAP-25–G protein complexes (Fasshauer et al. 1997). The G-protein \(\beta\gamma\) inhibits exocytosis downstream of priming through binding to the C-terminus of SNAP-25 (Gerachshenko et al. 2005), perhaps interfering with the Ca\(^{2+}\)--dependent binding to Syt I (Blackmer et al. 2005). Removal of this inhibition by 5-HT could be blocked by the EGFP–SNAP-25 dominant negative construct.

In conclusion, our data support that PKC phosphorylation of SNAP-25 is necessary, but probably not sufficient, for PDBu-mediated increases in transmitter release. Moreover, we provide suggestive evidence that SNAP-25 may play an important role in depression.
role in the block of calcium-secretion coupling that is important for HSD.

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