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Exocytotic mechanism studied by truncated and zero layer mutants of the C-terminus of SNAP-25

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The highly conserved SNARE proteins, SNAP-25, syntaxin and synaptobrevin, form a tight ternary complex, which is essential for exocytosis. Crystallization of this complex revealed a four-helix bundle with an unusual hydrophilic layer (zero layer) in its center. In order to evaluate the role of this layer in different kinetic components of secretion, we used the Semliki Forest virus (SFV) system to infect adrenal chromaffin cells with SNAP-25 Q174L, a point mutant in the zero layer. Using combined flash photolysis of caged calcium and membrane capacitance measurements, we investigated its effect on the exocytotic burst and sustained phase of exocytosis with high time resolution. Cells expressing SNAP-25 Q174L displayed a selective reduction in the sustained phase, while the two components of the exocytotic burst remained unaffected. Furthermore, the exocytotic response to the second flash was significantly reduced, indicating a decrease in refilling kinetics. We therefore conclude that the zero layer is critical for the formation of SNARE complexes, but that it plays no role in the dynamic equilibrium between the two exocytosis-competent vesicle pools.

Keywords: capacitance measurements/exocytosis/secretion/SNAP-25/SNARE complex

Introduction

The process of exocytosis is mediated by the fusion of vesicle membrane and plasma membrane. A highly conserved set of proteins, the SNAREs [soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) attachment protein receptors] were found to play a key role in inducing this fusion upon Ca^{2+} influx (Söllner *et al*., 1993; Südhof, 1995; Weber *et al*., 1998; Hilfiker *et al*., 1999). According to present theories, the assembly and disassembly of SNARE proteins is essential for SNARE function (Rothman, 1994; Nichols and Pelham, 1998). Syntaxin and SNAP-25, two SNAREs located at the plasma membrane, and synaptobrevin, a SNARE located at the synaptic vesicle membrane, have been shown to form a highly stable, ternary complex (Fasshauer *et al*., 1997; Sutton *et al*., 1998; Weis and Scheller, 1998). This complex can exist in two different conformations: a *trans* and a *cis* conformation (Hanson *et al*., 1997b; Lin and Scheller, 1997). It is largely accepted that SNARE complexes in the *trans* configuration play an important role in membrane fusion (Hanson *et al*., 1997a; Sutton *et al*., 1998). The ternary complex is comprised of four α-helices, each helix containing a leucine zipper-like layer consisting of 15 hydrophobic residues. Embedded within this layer is an ionic layer formed by three glutamines and one arginine residue, the so-called zero layer (Fasshauer *et al*., 1998; Sutton *et al*., 1998). Amino acids 150–206 of SNAP-25 form one of the four helices in the SNARE complex structure (Weimbs *et al*., 1997; Fasshauer *et al*., 1998).

High time resolution measurements in adrenal chromaffin cells revealed the existence of different kinetic phases of secretion (for a recent review, see Neher, 1998). A rapid increase in intracellular Ca^{2+} leads to an exocytotic burst followed by a sustained phase of secretion. The burst can be further resolved into two kinetically distinct components, suggesting the presence of two separate exocytosis-competent pools of vesicles (Heinemann *et al*., 1994). These pools most likely correspond to *trans*-SNARE complexes existing in a dynamic equilibrium between a loose and a tight state (Voets *et al*., 1999). While both states can support Ca^{2+}-triggered exocytosis, they can be distinguished through their sensitivity to either botulinum and tetanus toxins (Xu *et al*., 1998) or SNARE-specific antibodies (Xu *et al*., 1999). Moreover, recent experiments indicate that assembly of *trans*-SNARE complexes and membrane fusion are tightly linked (Chen *et al*., 1999).

In the current study, we investigated the effect of native and mutant SNAP-25 on secretion in adrenal chromaffin cells. Overexpression of wild-type SNAP-25 had no significant effect on secretion. Overexpression of SNAP-25 Δ9, in which the last nine C-terminal residues are missing, led to a selective reduction of the fast component of the exocytotic burst, in agreement with previous data on the cleavage of endogenous SNAP-25 by botulinum toxin A (BoNT/A) (Xu *et al*., 1998; Criado *et al*., 1999). Finally, overexpression of SNAP-25 Q174L, a point mutant in the zero layer of the SNARE complex, led to a reduction in the sustained phase, while the two components of the exocytotic burst remained unaffected. The results presented here imply that the replenishment rate of the exocytosis-competent vesicles in SNAP-25 Δ9- and SNAP-25 Q174L-overexpressing cells is reduced. Furthermore, our data indicate that the zero layer is not involved in the transition between the two states of SNARE complexes, but that it plays a critical role in the assembly of the SNARE complex.

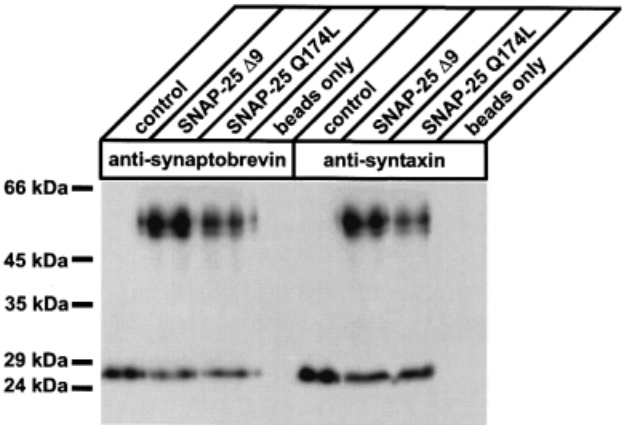

Fig. 1. Overexpressed SNAP-25 mutants are able to assemble into SNARE complexes *in vivo*. Immunoblot analysis of co-immunoprecipitations of endogenous SNAP-25 and overexpressed SNAP-25 mutants from Triton X-100-solubilized chromaffin cells. Control represents uninfected chromaffin cells (lanes 1 and 5), while in lanes 4 and 8 only the antibody-containing beads were loaded to estimate the background signal of the heavy and light chain derived from the IgGs. The infection efficiency was 12% for SNAP-25 Δ9 (lanes 2 and 6) and 15% for SNAP-25 Q174L (lanes 3 and 7), respectively. Immunoprecipitations were performed using monoclonal antibodies against synaptobrevin and syntaxin, respectively, which were covalently coupled to protein A–Sepharose.

Results

Overexpressed SNAP-25 mutants are able to assemble into SNARE complexes in vivo

We overexpressed two Semliki Forest virus (SFV)-based mutants of SNAP-25 in bovine chromaffin cells. In SNAP-25 Δ9, the last nine C-terminal amino acids had been deleted (see below). SNAP-25 Q174L carried a mutation in the zero layer of the C-terminal α-helix of SNAP-25 (see below). To verify expression, green fluorescent protein (GFP) was attached to the N-terminus of both constructs. Quantitative Western blot analysis showed that the ratio of overexpressed SNAP-25 Δ9 or SNAP-25 Q174L to endogenous SNAP-25 was ~25:1 (data not shown). In order to examine whether the overexpressed mutants were also able to form functional SNARE complexes *in vivo*, we performed immunoprecipitation experiments on infected chromaffin cells with synaptobrevin (VAMP)- and syntaxin 1-specific antibodies. Following SDS–PAGE and Western blotting, endogenous SNAP-25 and overexpressed SNAP-25 mutants were detected using a SNAP-25-specific antibody. As shown in Figure 1, the endogenous SNAP-25 in uninfected chromaffin cells yielded a single band at ~25 kDa, representing SNAP-25 molecules that had been incorporated into SNARE complexes in these cells. In both SNAP-25 Δ9- and SNAP-25 Q174L-overexpressing cells, an additional band with an apparent mol. wt of ~55 kDa could be detected. The detected band corresponds well with the predicted molecular weight of GFP–SNAP-25 Δ9 and GFP–SNAP-25 Q174L fusion proteins (52.4 kDa). Thus, our data demonstrate that these constructs could assemble with syntaxin and synaptobrevin (VAMP) into SNARE complexes *in vivo*.

Kinetic study of calcium-triggered secretion in SNAP-25-infected cells

We were first interested whether overexpression of wild-type SNAP-25 had any effect on catecholamine secretion. Therefore, we infected bovine adrenal chromaffin cells with an SFV-based SNAP-25 construct (see Materials and methods). In order to verify expression and to avoid any perturbation of the SNAP-25 C-terminus, GFP was attached to the N-terminus of SNAP-25. Chromaffin cells were held in the whole-cell patch–clamp configuration in order to dialyze the caged calcium compound into the cytosol and to measure membrane capacitance (Neher and Marty, 1982; Henkel and Almers, 1996). Secretion was elicited by flash photolysis of the caged calcium compound nitrophenyl-EGTA after ~3 min of dialysis. A second flash was applied 2 min after the first one. During the first 10 s after the flashes, we employed high time resolution capacitance measurements to record the secretory response (Figure 2A). Following a flash, calcium was elevated to levels between 10 and 20 μM. This elicited in control cells a robust capacitance increase with two distinct phases (Figure 2B), which we call the exocytotic burst and the sustained phase (Neher and Zucker, 1993; Heinemann *et al*., 1994; Xu *et al*., 1998). The exocytotic burst represents the fusion of those vesicles, which are in a release-ready state and require only elevation of $[Ca^{2+}]_i$ for exocytosis (Heinemann *et al*., 1993; Thomas *et al*., 1993; Gillis *et al*., 1996). It was postulated that the exocytotic burst is mediated by fusion-competent SNARE complexes (*trans* complexes), which may consist of either loosely or tightly assembled SNAREs. The sustained phase may represent the recruitment of additional vesicles that must undergo multiple steps of priming and maturation before they are ready for fusion (Heinemann *et al*., 1993; Thomas *et al*., 1993; Henkel and Almers, 1996; Xu *et al*., 1998). Therefore, in the sustained phase, the capacitance increases at a much slower rate with a time constant in the range of 5–30 s (Moser and Neher, 1997; Xu *et al*., 1998). In Figure 2B and C, the capacitance increase in response to the first flash was slightly larger in SNAP-25-overexpressing cells than in control cells. However, the response to a second flash given 2 min after the first one was ~30% smaller in infected cells. Since we also observed a 30% reduction in the second flash response of cells overexpressing GFP alone (see Figure 8), this effect most likely results from the infection method and not from a specific effect of SNAP-25.

Previous work has shown that the exocytotic burst can be further resolved into two distinct components when analyzed at high time resolution (Thomas *et al*., 1993; Heinemann *et al*., 1994; Xu *et al*., 1998; Voets *et al*., 1999). We therefore performed a similar kinetic analysis in order to define which of the kinetically defined phases of the burst was affected by overexpression of SNAP-25. For this analysis, we fitted C_m traces from cells overexpressing SNAP-25 and from control cells with a triple exponential. The first exponential component represented the fast burst, the second component represented the slow burst (Figure 3). In response to the first flash, the amplitudes and time constants of the two components in control cells were similar to those of infected cells. The time constants of the fast and slow burst components were 40.5 ± 6.5 ms (n = 19) and 360.4 ± 52.5 ms (n = 23) for control cells, and 40.0 ± 5.3 ms (n = 19) and 425.4 ± 68.2 ms (n = 23) for SNAP-25-overexpressing cells, respectively. Detailed comparison of the normalized C_m responses (Figure 3A), the two components of the

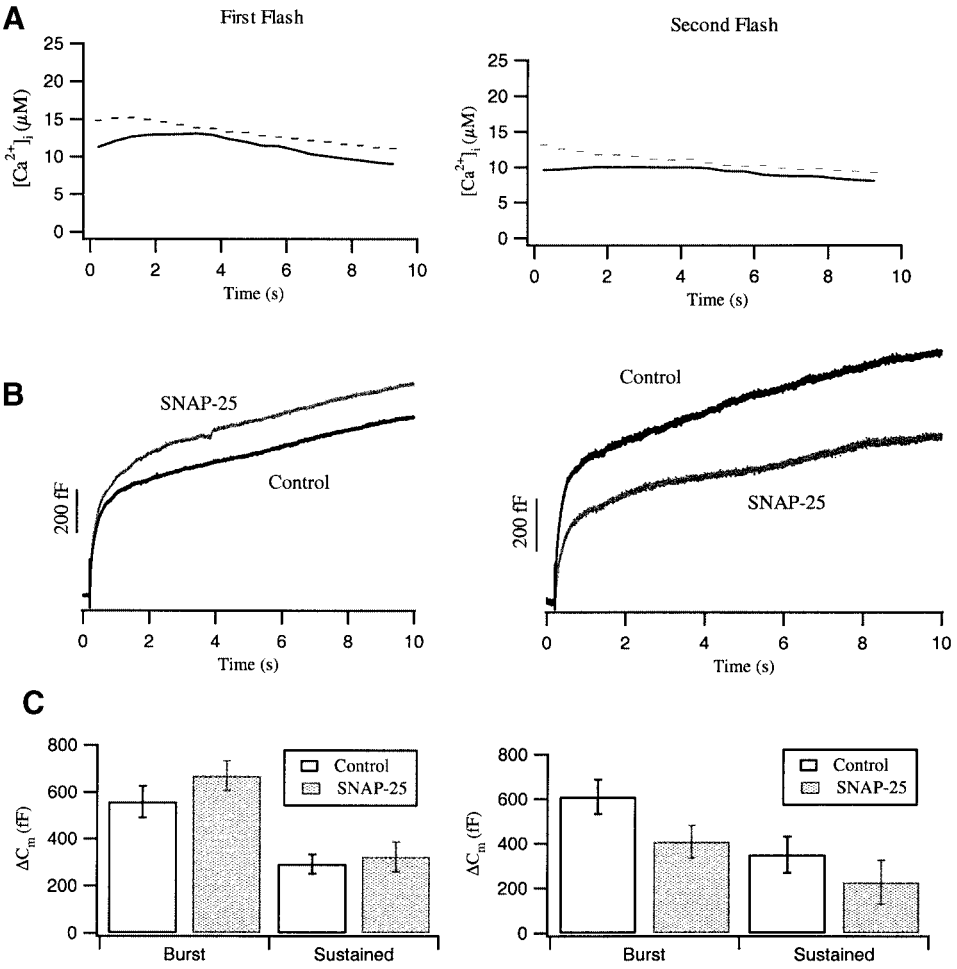

Fig. 2. Averaged, exocytotic responses to two consecutive flashes (first flash, left panel; second flash, right panel) in control and SNAP-25-overexpressing cells. (**A**) The averaged $[Ca^{2+}]_i$ level resulting from flash photolysis of the caged calcium compound nitrophenyl-EGTA in control (solid line, $n = 23$) and SNAP-25-overexpressing cells (dashed line, $n = 26$). (**B**) Corresponding capacitance traces in response to the first and second flash from paired experiments. (**C**) Averaged amplitudes of the exocytotic burst and the sustained phase for control (open bar) and SNAP-25-overexpressing cells (shaded bar). The amplitudes of the exocytotic burst were measured as the C_m increase during the first 1.8 s after each flash, the sustained phase was measured as the C_m increase between 1.8 and 9.8 s after each flash. The kinetic phases in response to the first flash were not significantly different. Values are given as the mean ± SE.

burst (Figure 3B) and the initial rate of C_m increase versus $[Ca^{2+}]_i$ in the burst (Figure 3C) revealed no significant difference between SNAP-25-overexpressing cells and control cells. It demonstrated that overexpression of SNAP-25 did not change the kinetic properties of the exocytotic burst in response to the first flash. We therefore conclude that overexpression of SNAP-25 does not significantly modify the secretion behavior in adrenal chromaffin cells, in agreement with a recent report by Criado *et al*. (1999). It should be noted, though, that a reduction of the autaptic response occurred in cultured rat hippocampal neurons infected with SNAP-25 (Owe-Larsson *et al*., 1999).

Overexpression of SNAP-25 Δ9 leads to a selective reduction of the fast burst component

Based on the structure of the SNARE complex, amino acids 150–206 of the C-terminus of SNAP-25 form one of the four helices in the SNARE complex structure. In previous studies, cleavage of the last nine amino acids from the C-terminus of SNAP-25 by BoNT/A reduced both the fast component of the exocytotic burst and the sustained phase (Xu *et al*., 1998). Also, BoNT/A-cleaved SNAP-25 is unable to form an SDS-resistant SNARE complex (Hayashi *et al*., 1995; Chen *et al*., 1999), demonstrating that the C-terminus of SNAP-25 functions to stabilize the SNARE complex.

We investigated the effect of the SNAP-25 C-terminus on exocytosis by overexpressing SNAP-25 Δ9 in chromaffin cells. As for wild-type SNAP-25, GFP was attached as a fluorescent marker to the N-terminus of SNAP-25 Δ9. A similar truncation construct was used by Criado *et al*. (1999) and was shown to reduce the rate of secretion, as measured by amperometry. As shown in Figure 4, the amplitudes of the exocytotic burst and the sustained phase in response to the first flash were 586.5 ± 71 and 309.9 ± 61.5 fF for control cells ($n = 28$), and 397.1 ± 50 and 109 ± 25.9 fF for SNAP-25 Δ9-overexpressing cells ($n = 27$). The reductions of the sustained phase in the first flash and of both phases in the second flash were statistically significant. (*t*-test, $p < 0.01$).

Kinetic analysis of the exocytotic burst in response to the first flash revealed a prominent slow-down of secretion in SNAP-25 Δ9-overexpressing cells (Figure 5A). When

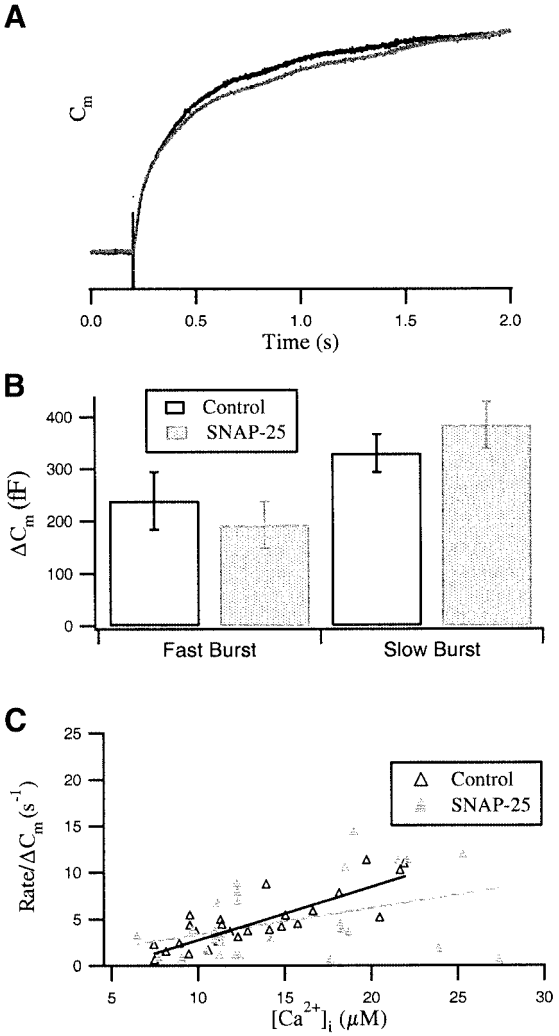

Fig. 3. The exocytotic burst in response to the first flash is not changed in SNAP-25-overexpressing cells. (**A**) Normalized C_m traces of control (dark line) and SNAP-25-overexpressing cells (light line) are very similar during the time course of the exocytotic burst (the first 2 s after the flash). (**B**) Comparison of the amplitudes of the fast and the slow burst components for the first flash. The values for control (open bar) and SNAP-25-overexpressing cells (shaded bar) are not significantly different. (**C**) The initial rate of C_m increase is plotted against $[Ca^{2+}]_i$ for control (△) and SNAP-25-overexpressing cells (▲). Data were normalized with respect to the C_m increase (ΔC_m) during the first 2 s after the flash. The solid lines represent the computerized fit for each data set. No significant change was observed in the $[Ca^{2+}]$ dependence of the initial rate constants.

we fitted the C_m traces of control cells with a triple exponential, 20 out of 28 flash responses could be adequately fitted. In contrast, 18 out of 27 SNAP-25 Δ9-overexpressing cells could be fitted by a double exponential, indicating a selective loss of one exocytotic component. The amplitude of the fast and slow burst components was 265.4 ± 62.3 and 227.1 ± 35.3 fF in control cells, and 54.6 ± 23 and 252.8 ± 43 fF in SNAP-25 Δ9-overexpressing cells, respectively (Figure 5, see also Table I). From these data, it was evident that the fast burst component was reduced by 80% in SNAP-25 Δ9-overexpressing cells, whereas the slow burst component remained unaffected (Figure 5B). As a consequence, the initial rate of capacitance increase was slower in SNAP-25 Δ9-overexpressing cells compared with control cells (Figure 5C). Our results are consistent with the effect reported for BoNT/A, which impairs exocytosis without affecting assembly of SNARE complexes (Hayashi *et al*., 1995; Otto *et al*., 1995; Bruns *et al*., 1997). Since this retardation was not connected to a reduction of the intracellular Ca^{2+} concentration (Figure 4A), we suggest that the truncated form of SNAP-25 is compromised in mediating a particularly rapid interaction between calcium sensor and release machinery for catecholamine release. We speculate that the formation of tight *trans* complexes is inhibited in SNAP-25 Δ9-overexpressing cells (see Discussion). The reduction of the sustained phase, which represents the refilling and subsequent fusion of vesicles of the release-ready pool during the time of high intracellular calcium (10 s), implies that the rate of this refilling process is decreased too. This assumption is supported by the largely reduced amplitudes of both exocytotic burst and sustained phase in the second flash response of SNAP-25 Δ9-overexpressing cells (Figure 4C). Finally, the similarity to results reported on BoNT/A-treated chromaffin cells in which the endogenous SNAP-25 is cleaved (Xu *et al*., 1998) provides evidence that the virus-based overexpression strategy yields physiologically functional SNAP-25 Δ9 molecules.

The zero layer of the SNARE complex is critical for complex formation, but not for the last step of membrane fusion

Crystallization of the SNARE complex revealed a hydrophilic layer ('zero layer') embedded in the center of a four-helix bundle (Sutton *et al*., 1998). It was shown recently that a point mutation of one of the hydrophilic residues, SNAP-25 Q174A, decreased the thermal stability of the SNARE complexes (Chen *et al*., 1999).

In order to understand the role of the zero layer in exocytosis, we overexpressed SNAP-25 Q174L, a zero layer mutant in the C-terminal helix of SNAP-25, in chromaffin cells. In response to the first flash, only a small reduction in the exocytotic burst was detected in these cells (779 ± 75 fF for control; 597 ± 61 fF for SNAP-25 Q174L), while the sustained phase was significantly reduced by 48% (434 ± 85 fF for control; 226 ± 40 fF for SNAP-25 Q174L) (Figure 6B, left panel). The reduction in the sustained phase already indicated a decrease in the refilling kinetics. This potential decrease became more evident in the C_m response to a second flash given 2 min after the first one (Figure 6B and C, right panel). While in control cells the amplitudes of the exocytotic burst and the sustained phase were comparable between the two flashes (848 ± 101 and 629 ± 97 fF for the second flash), SNAP-25 Q174L-overexpressing cells displayed a significant reduction by 56% for the exocytotic burst and by 35% for the sustained phase (260 ± 39 and 147 ± 41 fF for the second flash). If one assumes that physiological refilling of release-competent pools requires the formation of new SNARE complexes, these data imply that the zero layer plays an important role in the formation of these complexes.

The kinetic analysis of the exocytotic burst in response to the first flash confirmed that the normalized C_m responses of control and SNAP-25 Q174L-overexpressing cells are virtually indistinguishable (Figure 7A). Furthermore, the amplitudes of both exocytotic burst components

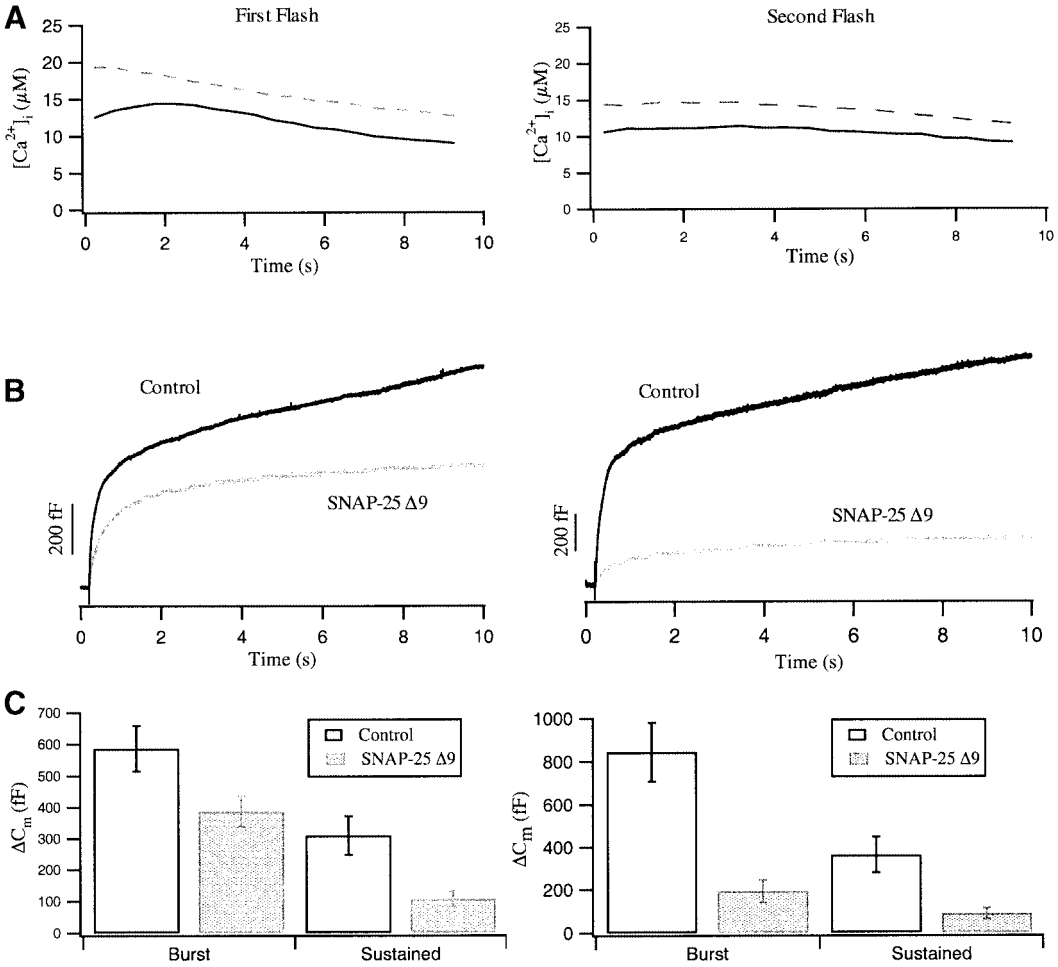

Fig. 4. SNAP-25 Δ9-overexpressing cells display a reduction in the exocytotic burst and the sustained phase. (**A**) The averaged $[Ca^{2+}]_i$ level in control (solid line, $n = 28$) and SNAP-25 Δ9-overexpressing cells (dashed line, $n = 27$) in response to the first (left panel) and second flash (right panel). (**B**) Corresponding capacitance traces in response to the first and second flash from paired experiments. (**C**) Averaged amplitudes of the exocytotic burst and the sustained phase for control (open bar) and SNAP-25 Δ9-overexpressing cells (shaded bar). The reductions of the sustained phase in the first flash and of both phases in the second flash were statistically significant (*t*-test, $p < 0.01$).

and the initial rate constant of the exocytotic burst on $[Ca^{2+}]_i$ were not altered (Figure 7B and C). We can therefore conclude that the zero layer, in contrast to the last nine amino acids at the C-terminus of SNAP-25, plays no role in the dynamic equilibrium between the two exocytotic burst components.

Figure 8 summarizes the amplitudes of the kinetic components for all proteins used in this study. The overexpression data of GFP alone were added to demonstrate that it did not cause a significant alteration in any of the kinetic components. This finding is in agreement with recent reports, which demonstrated that the morphology and the basic physiological features of chromaffin cells were unaffected by the infection with SFV-based constructs (Ashery *et al.*, 1999; Duncan *et al.*, 1999). Also, Figure 8 shows that although the sustained phases in all types of infected cells were smaller than their corresponding exocytotic burst, only in SNAP-25 Δ9- and SNAP-25 Q174L-overexpressing cells were the sustained phases significantly reduced when compared with those of control cells (*t*-test, $p < 0.01$ and $p < 0.05$, respectively) (Figure 8A). In the second flash, both phases were reduced significantly in SNAP-25 Δ9- and SNAP-25 Q174L-overexpressing cells (*t*-test, $p < 0.001$), but not in GFP- and wild-type SNAP-25-overexpressing cells (Figure 8B). If we focus on the two exocytotic burst components and normalize the data with their own control values of fast and slow burst, respectively, we find that only in SNAP-25 Δ9-overexpressing cells was the fast burst component significantly reduced, whereas the slow burst component was unaffected for all types of infected cells (Figure 8C). Even higher time resolution of the C_m responses of the first flash showed that in SNAP-25 Δ9-overexpressing cells there was a 4.6 ms delay of the exocytotic burst (Figure 9A), while there was no delay in SNAP-25 Q174L-overexpressing cells (Figure 9B). This further demonstrates that the mutant of the zero layer did not affect the ability of SNAREs to form tight *trans* complexes.

Discussion

The formation and stability of the ternary SNARE complex consisting of synaptobrevin, syntaxin and SNAP-25 are believed to play a central role in the molecular mechanism underlying exocytosis. The C-terminus of SNAP-25, which contributes one helix to the four-helix bundle, has been implicated in the final step of calcium-triggered exocytosis (Chen *et al.*, 1999). However, the biochemical assay that

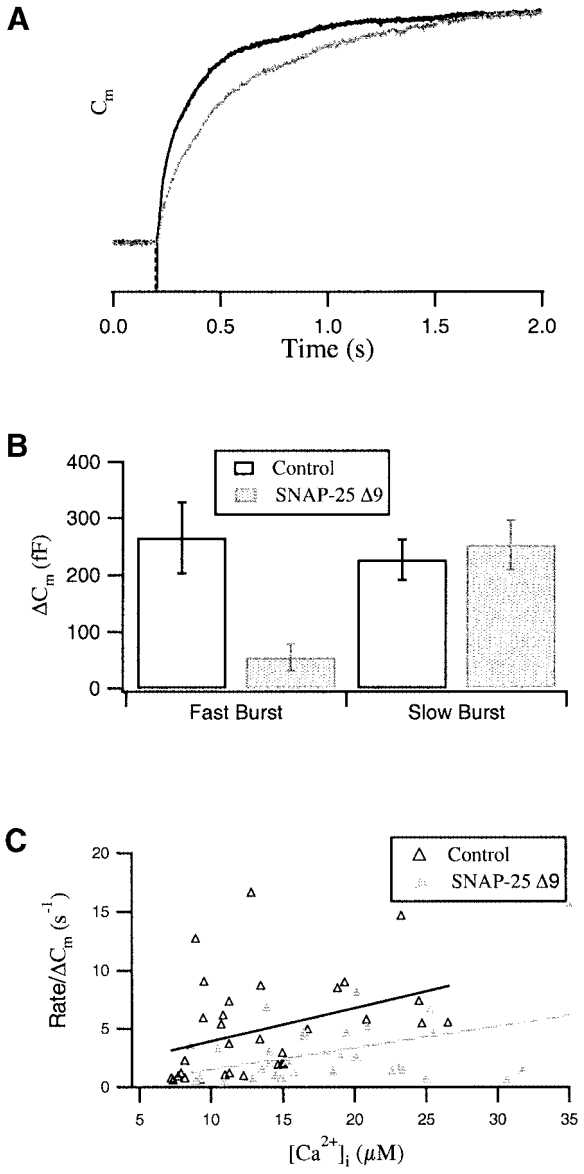

Fig. 5. The fast burst component is selectively reduced in SNAP-25 Δ9-overexpressing cells. (**A**) Normalized C_m traces of control (dark line) and SNAP-25 Δ9-overexpressing cells (light line) display a different time course within the first 2 s after the flash. (**B**) Comparison of the amplitudes of the fast and the slow burst components for the first flash. The fast burst component was reduced by ~80% in SNAP-25 Δ9-overexpressing cells (*t*-test; $p < 0.01$), while the slow burst component was unaffected. (**C**) The initial rate of C_m increase is plotted against $[Ca^{2+}]_i$ for control (△) and SNAP-25 Δ9-overexpressing cells (▲). Data were normalized with respect to the C_m increase (ΔC_m) during the first 2 s after the flash. The lines represent the computerized fit for each data set. The initial rate in SNAP-25 Δ9-overexpressing cells was slightly lower than in control cells.

was used in this study could not resolve different kinetic components of exocytosis. For this purpose, we used high time resolution capacitance measurements and molecular biological methods to study the functional effect of SNAP-25 C-terminal mutants on the very final membrane fusion stages of exocytosis and to establish links between the distinct kinetic components and molecular processes.

Both loosely and tightly assembled SNARE complexes are fusion competent

Before a vesicle can fuse with the plasma membrane, it has to translocate to the plasma membrane ('docking') and then undergo one or more maturation steps ('priming'). Although the molecular definition of docking remains unclear, it has been shown that when neurotoxins were injected into squid nerve terminals, vesicles remained tethered or even accumulated further at the active zone (Hunt *et al*., 1994). Thus, SNARE complex formation does not seem to be required for morphological docking. However, since neurotoxin treatment abolishes neurotransmitter release, the SNARE complex is required at a later, post-docking step in neuronal and neuroendocrine systems.

Previous work suggested that only SNARE complexes in the *trans* conformation, but not in the *cis* conformation, are fusion competent (Ryan, 1998; Ungermann *et al*., 1998). Since the formation of a SNAP-25–syntaxin complex greatly increases their individual affinity for synaptobrevin (Fasshauer *et al*., 1997; Sutton *et al*., 1998; Fiebig *et al*., 1999), it was suggested that *trans* complex formation is preceded by the formation of a syntaxin–SNAP-25 dimer. While uncomplexed or loosely assembled SNARE proteins are sensitive to proteolysis by neurotoxins, the fully assembled SNARE complex is resistant to proteolysis. Therefore, it has been postulated that the *trans* complex oscillates between two states: a loose, toxin-sensitive state and a tight, SDS-resistant state (Rizo and Südhof, 1998; Sutton *et al*., 1998; Fiebig *et al*., 1999). Furthermore, these states can be distinguished by biophysical methods (Xu *et al*., 1998, 1999).

Model of secretion in neuroendocrine cells and its corresponding kinetics

The results obtained in this study can be explained by hypothesizing an equilibrium between the releasable vesicle pools and a depot vesicle pool. They are highly compatible with a refined model (Voets *et al*., 1999) based on the original two-step model suggested by Heinemann *et al*. (1993).

$$\text{depot} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{SRP} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{RRP}$$
$$\text{SRP} \xrightarrow{\gamma_1} \text{C} \qquad \text{RRP} \xrightarrow{\gamma_2} \text{C}$$

Scheme 1

In this scheme, the depot pool is considered to be a large reserve pool of vesicles that can prime into the slowly releasable pool (SRP), which represents SNARE proteins that have formed loose *trans* complexes. Vesicles in the SRP either return to the depot pool or go to the readily releasable pool (RRP), which represents SNARE proteins that have formed tight *trans* complexes. Finally, vesicle fusion, which is represented by a transition to pool C, contributes to the increase of membrane capacitance. Fusion-competent SNARE complexes appear to be in a dynamic equilibrium between the two distinct states, which correspond in physiological terms to the two kinetic components of the exocytotic burst upon elevation of $[Ca^{2+}]$. Vesicles in the SRP fuse with a typical rate

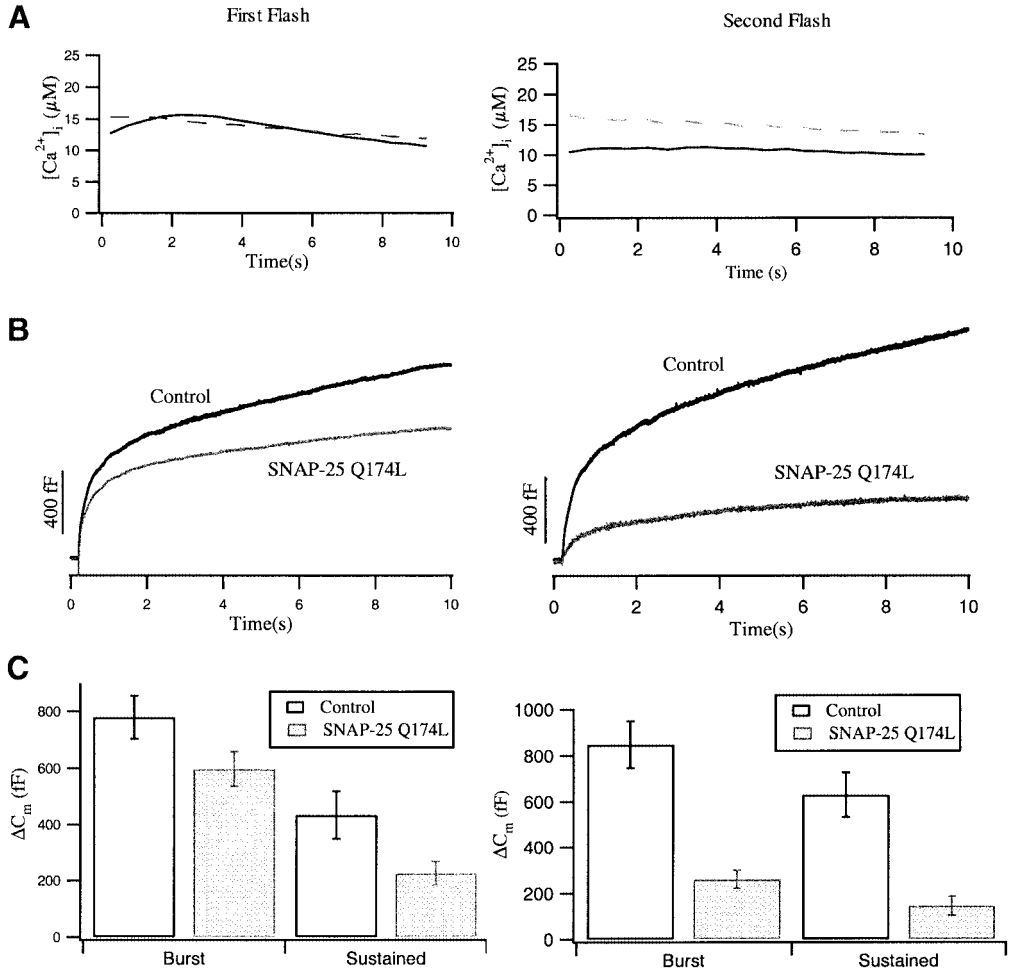

Fig. 6. SNAP-25 Q174L-overexpressing cells display a reduction of the sustained phase in response to the first flash (left panel) and a reduction of both burst and sustained phase in response to the second flash (right panel). (**A**) Average $[Ca^{2+}]_i$ level in control (solid line, $n = 23$) and SNAP-25 Q174L-overexpressing cells (dashed line, $n = 26$). (**B**) Corresponding capacitance traces in response to the first and second flash from paired experiments. (**C**) Averaged amplitudes of the exocytotic burst and the sustained phase for control (open bar) and SNAP-25 Q174L-overexpressing cells (shaded bar). In the first flash response (left panel), only the sustained phase was significantly reduced (*t*-test, $p < 0.05$), while in the second flash response (right panel) both exocytotic burst and sustained phase were significantly reduced (*t*-test, $p < 0.01$).

Table I. Amplitudes of fast and slow burst in paired experiments

	Fast burst (fF)		Slow burst (fF)	
	Control	Infected	Control	Infected
SNAP-25 wt	238.7 ± 54.7 (19)	192.8 ± 44.5 (26)	329.2 ± 36.2 (23)	383.7 ± 45.0 (26)
SNAP-25 Δ9	265.4 ± 62.3 (28)	54.6 ± 23.0 (27)	227.1 ± 35.3 (28)	252.8 ± 43.0 (27)
SNAP-25 Q174L	156.8 ± 29.4 (23)	137.6 ± 34.6 (26)	432.2 ± 49.3 (23)	357.3 ± 49.9 (26)

Values given are the mean ± SE. The values in parentheses represent the number of cells.

constant of ~3 s^{-1} (γ_1 in the model) while vesicles in the RRP fuse with a typical rate constant of ~30 s^{-1} (γ_2 in the model). During Ca^{2+}-evoked secretion, the rate of replenishment of vesicles from the depot pool to the SRP will be increased (Heinemann *et al*., 1993).

Our experiments revealed that the fast burst component was selectively reduced in the SNAP-25 Δ9-infected cells while the size and the rate constant of the SRP remained unaffected (see Figure 5). According to our model, such a change can be explained by a destabilization and resulting reduction of the RRP. It is important to note that our data obtained with the SFV-based overexpression of exogenous SNAP-25 Δ9 are in excellent agreement with previous studies using BoNT/A to cleave endogenous SNAP-25 (Xu *et al*., 1998). Since only 4% of the SNARE complexes in infected cells might contain native SNAP-25 (see Results), a contamination of our kinetic analysis by such a small fraction of native SNARE complexes would be hidden in the scatter between different cells. Thus, we conclude that the presence of endogenous SNAP-25 most

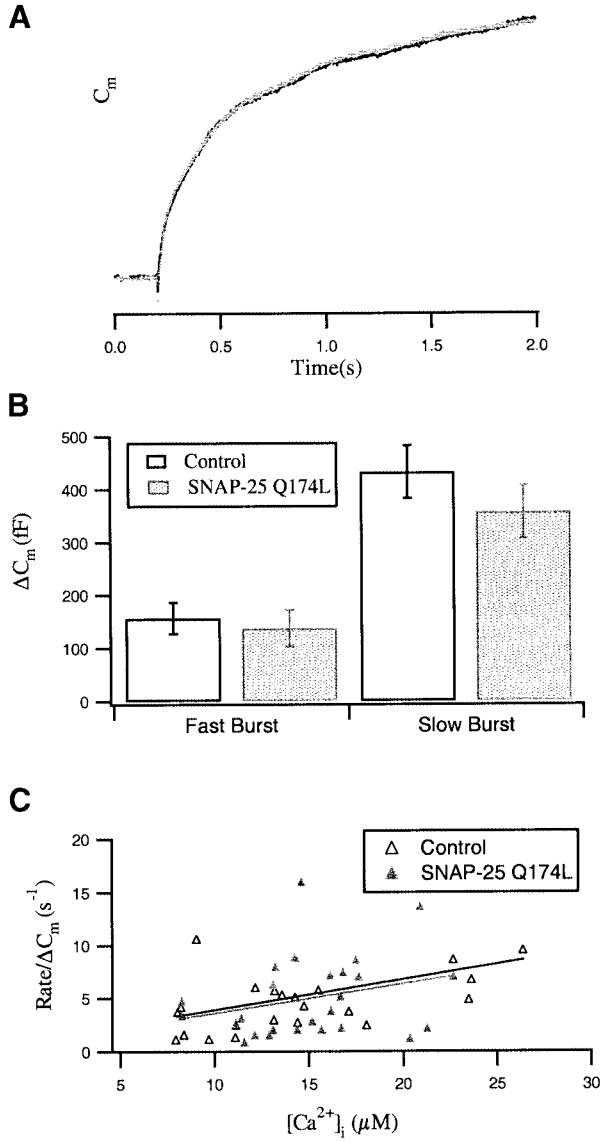

Fig. 7. The equilibrium between fast and slow burst component is unaffected in SNAP-25 Q174L-overexpressing cells. (**A**) Normalized C_m traces of control (dark line) and SNAP-25 Q174L-overexpressing cells (light line) are indistinguishable within the time course of the exocytotic burst (the first 2 s after the flash). (**B**) Comparison of the amplitudes of the fast and the slow burst components for the first flash. The values for control (open bar) and SNAP-25 Q174L-overexpressing cells (shaded bar) are not significantly different. (**C**) The initial rate of the C_m increase is plotted against $[Ca^{2+}]_i$ for control (△) and SNAP-25 Q174L-overexpressing cells (▲). Data were normalized with respect to the C_m increase (ΔC_m) during the first 2 s after the flash. The solid lines represent the computerized fit for each data set. The $[Ca^{2+}]$ dependence of the initial rate constant in SNAP-25 Q174L-overexpressing cells was almost identical to that in control cells.

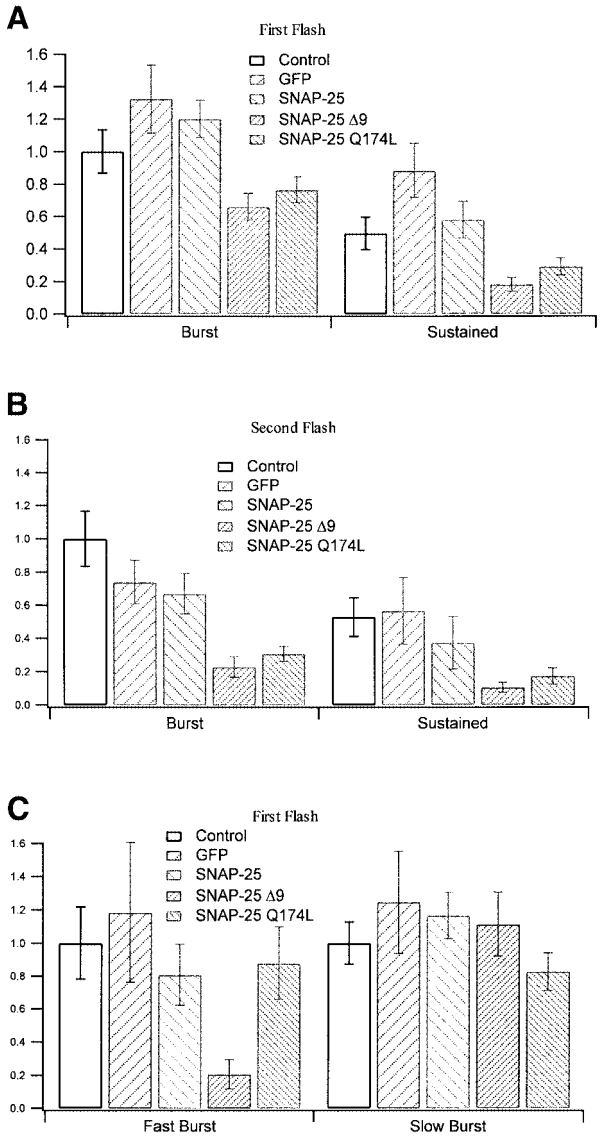

Fig. 8. Summary of the amplitudes of the different kinetic components of control and GFP-, SNAP-25-, SNAP-25 Δ9-, SNAP-25 Q174L-overexpressing cells (n = 83, 10, 26, 27 and 26, respectively). (**A**) The amplitudes of the exocytotic burst and the sustained phase in response to the first flash. GFP represents cells overexpressing GFP alone. Only in SNAP-25 Δ9- and SNAP-25 Q174L-overexpressing cells was the sustained phase significantly reduced (t-test, $p < 0.01$ and $p < 0.05$, respectively). (**B**) The amplitudes of the exocytotic burst and the sustained phase in response to the second flash. Although the amplitudes of the burst and sustained phase in infected cells were smaller than those of control cells, only in SNAP-25 Δ9- and SNAP-25 Q174L-overexpressing cells were both components significantly reduced (t-test, $p < 0.001$). The data in (A) and (B) were normalized to the amplitude of the exocytotic burst in control cells. (**C**) Summary of the amplitudes of the fast and slow burst components in response to the first flash. Only in SNAP-25 Δ9-overexpressing cells was the fast burst reduced. The data were normalized to the amplitudes of the fast and slow burst components in control cells, respectively.

likely did not influence our experiments and conclusions. Our data on the overexpression of SNAP-25 Δ9 are also in agreement with a recent report by Criado *et al.* (1999) using C-terminal deletion mutants of SNAP-25 and studying secretion by amperometry. Furthermore, recent work using a SNAP-25-specific antibody also showed a block of the fast exocytotic burst component, providing additional evidence for the existence of loose and tight SNARE complexes (Xu *et al.*, 1999).

A potential role for the zero layer in Ca^{2+}-dependent exocytosis

Since crystallization of the SNARE complex revealed the existence of a hydrophilic layer in the center of the four-helix bundle, there have been several hypotheses about the physiological function of this zero layer. One current

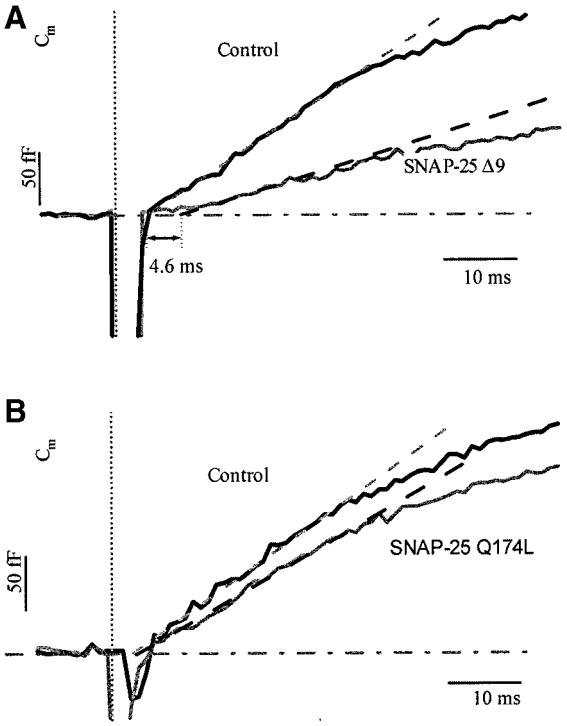

Fig. 9. The C_m responses in SNAP-25 Δ9-overexpressing cells display a delayed onset of the exocytotic burst in response to the first flash. (**A**) C_m responses of control (solid line, $n = 28$) and SNAP-25 Δ9-overexpressing cells (gray line, $n = 29$). The initial slopes are displayed in dashed lines, a 4.6 ms delay of the exocytotic burst in the SNAP-25 Δ9-infected condition was observed. (**B**) C_m responses of control (solid line, $n = 23$) and SNAP-25 Q174L-overexpressing cells (gray line, $n = 26$). No delay was found, which indicates that the mutant of the zero layer does not affect the formation of tight *trans* complexes.

hypothesis emerged from the observation that the leucine zipper layers act as a water-tight seal to shield the ionic interactions from the surrounding solvent. This seal might then further stabilize the four-helix oligomeric state and register of the complex by decreasing the local dielectric, thus enhancing electrostatic interactions within the ionic layer. Alternatively, when another protein such as α-SNAP or NSF might be able to puncture this seal, the ionic layer would be exposed to solvent and thereby facilitate disassembly of the complex (Sutton *et al*., 1998). However, biochemical data using SNARE complexes in which all four residues of the hydrophilic zero layer have been mutated to leucines showed that the sensitivity to NSF is increased (U.Matti and T.Binz, unpublished experiments), arguing against this hypothesis. On the other hand, it has been shown that mutations at the zero layer position of the C-terminal end of SNAP-25 decrease the melting point of the ternary complex (Chen *et al*., 1999; U.Matti and T.Binz, unpublished experiments), which is indicative of a decreased stability. In support of this hypothesis, a SNAP-25 Q53,174L double mutant displayed an even lower melting point and resulted in no detectable physiological effect (S.Wei, U.Matti and E.Neher, unpublished experiments). Our physiological measurements now show that the zero layer mutant in the C-terminus of SNAP-25, SNAP-25 Q174L, does not influence the dynamic equilibrium between loose and tight states (see Figure 7). However, it does decrease k_1, the replenishment rate of the release-ready vesicle pools (see Figures 6 and 8). We therefore conclude that the zero layer plays an important role in making vesicles available for fusion, probably by keeping the leucine zipper in register during the formation of *trans*-SNARE complexes. In addition, our data show that this decrease in stability has no physiological significance for the transition between loose and tight *trans*-SNARE complexes.

Materials and methods

Generation of SNAP-25 constructs

The viral vector pSCA1 (DiCiommo and Bremner, 1998) was modified by the introduction of an oligonucleotide cassette into its *Xma*I site to generate singular *Cla*I and *Bss*HII restriction sites. The gene coding for GFP-5 (cDNA kindly provided by G.Hobom, Giessen) was amplified by PCR to generate a 5′ *Bgl*I and a 3′ *Bam*HI site, and inserted into the *Bam*HI site of the modified pSCA1, yielding pSCA1-GFP. The coding sequence for murine SNAP-25A was cloned into the *Bam*HI–*Cla*I-cleaved pSCA1-GFP using PCR. The C-terminal truncated mutant SNAP-25 Δ9 was made in the same manner, introducing a stop codon at amino acid position 198 of SNAP-25. The zero layer mutant SNAP-25 Q174L was generated by site-directed mutagenesis. The sequence of all constructs was verified by DNA sequencing.

Cell preparation and solutions

Chromaffin cells from bovine adrenal glands were prepared and cultured as described (Ashery *et al*., 1999). Cells were used 1–2 days after preparation. The external bathing solution for experiments contained (in mM): 150 NaCl, 2.8 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 2 mg/ml glucose pH 7.2 (320 mOsm). Pipet solution was prepared as described by Xu *et al*. (1997) and contained (in mM): 110 Cs-glutamate, 5 nitrophenyl-EGTA, 4 $CaCl_2$, 2 MgATP, 0.3 GTP, 0.5 furaptra, 35 HEPES; osmolarity was adjusted to 310 mOsm. Chemicals used were: nitrophenyl-EGTA (Molecular Probes, Eugene, OR), fura-2 (Texas Fluorescence Labs, Austin, TX), furaptra (Molecular Probes, Eugene, OR), $CaCl_2$ (Sigma, St Louis, MO) and ATP (Boehringer Mannheim, Germany). The basal $[Ca^{2+}]_i$ was measured as 100–300 nM in separate experiments in which furaptra was replaced by fura-2. All experiments were performed at 32°C.

Infection and GFP detection

Virus production was performed as described (Ashery *et al*., 1999). An aliquot of frozen virus (450 µl) was thawed and 450 µl of Dulbecco's modified Eagle's medium without ITS-X were added. To activate the virus, 100 µl of chymotrypsin (2 mg/ml; Boehringer Mannheim, Germany) were added and incubated for 30–50 min at room temperature. Then, 110 µl of aprotinin (6 mg/ml; Boehringer Mannheim, Germany) were added to inactivate chymotrypsin and incubated for 3–5 min at room temperature. Infection was performed on cultured cells 3–24 h after preparation. Medium volume was reduced to 0.25–0.5 ml per 35 mm plate and 0.5–1 ml of the activated virus was added. After incubation for 1–2 h at 37°C, the virus-containing medium was replaced with 2 ml of conditioned medium. Initial GFP detection was performed with an IX70 inverted microscope (Olympus Optical Co., Japan) using a GFP filter set (AHF Analysentechnik, Tübingen, Germany).

Immunoprecipitation experiments

Monoclonal antibodies against synaptobrevin (Cl 69.1; Edelmann *et al*., 1995) or syntaxin (HPC-1; kindly provided by Dr C.Barnstable, NH) were covalently coupled to protein A–Sepharose (Pharmacia, Sweden) using the cross-linker dimethylpimelimidate (Pierce). Unbound antibodies were removed by alternating washes with 100 mM $NaHCO_3$, 500 mM NaCl pH 8.3 and 100 mM glycine, 500 mM NaCl pH 2.5. Uninfected and infected chromaffin cells were harvested and solubilized in 400 µl of extraction buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitors (10 µg/ml soybean trypsin inhibitor, 1 µg/ml pepstatin, 11 µg/ml benzamidine, 1 µg/ml antipain, 1 µg/ml leupeptin and 0.1 mM phenylmethylsulfonyl fluoride)] for 30 min at 4°C. Lysates were clarified by centrifugation at 200 000 *g* for 10 min. Supernatants were split into two aliquots and incubated for 2 h with the protein A–Sepharose containing the covalently cross-linked antibodies against synaptobrevin and syntaxin, respectively. After incubation, the Sepharose was washed eight times with extraction

buffer. The immunoprecipitates were analyzed by 12% SDS–PAGE and immunoblotting using a polyclonal antibody against SNAP-25 (Blasi *et al*., 1993).

Photolysis of caged Ca^{2+} and $[Ca^{2+}]_i$ measurements

Flashes of UV light were generated as described by Heinemann *et al*. (1994) and coupled to the epifluorescence port of an inverted Zeiss IM35 microscope with a 100× oil immersion objective (NA 1.3) (Zeiss, Oberkochem, Germany). The cell under study was located in the center of the illuminated area. The fluorescence detection area was adjusted to cover only the diameter of the cell, and the fluorescence was measured with a photomultiplier tube. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio R according to the method of Grynkiewicz *et al*. (1985).

Whole-cell patch–clamp and capacitance measurements

Conventional whole-cell recordings were performed with sylgard-coated 2–3 MΩ pipets. Series resistance ranged from 4 to 12 MΩ. An EPC-9 patch–clamp amplifier was used together with the Pulse software package (HEKA Electronics, Lambrecht, Germany). Capacitance (C_m) measurements were performed using the Lindau–Neher technique implemented as the 'sine+dc' mode (Gillis, 1995) of the software lock-in extension of Pulse, which allowed long duration C_m measurements in a single sweep. A 1000 Hz, 50 mV peak-to-peak sinusoid voltage stimulus was superimposed onto a DC holding potential of –70 mV. The capacitance traces were imported to IGOR Pro (WaveMetrics, Inc., Lake Oswego, OR). The analyses were conducted on a PC using IGOR Pro. Unless stated otherwise, the data are given as the mean ± SE.

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