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v-SNARE Actions during Ca^{2+}-Triggered Exocytosis

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SUMMARY

Assembly of SNARE proteins between opposing membranes mediates fusion of synthetic lipo-somes, but it is unknown whether SNAREs act during exocytosis at the moment of Ca^{2+} in-crease, providing the molecular force for fusion of secretory vesicles. Here, weshowthatexecu-tion of pre- and postfusional Steps during chromaffin granule exocytosis depends crucially on a short molecular distance between the com-plex-forming SNARE motif and the transmem-brane anchor of the vesicular SNARE protein synaptobrevin II. Extendingthejuxtamembrane region of synaptobrevin by insertion of flexible "linkers" reduces priming of granules, delays initiation of exocytosis upon stepwise elevation of intracellular calcium, attenuates fluctuations of early fusion pores, and slows rapid expansion of the pore in a linker-length dependentfashion. These observations provide evidence that v-SNARE proteins drive Ca^{2+}-triggered mem-brane fusion at millisecond time scale and sup-port a model wherein continuous molecular pulling by SNAREs guides the vesicle through-out the consecutive stages of exocytosis.

INTRODUCTION

To accomplish fusion, membranes must overcome large energy barriers created by local dehydration of polar phospholipid headgroups and membrane deformation. Surmounting these barriers requiresthe action of proteins with special capabilities to Iower the energy costs. The im-portant role of SNARE proteins has been shown in various membrane trafficking pathways (Jackson and Chapman, 2006; Jahn and Scheller, 2006). Reconstitution experi-ments in liposomes suggested that heterotrimeric SNARE complexes are able to promote fusion, albeit at a slow time scale (Weber et al., 1998; Nickeletal., 1999; Pobbati et al., 2006). In general, at least one SNARE protein is anchored in the vesicular membrane (v-SNARE), whereas another SNARE is anchored in the target membrane (t-SNARE). By binding one another, SNAREs may force these membranes into close proximity, resulting in hemi-fusion (mixing of the outer lipid monolayers) and ultimately leading to aqueous content mixing äs observed for in vitro fusion (Giraudo et al., 2005; Reese et al., 2005; Xu et al., 2005). Yet, the behavior of reconstituted fusion Systems varies widely across laboratories and recent data have challenged the idea that SNARE assembly triggers fusion between synthetic membranes (Bowen et al., 2004; Den-nison et al., 2006). Even more unclear is whether this sce-nario holds for rapid Ca^{2+}-triggered exocytosis and if so, at which stages the exocytotic process depends on me-chanical strain between the complex-forming SNARE do-main and the transmembrane domain (TMD). To address these issues in vivo, we expressed synaptobrevin II (sybll) mutant proteins carrying an extended juxtamembrane region in mouse chromaffin cells that are genetically defi-cient for sybll andcellubrevin (double-knockout cells, dko) and nearly devoid of secretion (Borisovska et al., 2005). In following this strategy, we intended to increase the phys-ical distance between the SNARE domain and the TMD and studied Ca^{2+}-dependent exocytosis mediated by v-SNARE mutants in a gain-of-function approach. By using a combination of high-resolution membrane capac-itance measurements, amperometry and photolytic "un-caging" of intracellular calcium, we track v-SNARE function at the millisecond time scale from pre- to postfusional stages that either do not exist or cannot be detected in in vitro assays. Our results indicate multiple v-SNARE actions from granule priming to membrane merger and suggest that persistent molecular straining by SNAREs guides the vesicle on the way toward complete fusion. They further provide experimental support that v-SNAREs exert force on membranes to initiate fusion at the moment of the intracellular Ca^{2+} increase, meeting the speed re-quirement of regulated exocytosis.

RESULTS

Priming and Fast Stimulus-Secretion Coupling Demand a Tight Molecular Link between SNARE Domain and Transmembrane Anchor of Sybll

The insertion of a flexible, 11 amino acid linker in the juxtamembrane region of sybll only moderately dimin-ished fusion (<20%) between artificial liposomes (McNew

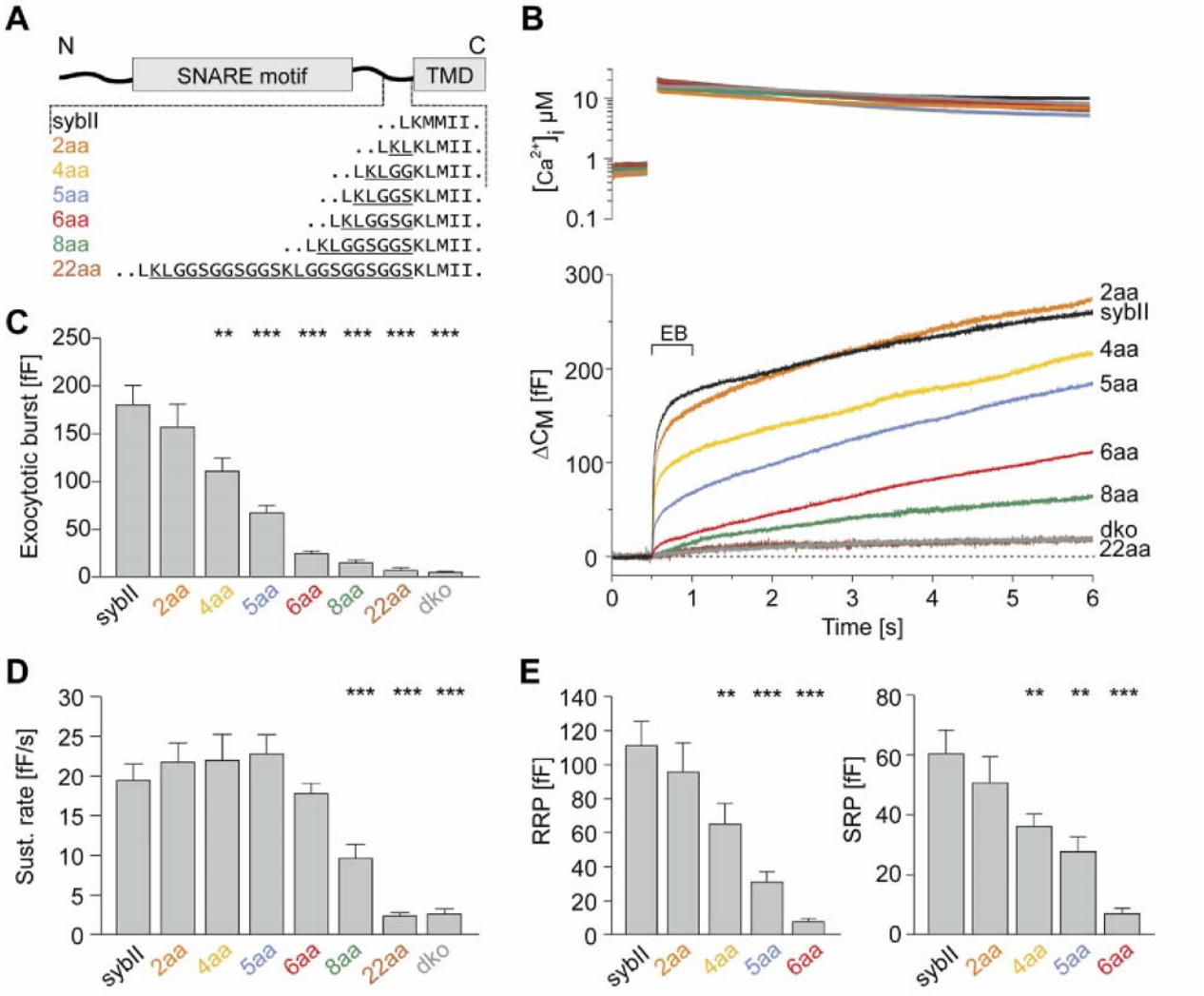


Figure 1. Extending the Juxtamembrane Region of Sybll Gradually Reduces the Flash-Evoked Capacitance Response

(A) Schematicviewof sybll domains. SNARE motif and transmembranedomain (TMD) are boxed. Underlined aminoacid sequencesshowtheinsertions intended to increase the physical distance and/orflexibility between SNARE domain and TMD.

(B) Averageflash-evoked [Ca^{2+}]j increases (upper panel) and corresponding capacitance responses (Iower panel) of dko cells expressing sybll (n = 25) or mutated sybll variants. Amino acid insertions of increasing length (2 aa, n = 13; 4 aa, n = 23; 5 aa, n = 30; 6 aa, n = 41) gradually decrease the exocytotic burst (EB). Longer insertions (e.g., 8 aa, n = 18) also attenuate the sustained phase of secretion. The 22 aa mutant (n = 10) fails to support any exocytosis when compared with dko (n = 17). Flash, t = 0.5 s.

(C and D) Linker-length dependence of the exocytotic burst size (EB, measured 0.5 s after flash) and the sustained phase of secretion.

(E) Both, RRP(left panel) äs well asSRP(right panel) gradually decrease with increasing linker length. **p< 0.01, ***p < 0.001 versus sybll, one-way analysis of variance.

et al., 1999). Surprisingly, expression of a similar mutant protein (8 amino acid insertion, 8 aa) in dko cells fails to re-store the initial capacitance increase (also referred to äs exocytotic burst component, EB) that is seen with the wild-type protein in response to the step-wise Ca^{2+} Stimulus (Figures 1A and 1 B). Decreasing the length of the insertion to 6,5, 4, and 2 amino acids (6 aa, 5 aa, 4 aa, and 2 aa) progressively increases the ability of the mutant protein to rescue the capacitance Signal (Figures 1B and 1C). With the 2 aa insertion, a nearly complete restoration of the flash-evoked response has been reached. For further analyses, the capacitance response from each cell was approximated by the sum of two exponentials and a linear rate to estimate the size of the fast (readily re-leasable pool, RRP) and the slow (slowly releasable pool, SRP) phase of release within the exocytotic burst äs well äs the subsequent sustained release phase (Experimental Procedures). Both components of the exocytotic burst, RRP and SRP, are similarly affected by the amino acid insertions (Figure 1 E), indicating that linker mutants interfere with the establishment or maintenance of these release-ready states. Thus, the exocytotic burst, a measure of

the number of primed granules (Rettig and Neher, 2002), exhibits a stringent length requirement of the juxtamembrane region. In contrast, the subsequent sustained re-lease phase decreases significantly only with insertions longer than 6 aa and is fully abolished with further exten-sion of the juxtamembrane region (e.g., 22 aa, Figure 1D). The latter phase of release may either reflect the genera-tion of newly primed vesicles that undergo fusion äs long äs calcium is high (Voets et al., 1999) or, analogous to neuronal secretion, represent a slow asynchronous form of exocytosis devoid of molecular factors, like complexin, which is required for exocytosis synchronization (Reim et al., 2001). In any case, the initial (synchronous) and subsequent (asynchronous) release phase similarly depends on v-SNARE proteins, äs illustrated by their strong block in dko cells. To investigate whether linker mutants assem-ble into SNARE complexes in vivo, we studied the effect of the 22 aa linker on exocytosis of wild-type cells. Overex-pression of the mutant protein reduces exocytosis by more than 60% compared with sybll expression (see Figure S1 in the Supplemental Data available with this article online). This demonstrates that the 22 aa mutant is able to compete with endogenous sybll and to bind its cognate SNARE partners, but forms complexes that are nonpro-ductive for fusion, asjudgedfrom itsinabilityto restore secretion in dko cells. The remaining exocytosis in wild-type cells expressing the 22 aa mutant is likely mediated by endogenous sybll, emphasizing the importance of a genetic "null" background for the analysis of v-SNARE function. Taken together, a short molecular distance or high strain between the complex-forming domain and the TMD of the v-SNARE protein is needed for release readiness of chromaffin granules, whereas longer extensions of the juxtamembrane region abolish their fusion competence. The latter observation is compatible with a recent study, showing that expression of sybll mutants with similarly long amino acid insertions (e.g., 24 aa) in sybll-deficient neurons falls to restore destaining of styryl dye labeled synapses upon Stimulation with high-potassium applica-tion (Deak et al., 2006).

A key question toward the understanding of SNARE function in exocytosis is whether these proteins act at the moment of the Ca^{2+} rise. To address this issue, we an-alyzed how lengthening of sybll's juxtamembrane region affects exocytosis timing. Averaged sybll and mutant re-sponses with a sizable exocytotic burst (>20 fF) are shown in Figure 2A and displayed on an extended time scale (first 40 ms after the flash) in Figure 2B. Increasing the linker length causes a systematic increase in the time lag between UV-flash and onset of the capacitance Signals trig-gered by nearly identical postflash [Ca^{2+}], (Figure 2D). Scaling of the capacitance Signals to the exocytotic burst size measured with sybll (0.5 s after the flash) shows that the delayed onset of mutant secretion is followed by a capacitance increase with unchanged kinetics (Figure 2C). Apparently, mutant secretion is shifted to latertimes after the flash, suggesting that readily releasable vesicles equipped with a mutant protein fuse with the plasma membrane upon Ca^{2+} elevation after an additional time lag. Kinetic rates of the exocytotic burst components (re-ciprocal of the exponential time constants tppp and tsRp) äs well äs the latency between UV-flash and Start of secretion were estimated by exponential fitting of the individual cellular responses. These results confirm the finding that amino acid insertions confer a significant and graded delay in exocytosis (sybll: 4.1 ± 0.5 ms; 4 aa: 5.8 ± 0.5 ms;
5 aa: 8.4 ± 0.7 ms; 6 aa: 14.0 ± 1.3 ms, Figure 2E). In good agreement with the averaged recordings, the rates of exocytosis from RRP and SRP resemble closely those of the sybll-mediated Signals (Figures 2F and 2G; $p > 0.7$).

For chromaffin cells, the exocytotic delay is largely de-termined by the kinetics of Ca^{2+} binding to synaptotagmin l serving äs the primary Ca^{2+} sensor for exocytosis (Voets et al., 2001; Nagy et al., 2006). As shown in Figure 2H, the delay of sybll Signals strongly decreases with increasing [Ca^{2+}]i. For 6 aa mutant responses, however, exocytotic delays are prolonged overthe entire ränge of investigated Ca^{2+} concentrations. In contrast, the Ca^{2+}-dependent rates of exocytosis from RRP are similar to those of sybll responses (Figures 2H and 2l). Notably, adding a constant time lag of 9 msec to the measured sybll delays reveals a reasonable overlap with the distribution of mutant delays (Figure 2H). Even at high [Ca^{2+}]i, in the ränge of 50-70 nM,
6 aa mutant exocytosis maintains a clear time lag of 6-10 msec compared with sybll. These findings strongly sug-gest that linker mutations prolong the latency between Stimulus and onset of exocytosis in a Ca^{2+}-independent fashion. To substantiate this observation, we determined the Ca^{2+} threshold of sybll and mutant secretion using a ramp-like calcium Stimulus (Sorensen et al., 2002). In these experiments, intracellular calcium is slowly "unc-aged" by alternating Illumination with 340 and 380 nm us ing the monochromator. The comparison of the capaci tance Signals in response to the rise of [Ca^{2+}], reveals similar Ca^{2+} thresholds for sybll- and 6 aa-mediated se cretion (Figure 3). Taken together, these results show that amino acid insertions immediately preceding the TMD cause an additional delay in stimulus-secretion cou-pling without altering the apparent Ca^{2+} sensitivity of exo cytosis. The most straightforward Interpretation of these observations is that at the moment of the Ca^{2+} rise, SNARE proteins exert force on membranes to initiate fu sion, whereas linker insertions partially dissipate this force leading to a delayed reaction. In contrast, expression of a v-SNARE variant, which carries two helix-breaking proline residues (PP mutant) instead of a flexible linker, enables a nearly complete rescue of the capacitance re sponse with no changes in the exocytotic delay (Figure S1). Thus, distance and/orflexibility rather than helical continu-ity between the SNARE domain and the TM D (Sutton et al., 1998) are responsible for the linker-length dependent ef-fects on pool size and exocytosis onset. Furthermore, v-SNARE mutants do not differfrom sybll protein regard-ing the level or pattern of protein expression in dko cells, suggesting that extension of the protein's juxtamembrane region causes the observed phenotypes (Figure S2).

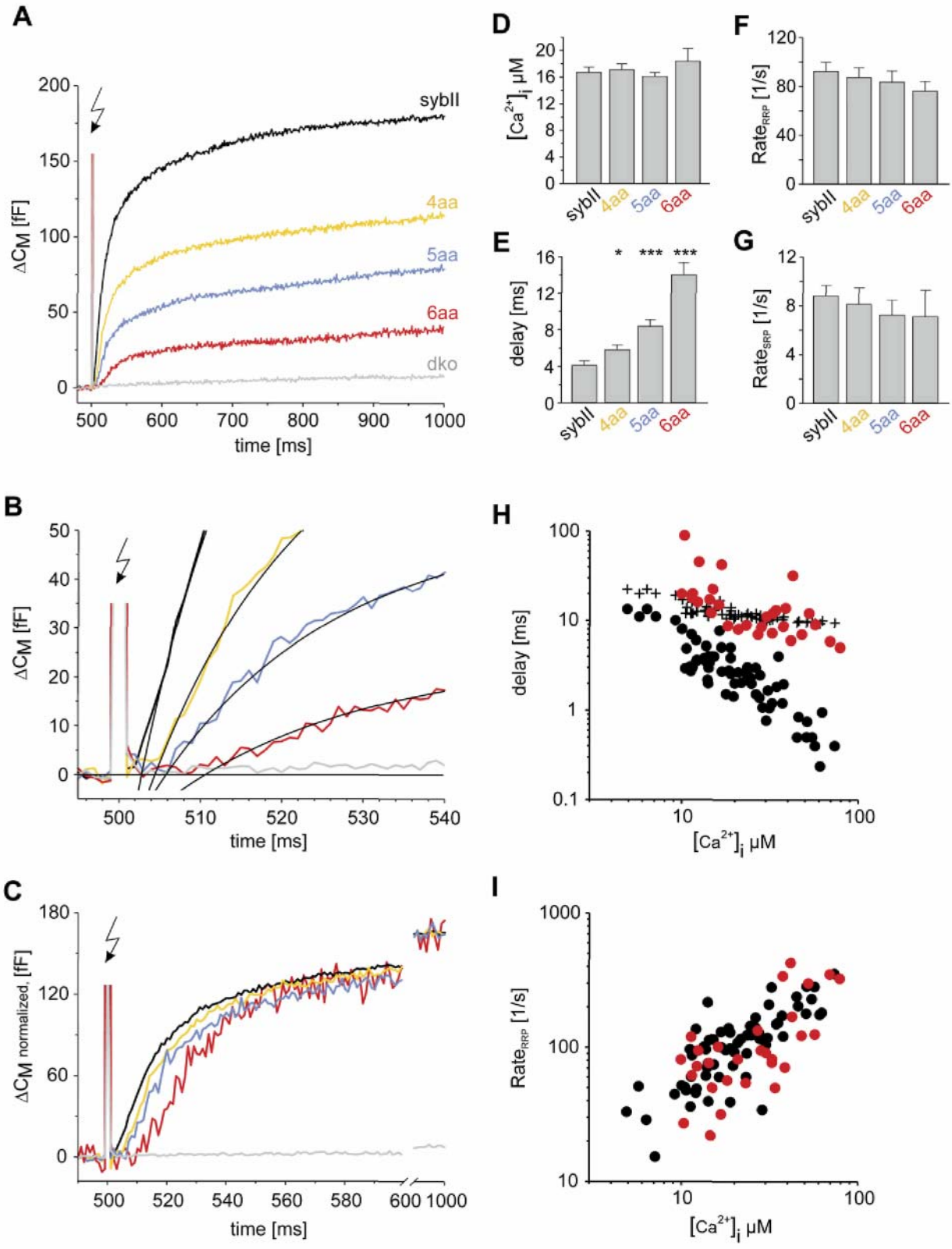


Figure 2. Extension of Sybll's Juxtamembrane Region Delays Granule Exocytosis without Changing the Ca^{2+} Dependence of Secretion

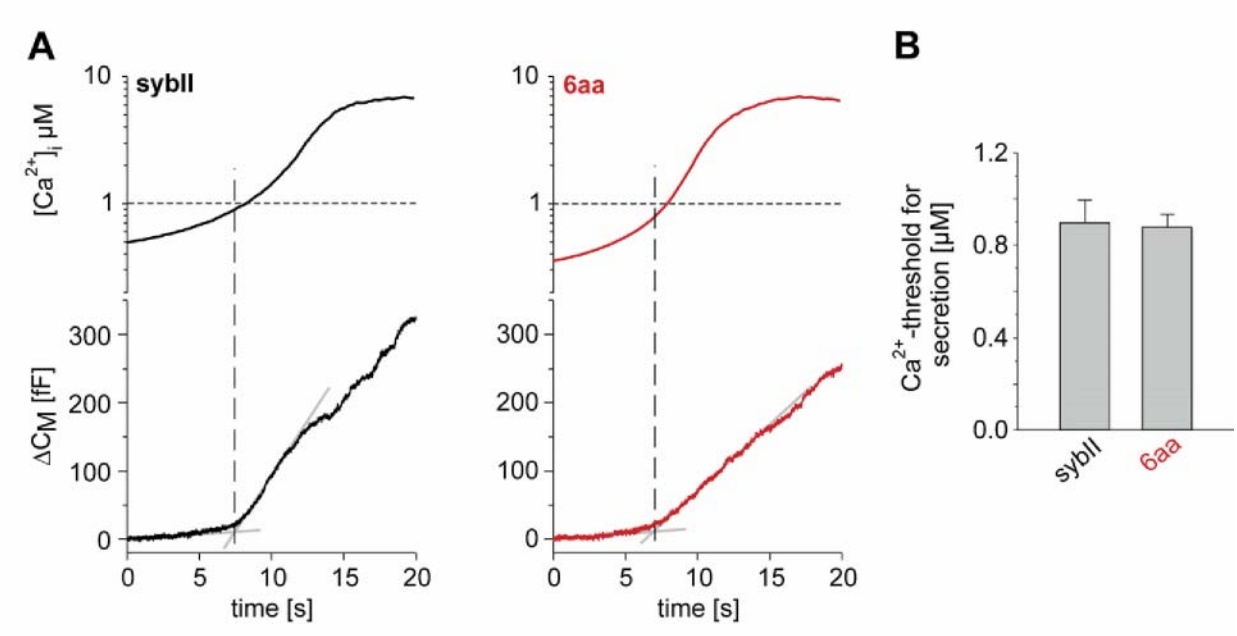


Figure 3. Linker Mutations Do Not Affect the Apparent Ca^{2+} Threshold of Secretion

(A) Exemplary recordings of the capacitance Signals (Iower panels) of dko cells expressing sybll (left) or 6 aa mutant (right) in response to a slow, ramp-like rise in [Ca^{2+}] (upper panels). The point of intersection between two linear regressions (gray lines) approximating the baseline (1-3 s) and the steep rise of the capacitance response defines the threshold for secretion (dashed line).

(B) The Ca^{2+} thresholds for secretion of sybll and 6 aa expressing cells are indistinguishable (sybll, n = 20; 6 aa, n = 26, p > 0.8, Students t test).

Postf usional Control of Quantal Discharge by Sybll

Encouraged by the above findings, we studied the func-tional impact of linker mutations at the resolution level of single vesicle release using amperometry. In these exper-iments, exocytosis was stimulated by intracellular perfu-sion with high Ca^{2+}-containing solution (19 u,M free cal-cium) and monitored simultaneously with membrane capacitance measurements and carbon fiber amperometry. This allowed us to gather a large number of exocytotic events that could be resolved äs well-separated ampero-metric spikes. Upon the Start of intracellular Ca^{2+} perfu-sion, sybll expressing cells respond with a strong increase in the frequency of amperometric Signals äs well äs in membrane capacitance (Figure 4A). In comparison, linker mutants carrying long amino acid insertions (e.g., 14 aa) promote a reduced secretory response to such a Ca^{2+} Stimulus. Both assays for secretion, membrane capacitance and amperometry, reveal a linker-length dependent attenuation in exocytotic activity and a complete loss of secretion with the 22 aa insertion (Figure 4C). These re-sults support several important conclusions. The close correspondence between the two independent measurements (r = 0.99, p < 0.001) shows that the observed changes in the capacitance Signal are due to alterations in granule exocytosis. Furthermore, they demonstrate that Ca^{2+}-triggered asynchronous exocytosis, measured here äs perfusion-induced capacitance increase, is less sensitive to lengthening of the juxtamembrane region than the exocytotic burst component in the flash experi-ment (Figure 4E). This may suggest that the two forms of

(A) Ca^{2+}-triggered capacitance Signals during thefirst 500 ms after the flash. Data are averaged from dko cells expressing sybll (n = 25) or its mutated variants (4 aa, n = 22; 5 aa, n = 19; 6 aa, n = 14) with an exocytotic burst size > 20 fF in response to average postflash [Ca^{2+}] ranging from 16.1 to 18.4 nM äs shown in (D). Secretion from dko cells (n = 17) in response to similar [Ca^{2+}] (15.0 ± 1.4 nM) is nearly abolished (<7% of sybll-mediated secretion at 0.5 s after the flash). Arrow indicates flash artifact.

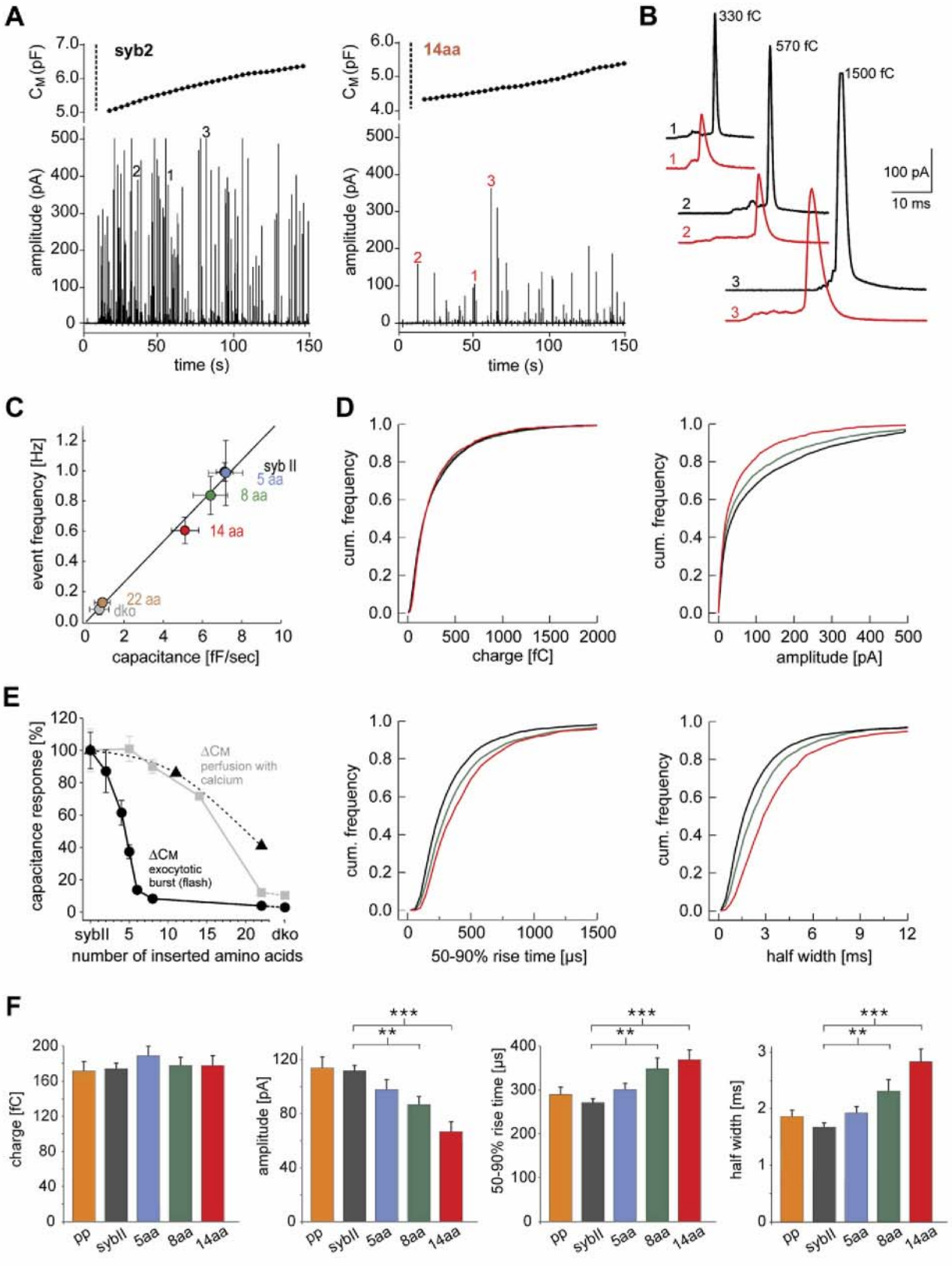
(B) Extended scaling of capacitance responses shown in (A) during the first 40 ms after flash. Compared with sybll (black), insertion of 4 (yellow), 5 (blue), or 6 (red) amino acids progressively delays the onset of secretion defined äs intersection point between the back extrapolated fast exponen-tial (black lines) and the baseline. (C) The delayed onset of mutant secretion is followed by a capacitance increase with rates similar to those of sybll. Capacitance Signals (äs shown in A) are normalized to the exoxytotic burst size of the sybll response (0.5 s after the flash) after subtraction of the sustained release component (see Figure 1D) and of the background Signal measured in dko cells.

(D) The average postflash [Ca^{2+}] values for the responses shown in (A) are nearly identical.

(E) The mean exocytotic delay determined from fitting individual cellular responses increases significantly with linker extension. *p < 0.05, ***p < 0.001, one-way analysis of variance versus sybll.

(F and G) Mean rates of RRP and SRP exocytosis measured for sybll and linker mutants.

(H and l) Stimulus-secretion coupling of sybll (black circles, n = 64) and 6 aa mutant (red circles, n = 30) exocytosis äs a function of flash-induced [Ca^{2+}]j. The delayed onset of mutant secretion persists over the entire ränge of calcium concentrations tested. Adding 9 ms to each sybll delay (black crosses) creates a reasonable overlap with the distribution of mutant delays. The rates of RRP exocytosis measured for sybll and 6 aa are similar.


A
syb2
14aa
C_M (pF)
amplitude (pA)
time (s)
B
330 fC
570 fC
1500 fC
100 pA
10 ms
C
event frequency [Hz]
capacitance [fF/sec]
syb II
5 aa
8 aa
14 aa
22 aa
dko
D
cum. frequency
charge [fC]
amplitude [pA]
E
capacitance response [%]
ΔCM perfusion with calcium
ΔCM exocytotic burst (flash)
sybII
dko
number of inserted amino acids
50-90% rise time [µs]
half width [ms]
F
charge [fC]
amplitude [pA]
50-90% rise time [µs]
half width [ms]
pp
sybII
5aa
8aa
14aa
***
**

v-SNARE-dependent exocytosis, synchronous and asyn-chronous exocytosis, are molecularly different, äs will be discussed below. In addition, the delayed onset of secre-tion seen with short insertions (Figure 2) together with the strong block of exocytosis by longer insertions (Figure 4E) indicates that increasing the physical distance between the SNARE domain and the TMD progressively inhibits fu-sion. Interestingly, amperometric Signals mediated by longer linker mutants differ not only in frequency, but also show clear changes in the kinetics of transmitter dis-charge from single vesicles, compatible with the pheno-type of a fusion mutant. As depicted for pairs of exemplary events with similar Charge in Figure 4B, Signals recorded upon expression of the 14 aa mutant protein (red) are characterized by significantly Iower amplitudes and slower discharge kinetics when compared with sybl l-mediated events (black). A comparison of the frequency distribu-tions of the parameters äs well äs of the cell-weighted averages confirms the strong and significant reduction in event amplitude measured for the 8 aa mutant that is even further decreased for the 14 aa mutant (Figures 4D and 4F). In close correlation, rise time and half width of the mutant spikes are gradually prolonged without changing the event Charge. For the PP mutant, no changes in the event properties are found. The preserved quantal size äs well äs the close correlation between membrane capaci-tance measurement and amperometric event frequency counter the possibility that alterations of the release time course are due to premature closure of the fusion pore, but instead suggest slower fusion pore expansion. Thus, tight coupling between the SNARE domain and the TMD of sybl l ensures rapid transmitter discharge from single vesicles.

Amperometric events are often preceded by a "foot" Signal (also referred to äs prespike Signal), which reflects the trickle of transmitter through a narrow, slowly expand-ing fusion pore, before its subsequent rapid expansion allows bulk release of neurotransmitter (Chow et al., 1992; Bruns and Jahn, 1995; Albillos et al., 1997). To test whether these distinct phases of transmitter discharge are similarly affected by linker mutations, we re-stricted our analysis to events preceded by a foot Signal. Indeed, insertions of 5, 8, and 14 amino acids gradually prolong the fast phase of transmitter release, indicating that extension of the juxtamembrane region impairs the final, rapid expansion of the fusion pore (Figure S3). In con-trast, only a slight reduction in amplitude and Prolongation of the prespike Signals is observed. Neither the frequency of prespike Signals nor their Charge is significantly changed. It is possible that dilation of the fusion pore, in its early stage, is largely determined by the dynamics of lipid flow restricted either by areas of high membrane curvature or by a "fence" of hindering transmembrane anchors, äs one may suggest from experiments with different v-SNARE TMDs that change the lifetime of the early fusion pore (Borisovska et al., 2005). This scenario would also be compatible with previous findings, showing that synaptotagmin regulates initial fusion pore expansion (Wang et al., 2001), presumably due to stabilization of SNAREs (with their TMDs) around the site of fusion or di-rectly caused by its strong electrostatic interactions with phospolipids (Arac et al., 2006; Bhalla et al., 2006; Zim-merberg et al., 2006). Taken together, linker mutants delay exocytosis Initiation and alter final fusion pore enlarge-ment, but appear to leave the intermittent phase of slower pore expansion unchanged.

Sybll Mediates Rapid Fluctuations of the Early Fusion Pore

The above findings motivated us to analyze prespike Signals more closely. Prespike Signals often exhibit rapid fluctuations that clearly exceed the baseline noise (Zhou et al., 1996), but the molecular mechanism underlying these events is not understood. As shown in Figure 5A, fluctuations during the foot Signal are short-lived events with durations of less than a millisecond, almost like "sub-spikes," and may document the unsuccessful attempt to widen the pore. To study whether v-SNARE proteins affect this fusion pore jitter, we determined the frequency of rapid current changes during the prespike Signal by count-ing suprathreshold deflections of the current derivative (Figure 5A). To identify fluctuations äs discrete events

Figure 4. Catecholamine Release from Chromaffin Granules Changes in a Linker Length-Dependent Fashion

(A) Measurements of membrane capacitance (upper panels) and simultaneous amperometric recordings (Iower panels) from dko cells expressing sybll (left)or 14 aa linker mutant (right, amino acid insertion: KLGGSGGSGGSGGS). Dashed line indicates cell openingthat initiates intracellular per-fusion with high Ca^{2+}-containing solution via the patch pipette.

(B) Pairs of transient oxidation currents with similar Charge taken from the recordings in (A) äs indicated by the numbering. Sybll-events (black) exhibit a faster release time course than 14 aa Signals (red) and occasionally saturatetheamplifier.

(C) Correlation between mean event frequency and rate of capacitance increase measured for sybll and mutant proteins. Continuous line, linear re-gression ($r = 0.99$, $p < 0.001$). Frequency data for amperometric events (>4 pA) were determined between 15 and 115s after cell opening and collected from the following number of cells: sybll (63), 5 aa (11), 8 aa (28), 14 aa (23), 22 aa (24), and dko (11).

(D) Properties of release events (>7 pA) mediated by sybll (black, n = 6119), 8 aa mutant (green, n = 2345) or 14 aa mutant (red, n = 1311), displayed äs cumulative frequency distributions for the indicated parameters.

(E) Flash-evoked (exocytotic burst component, Figure 1C) and perfusion-induced capacitance response (AC_M, 100 s) äs a function of linker length. Data of linker-dependent liposome fusion activity (dashed line) are taken from published results (McNew et al., 1999). Data are normalized to the cor-responding sybll response.

(F) Extension of the juxtamembrane region (5aa, 8aa, and 14aa) gradually reduces event amplitude and prolongs transmitter discharge without af-fecting quantal size. No changes occur with insertion of two proline residues (PP). Values are given äs mean for peak amplitude and äs median for Charge, 50%-90% rise time and half width determined from the parameter's frequency distribution foreach cell. Data were averaged from cells with > 40 events: sybll (59), 5 aa(11), 8 aa (25), 14 aa(17), PP(14). $^{**}p < 0.01$, $^{***}p < 0.001$, one-way analysis ofvariance versus sybll.

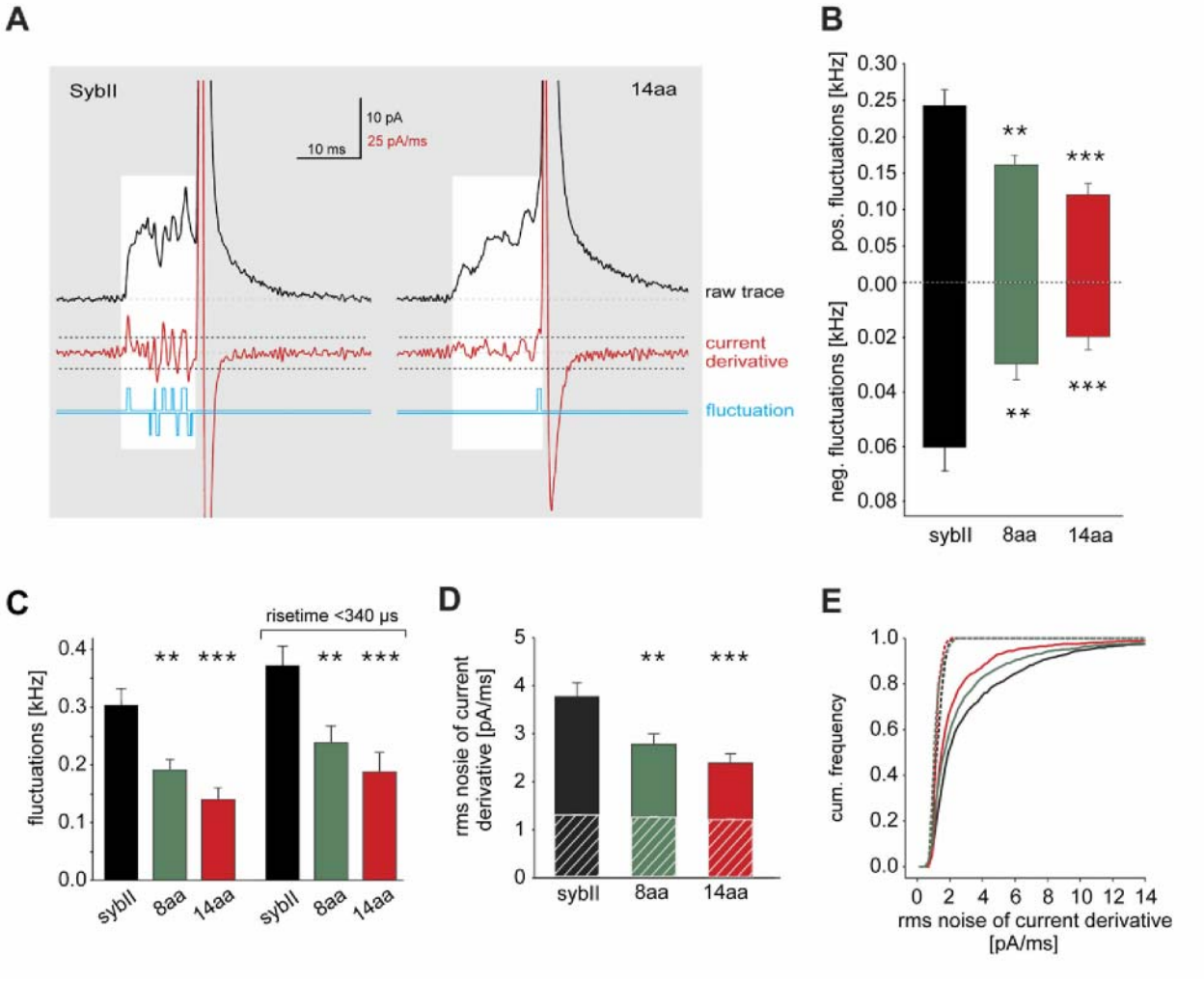


Figure 5. Linker Mutations Diminish Fluctuations of the Early Fusion Pore

(A) Exemplary analysisof current fluctuations during the prespike phase(highlighted area) of transmitter discharge from a chromaffin granule. In the current derivative (red trace), positive and negative excursions beyond the threshold (dashed lines = ±4 SD of baseline noise) were counted äs fluc tuations shown in the Iower trace (blue). Fluctuations occur more frequently in sybll- (left) than in 14 aa-mediated events (right). The displayed current transients exhibit a similar total Charge and 50%-90% rise time (sybll: 445 fC, 320 ns; 14 aa: 470 fC, 280 ns) showing that the different fluctuation behavior is not due to differences in diffusional smearing.

(B) Mean frequency of positive and of negative fluctuations decreases with increasing linker length. Data were averaged from dko cells expressing sybll (n = 23), 8 aa mutant (n = 20) and 14 aa mutant (n = 16). Note the different scaling for positive and negative fluctuations.

(C) The average fluctuation frequency (sum of positive and negative fluctuations) of all events with an amplitude > 7 pA äs well äs of events with spike rise times < 340 ns decreases in a linker-length dependent fashion.

(D) Mean rms noise of the current derivative during the prespike Signal. Hatched bars give values of corresponding background noise. Note that the rmsvalueof the current derivative isathreshold-independent parameter of current noise and is resistantto differences in the average slope among prespike Signals.

(E) Cumulative frequency distribution of rms noise for prespike Signals (continuous lines) and baseline Segments (dashed lines). Data were collected from cells/prespike Signals measured for sybll (black) 23/1541,8 aa (green) 20/1016 and 14 aa (red) 16/768. **p < 0.01, ***p < 0.001, one-way analysis of variance versus sybll.

during the prespike with reasonable fidelity, foot Signals with a minimum duration of 2 ms were analyzed. Both positive and negative going deflections were detected. Their frequencies are strongly diminished by extending the length of the juxtamembrane region (Figure 5B). On average, the fluctuation frequency is significantly reduced from 300 ± 30 Hz for sybll-mediated events to 190 ± 18 Hz for the 8 aa mutant and even further attenuated for the 14 aa mutant (140 ± 20 Hz, Figure 5C). An alternative source of rapid current changes in the record might be randomly occurring noise fluctuations. The "false" event rate (l_f), based on random noise, is given by $A_f = f_c e^{-\Theta^2/2rms^2}$ (Colquhoun and Sigworth, 1995). For nearly identical baseline noise (rms) of about 1.34 pA/ms in sybll and mutant recordings (Figure 5D, hatched bars), a thresh-old (8) of 6 pA/ms and an effective bandwidth (f_c) of

1.2 kHz, the "false" event rate is 0.05 Hz representing less than one thousandth of the observed fluctuation frequen-cies. Moreover, a similar phenotype is observed when we selected amperometric spikes with fast rise times (<340 ns), showing that properties of the release process rather than experimental inconsistencies like diffusional broadening are responsible for the observed differences in fluctuation frequency (Figure 5C). As shown in Figure 5A, current changes during the prespike Signal of the 14 aa mutant often remain subthreshold due to their slower ki-netics. To corroborate our observations, we also deter-mined the root-mean-square (rms) noise of the current de-rivative during the prespike Signal, serving äs a threshold independent parameter for fusion pore jitter. In good agreement with our fluctuation analysis, the 8 aa and the 14 aa insertion strongly diminishes rms noise of prespike Signals (Figure 5D). The frequency distributions of rms values illustrate higher current noise during the prespike Signal (Figure 5E, continuous lines) compared with baseline noise (dotted lines). Furthermore, they demonstrate the graded reduction of rms values for 8 aa and 14 aa Signals (Figure 5E). Taken together, v-SNARE action directly controls f luctuations of the initial fusion pore. It is likely that "linkers," äs flexible intermittent elements between the SNARE domain and the TMD, attenuate high frequency oscillations of the molecular machinery, illustrating the at-tempt of the SNARE engine to widen the pore.

DISCUSSION

The assembly of SNARE complexes is thought to bring vesicle and plasma membrane into close apposition and to mediate membrane fusion. This hypothesis is largely based on in vitro evidence, showing on one hand that syn-aptobrevin and syntaxin bind with their membrane-proxi-mal parts in a parallel fashion (Hanson et al., 1997; Lin and Scheller, 1997), and on the other hand that SNARE pro-teins can induce fusion between synthetic liposomes (Weber et al., 1998; Nickel et al., 1999; Pobbati et al., 2006). When and how SNAREs work in vivo is still under debate. In particular, evidence for hallmarksof the SNARE hypothesis, that these proteins provide the molecular force for fusion of secretory organelles and act at the moment of intracellular Ca^{2+} increase has not been presented. Our experiments show how key properties of the exocytotic mechanism gradually change by increasing the intra-molecular distance between SNARE domain and TMD and provide the first evidence for millisecond action of SNAREs in mediating Ca^{2+}-triggered membrane fusion. Using a combination of high-resolution techniques, we track v-SNARE function from pre- to postfusional stages of exocytosis and delineate a continuous line of v-SNARE actions from priming to complete membrane merger (Figure 6).

According to the hypothesis of N- to C-terminal "zip-ping" of SNAREs during exocytosis, priming of vesicles is assumed to coincide with initial N-terminal SNARE com-plex assembly (Hua and Charlton, 1999; Borisovskaetal., 2005; Sorensen et al., 2006). Interestingly, linker muta-tions gradually reduce the burst component of granule exocytosis, showing that length and/or flexibility of the juxtamembrane region, atthe C-terminal end of sybll'scy-toplasmic domain, are critical for granule priming. Given this observation, one might speculate that linker-mediated attenuation of the exocytotic burst simply reflects ineffi-cient targeting of the mutant protein to chromaffin gran-ules or its inability to assemble with SNARE partners in an unperturbed fashion. Several lines of evidence render these possibilities unlikely. First, immunofluorescence analyses reveal no differences in the level or pattern of protein expression between sybll and its mutants. Sec-ond, overexpression of the 22 aa mutant in wild-type cells strongly suppresses exocytosis, providing functional evidence for correct protein targeting and suggesting that even such long linker mutants are able to interact with their cognate SNARE partners. Furthermore, exocytotic activity in response to a long-lasting calcium Stimulus, äs mea-sured in our perfusion experiments, is nearly unaffected by the 8 aa insertion, which abolishesthe exocytotic burst. This indicates a particularly stringent length requirement of the juxtamembrane region for granule priming and con-trasts a general interference with SNARE complex assembly. In the context of trans-SNARE complexes, our results rather emphasize the importance of a short distance between vesicle and plasma membrane for establishment or maintenance of the release-ready state. Since an insertion of 8 amino acids suffices to eliminate the exocytotic burst, an increase in intermembrane distance of ~2-3 nm appears to be critical. This compares well with di-mensions of an intermembranous stalk in which the two contacting monolayers become continuous via an hour-glass-shaped membrane structure (Yang and Huang, 2002; Chernomordikand Kozlov, 2005), making it possible that primed vesicles are not only closely associated with the plasma membrane, but are hemifused organelles held in place by molecular straining of SNARE complexes and awaiting the calcium Stimulus. Together with synapto-tagmin's ability to buckle membranes of liposomes (Mar-tens et al., 2007), it is conceivable that close arrangement of SNAREs and synaptotagmin around the fusion site sta-bilizes secretory organelles in the hemifused state. Such a scenario would also agree with recent studies, showing that cortical granules of the sea urchin are hemifused with the plasma membrane prior to fertilization (Wong et al., 2007). Regardless of the exact underlying mechanism, our results demonstrate that even early stages of exocytosis, such äs priming, demand a remarkably tight molecular link between the SNARE motif and the transmembrane anchorof sybll.

Timing of Ca^{2+}-triggered secretion is considered to be controlled by synaptotagmin (Chapman, 2002). Disruption of the synaptotagmin l gene abolishes synchronized transmitter release (Geppert et al., 1994) and Ca^{2+}-binding mutants change the secretory delay äs well äs the appar-ent Ca^{2+} sensitivity of secretion (Fernandez-Chacon et al., 2001; Sorensen et al., 2003). In the same line, mutations in

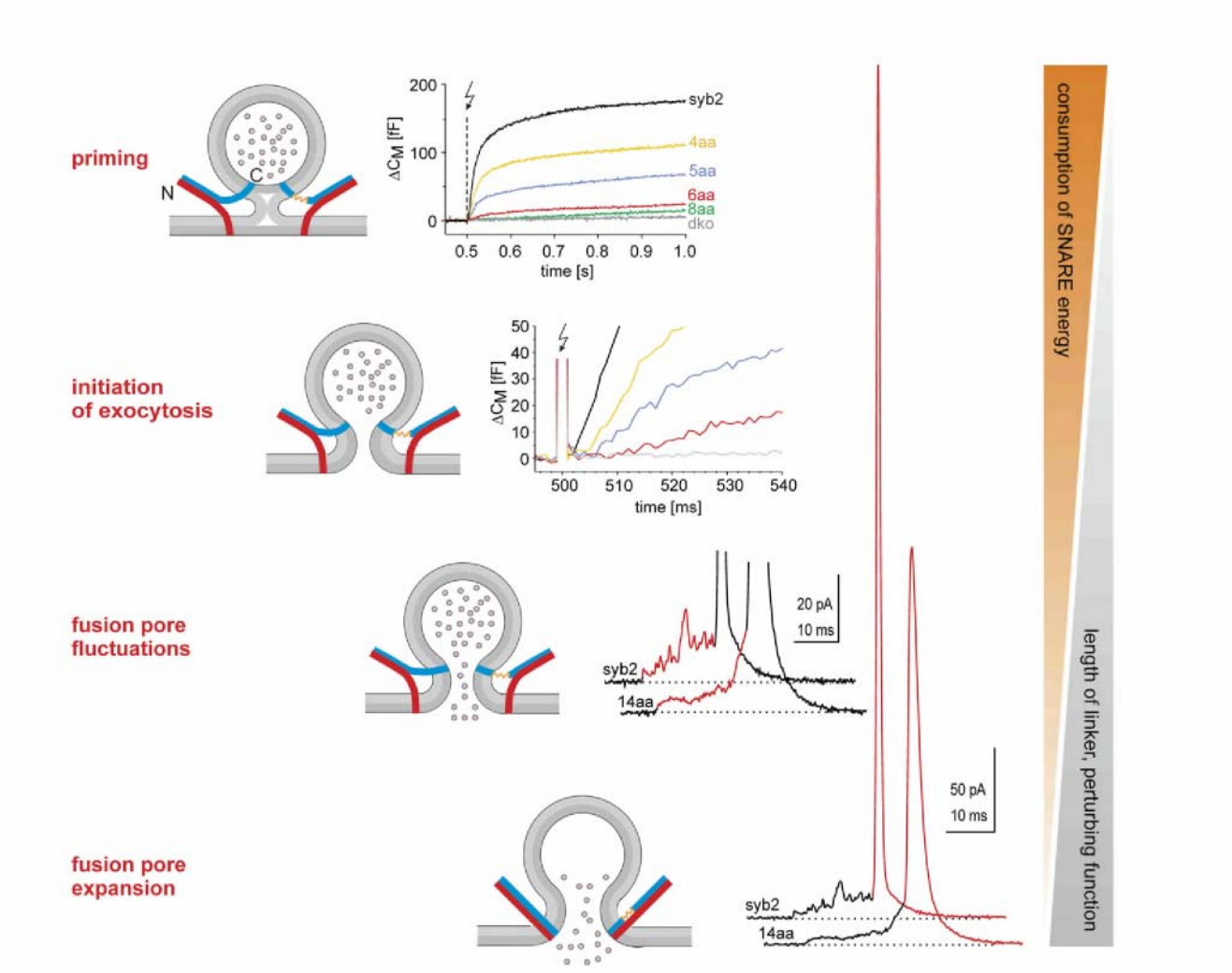


Figure 6. Hypothetical Model of SNARE Action throughout Exocytosis

The combined data set illustrates multiple v-SNARE actions from priming to complete membrane merger. Note that the length of the linker, which suffices to perturb function, increases from early to late stages of exocytosis. This may parallel the progressive conversion of frans-SNARE complexes to their c/s-orientation reflecting high and Iow energy states of the fusion machinery, respectively.

the C-terminal end of the SNARE complex or truncated versions of SNAP-25 affect exocytosis triggering and also the Ca^{2+} sensitivity of secretion, which may indicate defective synaptotagmin binding to the SNARE complex (Sakaba et al., 2005; Sorensen et al., 2006). Recent exper-iments with sybll-deficient neurons have shown that long insertions of 12 or 24 amino acids between the SNARE motif and the TMD interfere with synaptobrevin's ability to mediate Ca^{2+}-evoked exocytosis (Deak et al., 2006). Yet, even the shortest mutation used in this study is too long to restore efficient synchronized exocytosis, preclud-ing the observation of linker length-dependent changes in Ca^{2+}-triggered secretion. Ourresultsshowthatextending the length of the v-SNARE's juxtamembrane region grad-ually delays the onset of exocytosis, without affecting its Ca^{2+} sensitivity. Linker mutants neither alter the Ca^{2+}-de-pendent rates of capacitance increase (rate of RRP and SRP) nor the Ca^{2+} threshold for secretion indicating a syn-aptotagmin-independent change in exocytosis timing. Since synaptotagmin l binds to the carboxy-terminal end of the SNARE complex (Zhang et al., 2002; Dai et al., 2007), about 10-20 amino acids upstream of the site of insertions, it is unlikely that linker mutations destabilize the membrane-proximal part of the SNARE complex or change its overall conformation that should affect binding of synaptotagmin and the Ca^{2+} sensitivity of secretion. Thus, the linker phenotype highlights a postcalcium action of v-SNAREs in stimulus-secretion coupling and suggests that SNAREs are the proteins that exert force on mem-branes to initiate fusion. Taken together, these results indicate that v-SNAREs drive Ca^{2+}-triggered membrane fusion at the millisecond time scale.

Mechanisms that control fusion pore expansion deter-mine the rate of transmitter release from the fusing organ-elle and thus strength and f idelity of chemical signaling between cells. Previous studies on norepinephrine release

from PC12 cells suggested that mutations within the hydrophobic layers throughout the SNARE complex change the lifetime of the early fusion pore, albeit without any pref-erence for C- or N-terminal location within the SNARE motif (Man and Jackson, 2006). This finding has been interpreted äs evidence against vectorial "zipping" of SNAREs and led to the Suggestion that partial disassem-bly of the SNARE complex governs initial fusion pore ex-pansion. Our results are difficult to reconcile with such a scenario. First, they show that linker extensions attenu-ate fluctuations of the early fusion pore, suggesting that molecular tension on the TM D of sybll persists even after Initiation of exocytosis. Second, they slow down the fast phase of transmitter discharge from granules, indicating that v-SNARE action is continued throughout the final, rapid expansion of the pore. Thus, SNARE proteins not only provide sufficient energy to initiate exocytosis, but also to accelerate merger of membranes. In contrast to the clear changes in the properties of the amperometric spike, we failed to observe similarly significant alterations for the lifetime of the early fusion pore. Considering the ki-netic differences between prespike (slow) and spike phase (fast), it seems likely that distinct mechanisms gov-ern their overall time courses. An attractive explanation could be that interactions of synaptotagmin with t-SNARE proteins, hinder early fusion pore expansion, äs has been suggested by experiments in PC12 cells (Wang et al., 2001; Bai et al., 2004). With progressive pore widening these interactions are diminished, enabling an unhindered pull of SNAREs on their TMDs to complete membrane merger. Overall, our observations support a picture wherein mechanical coupling between v- and t-SNARE proteins motorizes exocytosis throughout its different stages. Therefore, it seems likely that continuous molecular strain-ing by SNARE proteins together with synaptotagmin's ability to control SNAREs and phospholipids guide the vesicle on the way toward complete fusion.

Based on these results, it Stands to reason that short linkers should dissipate force generated by SNARE as-sembly less effectively than long linkers. In comparison with the wild-type protein, short insertions like the 5 aa mutant, have a greater impact on priming (>2-fold reduc-tion) and stimulus-secretion coupling (2-fold increase) than on later stages of exocytosis, such äs fusion pore en-largement (12% increase in 50%-90% rise time). Long insertions (e.g., 14 aa), on the other hand, efficiently perturb the latter process, pointing to its Iower energy state. Thus, the differential attenuation of early and late stages of exocytosis by short and long linkers draws a downhill-oriented energy landscape that may reflect the progressive conversion of energy-providing SNARE complexes from their frans- to the c/s-orientation (Iow energy, all proteins in the same membrane). Although synchronous and asynchronous exocytosis absolutely depend on the func-tion of v-SNAREs, äs demonstrated by their nearly complete block in dko cells, they strongly differ regarding their sensitivity to extensions of the v-SNARE's juxtamembrane region (Figure 4E). This suggests that synchronous exocytosis demands more force from the SNARE machinery than asynchronous exocytosis. The resistance of asynchronous exocytosis to short extensions fits well with that of liposome fusion activity solely promoted by heter-otrimeric SNARE complexes (Figure 4E). Perhaps other proteins like complexin, which are needed for rapid stimulus-secretion coupling and arrest fusion of liposomes at hemifusion (Reim et al., 2001 ;Giraudo et al., 2006; Schaub et al., 2006; Tang et al., 2006), produce extra energy costs paid by the SNARE engine äs a tribute for synchronization. In summary, our observations pinpoint the function of v-SNARE proteins throughout different stages of exocytosis and provide in vivo evidence for the role of SNAREs in executing Ca^{2+}-triggered membrane fusion. Yet, elucidat-ing the function of SNARE proteins in the context of their supramolecular organization together with other scaffold-ing proteins is required for a true understanding of the mechanisms of transmitter release.

EXPERIMENTAL PROCEDURES

Mutant Mice and Cell Culture

Experiments were performed with embryonic chromaffin cells that were prepared at E17.5-E18.5 from double-v-SNARE knockout mice and cultured äs described (Borisovska et al., 2005). Recordings were done at room temperature on days 1 -3 in culture and 6-7 hr after infec-tion of cells with virus particles.

Viral Constructs

cDNAsencoding for sybll and its mutants were subcloned intotheviral plasmid pSFV1 (Invitrogen) upstream of an independent open reading frame that encodes for enhanced green fluorescent protein (EGFP). EGFP labeling was used to identify infected cells. Mutant constructs carrying either amino acid insertions of different length or a double-proline insertion at amino acid position 94 were generated using the overlapping primer method äs described (Higuchi, 1989). Note that Substitution of lysine residues (K91A and K94A) with alanine residues causes missorting of the expressed sybll variant, similar to that ob-served with the truncated TMD shown in Figure S2. Therefore, we kept the membrane proximal lysine residue (aa 94) downstream äs well äs the KNLK motif upstream of the insertion. This was best accomplished by generating two KL sites, which encode for Hindill restriction sites and allow in turn for a primer-based elongation of the inserted amino acid Stretch to facilitate the cloning process äs de-scribed previously (McNewet al., 1999). All mutations were confirmed by DNA sequence analysis. Virus particles were produced äs described (Ashery et al., 1999).

Whole-Cell Capacitance Measurements and Amperometry

Whole-cell membrane capacitance measurements and photolysis of caged Ca^{2+} äs well äs ratiometric measurements of $[Ca^{2+}]j$ were performed äs described (Borisovska et al., 2005). The extracellular Ringer's solution contained (in mM): 130 NaCI, 4 KCI, 2 $CaCI_2$, 1 $MgCI_2$,10 HEPES, 30 Glucose, pH 7.3 with NaOH. The pipette solution for flash experiments contained (in mM): 110 Cs-Glutamate, 8 NaCI, 3.5 $CaCI_2$, 5 NP-EGTA, 0.2 Fura-2, 0.3 Furaptra, 2 MgATP, 0.3 Na_2GTP, 40 HEPES, (pH 7.3), 320 mOsm. For Ca^{2+}-ramp experiments, cells were perfused with the same solution, but with 50% Ca^{2+}-loaded NP-EGTA (7.5 NP-EGTA and 3.5 Ca^{2+}) to Iower the rest-ing level of $[Ca^{2+}]$ before starting the ramp protocol. The flash-evoked capacitance response was approximated with the following function: $f(x) = A_O + A, (1 - \exp [-t/_{TI}]) + A_2 (1 - \exp [-t/T_2]) + kt$ where A_0 represents the cell capacitance before the flash. The parameters $A_{1;}$ T-I, and A_2, T_2 represent the amplitudes and time constants of RRP

and SRP, respectively. The exocytotic delay is defined äs the time be-tween theflash and the intersection point of the back-extrapolated fast exponential with the baseline (Figure 2B). For Ca^{2+} Infusion experi-ments, the pipette solution contained (in mM): 110 Cs-Glutamate, 8 NaCI, 20 DPTA, 5 $CaCI_2$, 0.2 Fura-2, 0.3 Furaptra, 2 MgATP, 0.3 Na_2GTP, 40 HEPES, pH 7.3, 19 nM free calcium. Amperometric recordings with an EPC7 amplifier (HEKA Electronic, Inc.) and carbon fiber electrodes (5 um diameter, Amoco) were performed äs previously described (Bruns, 2004). Current Signals were filtered at 2 kHz and dig-itized gap-free at 25 kHz. Amperometric spikes with a Charge ranging from 10-5000 fC and peak amplitude > 4 pA were selected for frequency analysis, while an amplitude criterion of > 7 pA was set for the analysis of singlespikecharacteristics. The Start of thefoot Signal is defined äs the time point where the current amplitude exceeds two times the Standard deviation of the average baseline noise and it ends at the inflection point (determined from maximum of the current second derivative) between the slowly increasing foot Signal and the rapidly increasing spike current. For foot flicker analysis, the current derivative was again filtered at 1.2 kHz and fluctuations exceeding the threshold of ± 6 pA/ms (~4 times SD of baseline noise) were counted. The number of suprathreshold fluctuations divided by the corresponding foot duration defines the fluctuation frequency. Values are given at mean ± s.e.m. if not indicated otherwise.

Immunocytochemistry

Chromaffin cells were processed 6 hr after virus infection for immuno-labeling äs described (Hannah et al., 1998). Epifluorescence pictures were acquired with an AxioCam MRm-CCD camera (Carl Zeiss, Inc.) and analyzed with Metamorph Software (Universal Imaging Inc.). The mouse monoclonal antibody against sybll (69.1, antigen epitope amino acid position 1-14) was kindly provided by R. Jahn (Göttingen, Germany). For quantification, the average intensity of the fluorescent immunolabel was determined within an area of interest comprising the outer cell perimeter.

Author Contribution

Maria Borisovska and Jaideep Kesavan have contributed equally to this work.

Supplemental Data

Supplemental Data include three figures and Supplemental Refer-ences and can be found with this article online at http://www.cell. com/cgi/content/full/131 /2/351 /DC1 /.

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