Linear motion before vesicle fusion characterizes sustained secretion in chromaffin cells.
Linear motion before vesicle fusion characterizes sustained secretion in chromaffin cells
ABSTRACT

The readily releasable pool (RRP) of secretory vesicles primed for fusion is limited and requires refilling from an unprimed pool to sustain exocytosis. While primed vesicles are physically docked to the membrane, the location of unprimed vesicles is less clear. Unprimed vesicles may be located at a distance from the membrane requiring translocation before priming steps can occur. Here, we show that mobile vesicles support almost 40% of high-potassium triggered exocytosis in wild-type (WT) cells. We found similar results in synaptotagmin-1 (Syt-1) and DOC2AB null mutant mouse lines with reduced RRP sizes. This supports the hypothesis that high-potassium triggered vesicle fusion from the unprimed pool requires transport towards the membrane before fusion. In addition we found that Syt-1 null mutant cells have more mobile vesicles located at greater distance from the membrane than WT cells underscoring its role as major vesicle docking protein.

Tony Cijsouw, Matthejs Verhage & Ruud F. Toonen
4.1 INTRODUCTION

Exocytosis of neuropeptide containing vesicles in neuroendocrine cells is highly regulated and triggered by depolarization-induced Ca2+ influx. Vesicles follow sequential, but reversible, biochemical maturation steps prior to fusion with the membrane. Vesicles arrive at the cell periphery, dock with the target plasma membrane, and undergo priming to become fusion competent. Docking requires the interaction of vesicle-bound synaptotagmin with a SNAP-25/syntaxin-1 complex, the target SNARE (t-SNARE) acceptor complex, and Munc18-1 (de Wit et al., 2009). Several priming proteins (Munc13-1, CAPS1-2) (Becherer and Rettig, 2006) facilitate the interaction of the vesicle SNARE (v-SNARE), synaptobrevin, with the t-SNARE acceptor complex forming a trans-SNARE complex between the vesicle and the plasma membrane that executes membrane fusion upon calcium influx (Walter et al., 2010).

Based on their release probability, secretory vesicles populate separate vesicle pools. Vesicles can exchange between pools with distinct kinetics (Becherer and Rettig, 2006; Stevens et al., 2011). Calcium influx triggers acute release that consists of two distinct phases and uses docked and primed vesicles from two releasable pools: a readily releasable pool (RRP) and a slowly releasable pool (SRP). When calcium levels remain sufficiently high, vesicles from an unprimed pool (UPP) or reserve pool undergo continuous priming into the releasable pool(s) and fuse forming the sustained phase of release (Sorensen, 2004; Stevens et al., 2011; Voets et al., 1999). The RRP and the SRP only comprise a fraction of the docked vesicles (Parsons et al., 1995; Steyer et al., 1997). It remains unclear if vesicles in the UPP are also docked at the membrane, or are located at distance from the membrane (Alvarez and Marengo, 2011; Voets et al., 1999). Release of such distant vesicles would, in addition to docking and priming, require vesicle transport to the membrane.

To gain better understanding of vesicle behavior near the membrane, optical techniques and quantitative analysis have been developed to directly study motion of single vesicles. Using wide field, confocal, and total internal reflection fluorescence microscopy (TIRFM) dynamics of fluorescently labeled secretory vesicles in insulin-secreting beta cells (Ohara-Imaizumi et al., 2002), PC12 cells (Zhang et al., 2008), and chromaffin cells (Oheim et al., 1998a; Oheim and Stuhmer, 2000) have been analyzed. These analyses identified a variety of types of motions prior to vesicle release in the different neuroendocrine cells. For example, release of neuropeptides from PC12 growth cones is mediated by the speed of vesicle diffusion of mobile cytoplasmic vesicles (Burke et al., 1997; Han et al., 1999), while TIRFM analysis of vesicle behavior in bovine chromaffin cells showed that vesicles become more restricted in motion towards the plasma membrane and do not display free diffusion (Johns et al., 2001; Oheim and Stuhmer, 2000).

TIRF imaging has been instrumental to study vesicle behavior near the plasma membrane. TIR of an excitation beam on a glass-aqueous interface generates an evanescent wave (EW) that propagates perpendicular into the aqueous phase and decays exponentially within 100-300 nm (Axelrod, 1981) providing excellent signal-to-noise of events occurring at the plasma membrane. Initial studies found that many vesicles in the EW were almost completely immobile (Johns et al., 2001; Oheim et al., 1998a; Oheim and Stuhmer, 2000; Steyer et al., 1997), and provided evidence that docked and immobile vesicles form the initial pool of vesicles undergo exocytosis upon stimulation (Oheim et al., 1998b). Recent studies confirmed these findings, but also showed that new vesicles in the EW comprise a part of the fusion events during stimulation (Allersma et al., 2004). Improved analysis additionally showed that vesicles display significant lateral motion.
immediately preceding fusion (Allersma et al., 2006; Degtyar et al., 2007) or in axial direction (Karatekin et al., 2008). Moreover, molecular states of vesicles were characterized by their mobility: primed vesicles were nearly immobile, docked vesicles displayed confined motion and undocked vesicles showed directed motion (Nofal et al., 2007). Increased priming was correlated with an increased immobilization from new vesicles arriving at the membrane (López et al., 2009; Yizhar and Ashery, 2008; Zhang et al., 2008). This suggests that the UPP is located at a distance from the membrane and that mobile vesicles refill the releasable pools during stimulation.

Here, we studied dense core vesicle (DCV) dynamics and fusion in mouse embryonic chromaffin cells to understand the role of vesicle motion in exocytosis. We hypothesized that the UPP constitutes a pool of vesicles at distance from the membrane and that refilling of releasable pools from the UPP requires vesicle transport towards the membrane. We used two null mutant mouse

**Figure 4.1 Dense core vesicle dynamics in docking deficient chromaffin cells.** A, Schematic representation of a reporter for DCV dynamics. A dense core vesicles (DCV) expressing NPY-Venus increases in fluorescence when entering the evanescent wave (blue gradient) caused by total internal reflection of the excitation light (not shown). Maximum fluorescence intensity is reached nearest to the point of origin of the evanescent wave, here the membrane of the cell (footprint) that is attached to the glass coverslip. Changes in intensity (right panel, green line) are used to calculate relative changes in axial distance (right panel, black line). B, TIRF imaging reveals fluorescent DCVs (open arrowheads show examples) in the footprint (dashed line) of a chromaffin cell. A time-lapse recording reveals stationary (open arrowheads) and mobile (closed arrowheads) vesicles. Vesicles were tracked using custom written software and extracted coordinates (bottom panel, +) were overlaid on footprint image (bottom panel). Zoomed region with overlaid coordinates (+) shows 6 initial (t = 0 s) detected vesicles during a fraction of the time-lapse recording. Detection routine fails to detect dim vesicles (2, t = 9.3 s) or nearby vesicles (6, t = 9.3 s). A new vesicle is counted when the vesicle is detected again (8, t = 33.3 s). When a vesicle gets dim it is possible the detection routine erroneously counts a nearby appeared vesicle as an old vesicle having moved (8, t = 33.9 s). Scale bar top panel 2.5 µm, Scale bar bottom panel 1 µm. C, Intensity of vesicle #4 in B over time shows overall decrease due to imaging bleaching of NPY-Venus and variations among that downward trend due to vertical movement. If detection of a puncta was lost and moments later regained the routine reported a new vesicle. The detection routine has split up this vesicle erroneously into 6 separate traces (shades of grey) and one time tracked the wrong vesicle (dashed rectangle). D, Left panel: scatter plot of Syt1, WT and KO chromaffin cells footprint size and number of detected NPY-Venus vesicles within the footprint illuminated by the evanescent wave. Right panel: Vesicle density and footprint area (inset) of Syt1WT and KO chromaffin cells during first second of image acquisition. Per cell the vesicle density (detected vesicles per footprint area) was averaged over first 1 s (34 frames). (Mann-Whitney test: * p < 0.05; Syt1 WT: d = 0.0663 ± 0.0108 vesicles/µm2 N = 18 cells; Syt1 KO: d = 0.128 ± 0.0146 vesicles/µm2, N = 26 cells). E, Distance distribution of vesicles near the membrane of Syt1 WT and KO chromaffin cells during first second of image acquisition. Per cell the intensity of each detected vesicles was averaged over first 1 s (33 frames) and the distance determined relative to the brightest vesicle (set to 0 nm, see M&M). Inset: cumulative plot of relative frequency distributions (Kolmogorov-Smirnov two-sample test: *** p < 0.001; Syt1 WT N = 18 cells, n = 470 vesicles; Syt1 KO N = 26 cells, n = 740 vesicles). F, Residency time distribution of vesicles in the evanescent wave during stimulated exocytosis. Stimulated exocytosis is defined as period of membrane depolarization (t = 10 s to 40 s) by 60 mM KCl application and succeeding 60 s (t = 40 s to 100 s). Lifetime frequencies are normalized to footprint area and total observation time in Syt1 WT and KO chromaffin cells. (Syt1 WT N = 18 cells, n = 2613 vesicles; Syt1 KO N = 26 cells, n = 3361 vesicles). Arrows point to sub-populations in the distribution. G, Residency time distribution of vesicles in the evanescent wave after stimulated exocytosis (t = 100 s to 190 s). Lifetime frequencies are normalized to footprint area and total observation time in Syt1 WT and KO chromaffin cells. (Syt1 WT N = 18 cells, n = 1932 vesicles; Syt1 KO N = 26 cells, n = 2841 vesicles). Arrows point to sub-populations in the distribution.

*Figure 4.1 and legend continue on next pages >>>*
DCV dynamics and fusion in chromaffin cells

Fluorescence Distance

NPY-Venus

Distance from brightest vesicle (nm)
Frequency (vesicles/cell)

0%
20%
40%
60%
80%
100%

0 150 300 450

Residency time at membrane (s)
Hitrate (vesicles/µm²/min.)

10 - 100 s

Residency time at membrane (s)
Hitrate (vesicles/µm²/min.)

100 - 190 s

Syt1 WT
Syt1 KO

Footprint size (µm²)
Number of vesicles per footprint

97 +/- 7 µm²
125 +/- 11 µm²

0 50 100 150 200 250

Density (vesicles/µm²)

0.00
0.04
0.08
0.10
0.16

0.1
0.15

0.15
0.00
0.05
0.15

10 - 100 s

100 - 190 s

Syt1 WT
Syt1 KO
lines with altered vesicle pools and/or refilling rates to test this hypothesis. Synaptotagmin-1 (Syt-1) null mutants have a reduced number of docked vesicles (de Wit et al., 2009) and a smaller RRP but a normal SRP (Voets et al., 2001). DOC2 null mutants also have a smaller RRP and release is shifted towards the sustained phase due to increased but incomplete refilling (Pinheiro et al., 2013). We show that mobile vesicles support ~ 40 % of exocytosis in wild-type (WT) cells. We found similar results for Syt-1 null mutants, where mobile DCV reside at a distance from the membrane and for DOC2AB null mutants where release is more synchronized with stimulation. Hence, mobile vesicles undergo stimulated exocytosis independent of calcium sensors Syt-1 and DOC2AB in embryonic chromaffin cells.

4.2 RESULTS

4.2.1 VESICLE LOCALIZATION AND DYNAMICS IN ABSENCE OF DOCKING FACTOR SYNAPTOTAGMIN-1

To investigate the role of Syt-1 in DCV behavior in living cells, we used TIRF imaging of DCVs labeled with neuropeptide Y fused to Venus (NPY-Venus; (Nagai et al., 2002) in Syt-1 wild-type and null mutant embryonic chromaffin cells. The fluorescence intensity of NPY-Venus labeled DCVs increases when vesicles move towards the plasma membrane (Figure 4.1A). Within the cellular

>>> H. Average vesicle abundance at any given time (snapshot) during stimulated exocytosis. Inset: vesicle abundances at any given time were grouped into un-retained visitors (residency time < 1 s), short-retained (1 – 10 s), and long-retained (> 10 s) vesicles (Mann-Whitney tests with FDR correction: *, p < 0.05; Syt1 WT N = 18 cells, n = 2613 vesicles; Syt1 KO N = 26 cells, n = 3361 vesicles). I, Average vesicle abundance at any given time (snapshot) after stimulated exocytosis. Inset: vesicle abundances at any given time were grouped into un-retained visitors (residency time < 1 s), short-retained (1 – 10 s), and long-retained (> 10 s) vesicles (Mann-Whitney tests with FDR correction: *, p < 0.05; Syt1 WT N = 18 cells, n = 1932 vesicles; Syt1 KO N = 26 cells, n = 2841 vesicles). J, Distance distribution of vesicles, grouped into un-retained visitors (residency time < 1 s), short-retained (1 – 10 s), and long-retained (> 10 s), near the membrane of Syt1 WT chromaffin cells during first second of image acquisition. Inset: cumulative plot of relative frequency distributions (Kolmogorov-Smirnov two-sample test with FDR(9) correction: *** p < 0.001 for long-retained against short-retained and un-retained visitors; others not significant). Note: FDR correction for total 9 comparisons in Figure J, K, L, K, Distance distribution of vesicles, grouped into un-retained visitors (residency time < 1 s), short-retained (1 – 10 s), and long-retained (> 10 s), near the membrane of Syt1 KO chromaffin cells during first second of image acquisition. Inset: cumulative plot of relative frequency distributions (Kolmogorov-Smirnov two-sample test with FDR(9) correction: *** p < 0.001 for long-retained against short-retained and un-retained visitors; others not significant). Note: FDR correction for total 9 comparisons in Figure J, K, L, L, Distance distribution of visitors (left top), short-retained (right top), and long-retained (left bottom) vesicles near the membrane of WT compared with Syt1 KO chromaffin cells (data from J and K). Inset: cumulative plot of relative frequency distributions (Kolmogorov-Smirnov two-sample test with FDR(9) correction: *** p < 0.001). Note: FDR correction for total 9 comparisons in Figure J, K, L, M, Lifetime characterization of brightest vesicles. Lifetime status, visitors (<2.5 s), short-retained (2.5 s – 10 s), long-retained (> 10 s), of the brightest vesicle in each cell. Median lifetime of long-retained vesicles is included. A significant association between genotype and lifetime status (visitors, short-retained, long-retained) was observed p < 0.05 Fisher’s Exact test. The odds of finding a brightest vesicle in the long-retained group were 5.5 times higher for Syt-1 null mutant cells than for wild-type cells. A lifetime of long-retained vesicles in Syt-1 null mutant was not significantly different from WT according to Mann-Whitney U = 92.0, p > 0.05. Data plotted as means ± SEM.
DCV dynamics and fusion in chromaffin cells

**Residency time at membrane (s)**

- **10 - 100 s**
- **100 - 190 s**

**Distance from brightest vesicle (nm)**

- **Visitors**
  - < 2.5 s
  - 2.5 - 10 s
  - > 10 s

- **Short-retained**
  - < 2.5 s
  - 2.5 - 10 s
  - > 10 s

- **Long-retained**
  - > 10 s

**Frequency (vesicles/cell)**

- **Syt1 WT**
- **Syt1 KO**

**Status**

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<th>Lifetime</th>
</tr>
</thead>
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<td>&lt; 2.5 s</td>
<td>5 (28%)</td>
<td></td>
</tr>
<tr>
<td>2.5 - 10 s</td>
<td>4 (22%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 10 s</td>
<td>9 (50%)</td>
<td>40.2 s</td>
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<tr>
<td>Total</td>
<td>18 (100%)</td>
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**Syt1 KO**

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<th>Lifetime</th>
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</thead>
<tbody>
<tr>
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<td>3 (12%)</td>
<td></td>
</tr>
<tr>
<td>2.5 - 10 s</td>
<td>1 (4%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 10 s</td>
<td>22 (85%)</td>
<td>69.2 s</td>
</tr>
<tr>
<td>Total</td>
<td>26 (100%)</td>
<td></td>
</tr>
</tbody>
</table>
footprint illuminated by the evanescent wave \((d_{ew} = 120 \text{ nm})\) (Toonen et al., 2006) several dynamic fluorescent puncta representing DCVs were observed over time (Figure 4.1B, top row). We used an automated software routine to detect these puncta and determine their coordinates (Figure 4.1B, bottom row) and fluorescence intensity (Figure 4.1C) over time. We previously found that fewer DCVs were docked at the membrane in electron microscopy preparations of fixed Syt-1 null mutant chromaffin cells compared with wild-type (de Wit et al., 2009). To test this in living cells, we measured the number of fluorescent vesicles per footprint in single frames (Figure 4.1D). We found significantly more fluorescent vesicles close to the plasma membrane per unit area of footprint (Figure 4.1D) in Syt-1 null mutant cells. In addition, Syt-1 null footprints were smaller than wild-type (WT) cells (Figure 4.1D). To determine the DCV density distribution we calculated the axial position of each individual punctum relative to the brightest vesicle at the footprint (assumed closest to or at the membrane). The average intensity of the brightest vesicles was similar in WT and Syt-1 null mutant cells (WT: 2715 a.u. ± 533.8 SEM; Syt-1 KO: 3303 a.u. ± 391.9 SEM; \(p>0.05\) t-test). We found that the relative distance of DCVs was significantly increased in Syt-1 null compared with WT cells (Figure 4.1E). This suggests that, in absence of Syt-1, more DCVs populate the area illuminated by the EW, but on average reside further away from the membrane.

To analyze the dynamic behavior of vesicles during and after stimulation, we acquired TIRF images with 0.3 s interval for 190 seconds, and applied 60 mM K+ for 30 s (at \(t = 10\) s) to depolarize the membrane. We measured the number of DCVs that appeared at the footprint and how long they resided during periods of the recording. To show all residency times in one histogram, residency times were sorted in logarithmically increasing bin sizes (Figure 4.1F and G). Distinct peaks in logarithmic distributions indicate different states with specific residency times (Sigworth and Sine, 1987; Toonen et al., 2006). We analyzed residency times during the period of membrane depolarization and the subsequent 60 s and found several peaks for stimulated Syt-1 null and WT cells (Figure 4.1F, arrows). The first state, with a life time shorter than 2.5 s most likely represents vesicles that move in and out of the evanescent wave without or with brief membrane interaction (visitors). One or two states could be observed between 2.5 s and 10 s and possibly indicate a form of minimal retention of these vesicles at their target: short-retained, or weak tethering (Toonen et al., 2006). A pronounced tail in the residency time distribution (> 10 s in Figure 4.1F) suggests that one or more long-retained states exist. Similar results were found for un-stimulated WT and munc18-1null mutants (Toonen et al., 2006). This suggests that these states are independent of

**Figure 4.2 Dense core vesicle fusion in chromaffin cells**  
A, Schematic representation of a reporter for DCV exocytosis. NPY-pHluorin expressed in acidic DCV is quenched by protons and therefore not visible while approaching the membrane (1, 2, 3). Exocytosis causes efflux of protons (4) fluorescence of pHluorin sharply rises (4). B, Three footprints (dashed areas) of chromaffin cells imaged using TIRF microscopy show two fusion events (open arrowheads), upon membrane depolarization, during a time-lapse recording. Scale bar 5 µm. C, A time series of the fusion events in B. Fusion event II is completely shown, while fusion event I shows beginning, one middle frame and end. Scale bar 1 µm. D, Fluorescence intensity analysis of fusion events in C. Automated detection of start of fusion event is marked by blue square. Membrane depolarization by superfusion of 60 mM K+ is indicated by grey area. 32% showed intensity profile similar to I (discharge) and 65% showed a profile similar to II (display) (N = 31 cells, \(n = 95\) vesicles). E, Frequency distribution (left axis) and cumulative frequency distribution (right axis) of fusion events measured using NPY-pHluorin in chromaffin cells. Grey area indicates time of 60 mM K+ superfusion. Inset: Cumulative frequency distribution of display and discharge fusion events Kolmogorov-Smirnov two-sample test: n.s. \(p > 0.05\). Note: spontaneous fusion events were not included in cumulative distributions.
DCV dynamics and fusion in chromaffin cells

A

B

C

D

E

115
depolarization-induced calcium influx. Indeed, similar peaks in distribution of residency times were observed during a period when calcium levels were back to base-line —60 s to 150 s after end of stimulation (Figure 4.1G, t = 100 – 190 s). The residency time distribution of Syt-1 null cells showed more pronounced peaks than control cells at residency times > 2.5 s (Figure 4.1F and G, > 2.5 s) suggesting that DCVs that enter the EW are longer retained in absence of Syt-1.

To understand the vesicle distribution with respect to their residency times at any given moment, we averaged the total residency time within a vesicle bin over the total time of that period (Figure 4.1H and I, both 90 s). Hence, we acquired an estimate of the abundance of a residency time in a cell at any time point (snapshot), comparable with the snapshot in time of vesicle density in Figure 4.1D and electron microscopical analysis of fixed cells (de Wit et al., 2009). Electron micrographs of Syt-1 null cells show a decrease in docked vesicles (de Wit et al., 2009). Our snapshot analysis suggests that, irrespective of secretion, retained vesicles (residency times > 2.5 s) dominate in Syt-1 null mutant cells (Figure 4.1H and I). Based on the hit rate histogram (Figure 4.1F and G), vesicles were grouped into three classes (Figure 4.1H and I, inset: visiting vesicles with residency times smaller than 2.5 s; short-retained vesicles, between 1 and 10 s; and long-retained > 10 s). Indeed, the long-retained vesicles were significantly more abundant in Syt-1 null mutant cells (Figure 4.1H and I, inset). Long-retained vesicles in WT and Syt-1 null mutant cells were significantly closer to the brightest vesicle (Figure 4.1J and K) than short-retained and visitor vesicles. All visitor and retained vesicles were located significantly further away from the brightest vesicle in Syt-1 null mutant cells (Figure 4.1L). Additionally, we found that the brightest vesicle at the start of the experiment was 5.5 times more likely to be long-retained in absence of Syt-1 (Figure 4.1M). Together, these results suggest that two spatial pools exist: stable vesicles close to the membrane and mobile vesicles at distance from the membrane. In absence of Syt-1 these pools reside further from the membrane and more DCVs reside in a stable pool compared to WT cells. The size of this pool does not depend on calcium influx.
DCV dynamics and fusion in chromaffin cells
4.2.2 A METHOD TO CHARACTERIZE PRE-FUSION DYNAMICS OF DCVS IN CHROMAFFIN CELLS

To analyze the dynamics of DCVs prior to their secretion we developed an optical probe that reports vesicle movement and fusion. The sensitivity of many fluorescent proteins to protons can be used to detect vesicle fusion with the plasma membrane (Miesenböck et al., 1998). Due to the acidic vesicle lumen, cargo labeled with the pH sensitive EGFP variant pHluorin is quenched until fusion pore opening when the vesicular pH sharply rises due to the efflux of protons into the extracellular fluid (Figure 4.2A). First, we investigated the properties of DCVs labeled with such a secretion reporter, NPY fused to pHluorin (NPY-pHluorin) (Figure 4.2A). Using high frequency TIRF imaging (50 Hz), we observed relatively long lasting fusion events (display events) and short fusion events (discharge events) upon membrane depolarization with a high K+ buffer (Figure 4.2B and C) (Perrais et al., 2004) with very few events before (5.3%) and after (21.1%) stimulation. Display events were characterized by several seconds of approximately constant fluorescence intensity (Figure 4.2D, event I), while discharge events displayed an initial rise followed by an immediate decrease in fluorescence (Figure 4.2D, event II), often accompanied by a ‘cloud’ of fluorescence that radially dispersed (Figure 4.2B and C). The majority (68%) of all fusion events were display events (Figure 4.2E), in contrast to adult bovine chromaffin cells (Perrais et al., 2004). Both types of events occurred with a similar time-lag after membrane depolarization and had similar fusion time distributions (Figure 4.2E), in line with previous results (Perrais et al., 2004). Hence, NPY-pHluorin is a reporter of stimulated exocytosis in embryonic chromaffin cells.

To investigate pre-fusion dynamics of secretory DCVs, we fused mCherry to NPY-pHluorin (NPY-pHluorin-mCherry or NPY-mCherry-pHluorin). DCVs labeled with NPY-mCherry-pHluorin moving towards the membrane show an increase in mCherry fluorescence intensity, which can be transformed to relative axial distance up to the moment of vesicle fusion (Figure 4.3A). We observed extracellular pHluorin puncta on naïve chromaffin cells expressing NPY-mCherry-
DCV dynamics and fusion in chromaffin cells

**A**

-900 ms

slope = 8 nm/s

p = 0.417

**B**

slope = -91 nm/s

p = 0.005

**C**

p < 0.001

slope = 73 nm/s

**D**

last step = 147 nm

p = 0.007
pHluorin (Figure 4.3B, left column). This suggested that the DCV cargo after spontaneous fusion events had not dissolved into the extracellular buffer. Indeed, also during stimulated fusion events (Figure 4.3B, middle and left column), post fusion intensities of pHluorin remained high for extended periods (Figure 4.3C). However, the onset and distribution of vesicle fusion events reported by NPY-mCherry-pHluorin were identical to those reported by NPY-pHluorin (Figure 4.3D). This shows that NPY-mCherry-pHluorin does not affect vesicle fusion probability and timing, and that, despite post-fusion accumulation, NPY-mCherry-pHluorin can be used to study pre-fusion dynamics of DCVs.

### 4.2.3 STATIONARY AND MOBILE VESICLES FUSE WITH THE MEMBRANE

Next, we determined axial movement of NPY-pHluorin-mCherry labeled DCVs before exocytosis by analyzing changes in mCherry fluorescence up to 1 second before fusion using TIRF imaging. Variations in mCherry fluorescence were observed (Figure 4.4A-D, top panel) and analyzed (Figure 4.4A-D, bottom left panel) up to the moment of a sharp rise in pHluorin fluorescence (Figure 4.4, blue box and line). A sharp rise in pHluorin fluorescence was often accompanied by a similar, although often smaller, rise in mCherry fluorescence (Figure 4.4 A-C, bottom left panel), likely due to the pH sensitivity of mCherry (pKa <4.5, (Shaner et al., 2004)). Therefore, mCherry intensities were transformed to distances relative to the last frame before fusion and linear regression analysis was used to determine changes in distance up to 1 s prior to fusion (Figure 4.4A-D, bottom right panel). Linear regression analysis determines if changes in distance over time can be described by linear motion, which may correspond to the assumed constant speed of motor proteins (Bier, 2003; Hill et al., 2004; Kolomeisky and Fisher, 2003; Uhde et al., 2004) responsible for DCV delivery to the plasma membrane (Chan et al., 2010; Trifarò et al., 2008). A significant slope defines a moving vesicle, a non-significant slope a stationary vesicle (Figure 4.3F). Several different mCherry intensity changes prior to fusion were observed: 1) 62% of the fusion events in WT cells (Table 1) did not show a significant change in mCherry fluorescence 1 s prior to fusion (Figure 4.4A). 2) Almost one third of the DCVs (Table 1) showed a significant slope prior to fusion (Figure 4.4B and C), with the majority showing a positive slope. 3) A small minority of DCVs revealed a relatively large step in mCherry fluorescence, in between the last and single last frame prior to fusion. Their intensity, and hence their calculated z-distance at t = 0 s was significantly different than the preceding 29 values (Table 1 and Figure 4.4D). Together, this shows that both stationary and mobile vesicles fuse with the membrane upon stimulation and that almost 40 % of all DCVs are mobile in the second prior to fusion.
DCV dynamics and fusion in chromaffin cells

>> included in cumulative distributions. B, Average vesicle fusion per cell, weighted for footprint size binned in period during (t = 0 – 10 s) and after (t = 10 – 60 s) stimulation. (Mann-Whitney tests, with FDR (6) correction, to test for significance against wild-type: *, p < 0.05. FDR correction for 6 comparisons together with Figure 4.5B). C, Frequency distribution of significant slopes of all fusion events. Number indicates middle of bin (width 25 nm/s). D, Schematic representation of vesicle behavior before fusion. A significant negative slope (top left panel) indicates a vesicle approaching the membrane before fusion, a non-significant slope (slope = 0, top right panel) indicates a stationary vesicle, a significant positive slope (bottom left panel) indicates a vesicle moving up before fusion, and a significant last step (step > 0, bottom right panel) indicates a stationary vesicle approaching or leaving the membrane in the final step before fusion. Data plotted as means ± SEM.
4.2.4 DCV DYNAMICS AND FUSION IN CHROMAFFIN CELLS WITH ALTERED POOL DYNAMICS

Experiments combining flash photolysis of caged Ca\textsuperscript{2+} with patch-clamp measurements of membrane capacitance have identified various vesicle pools with different fusion readiness (Stevens et al., 2011). The almost instantaneous increase in cytosolic Ca\textsuperscript{2+} after photolysis triggers an exocytotic burst followed by a sustained phase of exocytosis. The slower sustained phase may be composed of vesicles that are unprimed and possibly undocked from the membrane. To test this, we analyzed DCV fusion and pre-fusion dynamics after membrane depolarization with 60 mM K\textsuperscript{+} in WT chromaffin cells and cells that, in absence of Syt-1, only have a sustained phase of DCV release (Voets et al., 2001), and in DOC2AB null mutant cells where release is shifted towards the sustained phase (Pinheiro et al., 2013). We found that the total number of fusion events per footprint area in Syt-1 and DOC2AB null mutant cells was similar to WT, but fusion in DOC2AB null mutants appeared more synchronized with stimulation (Figure 4.5A). Indeed, DCV release onset time was significantly faster when DOC2AB was absent (Table 4.1) and approximately 2 times more fusion events occurred during membrane depolarization compared with WT and Syt-1 null mutant cells (Figure 4.5B). Hence, release in DOC2AB null mutant cells was more synchronized with membrane depolarization (Figure 4.5A and B).

Table 4.1 Characterization of DCV dynamics and fusion

<table>
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<th>Status</th>
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<th>Slope</th>
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<tr>
<td></td>
<td>Stationary</td>
<td>26 (62%)</td>
<td>9.7 (s)</td>
<td>-4.5 (nm/s)</td>
</tr>
<tr>
<td></td>
<td>Moving</td>
<td>16 (38%)</td>
<td>7.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Up</td>
<td>7</td>
<td>5.9</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>6</td>
<td>10.3</td>
<td>-55.6</td>
</tr>
<tr>
<td></td>
<td>Step</td>
<td>3</td>
<td>6.2</td>
<td>147; -62; -41</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>49 (15 cells)</td>
<td>9.3</td>
<td>-2.0</td>
</tr>
</tbody>
</table>

|         | Stationary| 27 (79%)     | 5.8     | 6.6        | -          |
|         | Moving    | 7 (21%)      | 12.1    | -          |
|         | Up        | 4            | 12.6    | 37.1       | -          |
|         | Down      | 3            | 4.2     | -38.6      | -          |
|         | Step      | -            | -       | -          |
|         | Total     | 44 (18 cells)| 8.3     | 7.2        | -          |

|         | Stationary| 34 (77%)     | 5.8     | 0.9        | -          |
|         | Moving    | 10 (23%)     | 3.1     | -          |
|         | Up        | 7            | 4.6     | 30.8       | -          |
|         | Down      | 3            | 2.3     | -51.9      | -          |
|         | Step      | -            | -       | -          |
|         | Total     | 48 (15 cells)| 5.4*   | 2.5        | -          |

\* No significant association between genotype and behavior (stationary or moving) \(\chi^2 (2) = 3.682, p > 0.05\).
\* Fusion time of DOC2AB KO was significantly different from WT according to Mann-Whitney \(U = 861.5, p < 0.05\) (Bonferroni corrected).
Fusion events where no vesicle could be detected before or after fusion using mCherry fluorescence were excluded from mobility analysis.
To test if release after refilling corresponds with release from a mobile pool we analyzed vesicle mobility before fusion. The ratio of stationary and moving DCVs was not different between WT, Syt-1 and DOC2AB KO chromaffin cells (Table 4.1; χ² (2) = 0.754, p > 0.05). Also, the distribution of fusion events from mobile and stationary vesicles was rather broad in all genotypes with no specific correlation of vesicle movement to onset time of fusion (Figure 4.5A). These results suggest that mobile DCVs support all phases of exocytosis triggered by 60 mM K⁺.

4.3 DISCUSSION

DCVs are actively transported to the cell membrane where they dock and prime before fusion with the plasma membrane. Docking, priming and fusion mechanisms have been extensively studied and a general understanding of the last steps of exocytosis has emerged (Alvarez and Marengo, 2011; Mohrmann and Sorensen, 2012; Stevens et al., 2011). Likewise, active transport towards the membrane is highly investigated (Park et al., 2009). However, what happens at the interface of these two processes and the role of vesicle movement prior to exocytosis is incompletely understood (Kogel et al., 2010). Here, we investigated the role of vesicle motion in the last steps of exocytosis in WT chromaffin cells compared to null mutant chromaffin cells with decreased secretion from docked vesicles, and null mutant chromaffin cells with increased refilling of primed vesicles. We find that mobile vesicles support a large fraction of secretion in all genotypes. This supports a model in which high-potassium triggered release requires vesicle translocation to the membrane prior to fusion.

4.3.1 A MEMBRANE DISTAL VESICLE POOL IN CHROMAFFIN CELLS LACKING BURST SECRETION

With TIRF imaging we found that in absence of Syt-1, secretory vesicles in living cells reside at greater distance from the membrane (Figure 4.1E). This result complements findings from EM preparations that show that Syt-1 is necessary to morphologically dock vesicles to the membrane (de Wit et al., 2009) and supports the hypothesis that Syt-1 is a docking factor for DCVs in living chromaffin cells.

In addition, we found that Syt-1 deficient chromaffin cells have more fluorescently labeled vesicles at a distance from the membrane (Figure 4.1D) and more long retained vesicles (Figure 4.1F-I). The increased DCV density appears to be largely resulting from the larger contribution of long-retained vesicles. DCVs with long residency times may be docked to the membrane (Allersma et al., 2004; Toonen et al., 2006) or tethered at some distance from the membrane. Because Syt-1 deficiency impairs vesicle docking it is likely that these long-retained vesicles are tethered to structures at a distance from the membrane, which is in agreement with the increased separation of long-retained vesicles in absence of Syt-1 (Figure 4.1L). However, in absence of Syt-1 most of the brightest vesicles are highly stable (Figure 4.1M), even throughout stimulation. These vesicles may be “dead-end” docked vesicles (Hugo et al., 2013), which suggests that Syt-1 has a role in the formation of productive SNARE complexes. The structures where long-retained vesicles may be tethered to may be the cortical F-actin, which contains open cage-like structures that restrict vesicle dynamics (Giner et al., 2007; Giner et al., 2005). Some synaptotagmin like proteins have been associated with actin modulation (Pilot et al., 2006) or myosin based transport (Roux et al.,
2009) and even Syt-1 may directly modulate actin dynamics (Johnsson and Karlsson, 2012). However, we did not find an effect of K+-stimulation, which modulates the F-actin ring (Burgoyne and Cheek, 1987; Cheek and Burgoyne, 1986), on the number of long-retained vesicles (Figure 4.1F and G).

We also found a smaller footprint in Syt-1 KO cells (Figure 4.1D). Cell morphology and total number of vesicles were not grossly affected in EM preparations of Syt-1 KO chromaffin cells (de Wit et al., 2009) and other secretion deficient chromaffin cells have normal footprint size (Toonen et al., 2006). Hence, this appears to be a Syt-1 specific effect on footprint size that cannot be explained by decreased release of DCVs. Syt-1 deletion may affect secretion of extracellular matrix proteins or their receptors via exosomes or lysosomes (Février and Raposo, 2004; Martinez et al., 2000), causing decreased cell adhesion to the substrate. Syt-1 deletion may also alter clustering of PIP2 at the plasma membrane and affect actin polymerization (Johnsson and Karlsson, 2012). However, both possibilities will require further testing.

The RRP in Syt-1 null mutants is almost absent, in line with a dramatically reduced number of docked vesicles (de Wit et al., 2009; Voets et al., 2001). However, the SRP is mostly intact (Voets et al., 2001). If indeed the SRP comprises undocked vesicles that require transport and priming prior to fusion, the larger distant vesicle pool in Syt-1 null mutant cells has a lower vesicular release probability than in WT cells. Hence, our data support a model in which Syt-1 controls the number of docked DCVs at the plasma membrane and is rate-limiting for the final approach of DCVs from the SRP towards the membrane.

4.3.2 A POOL OF VESICLES UNDERGOING MEMBRANE TRANSLOCATION AND FAST FUSION SUPPORTS SUSTAINED SECRETION

We found that almost 40% of vesicles displayed significant displacement in the 1 second before fusion (Table 1). The majority of mobile vesicles we observed could be described by a linear function, in line with directed vesicle motion near the membrane reported before (Duncan et al., 2003; Karatekin et al., 2008; Oheim and Stuhmer, 2000; Steyer and Almers, 1999; Steyer et al., 1997). The majority of mobile vesicles moved distances larger than the 12 to 24 nm length of a partially-zippered trans-SNARE complex bridging a vesicle and target membrane (Sutton et al., 1998) and larger than the 20 nm step towards the membrane observed for SNARE-complex-tethered vesicles just before fusion (Karatekin et al., 2008). Hence, docking and priming had to occur within fractions of a second prior to fusion. Electrophysiological measurements of chromaffin cells suggest that priming can take up to 10 s (Xu et al., 1998) and typical dwell times before fusion in human carcinoid cells are similar (Karatekin et al., 2008). However, insulin filled vesicles fuse within 50 ms upon reaching the plasma membrane (Ohara-Imaizumi et al., 2007; Ohara-Imaizumi et al., 2004) and Allersma et al. (Allersma et al., 2006) concluded that the interaction of the vesicle with the t-SNAREs leading to fusion occurs within 100 ms of fusion. Hence, docking and fusion can occur relatively fast. In conclusion, our findings are in line with the notion that a mobile pool of vesicles supports a significant fraction of exocytosis triggered by K+-mediated membrane depolarization. The timescale of our measurements (1 sec) leaves sufficient time for docking, priming and fusion of dynamic vesicles in chromaffin cells.
Release upon a high K+ stimulation in absence of Syt-1 was similar to WT (Figure 4.5). This does not seem to be in agreement with earlier findings using Ca2+ uncaging where deletion of Syt-1 strongly reduces the RRP (Voets et al., 2001). However, high K+ application causes a sustained, relatively low elevation in intracellular Ca2+ compared to flash photolysis (O’Sullivan et al., 1989). Simulations using a kinetic model for secretion of DCV in chromaffin cells, suggest that during prolonged elevated Ca2+ the majority of exocytosis occurs via the SRP and deficits of the RRP may not be visible (Sorensen, 2004). Our results show that high K+ application in majority probes release from the SRP. Indeed, the SRP in Syt-1 null mutants is similar to wild-type (Voets et al., 2001). In DOC2AB null mutants vesicle fusion was more synchronized with the high K+ stimulation, which is in line with earlier results in which deletion of DOC2A resulted in enhanced refilling of the SRP (Pinheiro et al., 2013). Syt-1 null mutants and DOC2AB null mutants had similar percentages of mobile vesicles as control. This supports the conclusion that high K+ stimulation probes secretion via the SRP. Short depolarizations that produce short lasting [Ca2+] jumps may probe mostly the RRP and would be a suitable paradigm to test if vesicles fusing from the RRP are stationary vesicles (Sorensen, 2004). In conclusion, our results support a model where vesicles from an unprimed pool at a distance from the membrane continuously exchange with releasable vesicles at the membrane.

4.4 EXPERIMENTAL PROCEDURES

4.4.1 CHROMAFFIN CELL CULTURE

Dissociated chromaffin cell cultures were obtained from the adrenal glands of E18 mice as described previously (Toonen et al., 2006). In brief, isolated adrenal glands were placed in Locke’s solution (containing in mM: NaCl: 154; KCl: 5.6; NaH2PO4: 0.85; Na2HPO4: 2.15; Glucose: 10; pH 7.4) and cleared from connective tissue. Adrenals were incubated in enzyme solution (DMEM containing 1 mM CaCl2, 0.5 mM EDTA, 0.1 mM L-Cysteine, 20-25 units/ml papain and 5 µg/ml DNAse) for 45 minutes at 37˚ C. Inhibitor solution (20% FCS, albumin trypsin-inhibitor and 5 µg/ml DNAse) was added to the enzyme solution and incubated for 10 minutes at 37° C. The adrenals were triturated in enriched DMEM (DMEM containing 1% insulin-transferrin-selenium and 0.1% Pen/Strep) containing 5 µg/ml DNase. The cell suspension was centrifuged for 5 minutes at room temperature and resuspended in pre-warmed enriched DMEM. Cells were plated on rat tail collagen-coated coverslips at 50 µl/cover slip and placed for 30-60 minutes in the incubator (37°C and 10% CO2) to allow cell attachment. Finally, 1 ml of enriched DMEM was added per coverslip.

4.4.2 LIVE CELL IMAGING: TOTAL INTERNAL REFLECTION MICROSCOPY

Experiments for Figure 4.1 and 4.2 were performed on an inverted Zeiss microscope equipped with a TIRF condenser (TILL Photonics) to view footprints of chromaffin cells under TIRF illumination. Excitation light at 473 nm was provided by an argon laser (National Laser Company) coupled into a single fiber-optic cable that was connected to the TIRF condenser. The laser was focused into the back focal plane of a high-numerical-aperture lens (X100; numerical aperture = 1.46) designated for TIRFM imaging (Zeiss). The characteristic 1/e depth d for the evanescent field was measured at ~120 nm (Toonen et al., 2006). The microscope was equipped with appropriate
filter set for GFP imaging, and a 1.6x relay lens. Time-lapse live imaging was captured using a Roper Scientific Cascade EMCCD camera controlled by MetaMorph software (Universal Imaging).

Experiments for Figure 4.3 to 4.5 were performed on a custom-built Tandem Illumination Microscope (TIM, Olympus) equipped for dual color TIRF imaging. We used the inverted part for guidance of a 488 nm solid-state laser, and a 561 nm solid-state laser (Olympus) where applicable, through a 60x oil immersion TIRF objective (NA 1.49) to obtain TIRF illumination, with an additional 1.6x magnification. The characteristic 1/e depth for the evanescent field was measured at 488 nm and adjusted at 561 nm to ~145 nm. We used an additional dual-color beam splitter (Dual View, Optical Insights) before a Hamamatsu EM–CCD camera (Hamamatsu City, Japan) to allow simultaneous imaging of mCherry and pHluorin where applicable. Time-lapse live imaging was controlled by Excellence software (Olympus).

4.4.3 CONSTRUCTS AND TRANSFECTION

Chromaffin cells in primary culture were infected with Semliki Forest Virus infectious particles (Ashery et al., 1999) encoding NPY-Venus (Nagai et al., 2002), NPY-pHluorin (Venus replaced with pHluorin, (Miesenböck et al., 1998)), NPY-pHluorin-mCherry (mCherry (Shaner et al., 2004) fused in frame to pHluorin) or NPY-mCherry-pHluorin. Experiments were performed 8–16 h after infection.

4.4.4 ANALYSIS

For quantification in Figure 4.1, TIFF images were loaded into IGOR Pro v5.0.5A to automatically detect and track vesicles over time, and analyze movement and intensity by a home-written macros (vesicle detection v1.81) (Friedrich et al., 2008). Occasionally, a single vesicle was erroneously registered as multiple short traces, resulting in a slight overestimation of the number of retained vesicles and underestimation of their residency time (Figure 4.1C). Footprint size was measured by manually drawing a perimeter in IGOR Pro. Relative distance ($Z$) of a vesicle ($ves_i$) to the brightest vesicle in the cell were calculated according to

$$Z(ves_i) = -d \cdot \ln\left(\frac{I(ves_i)}{I_{brightest}}\right)$$

With $I_{brightest}$ the background corrected mCherry intensity of brightest vesicle per chromaffin cell, $I(ves_i)$ the background corrected intensity of a vesicle, and $d$ the characteristic 1/e depth for the evanescent field. The transformation of intensity to distance leads to a skewed representation of relative vesicle position, because DCV brightness is also a function of vesicle diameter, dye content, and cellular environment (Johns et al., 2001; Oheim and Stuhmer, 2000). Hence, the observed distribution presents a general relative change in vesicle position. Hitrate and snapshot residency times were calculated according to (Toonen et al., 2006).

For quantification in Figure 4.2 to 4.5 vesicle intensities were measured in ImageJ (NIH) by placing a rectangular ROI around a vesicle (and besides the vesicle for background measure). Distance of a vesicle, in each frame up to one second before fusion, relative to the membrane was calculated according to

$$Z(t_i) = -d \cdot \ln\left(\frac{I(t_i)}{I_0}\right)$$
With $I_0$, the background corrected mCherry intensity of a vesicle one frame before fusion. Intensity traces were corrected for background by subtracting the average background intensity over the 1 s before fusion. No significant change in mCherry fluorescence prior to fusion indicates that vesicles had no significant change in axial distance prior to fusion (Oheim and Stuhmer, 2000); a significant negative slope suggests that vesicles move towards the glass-water interface before fusion (Karatekin et al., 2008), while a significant positive slope suggest that vesicles move away from the glass-water interface; a significant step-like change suggest a sudden move towards or away from the glass-water interface (Ohara-Imaizumi et al., 2004). Moment of fusion was automatically detected in an intensity time series using custom written routines in MS Excel when background corrected pHluorin intensity was, for 3 consecutive frames, above 2 x the standard deviation of 50 previous frames. Cells were excluded from analysis when no fusion event was detected. Vesicles were excluded from distance analysis when no mCherry fluorescence was detected after background correction.

Differences between two groups were tested for significance using a Student’s t test for unpaired data when data passed a normality test (Kolmogorov-Smirnov) and a Mann-Whitney (M-W) test when not. For testing significant differences in amount of vesicles per group under different conditions the Pearson’s chi-square test or Fisher’s Exact test was used. Distributions were tested for significance using Kolmogorov-Smirnov two-sample test. P-values were adjusted using a False Discovery Rate (FDR) correction (Benjamini and Hochberg, 1995) for number (#) of multiple comparisons, in short FDR (#) corrections. Differences were regarded significant when p < 0.05. Statistics were performed in SPSS (IBM). All data plotted as mean ± SEM (error bars or shaded area) in arbitrary units.

REFERENCES


