The plasma membrane of Munc18-1 deficient chromaffin cells contains increased levels of PI(4,5)P$_2$

Julia Kurps$^1$, Jan R.T. van Weering$^2$, Matthijs Verhage$^{1,2}$

$^1$Department of Functional Genomics (VU)
$^2$Department of Clinical Genetics (VUmc)
Center for Neurogenomics and Cognitive Research
VU University Amsterdam and VU Medical Center
1081 HV Amsterdam, The Netherlands
Abstract

The regulation of the cortical F-actin network in adrenal chromaffin cells is Munc18-1 dependent. Since there is no evidence for a direct interaction between both proteins, we suggest an indirect pathway via the phosphoinositol PI(4,5)P$_2$, which regulates a multitude of actin-regulating proteins and can be linked to Munc18-1. We tested different PI(4,5)P$_2$-reporters and identified the lentiviral expression of PH-PLC$\delta$-eGFP as optimal probe for our assays. The levels of PH-PLC$\delta$ at the plasma membrane were highly increased in fixed munc18-1 null CCs compared to wild type, and we confirmed these results in living cells both at the equatorial plane and at the cell footprint. Furthermore, the absence of Munc18-1 resulted in a slight increase in the mobility of PH-PLC$\delta$-eGFP. First attempts using super-resolution microscopy suggested a clear co-localization of PI(4,5)P$_2$ patches at the plasma membrane with Syntaxin1a and filamentous actin. Together these results suggest that the loss of Munc18-1 results in an increase of plasma membrane PI(4,5)P$_2$ levels, which might form a plausible explanation for the previously observed cortical F-actin phenotype in munc18-1 null CCs.
Introduction

Munc18-1 deficiency in adrenal MCCs results in an increased cortical F-actin network (Toonen et al., 2006). We characterized distinct residues and structural domains of Munc18-1 and their effect on F-actin (Chapter 4). However, the underlying molecular mechanisms remain not fully understood.

To gain a deeper understanding of the molecular link between Munc18-1 and the cortical F-actin network, we considered proteins that intersect both interactomes. The F-actin phenotype that we observe in munc18-1 null CCs might be attributable to distinct speculative scenarios or a combination of several: (I) increased actin polymerization (e.g., by crosslinking of actin fibers via over active MARCKS) or (II) hampered actin depolymerization (e.g., by inhibition or inactivation of scinderin (Rodríguez Del Castillo et al., 1990; Vitale et al., 1991)) or cofilin (Arber et al., 1998; Birkenfeld et al., 2001). However, to our knowledge no actin-regulating factor is known to interact with Munc18-1 directly, most of the actin-regulating proteins bind the phospholipid phosphatidylinositol 4,5-bisphosphate PI(4,5)P$_2$ (for review see Caroni, 2001; Takenawa and Itoh, 2001; Yin and Janmey, 2003; Janmey and Lindberg, 2004; Di Paolo and De Camilli, 2006; Saarikangas et al., 2010; Kwiatkowska, 2010). PI(4,5)P$_2$ recruits actin-regulating proteins to the PM and it was shown that several proteins involved in actin polymerization (e.g., MARCKS) and de-polymerization (e.g., scinderin) are PI(4,5)P$_2$ dependent (Glaser et al., 1996; Zhang et al., 1996).

Munc18-1 binds PLD directly and inhibits its function (Lee et al., 2004) and unpublished data by Angeli Möller, MDC, Berlin). PLD stimulates a PI4P5kinase and is therefore involved in the generation of PI(4,5)P$_2$. The phospholipid can also be linked to the most important Munc18-1 interactor: Syntaxin1a (Bennett et al., 1992; Hata et al., 1993; Pevsner et al., 1994). Due to electrostatical interactions, PI(4,5)P$_2$ and Syntaxin1a form clusters in lipid membranes (Murray and Tamm, 2009; van den Bogaart et al., 2011).

Based on this evidence, we hypothesize that the actin-regulating role of Munc18-1 is attributable to increased PI(4,5)P$_2$ levels at the PM of adrenal CCs from munc18-1 null mice. To test this hypothesis, we compared PI(4,5)P$_2$ levels in wild type and munc18-1 null CC. We found an increase in PM-located PI(4,5)P$_2$ and a slight increase in PI(4,5)P$_2$ mobility in the absence of Munc18-1. These findings will enhance the understanding of the molecular interactome that connects Munc18-1 and the cortical F-actin network in adrenal CCs.
Results

Lentiviral expression of PH-PLCδ-eGFP reports PI(4,5)P₂ levels on the plasma membrane

We used different quantification methods to compare the levels of PI(4,5)P₂ at the PM of wild type and munc18-1 null MCCs. We first tested anti-PI(4,5)P₂ antibodies, which were successfully used by others (Milosevic et al., 2005) and resulted in a PM-localized staining as expected (fig5.6A). We subsequently co-stained PM-localized proteins, which interact with PI(4,5)P₂ and expected a high co-localization between both signals. However, the correlation between PI(4,5)P₂ and cortical F-actin (fig5.6Ai) and PI(4,5)P₂ and Syntaxin1a (fig5.6Av) at the PM was much lower than expected (fig5.6Aii: PI(4,5)P₂/F-actin: R=0.26; fig5.6Avi: PI(4,5)P₂/Syntaxin1a: R=0.08), whereas the correlation of the signals of PI(4,5)P₂ and Syt-1 (fig5.6Aiii) showed a moderate correlation (fig5.6Aiv: PI(4,5)P₂/Syt-1: R=0.64). To further test the anti-PI(4,5)P₂ antibody, we used Lentivirus particles to express the widely used PI(4,5)P₂ marker PH-PLCδ₁-eGFP (eGFP tagged plekstrin homology (PH) domain of phospholipase Cδ₁ (PLCδ₁) (Holz et al., 2000)) in HEK cells and compared the eGFP signal to the anti-PI(4,5)P₂ antibody staining (fig5.6B). The antibody staining (fig5.6Bi) did not overlap with the eGFP signal (fig5.6Bii). Together with the surprisingly low co-localization between PI(4,5)P₂ and known PI(4,5)P₂ interacting proteins, this observation led to the rejection of the antibody approach. Instead, we rely on the PH-PLCδ₁-eGFP expression as the best possible PI(4,5)P₂ marker in living cells.

Based on the relative short live time of CCs in culture, Semliki-Forest-virus (SFV) is commonly used to induce acute high expression of exogenous proteins (Sørensen et al., 2003b; de Wit et al., 2009). We infected CCs from wild type and munc18-1 null mice with SFV particles for 5-8 h (fig5.6C). Examination of the cell culture showed a high loss of cells, which was confirmed when images of fixed cells were acquired with the confocal microscope. CCs from both genotypes showed blebbing and abnormal amounts of PH-PLCδ₁-eGFP on intracellular structures (fig5.6Ci-fig5.6Civ). Since these artefacts can be caused by the SFV-mediated high-level acute expression of PH-PLCδ₁-eGFP, we decided to use the Lentivirus system to express the PH-PLCδ₁-eGFP construct at a more moderate level (Walter et al., 2014). Lentivirus expressed PH-PLCδ₁-eGFP did not induce cell loss or blebbing in CC cultures during the 30 h incubation interval (fig5.1).
Figure 5.1: Increased PI(4,5)P$_2$ levels on the plasma membrane in munc18-1 null chromaffin cells compared to wild type chromaffin cells. Ai. Confocal microscopy images of fixed MCCs expressing the PH-PLC$\delta_1$-eGFP construct, top: wild type MCC, bottom: munc18-1 null MCC. Aii-Aiv. Quantification of PI(4,5)P$_2$ signal in wild type (grey, N:3, n:27) and munc18-1 null MCCs (green, N:3, n:29). Aii. intensity: wild type: 21.25 ka.u. ± 2.46; munc18-1 null: 34.78 ka.u. ± 3.18; t-test, **, p = 0.0015). Aiii. thickness: wild type: 15.09 a.u. ± 1.06; munc18-1 null: 17.01 a.u. ± 0.9; t-test, n.s.). Aiv. density: wild type: 1.25 ka.u. ± 0.11; munc18-1 null: 1.93 ka.u. ± 0.16, t-test, **, p = 0.0012). Av. MCCs expressing the control Lentivirus construct PHmutPLC$\delta_1$-eGFP (K30A, K32A, W36N), which cannot bind PI(4,5)P$_2$ top: wild type MCC, bottom: munc18-1 null MCC. Avi. Quantification of eGFP intensity in cortical region (wild type: grey, N:1, n:19; 33.66 ka.u. ± 5.05; munc18-1 null: green, N:1, n:16; 38.77 ka.u. ± 7.45; t-test, n.s.). Bi. MCCs expressing PH-PLC$\delta_1$-mCherry, top: wild type MCC, bottom: munc18-1 null MCC. Bii-Biv. Quantification of PI(4,5)P$_2$ signal wild type (grey, N:1, n:13) and munc18-1 null MCCs (red, N:1, n:14). Bii. intensity: wild type: 12.02 ka.u. ± 1.22; munc18-1 null: 17.75 ka.u. ± 1.39; t-test, **, p = 0.005). Biii. thickness: wild type: 16.61 a.u. ± 1.59; munc18-1 null: 16.89 a.u. ± 1.52; t-test, n.s.). Biv. density: wild type: 0.64 ka.u. ± 0.06; munc18-1 null: 0.98 ka.u. ± 0.08; t-test, **, p = 0.003); shown are mean ± SEM; Scale bars: 2 μm.
PI(4,5)P_2 levels at the plasma membrane of \textit{munc18-1} null chromaffin cells are increased

MCCs from \textit{wild type} and \textit{munc18-1} null mice were infected with Lentivirus particles expressing PH-PLC\(\delta\)1-eGFP. After 30 h incubation the cells were fixed and images of the equatorial plane were acquired with the confocal microscope. We quantified signal intensity and thickness and compared the results of \textit{wild type} and \textit{munc18-1} null MCCs (fig5.1Ai). The signal intensity of PH-PLC\(\delta\)1-eGFP at the PM was increased by 64\% in \textit{munc18-1} null MCCs (fig5.1Aii), whereas the thickness was not significantly different between both genotypes (fig5.1Aiii). To exclude an expression-dependent increase in signal intensity, we measured the expression of a control construct, containing a mutation in the PH domain (Milosevic et al., 2005), which is unable to bind PI(4,5)P_2 (fig5.1Aiv). The overall intensity of this construct was not significantly different between MCCs from \textit{wild type} and \textit{munc18-1} null mice (fig5.1Av). We furthermore examined whether the fluorescent tag on the PI(4,5)P_2 reporter had an effect on the analysis outcome (fig5.1B). When we expressed PH-PLC\(\delta\)1-mCherry instead of PH-PLC\(\delta\)1-eGFP, we found similar results. In \textit{munc18-1} null MCCs, the signal intensity was almost doubled (increase of 48\%) compared to \textit{wild type} MCCs (fig5.1Bii). The signal thickness was not altered between both genotypes (fig5.1Biii). These data show that Munc18-1 deficiency in adrenal CCs results in increased levels of PH-PLC\(\delta\)1-eGFP at the PM, indicative of elevated PI(4,5)P_2 levels.

Two detection methods confirm increased intensity of PI(4,5)P_2 reported in living chromaffin cells

Equatorial plane
Since lipids are difficult to fix and artefacts can occur during this process, we performed similar experiments as in fig5.1, but now in living cells. First, we quantified the signal intensity of PH-PLC\(\delta\)1-eGFP at the equatorial plane of MCCs from both genotypes (fig5.2Ai). The increase in the signal intensity of PH-PLC\(\delta\)1-eGFP in the absence of Munc18-1 was confirmed with this method (fig5.2Aii). The signal intensity in \textit{munc18-1} null MCCs was highly increased compared to \textit{wild type} MCCs (\approx 194\%), however this difference was not significant. To calculate the signal intensity at the PM, we measured the intensity of three ROIs (fig5.2Aiii) and compared the signals in both genotypes (fig5.2Aiv). Both the average signal intensity in the entire cells and the intensity on the inside of the cells were higher (34\% and 26\% respectively) in \textit{munc18-1} null MCCs compared to \textit{wild type} cells (fig5.2Aiv). Due to the low number of independent observations in this experiment, no strong conclusions can be drawn. Furthermore, we observed high variation in signal intensities, which are probably due to motility of the living cells in combination with a high background intensity, which can be explained by the out-of-focus fluorescence that is included in the epi-fluorescence image. However, this limited dataset suggests that the observed PI(4,5)P_2 increase in fixed \textit{munc18-1} null MCCs, can be replicated in living cells. To confirm this suggestion we turned to the analysis of footprints in the TIRF plane.
Increased levels of PI(4,5)P$_2$ in munc18-1 null CCs

Footprint
The alterations in the amount of the PI(4,5)P$_2$ occur at the PM. So far we primarily analyzed PM-localized fluorescent signals in the equatorial plane. However, in this plane, the actual amount of PM is rather small compared to the overall size of the cell (≈ 5% of the total cell surface, when a cell diameter of 10 µm and the confocal microscopy z-resolution of 500 nm are assumed). Therefore, we applied total internal reflection fluorescence (TIRF) imaging to focus on the so-called footprint of the cell. The footprint shows the part of the cells PM which is attached to the coverslip and represents ≈ 10% of the total cell surface (fig5.2Bi). We used ROIs to quantify the signal intensity at the footprint (fig5.2Bii). Interference reflection microscopy (IRM) was used to confirm cell attachment on the glass coverslip (fig5.2Biii). The signal intensity in munc18-1 null MCCs was increased (78%) compared to wild type MCCs (fig5.2Biv). The increased PH-PLCδ1-eGFP signal intensity at the footprint of munc18-1 null MCCs confirmed the results gathered at the equatorial plane and the previous conclusions from fixed cells.

Mobility of PH-PLCδ1-eGFP rich membranes is increased in munc18-1 null chromaffin cells

We hypothesized that the increase in PI(4,5)P$_2$ levels may influence lipid mobility in the PM. We quantified the alterations in the PH-PLCδ1-eGFP signal intensity on the footprint over time. We used two approaches to calculate the mobility of PH-PLCδ1-eGFP. First, we defined mobility as difference between maximum and minimum intensity values and second, we compared signal intensities at different time points (for detailed descriptions see Material and Methods section). In wild type MCCs, a homogenous distribution of pixels with intensity alterations can be observed at the footprint. In munc18-1 null MCCs, the amount of such pixels is increased. Hence, both methods showed a slight mobility increase in MCCs from munc18-1 null mice compared to wild type MCCs (12% and 13%; fig5.3).
Figure 5.2: Confirmation of Munc18-1-dependent PI(4,5)P$_2$ phenotype in living cells

Ai. Epi-fluorescence microscopy images (average intensity Z-projection of a stack, consisting of 5 frames) of MCCs expressing PH-PLCδ1-eGFP, top: *wild type* MCC, bottom: *munc18-1 null* MCC. Aii. Quantification of PH-PLCδ1-eGFP intensity on the plasma membrane by application of three ROIs (*wild type*: grey, N:1, n:17; 43.33 a.u. ± 10.32; *munc18-1 null*: green, N:1, n:17; 127.47 a.u. ± 40.33; t-test; n.s.). Aiii. Visualization of the three ROIs (background, entire cell, inside of the cell) which were used to analyze signal intensities on the plasma membrane, left: *wild type* MCC, right: *munc18-1 null* MCC. Aiv. Analysis of average signal intensities in the three ROIs separately (green: entire cell, *wild type*: 945.92 a.u. ± 76.97; *munc18-1 null*: 1270.66 a.u. ± 71.57; light green: inside the cell, *wild type*: 902.59 a.u. ± 71.57; *munc18-1 null*: 1143.19 a.u. ± 135.10; t-test, n.s.; light grey: background, *wild type*: 480.90 a.u. ± 2.71; *munc18-1 null*: 443.45 a.u. ± 123.79, t-test, n.s.) (left: *wild type*, right: *munc18-1 null*). Bi. TIRF image of PH-PLCδ1-eGFP on footprint (left: *wild type*, right: *munc18-1 null*). Bii. Visualization of the ROIs (one ROI for background subtraction, three ROIs on the footprint used for analysis of signal intensity (left: *wild type*, right: *munc18-1 null*). Biii. IRM images of footprint area to determine proper cell to coverslip attachment (left: *wild type*, right: *munc18-1 null*). Biv. Quantification of signal intensity based on ROI analysis (*wild type*: grey, N: 2, n:28; normalized wild type to 100%, *munc18-1 null*: green, N:2, n:34, 177.92 % ± 32.93; t-test, **, p = 0.007); shown are mean ± SEM; Scale bars: 2 µm.
5. Increased levels of PI(4,5)P\textsubscript{2} in munc18-1 null CCs

Figure 5.3: No significant difference in mobility of PI(4,5)P\textsubscript{2}/PH-PLC\textgreek{d}-eGPF molecules. A. Mobility analysis 1, based on Z-projections of maximum and minimum intensities. Ai. Z-projection of 20 frames, based on maximum intensity in each frame (left: wild type; right: munc18-1 null). Aii. Z-projection of 20 frames, based on minimum intensity in each frame (left: wild type; right: munc18-1 null). Aiii. Image resulting from subtraction of "minimum projection" from "maximum projection" Z-projection (left: wild type; right: munc18-1 null). Aiv. Quantification of average intensity of Aiii (wild type: grey, N:1, n:17; 1121.65 a.u. ± 49.91; munc18-1 null: green, N:1, n:20, 1269.63 a.u. ± 46.81; t-test, *; p = 0.04). B. Mobility analysis 2, based on binning of frames and calculation of intensity differences between binned images. Bi. Inserts: Z-projection (average intensity) of binned frames 1-5 (top) and binned frames 6-10 (bottom), Image resulting from subtraction of insert(bottom) from insert(top): "difference1" (left: wild type, right: munc18-1 null). Bii. Inserts: Z-projection (average intensity) of binned frames 6-10 (top) and binned frames 11-15 (bottom), Image resulting from subtraction of insert(bottom) from insert(top): "difference2" (left: wild type, right: munc18-1 null). Biii. Inserts: Z-projection (average intensity) of binned frames 11-15 (top) and binned frames 16-20 (bottom), Image resulting from subtraction of insert(bottom) from insert(top): "difference3" (left: wild type, right: munc18-1 null). Biv. Quantification of average intensity difference (average of difference1-3): wild type: grey, N:1, n:17; 81.14 a.u. ± 3.25; munc18-1 null: green, N:1, n:20, 90.75 a.u. ± 3.68; t-test, n.s.; shown are mean ± SEM; Scale bars: 2 \textmu m.
First attempts of super-resolution microscopy analysis suggest clear correlations between PI(4,5)P₂ and F-actin signals and between PI(4,5)P₂ and Syntaxin1a signals at the plasma membrane

The resolution of conventional microscopy techniques (e.g., confocal, epi-fluorescence, TIRF) is not high enough to observe distinct PI(4,5)P₂ clusters in the PM. Therefore, we used Structured Illumination Microscopy (SIM) to investigate the co-localization of PI(4,5)P₂ clusters and F-actin (fig5.4A) or Syntaxin1a (fig5.5B). We acquired images of wild type MCCs which expressed PH-PLCδ1-eGFP and were stained for F-actin or Syntaxin1a. We performed a Z-projection to generate three dimensional representations of the MCCs and used single slices to determine the correlation between PH-PLCδ1-eGFP and F-actin (fig5.4Aiv) and between PH-PLCδ1-eGFP and Syntaxin1a in these example images (fig5.5Biv). As expected, both correlations were clearly positive (PH-PLCδ1-eGFP/F-actin: \( R = 0.64 \pm 0.09 \) (fig5.4Av); PH-PLCδ1-eGFP/Syntaxin1a: \( R = 0.73 \pm 0.09 \) (fig5.5Bv)). PM-localized clusters of PI(4,5)P₂ and F-actin or Syntaxin1a can be visualized using SIM. These first attempts indicate that SIM can be used for future research to uncover the organization of Munc18-1 dependent F-actin regulation.
5. Increased levels of PI(4,5)P$_2$ in *munc18-1* null CCs

**Figure 5.4:** Super-resolution microscopy reveals strong co-localization of PI(4,5)P$_2$ with F-actin and Syntaxin1a at the plasma membrane. **Ai**. SIM images of PI(4,5)P$_2$ clusters and cortical F-actin at the PM in two attached *wild type* chromaffin cells; cell on top is infected with Lentivirus which expresses PH-PLCδ1-eGFP. Ai. Z-projection of 107 frames of SIM images (left: PH-PLCδ1-eGFP; middle: cortical F-actin network; right: merge of both channels (green: PH-PLCδ1-eGFP, red: F-actin). Aii. Single slices/frames from the z-stack (left: PH-PLCδ1-eGFP; middle: cortical F-actin network; right: merge of both channels (green: PH-PLCδ1-eGFP, red: F-actin). Aiii. Polar Transformation images of both single channels (top: PH-PLCδ1-eGFP, middle: F-actin, bottom: merged channels). Aiv. Line scan showing the thickness of both signals (green: PH-PLCδ1-eGFP, red: F-actin) along the PM of both cells. Av. Scatterplot illustrating correlations of PH-PLCδ1-eGFP and F-actin signals, N:1, n:3, R=0.64 ± 0.09; p < 0.001.
Figure 5.5: Super-resolution microscopy reveals strong co-localization of PI(4,5)P₂ with F-actin and Syntaxin1a at the plasma membrane. Bi. SIM images for analysis of co-localization of PI(4,5)P₂ clusters and Syntaxin1a at the PM of a wild type chromaffin cell. Bi. Z-projection of 99 frames of SIM images (left: PH-PLCδ₁-eGFP; middle: Syntaxin1a; right: merge of both channels (green: PH-PLCδ₁-eGFP, blue: Syntaxin1a)). Bii. Single slices/frames from the z-stack (left: PH-PLCδ₁-eGFP; middle: Syntaxin1a; right: merge of both channels (green: PH-PLCδ₁-eGFP, blue: Syntaxin1a)). Biii. Polar Transformation images of both single channels (top: PH-PLCδ₁-eGFP; middle: Syntaxin1a; bottom: merged channels. Biv. line scan showing the thickness of both signals (green: PH-PLCδ₁-eGFP, blue: Syntaxin1a) along the PM of both cells. Bv. Scatterplot illustrating correlations of PH-PLCδ₁-eGFP and Syntaxin1a signals, N:1, n:2, R=0.73 ± 0.09; p < 0.001.
5. Increased levels of PI(4,5)P$_2$ in munc18-1 null CCs

**Discussion**

In the present study we show a robust increase in PM-localized PI(4,5)P$_2$ in munc18-1 deficient MCCs. This increase was shown in fixed cells as well as living cells and with different microscopy techniques. PI(4,5)P$_2$ was slightly more mobile in munc18-1 null MCCs. First attempts using super-resolution analysis suggest a clear co-localization of PI(4,5)P$_2$ with F-actin and Syntaxin1a at the PM of wild type MCCs.

**PH-PLCδ1: a widely used tool to monitor PI(4,5)P$_2$, but not without limitations**

To quantify levels of PM-localized PI(4,5)P$_2$, we used the fluorescently-tagged pleckstrin homology (PH) domain of PLCδ1 (Holz et al., 2000). Based on its high affinity to bind PI(4,5)P$_2$ (Lemmon et al., 1995), this construct is widely used and allows PI(4,5)P$_2$ monitoring and analysis in plasma sheets (Milosevic et al., 2005) and living (Stauffer et al., 1998; Balla and Varnai, 2002; Hammond et al., 2009) as well as fixed cells.

Despite its widespread application this method is poorly validated and has disadvantages that need to be taken into consideration. First, PH-PLCδ1 also has a high binding affinity for Inositol(1,4,5)-triphosphate I(1,4,5)P$_3$, a second messenger molecule that is generated by the PLCδ1-dependent hydrolysis of PI(4,5)P$_2$ (Michell, 1975; Berridge and Irvine, 1984), even higher than for PI(4,5)P$_2$ (Kd = 130nM vs 210nM respectively, Lemmon et al., 1995). I(1,4,5)P$_3$ is a soluble molecule that diffuses freely through the cytosol. It is unclear to what extent cellular I(1,4,5)P$_3$ levels and changes in these levels between experimental groups confound the conclusions of PH-PLCδ1 based assays. Since we only measured the signal at the PM, the uncertainty about the marker being bound to mobile I(1,4,5)P$_3$ instead of membrane-localized PI(4,5)P$_2$ might be limited.

Second, it was shown that the binding of PH-PLCδ1-GFP to PI(4,5)P$_2$ prevents the PLC-dependent hydrolysis of PI(4,5)P$_2$ (Wang et al., 2005). Such an inhibitory effect could alter signal transduction cascades in cells and again confound the conclusions of PH-PLCδ1 based assays. Third, since the only accessible component of PI(4,5)P$_2$ is its charged inositol head group, binding of the PH-PLCδ1 probe prevents interactions with other PI(4,5)P$_2$ binding proteins and thereby inhibits regulated secretion (Holz et al., 2000). The interpretation of our results might be influenced by any of these three confounding factors. For example, the slight increase in mobility in fig5.3 might be caused by an increased PI(4,5)P$_2$ turnover rate due to an up-regulation of PLC-dependent hydrolysis of excess PI(4,5)P$_2$. Another explanation might be increased competition for PH-PLCδ1-eGFP, since elevated levels of PI(4,5)P$_2$ might lead to an increased recruitment of actin-regulating proteins. To understand these complex processes and mechanisms, more experimental evidence is crucial. Most of all, validation of conclusions based on PH-PLCδ1-eGFP by independent methods is required. In principle, PI(4,5)P$_2$-specific antibodies might serve such a purpose. However, currently available antibodies appear not to be specific (fig5.6).

It might be possible to circumvent the problems associated with PH-PLCδ1-eGFP by not visualizing PI(4,5)P$_2$, but rather by modifying the amount of intracellular PI(4,5)P$_2$ levels and analyzing the consequences. Several strategies can be used to increase intracellular PI(4,5)P$_2$ levels, e.g., overexpression of phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) (Milosevic et al., 2005). PI4P5Ks are known to catalyze the phosphorylation of PI4P on the 5-position and thereby synthesize PI(4,5)P$_2$ (Carpenter and Cantley, 1990).
Unfortunately, the overexpression of PI4P5Ks was shown to result in abnormal actin structures (Rozelle et al., 2000), which makes this approach non-applicable for our experiments, since we are particularly interested in the cortical F-actin network. Methods to increase PI(4,5)P₂ levels more acutely are the application of Wortmannin or the chemical LY294002 which are commonly used PI3K inhibitors (Arcaro and Wymann, 1993; Vlahos et al., 1994). The most commonly used method to deplete PI(4,5)P₂ levels is the overexpression of full length phosphatases or their active domains (e.g., synaptojanin (Krauss et al., 2003; Milosevic et al., 2005)). A recently developed, very elegant method to alter the phospholipid metabolism at the PM makes use of light-induced dimerization of phosphoinositide-regulating proteins (kinases and phosphates) and proteins with membrane-targeting motifs (Idevall-Hagren and De Camilli, 2015). This approach allows a highly temporally and spatially controlled increase or decrease of phosphoinositide species at the PM, which might help to unravel to biological mechanisms involved. Besides those primarily imaging-based analysis methods, a multitude of biochemical techniques can be applied to determine PI(4,5)P₂ levels (e.g., high performance liquid chromatography (HPLC) (Boyle et al., 1990) and mass spectrometry (MS) (Bird, 1994; Han and Gross, 1994)). Since none of the currently known PI(4,5)P₂ quantifying tools is flawless, a combination of several methods and techniques will be necessary to unravel the multitude of functions in which this phospholipid and its derivates are involved.

Increased PI(4,5)P₂ levels at the plasma membrane and higher mobility in munc18-1 null chromaffin cells

The analysis of the signal intensity of PI(4,5)P₂-markers (PH-PLCδ₁-eGFP or PH-PLCδ₁-mCherry) showed an increase in fixed and alive MCCs from munc18-1 null mice compared to wild type mice (fig1-2). This increase in the absence of Munc18-1 can be explained by two scenarios: a better accessibility of PI(4,5)P₂ to the marker or a true increase in the amount of PM localized PI(4,5)P₂. The first option seems less likely, since the increase of the cortical F-actin network might leave less space for the marker to access PM localized PI(4,5)P₂. Furthermore, it might be possible that more actin-stabilizing proteins are bound to PI(4,5)P₂ and thereby hamper the binding of the probe to the lipid. Both aspects would result in an underestimation of PI(4,5)P₂ rather then the observed increased PI(4,5)P₂ levels in munc18-1 null cells. An increase in the amount of PI(4,5)P₂ on the PM of munc18-1 null MCCs could be caused by a variety of reasons. Our current working model (for detailed description see chapter 6 of this thesis) is based on the inhibitory role of Munc18-1 on PLD (Lee et al., 2004) and unpublished data by Angeli Möller, MDC, Berlin). However, other possibilities cannot be excluded at this stage. Alterations in the phosphoinositide metabolism (e.g., an increase in amount or activity of the main PI(4,5)P₂ generating kinase or a decrease in PI(4,5)P₂ phosphatases, PI(3,4,5)P₃ kinases) could also offer plausible explanations for the observed phenotype. Future experiments are necessary to test the PLD-based working model and clarify a potential involvement of other mechanisms. However, based on our results and the current interpretation, PI(4,5)P₂ might very well be the missing link that connects Munc18-1 as essential docking factor with the cortical F-actin network.
Future directions
The results that were presented in this study are consistent with our model, in which the increase of available PI(4,5)P$_2$ molecules at the PM and the alterations in the cortical F-actin network are directly related. Next, the directionality of this relationship needs to be determined: is the increase in PI(4,5)P$_2$ at the PM cause or consequence of the increased cortical F-actin network? The PI(4,5)P$_2$-dependent F-actin regulation was subject of several studies (for review see: Caroni, 2001; Takenawa and Itoh, 2001; Yin and Janmey, 2003; Janmey and Lindberg, 2004; Di Paolo and De Camilli, 2006; Saarikangas et al., 2010; Kwiatkowska, 2010) and is widely accepted. However, the converse dependency was also shown (Bittner and Holz, 2005). It also needs to be investigated how the absence of Munc18-1 results in an increase in PI(4,5)P$_2$ levels at the PM and whether PLD could be the link between the protein and the phospholipid. PI(4,5)P$_2$ clusters seem to co-occur with cortical F-actin and Syntaxin1a in wild type CCs and it will be very interesting to determine whether the co-localization is significantly altered in CCs from munc18-1 null mice. Based on the increased amount of both PI(4,5)P$_2$ and F-actin in munc18-1 null MCCs, one would expect an even higher co-localization here. On the other hand the co-localization between PI(4,5)P$_2$ and Syntaxin1a is likely to be lower in the absence of Munc18-1, since the redistribution of Syntaxin1a to the cytoplasm results in a reduced amount at the PM. It will be very interesting to see whether the remaining Syntaxin1a molecules will be hotspots for PI(4,5)P$_2$ clusters at the PM. Besides the imaging approaches that show spatial co-occurrence between the proteins and lipids of interest, it will be necessary to further investigate the functional interaction between the phospholipid PI(4,5)P$_2$ and F-actin or Syntaxin1a and determine whether those interactions are altered in a Munc18-1 dependent manner. Discussing a specific protein or subset of proteins at this stage is speculative, but pathway hypotheses that are consistent with the currently available data will be discussed in chapter 6 of this thesis.
Material and Methods

Laboratory animals
Generation of munc18-1 null mice was described earlier (Verhage et al., 2000). Embryos (E18) were obtained by caesarean section of pregnant females from timed breeding of heterozygotes. Laboratory animals were bred and housed according to Dutch governmental guidelines for animal welfare.

Chromaffin cell primary culture and HEK cell culture
Adrenal CCs from embryonic wild type and munc18-1 null mice (E18) were isolated as described previously (Sørensen et al., 2003b). The isolated CCs were cultured on glass coverslips coated with rat tail collagen. For a subset of the immunocytochemistry experiments we used the human embryonic kidney cell line 239T (HEK239T).

Semliki Forest Virus (SFV) infection
SFV particles were stored at -80 °C and were activated prior to usage. Activation was performed using chymotrypsin and aprotinin. 25 µl of activated SFV solution were added to the culture medium of each well. The MCCs were incubated for 5-8 hrs at 37 °C.

Lentivirus infection
8 µl of Lentivirus solution were added to the medium in each well to infect MCCs. The MCCs were incubated for 30-48 hrs at 37 °C.

Immunocytochemistry
Chromaffin cells were fixed with 4% paraformaldehyde (PFA) at DIV3. The cells were permeabilized by 5 min incubation in phosphate buffered saline (PBS) containing 0.5% Triton X-100. To block nonspecific binding, the cells were incubated for 30 min in PBS containing 0.1% Triton X-100 and 2% normal goat serum. All antibodies were diluted in this solution. The cells were incubated in the primary antibody solution for 1 h at room temperature (RT). After washing 3 times with PBS for 10 min, the cells were incubated in the secondary antibody solution for 1 hr at RT. The cells were washed 3 times in PBS and mounted on microscopy slides with Mowiol in preparation for the confocal microscopy and glycerol for the structured illumination microscopy, respectively.

Antibodies
Specific primary antibodies were used against Syntaxin1a (rabbit polyclonal, I379 (gift from T.C. Südhof lab), 1:1000), PI(4,5)P2 (mouse monoclonal, ADI-915-062, Enzo LifeScience, 1:1000) and Syt-1 (rabbit polyclonal, W855 (gift from T.C. Südhof lab), 1:2000). As secondary antibodies goat α-rabbit Alexa Fluor 647 and goat α-mouse Alexa Fluor 546 and 647 were used (Molecular Probes, 1:1000). The cortical F-actin network was stained with the conjugate rhodamine-phalloidin (R415, Molecular Probes), which does not require a secondary antibody.
Increased levels of PI(4,5)P\textsubscript{2} in munc18-1 null CCs

**5. Image Acquisition**

*Confocal Microscopy* Images of fixed and mounted samples were acquired using confocal microscopy and structured illumination microscopy. For confocal microscopy, the microscopy slides were imaged with a 63x plan-neofluor lens (numerical aperture 1.4, Carl Zeiss) on a Zeiss 510 Meta Confocal Microscope. An additional zoom factor of 5 was applied and the images were acquired with a frame size of 1024x1024 pixels. A single image of the equatorial plane of the MCCs was acquired.

*Structured Illumination Microscopy (SIM)* Structured illumination microscopy was performed with the Elyra PS.1 platform (Carl Zeiss), equipped with a 63x plan-apochromat lens (numerical aperture 1.46, Carl Zeiss). Here a z-stack covering the entire cell was acquired.

*Epi-fluorescence Microscopy* Coverslips were transferred into an imaging chamber and equilibrated in Ringer solution (2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 147 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 10 mM Glucose). The cells were imaged on an Olympus 1X81 microscope (Olympus), equipped with a Hamamatsu C9100 EM-CCD camera (Hamamatsu City, Japan) and a MT20 illumination unit (Olympus). Images were acquired with a 60x oil lens (NA 1.49) and at a frequency of 2 Hz.

*Total internal reflection Microscopy (TIRF)* For TIRF imaging we applied the same conditions as for epi-fluorescence microscopy. We used the same microscope, but in combination with an additional 1.6 x magnification and a 488 nm solid-state laser (Olympus).

**Image Analysis**

Image analysis was primarily executed with PlasMACC, which is implemented as a plugin in the image analysis software ImageJ. We determined intensity, thickness and density of fluorescent signals at the plasma membrane and in subplasmalemmal regions. Besides the analysis with PlasMACC, we also used several ROI-dependent analysis methods. To determine fluorescent signals at the PM of chromaffin cells, which were acquired with epi-fluorescence microscopy (fig5.2), we measured the average intensity of three specific ROIs: one ROI in the background, one ROI covering the entire cell surface and one ROI which only included the inside of the cell. For the signal quantification at the PM and in subplasmalemmal regions, we subtracted the average intensity of the inside of the cell as well as the background signal from the signal of the entire cell. For the analysis of the eGFP signal intensity on the cells footprint, we also used an ROI-based quantification method (fig5.3A). We first measured the average intensities of three ROIs which were randomly distributed on the footprint area. We determined the average intensity of those signals and afterwards subtracted the signal intensity in the background of the image.

In fig5.3 we determined the PI(4,5)P\textsubscript{2} mobility on the footprint with TIRF movies. First, we quantified the average intensity difference between two Z-projections of all 20 consecutive frames; one was based on maximal intensity (fig5.3Ai, ”maximum projection”) and the other one on minimal intensity (fig5.3Aii, ”minimum projection”) in each frame. We used the Image analysis software ImageJ to generate both Z-projections and to calculate the difference between both per cell. We subsequently determined the average intensity in both genotypes (wild type and munc18-1 null).
In the second approach, we merged 5 consecutive frames in 4 bins (bin1: frame 1-5; bin2: frame 6-10; bin3: frame 11-15; bin4: frame 16-20) and generated Z-projections based on the average intensity of those 5 frames. Afterwards we calculated the intensities of images which resulted from subtraction of bin2 from bin1 (difference1; fig5.3Ci; inserts: Z-projections frame 1-5 (top), frame 6-10 (bottom); intensity values in subtracted image), bin3 from bin2 (difference2; fig5.3Cii; inserts: Z-projections frame 6-10 (top), frame 11-15 (bottom); intensity values in subtracted image) and bin4 from bin3 (difference1; fig5.3Ciii; inserts: Z-projections frame 11-15 (top), frame 16-20 (bottom); intensity values in subtracted image). The three resulting differences were averaged. We performed Students t-tests to compare the described parameters between wild type and munc18-1 null CCs.
5. Increased levels of PI(4,5)P₂ in munc18-1 null CCs

Supplementary Material

**Figure 5.6: Less successful methodological approaches**

A. Comparison of anti-PI(4,5)P₂ antibody staining with staining against F-actin, Syt-1 and Syntaxin1a in wild type CCs. Ai. Co-staining: PI(4,5)P₂ (left) and F-actin (middle) and merge of both channels (right). Aii. line scans of PI(4,5)P₂ signal (green) and F-actin (red) at the PM. Aiii. Co-staining: PI(4,5)P₂ (left) and Syt-1 (middle) and merge of both channels (right). Aiv. line scans of PI(4,5)P₂ signal (green) and Syt-1 (red) at the PM. Av. Co-staining: PI(4,5)P₂ (left) and Syntaxin1a (middle) and merge of both channels (right). Avi. line scans of PI(4,5)P₂ signal (green) and Syntaxin1a (red) at the PM.


C. 5-8 hrs expression of PH-PLCδ1-eGFP through SFV particles in wild type (Ci, Cii) and munc18-1 null (Ciii, Civ) CCs.