Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 360 (2007) 609–614
BBRC
www.elsevier.com/locate/ybbrc

Interaction of Munc18 and Syntaxin in the regulation of insulin secretion

Yongming Dong [a,1], Qunfang Wan [a,1], Xiaofei Yang [a], Li Bai [a], Pingyong Xu [b,*]

[a] Joint Laboratory of the Institute of Biophysics & Huazhong University of Science and Technology, School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, PR China
[b] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

Received 4 June 2007
Available online 2 July 2007

Abstract

Syntaxin1A and Munc18-1 play essential roles in exocytosis. However, the molecular mechanism and the functional roles of their interaction in insulin secretion remain to be explored. Using membrane capacitance measurement, we examine effect of overexpressing Munc18-1 on exocytosis in pancreatic β cells. The results show that Munc18-1 negatively regulates vesicle fusion. To probe the interaction between Munc18-1 and Syntaxin1A, Munc18-1-Tdimer2 and EGFP-Syntaxin1A were co-transfected into INS-1 cells. FRET measurement confirmed that Munc18-1 interacted with wild type Syntaxin 1A, but not the constitutively open form (DM) of Syntaxin1A. Overexpressing DM in primary pancreatic β cells augmented insulin secretion, and this effect can overcome the inhibitory effect of Munc18-1 overexpression. We propose that Munc18-1 inhibitis the SNARE complex assembly by stabilizing Syntaxin1A in a closed conformation in vesicle priming process, therefore negatively regulates insulin secretion.

Keywords: Munc18; Syntaxin; Insulin secretion

The SNARE (soluble *N*-ethyl-maleimide sensitive factor attachment protein receptor) family plays fundamental roles in mediating membrane fusion [1]. SNAREs can be divided into two categories: the v-SNAREs located on carrier vesicles, and the t-SNAREs present on target compartments [2]. Syntaxin1A (Stx1A) is a t-SNARE predominantly located in the plasma membrane (PM) and has been shown to be essential for vesicle docking and fusion [3]. Munc18 isoforms are mammalian members of Sec1 and UNC-18-related proteins that are highly conserved from yeast to mammals. This protein family is thought to regulate the exocytosis in a SNARE-dependent way [4,5]. However, the exact role of Munc18-1 in secretion cells remains controversial. There has been considerable debate about whether Munc18-1 is involved only in secretory-vesicle docking step at the target membrane [6] or additionally in fusion step [7].

Zhang et al. recently reported that Munc18 was associated with Syntaxin and served as a negative regulator of exocytosis in insulin secretion cell line HIT-T15 cells [8]. However, pancreatic primary β cells differ from insulin secretion cell lines. For instance, Synaptagmin I is not expressed in primary β cells but is abundantly expressed in insulin secretion INS-1 cells [9–11]. So, key regulators controlling insulin secretion should be studied in these primary cells. The mechanism by which Munc18-1 interact with Stx1A and regulate insulin secretion in primary β cells remains unsolved. In the present study, we aim to clarify the functional role of Stx1A/Munc18-1 interaction in the fusion of insulin-containing granules to the plasma membrane in primary β cells transfected with a Recombinant Semliki Forest Virus (SFV) vector [12]. We overexpressed Munc18-1, wild type Stx1A and the open form mutant of Stx1A in primary rat pancreatic β cells, and examined roles of these proteins in insulin secretion.

* Corresponding author. Fax: +86 10 64871293.
E-mail address: pyxu@moon.ibp.ac.cn (P. Xu).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.06.107

Materials and methods

Plasmids construction. Stx1A fragment was amplified with Pfu DNA polymerase (Stratagen, La Jolla, CA), digested and ligated into pEGFP-C1 fusion vector (Clontech Laboratories, Palo Alto, CA) to get the corresponding N-terminal GFP-tagged fusion proteins. The double point mutant, which is an open form mutant of Stx1A (DM, L165A/E166A) was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and ligated into vector pIRES2-EGFP to make pDM-IRES-EGFP. EGFP-Munc18-1 fragment was ligated into pDM-IRES-EGFP to generate pDM-IRES-EGFP-Munc18-1. For construction of Munc18-Tdimer2, Munc18-1 PCR fragment was ligated into pcDNA3.1-TDimer2 (gift from Dr. R.Y. Tsien). To generate recombinant pSFV plasmids, the fragments EGFP-Stx1A, EGFP-Munc18-1, DM-IRES-EGFP, and DM-IRES-EGFP-Munc18-1 were cloned into plasmid pSFV1 (Invitrogen Life Science). Construct integrity was verified using DNA sequencing analysis provided by United Gene Holdings, LTD (Shanghai, China). Restriction enzymes and other standard reagents were purchased from New England Biolabs.

Cell culture. INS-1 cells were cultured as previously described [14]. Pancreatic β cells from adult male Wistar rats were prepared and cultured as described previously [15].

Unless otherwise noted, cells were superfused at room temperature in the external solution containing (in mmol/L): 135 NaCl, 2 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, pH 7.4.

Transfection. INS-1 cells were transfected with Lipofectamine 2000 (Invitrogen Life Science) as per manufacture's recommendations. Four microliters of the purified plasmid DNA was used for cells cultured on 6-well plates. Cells were used 2 days after transfection.

Recombinant SFV carrying the desired proteins were generated as previously described [12]. The virus-containing supernatant was titrated on pancreatic β cells in serum-free medium. Infection was continued for 1.5 h at 37 °C with 5% CO_2, then the virus solution was drained and the incubation was continued for 8–16 h before electrophysiology experiments.

Fluorescence imaging. Confocal laser scanning biological microscope (FV500, Olympus) was constructed on an inverted microscope (IX81, Olympus). Images were collected under a Zeiss α-Plan Fluar 100× (NA = 1.45) oil objective. Images were acquired and analyzed using FLUOVIEW 4.3 (Olympus).

For FRET study, CFP labeled Stx1A and YFP labeled Munc18-1 were imaged by confocal microscope. We also developed another FRET pair [16], EGFP/Tdimer2, to study the interaction between Stx1A (or open form mutant) with Munc18-1. Excitation light was selected using a fiber optical coupled monochromator (Polychrome IV; TILL Photonics GmBH, Germany). FRET signal was quantified by calculating the effective FRET efficiency following the equation

$$E_A = \gamma\left[\frac{I_F - \beta I_D}{\alpha I_A} - 1\right] \quad (1)$$

as we described previously [17].

Ca^{2+}-uncaging and $[Ca^{2+}]_i$ measurement. Flashes of UV light and fluorescence-excitation light were generated as described [18]. We used DM-nitrophen (DMN)-containing internal solutions consisted of (in mM): 110 CsGlu, 5 DMNP–EDTA, 8 NaCl, 3.6 $CaCl_2$, 2 MgATP, 0.3 GTP, 0.2 furaptra, and 35 Hepes (pH 7.2). The basal $[Ca^{2+}]_i$ was measured to be ~200 nM by fura-2. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio according to the equation: $[Ca^{2+}]_i = K_{eff} * (R - R_{min})/(R_{max} - R)$, where K_{eff}, R_{min}, and R_{max} are constants obtained from intracellular calibration as previously described [18].

Membrane capacitance (C_m) measurement. All electrophysiological experiments on overexpressed β cells were carried out 8–16 h after SFV infection at 30–32 °C. C_m measurements were conducted as previously described [19]. The C_m traces were imported to IGOR Pro (WaveMetrics, Lake Oswego, OR) and were fitted with a triple exponential function. The sustained component of release was measured as the C_m increase between 1 and 5 s after each flash.

The data are given as the mean ± SEM. Statistical significance was evaluated using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Overexpressing Munc18-1 reduced insulin secretion in primary rat β cells

To determine the function of Munc18-1 in insulin secretion, EGFP-Munc18-1 was overexpressed in primary rat pancreatic β cells using SFV expression system [12,13]. Exocytosis was elicited by flash photolysis and was monitored by C_m measurement. In response to the step-like $[Ca^{2+}]_i$ elevation, the C_m increase typically consists of three components obtained from a triple exponential fit, which we termed the fast burst, slow burst and sustained components as previously reported [20]. The fast burst and slow burst components represent fusion of the rapidly and slowly releasable pools, respectively [13,21], whereas the sustained component represents refilling of the releasable pools from a large depot pool of vesicles [13,22].

Comparing to non-transfected cells, we observed a significant reduction of exocytosis in β cells overexpressing Munc18-1 (Fig. 1). The sizes of the slow burst and sustained components were reduced by ~50% by Munc18-1 overexpression, whereas the fast burst component remained unaffected (Fig. 1A and C). The result suggests that Munc18-1 may inhibit the priming and refilling of vesicle pool. In basal condition, vesicles in rapidly releasable pool (RRP) have formed tight SNARE complex already, so RRP size cannot be reduced by overexpression of Munc18-1. To further confirm this hypothesis, second flash were used to trigger exocytosis 2 min after the first flash uncaging. After depleting the RRP pool in the first flash, an inhibition of the refilling step would be expected to reduce the fast burst component in second flash. We noted that both the fast and slow burst components of the second flash were reduced by ~50% in cells overexpressing Munc18-1, as well as for the sustained component (Fig. 1B and D). To explore whether Munc18-1 participates in the final fusion step, the kinetics of the fast burst and slow burst components were compared between control cells and cells overexpressed with Munc18-1. No significant difference was observed (data not shown).

In vivo FRET measurement between Stx1A and Munc18-1

To further identify whether spatially colocalized Stx1A and Munc18-1 interact with each other *in vivo*, we have employed the FRET technique [23]. First, we checked whether Munc18-1 interacts with wild type Stx1A using the most frequently used FRET pair CFP/YFP. Cytoplasmic-tagged CFP-Stx1A and Munc18-1-YFP were coexpressed in INS-1 cells. As shown in Fig. 2A, after photobleaching the YFP fluorescence using 514 nm laser, the intensity of CFP fluorescence increased accordingly at

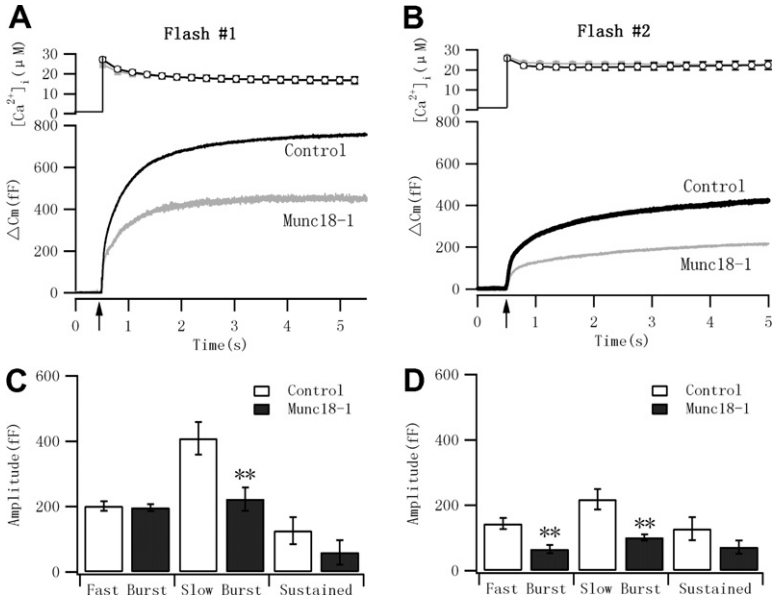

Fig. 1. Overexpression of Munc18-1 inhibits Ca^{2+}-triggered exocytosis in rat pancreatic β cells. (A,B) Averaged $[Ca^{2+}]_i$ and C_m increases (bottom) in response to the first and second flash from 33 control cells (black) and 21 cells overexpressing Munc18-1 (grey). Arrows indicate the onset of the flash. (C,D) Averaged amplitudes of the fast burst, slow burst and sustained components from control (open bar) and Munc18-1 overexpressing cells (filled bar) in response to the first and second flash, respectively. The sustained component was measured as the C_m increase between 1 and 5 s after flash. The fast burst and slow burst component were derived from the triple exponential fit. **Significant difference ($p < 0.01$, t-test) compared with paired control condition.

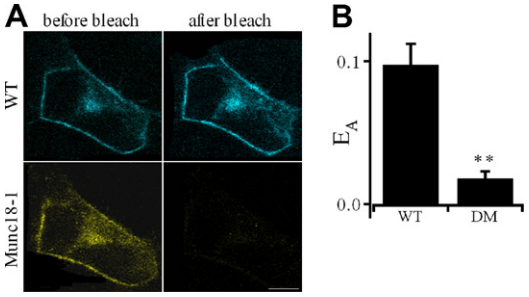

Fig. 2. *In vivo* interaction between Stx1A and Munc18-1. (A) Displayed are confocal fluorescence images of co-expressed CFP-Stx1A and Munc18-1-YFP in INS-1 cells. After photobleaching of YFP fluorescence using 514 nm laser, the fluorescent intensity of CFP showed significant increase, demonstrating an *in vivo* interaction of Munc18-1 with Stx1A. Bar, 5 μm. (B) The EGFP/Tdimer2 FRET pair was employed to assay the interaction between wild type Stx1A (WT) and DM with Munc18-1. The apparent FRET efficiency calculated according to Eq. (1) was used to quantify the FRET signal. **Significant difference ($p < 0.01$, t-test) compared with wild type FRET signal.

the PM, demonstrating an *in vivo* interaction of Munc18-1 with Stx1A. As the CFP/YFP pair are suboptimal for FRET measurement in our system, we have later developed an alternative pair, EGFP/Tdimer2, which displays superior wavelength separation of the emission spectra [16]. We quantified the FRET efficiency [17] between Stx1A-EGFP and Munc18-1-Tdimer2 according to Eq. (1). In agreement with our photobleaching experiment, we observed a significant FRET signal between Stx1A and Munc18-1 (Fig. 2B). In contrast, Munc18-1 and DM displayed a significantly reduced FRET signal, further supporting an impaired interaction between DM and Munc18-1.

Munc18-1 regulates insulin secretion in primary rat β cells through its interaction with Stx1A

To address roles of these two proteins in insulin secretion, the open form DM mutant was overexpressed in primary rat β cells. Interestingly, we identified overexpression of DM significantly augmented the sustained component as well as the slow burst component (Fig. 3A and B), indicating that DM, the open form Stx1A, can facilitate the formation of SNARE complex. As shown previously, Munc18-1 was a negative regulator of insulin secretion. However, co-expression of Munc18-1 with DM did not block the augmentary effect of DM, suggesting the inhibitory effect of Munc18-1 was due to its interaction with Stx1A. As a control, overexpression of wild type Stx1A did not significantly affect flash-evoked exocytosis in β cells (Fig. 3C and D).

Discussion

Munc18 is believed to play an important role in vesicle docking and fusion. There are various models concerning the role of Munc18 in regulating membrane fusion [4,5]. One of the models suggests that the association of Munc18 with Syntaxin stabilizes the closed form of Syntaxin, and thereby negatively regulates exocytosis [24]. This idea is supported by the observation that overexpression of UNC-18 in *Drosophila* inhibits neurotransmitter release

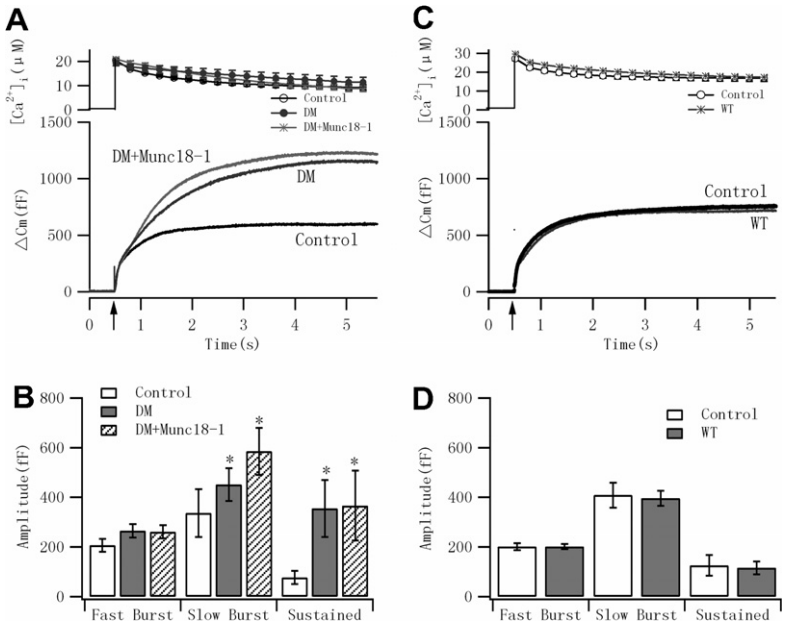

Fig. 3. Conversion of Stx1A from closed to open form is rate-limiting in insulin secretion. Shown are C_m responses of rat pancreatic β cell to flashes from paired experiments. Arrows indicate the onset of the flash. (A) Averaged $[Ca^{2+}]_i$ (top) and ΔC_m increases from 17 control cells, 27 cells overexpressed with DM and 16 cells co-overexpressed with DM and Munc18-1. (B) Summary of the amplitudes of the three exocytotic components from control cells, cells overexpressed with DM and DM + Munc18-1. *Significant difference ($p < 0.05$, t-test) as compared with paired control cells. (C) Averaged $[Ca^{2+}]_i$ (top) and ΔC_m increases from control cells ($n = 33$) and cells overexpressed with wild type (WT) Stx1A ($n = 25$). (D) No statistic significance was observed between the three exocytotic components after overexpression of Stx1A as compared with paired control cells.

in a Syntaxin-dependent manner [25]. Contrary to this model, however, overexpression of Munc18-1 increased exocytosis in chromaffin cells [6]. Moreover, deletion of Munc18-1 in mice resulted in a complete diminishment of neurotransmitter release [26]. Other models propose a role for Munc18 in the docking of vesicles to the PM [6,27] or in the final fusion after docking by interacting with Syntaxin [28] or the pre-assembly SNARE complex [29–31]. There are still a number of unanswered question regarding this docking and fusion issues.

In this study, we investigated the function of Munc18 in isolated primary insulin secretion β cell. The insulin response to glucose stimulation is characterized by a typical biphasic time course. It has been suggested that the biphasic release of insulin reflects the fusion of distinct subsets of secretory vesicles. The first phase of insulin secretion is attribute to the release of vesicles from the RRP vesicle pool, while the second phase involves mobilization of granules from the reserve pool [32,33]. The exact role of Munc18-1 in the multiple steps of insulin secretion has not been investigated. Taking advantage of the capability to dissect multiple components of exocytosis using caged Ca^{2+} release, we have studied the effect of overexpressed Munc18-1 on different components of secretion in primary cultured pancreatic β cells.

We demonstrated that the slow burst component as well as the sustained component of exocytosis was inhibited, whereas the fast burst component remains unaltered in response to the first flash. After depletion of RRP in first flash, the three components elicited by the second flash were more severely inhibited, suggesting that the overexpression of Munc18-1 inhibited the priming and recruitment of vesicle to the RRP pools (see Fig. 1). Whereas our result is in agreement with a negative role of Munc18-1 in insulin secretion [34], it is in contrast with the previous study in chromaffin cells where overexpression of Munc18-1 increased the docking of vesicles [35]. Against a role of Munc18-1 in the final Ca^{2+}-dependent binding steps prior to fusion, we demonstrated that the kinetics of the exocytotic burst was not altered by overexpression of Munc18-1. However, our data does not exclude possible effects of Munc18-1 on the kinetics of fusion pore expansion as demonstrated previously in chromaffin cells [28].

To get further understanding of the molecular mechanisms, we have tested the interaction between Stx1A and Munc18-1 *in vivo*. We provided direct evidence for the binding of Stx1A and Munc18-1 from *in vivo* FRET measurement, which is consistent with a recent study in HEK293 cells [36]. We found no colocalization of Munc18-1 with DM mutant on the surface membrane (data not shown). The FRET signal between DM and Munc18-1 is almost abolished (see Fig. 2B). Interestingly, DM is still able to form clusters at the PM as the wild type does (data not shown), implying that DM can localize at the correct position and assemble in clusters without help of Munc18-1. When we overexpressed the open form mutant, we observed a large increase in the slow burst and the sustained component (Fig. 3). Moreover, the augmentative effect of DM persists in the presence of Munc18-1. We also showed that DM aggregated into

clusters at the PM but could not interact with Munc18-1. Thus, we conclude that the inhibitory effect of Munc18-1 is mainly due to its interaction with Stx1A, i.e., by stabilizing Stx1A in the closed form. Intriguingly, both DM and Munc18-1 influence the slow burst without affecting the fast burst. The fast and slow burst components are supposed to represent vesicles in the rapidly and slowly releasable pool, which probably have already formed tight and loose ternary SNARE complexes, respectively [20]. As a control, overexpressing wild type Stx1A exerted no effect on exocytosis in pancreatic β cells (Fig. 3). The lack of effect of wild type Stx1A could not be due to an inefficiency in our overexpression system because the SFV system has been successfully employed to overexpress a number of proteins in a variety of cells [12,37]. The lack of effect is in contrast with the previous finding [38], but in agreement with a recent report that overexpression of Stx1A has no effect on the extent or time course of exocytosis in chromaffin cells [28]. A recent elegant experiment in Stx1A transgenic mice has suggested that elevated level of Stx1A inhibited insulin secretion by down-regulation of voltage-gated Ca^{2+} channels [39]. This effect, however, will be bypassed in our flash experiment because of homogenously elevated $[Ca^{2+}]_i$ independent of Ca^{2+} channels. Thus, this discrepancy may be explained, at least in part, by the difference in method of stimulation used.

In summary, our results provide direct evidence that the conversion of Stx1A from a closed form to an open form is critical for the formation of the slowly releasable pool, presumably by initiating the complexation of the *trans* SNARE complex. We propose that the opening of Stx1A in pancreatic β cells constitutes a rate-limiting step in insulin secretion and Munc18-1 negatively regulates this step.

Acknowledgments

We thank Dr. R.Y. Tsien (UCSD, USA) for kindly providing the cDNAs for Tdimer2, Dr. Y.C. Liu (University of Oklahoma, USA) for CFP-Stx1A and Munc18-1-YFP, and Dr. S.N. Yang (Karolinska Institute, Sweden) for INS-1 cells. This work was supported by National Science Foundation of China Grants 30670504.

References

[1] R. Jahn, T.C. Sudhof, Membrane fusion and exocytosis, Annu. Rev. Biochem. 68 (1999) 863–911.
[2] T. Sollner, S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, J.E. Rothman, SNAP receptors implicated in vesicle targeting and fusion, Nature 362 (1993) 318–324.
[3] S. Ferro-Novick, R. Jahn, Vesicle fusion from yeast to man, Nature 370 (1994) 191–193.
[4] R.F. Toonen, M. Verhage, Vesicle trafficking: pleasure and pain from SM genes, Trends Cell Biol. 13 (2003) 177–186.
[5] R.D. Burgoyne, A. Morgan, Membrane trafficking: three steps to fusion, Curr. Biol. 17 (2007) R255–R258.
[6] T. Voets, R.F. Toonen, E.C. Brian, H. de Wit, T. Moser, J. Rettig, T.C. Sudhof, E. Neher, M. Verhage, Munc18-1 promotes large dense-core vesicle docking, Neuron 31 (2001) 581–591.
[7] R.J. Fisher, J. Pevsner, R.D. Burgoyne, Control of fusion pore dynamics during exocytosis by Munc18, Science 291 (2001) 875–878.
[8] W. Zhang, A. Efanov, S.N. Yang, G. Fried, S. Kolare, H. Brown, S. Zaitsev, P.O. Berggren, B. Meister, Munc-18 associates with syntaxin and serves as a negative regulator of exocytosis in the pancreatic β-cell, J. Biol. Chem. 275 (2000) 41521–41527.
[9] A. Gut, C.E. Kiraly, M. Fukuda, K. Mikoshiba, C.B. Wollheim, J. Lang, Expression and localisation of synaptotagmin isoforms in endocrine β-cells: their function in insulin exocytosis, J. Cell Sci. 114 (2001) 1709–1716.
[10] J. Lang, M. Fukuda, H. Zhang, K. Mikoshiba, C.B. Wollheim, The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic β-cells: action of synaptotagmin at low micromolar calcium, EMBO J. 16 (1997) 5837–5846.
[11] M. Iezzi, G. Kouri, M. Fukuda, C.B. Wollheim, Synaptotagmin V and IX isoforms control Ca^{2+}-dependent insulin exocytosis, J. Cell Sci. 117 (2004) 3119–3127.
[12] S. Wei, T. Xu, U. Ashery, A. Kollewe, U. Matti, W. Antonin, J. Rettig, E. Neher, Exocytotic mechanism studied by truncated and zero layer mutants of the C-terminus of SNAP-25, EMBO J. 19 (2000) 1279–1289.
[13] T. Xu, T. Binz, H. Niemann, E. Neher, Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity, Nat. Neurosci. 1 (1998) 192–200.
[14] M. Asfari, D. Janjic, P. Meda, G. Li, P.A. Halban, C.B. Wollheim, Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines, Endocrinology 130 (1992) 167–178.
[15] X.L. Lou, X. Yu, X.K. Chen, K.L. Duan, L.M. He, A.L. Qu, T. Xu, Z. Zhou, Na^+ channel inactivation: a comparative study between pancreatic islet β-cells and adrenal chromaffin cells in rat, J. Physiol. 548 (2003) 191–202.
[16] X. Yang, P. Xu, T. Xu, A new pair for inter- and intra-molecular FRET measurement, Biochem. Biophys. Res. Commun. 330 (2005) 914–920.
[17] A. Hoppe, K. Christensen, J.A. Swanson, Fluorescence resonance energy transfer-based stoichiometry in living cells, Biophys. J. 83 (2002) 3652–3664.
[18] T. Xu, M. Naraghi, H. Kang, E. Neher, Kinetic studies of Ca^{2+} binding and Ca^{2+} clearance in the cytosol of adrenal chromaffin cells, Biophys. J. 73 (1997) 532–545.
[19] Q.F. Wan, Y. Dong, H. Yang, X. Lou, J. Ding, T. Xu, Protein kinase activation increases insulin secretion by sensitizing the secretory machinery to Ca^{2+}, J. Gen. Physiol. 124 (2004) 653–662.
[20] T. Xu, B. Rammner, M. Margittai, A.R. Artalejo, E. Neher, R. Jahn, Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis, Cell 99 (1999) 713–722.
[21] T. Voets, E. Neher, T. Moser, Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices, Neuron 23 (1999) 607–615.
[22] U. Ashery, F. Varoqueaux, T. Voets, A. Betz, P. Thakur, H. Koch, E. Neher, N. Brose, J. Rettig, Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells, EMBO J. 19 (2000) 3586–3596.
[23] L. Matyus, Fluorescence resonance energy transfer measurements on cell surfaces. A spectroscopic tool for determining protein interactions, J. Photochem. Photobiol. B 12 (1992) 323–337.
[24] B. Yang, M. Steegmaier, L.C. Gonzalez Jr., R.H. Scheller, nSec1 binds a closed conformation of syntaxin1A, J. Cell Biol. 148 (2000) 247–252.
[25] M.N. Wu, K.L. Schulze, T.E. Lloyd, H.J. Bellen, The ROP-syntaxin interaction inhibits neurotransmitter release, Eur. J. Cell Biol. 80 (2001) 196–199.
[26] M. Verhage, A.S. Maia, J.J. Plomp, A.B. Brussaard, J.H. Heeroma, H. Vermeer, R.F. Toonen, R.E. Hammer, T.K. van den Berg, M. Missler, H.J. Geuze, T.C. Sudhof, Synaptic assembly of the brain in the absence of neurotransmitter secretion, Science 287 (2000) 864–869.
[27] R.M. Weimer, J.E. Richmond, W.S. Davis, G. Hadwiger, M.L. Nonet, E.M. Jorgensen, Defects in synaptic vesicle docking in *unc-18* mutants, Nat. Neurosci. 6 (2003) 1023–1030.

[28] M.E. Graham, J.W. Barclay, R.D. Burgoyne, Syntaxin/Munc18 interactions in the late events during vesicle fusion and release in exocytosis, J. Biol. Chem. 279 (2004) 32751–32760.
[29] J. Shen, D.C. Tareste, F. Paumet, J.E. Rothman, T.J. Melia, Selective activation of cognate SNAREpins by Sec1/Munc18 proteins, Cell 128 (2007) 183–195.
[30] C. Rickman, C.N. Medine, A. Bergmann, R.R. Duncan, Functionally and spatially distinct modes of munc18-syntaxin 1 interaction, J. Biol. Chem. 282 (2007) 12097–12103.
[31] I. Dulubova, M. Khvotchev, S. Liu, I. Huryeva, T.C. Sudhof, J. Rizo, Munc18-1 binds directly to the neuronal SNARE complex, Proc. Natl. Acad. Sci. USA 104 (2007) 2697–2702.
[32] P. Rorsman, E. Renstrom, Insulin granule dynamics in pancreatic beta cells, Diabetologia 46 (2003) 1029–1045.
[33] P. Rorsman, L. Eliasson, E. Renstrom, J. Gromada, S. Barg, S. Gopel, The cell physiology of biphasic insulin secretion, News Physiol. Sci. 15 (2000) 72–77.
[34] A.I. Fletcher, R. Shuang, D.R. Giovannucci, L. Zhang, M.A. Bittner, E.L. Stuenkel, Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18, J. Biol. Chem. 274 (1999) 4027–4035.
[35] R.F. Toonen, O. Kochubey, H. de Wit, A. Gulyas-Kovacs, B. Konijnenburg, J.B. Sorensen, J. Klingauf, M. Verhage, Dissecting docking and tethering of secretory vesicles at the target membrane, EMBO J. 25 (2006) 3725–3737.
[36] J. Liu, S.A. Ernst, S.E. Gladycheva, Y.Y. Lee, S.I. Lentz, C.S. Ho, Q. Li, E.L. Stuenkel, Fluorescence resonance energy transfer reports properties of syntaxin1a interaction with Munc18-1 in vivo, J. Biol. Chem. 279 (2004) 55924–55936.
[37] M.U. Ehrengruber, K. Lundstrom, C. Schweitzer, C. Heuss, S. Schlesinger, B.H. Gahwiler, Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures, Proc. Natl. Acad. Sci. USA 96 (1999) 7041–7046.
[38] Y. Kang, X. Huang, E.A. Pasyk, J. Ji, G.G. Holz, M.B. Wheeler, R.G. Tsushima, H.Y. Gaisano, Syntaxin-3 and syntaxin-1A inhibit L-type calcium channel activity, insulin biosynthesis and exocytosis in beta-cell lines, Diabetologia 45 (2002) 231–241.
[39] P.P. Lam, Y.M. Leung, L. Sheu, J. Ellis, R.G. Tsushima, L.R. Osborne, H.Y. Gaisano, Transgenic mouse overexpressing syntaxin-1A as a diabetes model, Diabetes 54 (2005) 2744–2754.