PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2012, 39(6): 556~562
www.pibb.ac.cn

UNC-10 Regulates The Docking Step of DCV Exocytosis in *C. elegans**

FENG Wan-Juan[1)], ZHOU Wei[1)], ZHANG Yong-Deng[1)], WU Zheng-Xing[1)], XU Tao[1, 2)**]

([1)] *College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan* 430074, China;
[2)] *National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing* 100101, China)

Abstract Rim is an active zone protein which is suggested to be involved in the regulation of vesicle exocytosis and synaptic plasticity. The *C. elegans* genome encodes only one Rim gene, *unc-10*. In this study, we demonstrate that UNC-10 is involved in the dense core vesicle (DCV) exocytosis in *C. elegans* neurons. We find that *unc-10* mutants exhibit reduced peptide release *in vivo*. In IDA-1::GFP labelled ALA neurons, which serve as a good model for studying DCV exocytosis, mutations of UNC-10 lead to a visible reduction of the readily released pool (RRP) size. The analysis of vesicular docking by total internal reflection fluorescence microscopy (TIRFM) reveals that the loss of function of *unc-10* hinders the docking of DCVs in neurons. The above results provide evidence that UNC-10 is required for the exocytosis of DCVs and may be involved in the docking step of DCV release.

Key words *C. elegans*, dense core vesicle (DCV), UNC-10, exocytosis
DOI: 10.3724/SP.J.1206.2011.00374

Rim is originally identified as a protein that contains a putative Rab3A-effector domain at the N-terminus[1], and it has been shown to be involved in the regulation of vesicle exocytosis and synaptic plasticity[2-4]. Two isoforms of Rim (named Rim1α and Rim2α, designated simply Rim1 and Rim2, respectively) have been reported in mammals[5-6], and they share the same domain structures: (a) an N-terminal zinc-finger domain that interacts with the synaptic vesicle protein Rab3 and the active zone protein Munc13-1[1, 4, 7-8]; (b) a central PDZ domain that binds the C-terminus of ERCs (ELKS/Rab3-interacting molecule/CAST) [9-10]; and (c) two C-terminal C2 domains, of which the second, the C2B domain, binds to α-liprins[4]. Interactions with Rab3, Munc13-1, etc, suggest an essential role in secretory vesicle exocytosis.

Besides the synaptic vesicle (SV), there is another important vesicle called the dense core vesicle (DCV). Neuropeptides are packed into these DCVs and play critical roles in synaptic signalling. There are many differences in the properties of DCVs and SVs, suggesting that these two classes of secretory organelles employ different molecules during secretion. Whereas there are two isoforms of Rim (Rim1 and Rim2) in mammals, there is only one isoform of Rim gene (*unc-10*) in *C. elegans*. Therefore, *C. elegans* is a good model in which to study the function of Rim proteins in DCV exocytosis.

In this paper, we assessed neuropeptide release *via* measuring the fluorescence intensity of ANF-GFP in coelomocytes *in vivo*. We performed high resolution membrane capacitance(C_m) measurement in combination with Ca^{2+}-uncaging stimuli to monitor the amplitudes of exocytosis. We tracked the movements of single vesicles using optical measurements(TIRFM). Through the combination of these methods, we found that UNC-10 was required for DCV release and that it

*This work was supported by grants from National Basic Research Program of China (2010CB833701), The National Natural Science Foundation of China (30870564, 90913022), and Key Inndvative Project of Chinese Academy of Sciences (KSCX2-SW-224).
**Corresponding author.
Tel: 86-10-64888469, E-mail: xutao@ibp.ac.cn
Received: August 15, 2011 Accepted: September 19, 2011

regulated the docking and priming steps of DCV exocytosis.

1 Materials and methods

1.1 *C. elegans* strains

The nematode strains were maintained at 20℃, utilising standard methods[11–12]. The strains used in this study were as follows: *unc-10* (*md1117*), *ida-1*::*gfp* KM246[13], and *anf*::*gfp* EG3680[14]. We crossed KM246 and EG3680 with *unc-10*(*md1117*).

1.2 Behavioural assays

The number of body bends was sequentially recorded for five 20 s intervals. The data shown represent the mean of at least five animals for each genotype. Pharyngeal pumping was assayed by counting the pumping for five 1 min intervals. The data shown represent the mean of at least eight animals for each genotype. Defecation was observed under a dissecting microscope for the presence or absence of an expulsion event after each posterior body muscle contraction (pBoc) of the defecation cycle [15]. Ten animals from each strain were observed for 10 consecutive cycles. The data shown represent observations of at least five animals for 10 min.

1.3 Embryonic cell culture

Embryonic cells were isolated from nematode eggs and cultured as described[16–17]. Eggs were isolated from gravid adults following bleach treatment in a hypochlorite solution. Intact eggs were separated from debris by flotation on 30% sucrose. The egg shells were removed by incubation in 0.5 ml egg buffer containing 1 U/ml of chitinase (Sigma Chemical, St. Louis, MO) at 25℃ for 40～50 min. Following resuspension in Leibovitz's L-15 Media (Gibco, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (Gibco, Invitrogen) and antibiotics, the embryos were dissociated by gentle pipetting. Then, the intact embryos, clumps of cells, and larvae were removed by passing through a 5 μm Durapore syringe filter (Millipore, Bedford MA). Cells were plated on peanut lectin-coated (0.5 g/L, Sigma) cover glasses and maintained in L-15 medium. Cells were incubated at 20℃ in a humidified incubator. The culture medium was adjusted to 340 mOsm with sucrose and filter sterilised. Cells were cultured for 3～4 days.

1.4 The assay of ANF-GFP release

The ectopically expressed neuropeptide ANF-GFP was measured as the intensity of fluorescence in coelomocytes as described [14]. The imaging of immobilised worms was performed on an Andor Revolution XD laser confocal microscope system based on a spinning-disk confocal scanning head CSU-X1 (Yokogawa Electric Corporation) under the control of Andor IQ 1.91 software. The microscopic images were taken on an Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan) with a 60 × objective lens (NA =1.45, Olympus, Japan). Only the coelomocytes that were not masked by other tissues (gut and gonads) and did not abut with the body wall were captured. Images were displayed and analysed by Image-J 1.43b (Wayne Rasband, National Institute of Health, USA).

1.5 TIRFM imaging

Our TIRFM setup was constructed on an Olympus IX70 microscope equipped with a × 100 objective lens (NA = 1.45, Zeiss, Germany) based on the prismless and through-the-lens configuration by using a TILL TIRF condenser (TILL Photonics GmbH, LochhamerSchlag, Germany). A 488 nm laser was used for fluorescence excitation. Images were acquired at 40 Hz with a 25 ms exposure by iXonEM + 885 EMCCD (Andor Technology, Springvale Business Park, Belfast, United Kingdom) with a pixel size of 80 nm. We calculated the penetration depth of the evanescent field (d =109 nm) by measuring the incidence angle with a prism (n=1.516) with a 488 nm laser beam. Imaging was controlled by Andor IQ 1.91 software. Stacks of TIRF images were analysed by a self-written program in Mat-lab (Mathworks, Natick, MA, USA). Single vesicle detection was performed by the use of àtrous wavelet transformation with the level k = 3 and a detection level of ld = 1.0[18], producing a binary mask image. Only spots with areas larger than 4 (2 × 2) pixels were accepted as vesicles for further analysis. The fluorescence intensity and positions of the vesicles were determined using a 2D Gaussian fitting with a background offset. For tracking the vesicles, we used a robust single particle tracking algorithm provided by Jaqaman [19]. We defined a dwelling event as the 2D displacement of a vesicle less than 80 nm (1 pixel size of our CCD camera) for at least three consecutive frames (75 ms).

1.6 Statistics

All data were presented as the $\bar{x} \pm s$ with the indicated number of experiments. Statistical significance was evaluated by the Student's t test or the Mann-Whitney rank sum test according to the normality of datum distribution in SigmaStat 3.11

(Systat Software, Inc.). A significant difference is indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). All comparisons are made between paired experiments performed under the same conditions on the same days.

2 Results

2.1 UNC-10/Rim exhibits behavioural defects

unc-10 mutants were first isolated on the basis of defects in locomotion [11], but later were shown to exhibit broad nervous system dysfunction. We found that UNC-10 displayed various behavioural defects, including pharyngeal pumping, body bends and a slight deficit in defecation (Figure 1a, b, c and d). Similar defects have been observed with mutations affecting monoamines and neuropeptides.

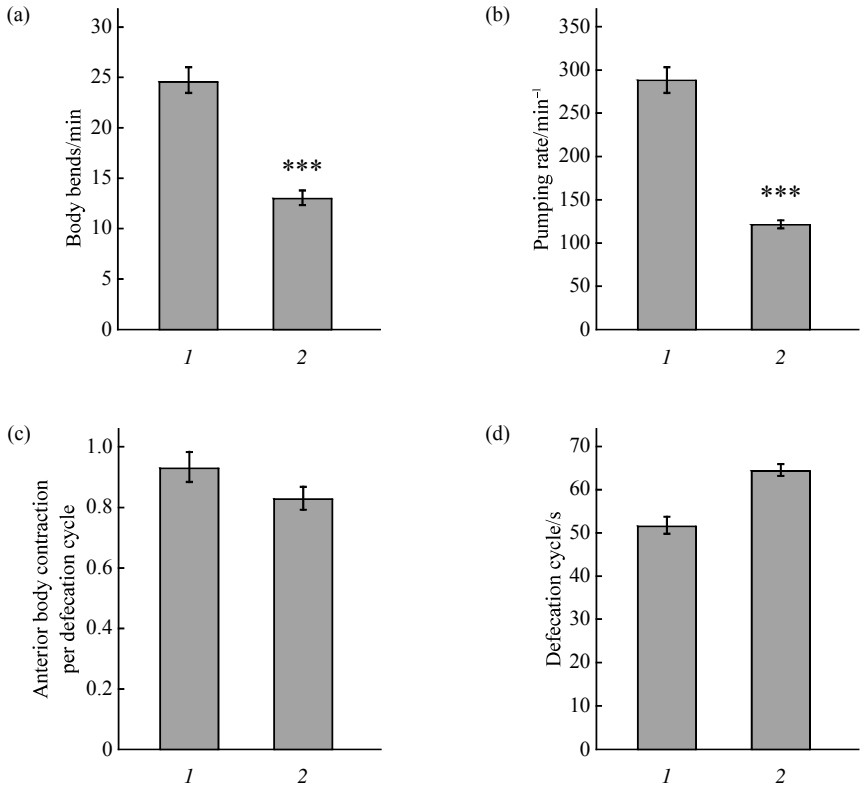

Fig. 1 Behaviour of *unc-10* mutants

(a) Body bends of wild type and *unc-10* mutants (per minute). (b) Pumping rate of wild type and *unc-10* mutants (per minute). (c) Defects in defecation were scored as failure of the enteric muscle contractions (EMCs) during the defecation process. The percentage of failure of EMCs was compared with that of wild-type and the *unc-10* mutants. (d) Average time interval between consecutive contractions of the posterior body-wall muscles (pBocs). Error bars indicate SEM. The asterisks indicate significant differences, as determined by Student's *t* test or the Mann-Whitney test (***$P < 0.001$). *1*: Wild type; *2*: *unc-10* (*md1117*).

2.2 UNC-10 is required to maintain the release of ectopically expressed ANF-GFP in neurons

To directly assess the function of UNC-10 *in vivo*, we quantified the accumulation of ANF-GFP in coelomocytes of *C. elegans*. The ectopic neuropeptide ANF-GFP, under the control of the pan-neuronal *aex-3* promoter, is expressed throughout the nervous system in the integrated strain (EG3680). ANF-GFP is released from neurons and then is endocytosed by coelomocytes [14], so it can be used as a reliable fluorescent reporter of DCV transport and exocytosis *via* the quantification of the accumulation of ANF-GFP in coelomocytes. We crossed the EG3680 strain with UNC-10 to generate neurons expressing ANF-GFP in a different genetic background (Figure 2a). We found that mutations in *unc-10* produced a large reduction (~60%) in peptide release (Figure 2b). To exclude the possibility of any influence on endocytosis in *unc-10*

mutants, we injected Rhodamine-conjugated dextran (10 ku) into the pseudocoelomic space and found that uptake by coelomocytes of *unc-10* mutants was not altered in comparison with wild type animals (Figure 2c). Taken together, Rim/UNC-10 might play an important role in regulating DCV release.

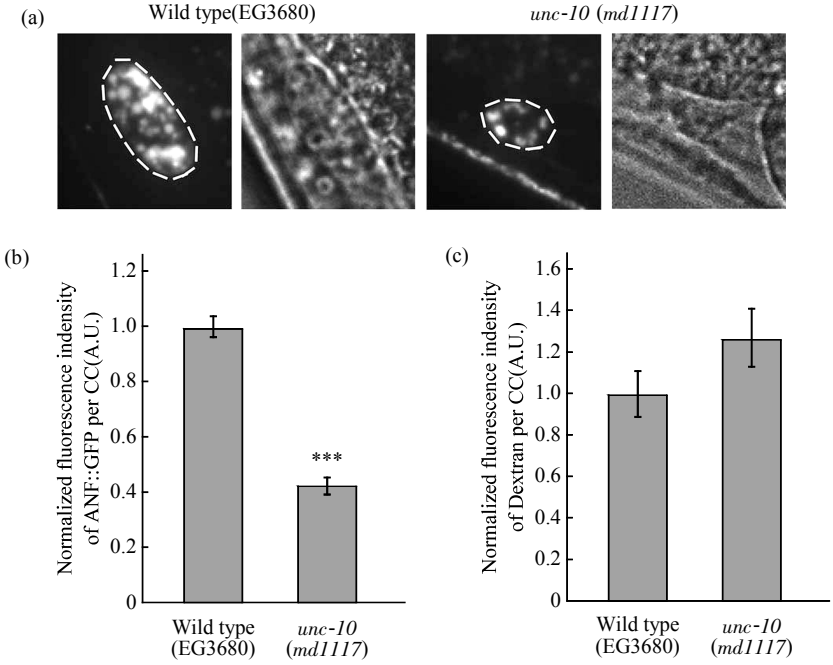

Fig. 2 UNC-10 was required for the release of ectopically expressed ANF-GFP in neurons

(a) Representative examples of ANF-GFP fluorescence and bright field images of coelomocytes in worms of the indicated strains. (b) Histograms showing the average total fluorescence intensity of ANF-GFP in coelomocytes in wild type and *unc-10* worms. (c) Histograms showing the average total fluorescence intensity of Dextran (10 ku) in coelomocytes from wild type and *unc-10* worms. Error bars indicate SEM. The asterisks indicate significant differences, as determined by Student's *t* test or the Mann-Whitney test (***$P < 0.001$).

2.3 UNC-10 is required for neuropeptide release in IDA-1::GFP labelled neurons

To directly assess the impact of losing UNC-10 on exocytosis, we utilised membrane capacitance (C_m) recording in worm neurons[20]. We carried out C_m recording from IDA-1::GFP-labelled ALA neurons because IDA-1 was a specific DCV marker [13]. As shown in Figure 3, flash photolysis in KM246(*ida-1*::*gfp* transgene) cells evoked a C_m increase of (13.4144 ± 1.5882) fF. We crossed the KM246 strain with *unc-10* mutants. Under similar step-like $[Ca^{2+}]_i$ elevations, the burst of exocytosis in the UNC-10 cells was significantly reduced to (6.905 ± 1.1377) fF (n=17, light gray trace), a 48% reduction. This result confirmed that UNC-10 was essential for the secretion of DCVs.

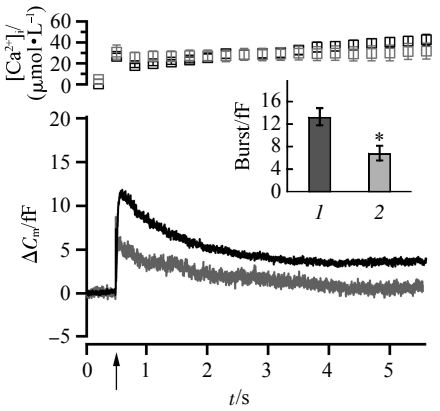

Fig. 3 The amplitudes of the exocytotic burst were compared between wild type (black bar) and *unc-10* (*md1117*) mutant (grey bar) cells

Error bars indicate SEM. The asterisks indicate significant differences, as determined by Student's *t* test or the Mann-Whitney test (*$P < 0.05$). *1*: Wild type; *2*: *unc-10* (*md1117*).

2.4 Active zone protein UNC-10 regulates the docking of DCVs

Since our above experiments revealed a role of UNC-10 in ANF-GFP secretion and DCV release (Figure 2 and Figure 3), we further tried to understand at which step UNC-10 might act. We employed time-lapse TIRFM to track the dynamic behaviour of individual DCVs. We also used IDA-1::GFP-labelled DCVs in ALA neurons (Figure 4a). Vesicles which entered the TIRF zone and remained still within one pixel (80 nm, in our CCD) for more than three consecutive frames (0.075 s) were called dwelling vesicles. One representative dwelling event was shown in Figure 4b. A vesicle appeared at 0.225 s and remained still within one pixel for 0.2 s. The distribution of dwelling time of DCVs was skewed, most of the data were within a short range (< 0.2 s) and only a few were within a long range (Figure 5c). A dwelling time less than 0.2 s was called short retained, while a dwelling time of more than 0.2 s was called long retained.

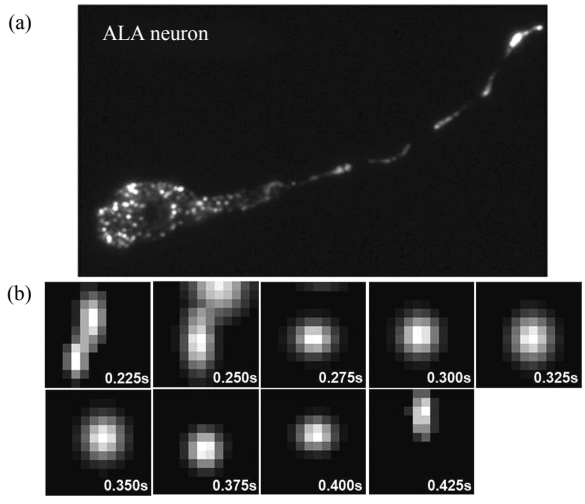

Fig. 4 Quantification of the behaviour of DCVs in the TIRF zone

(a) DCVs of IDA-1::GFP-labelled ALA neurons showed various punctuate fluorescent spots under TIRFM. (b) A vesicle appeared at 0.225 s and remained still within 1 pixel for 0.2 s in the TIRF zone.

In *unc-10* mutants, the snapshot vesicle density was not significantly different from that of wild type N2 worms (Figure 5a). This suggested that UNC-10 did not affect the translocation of DCVs into the TIRF zone. However, we did observe a significant reduction in the dwelling rate of DCVs(Figure 5b). Both the short- and long-retained DCVs were significantly reduced in *unc-10* mutants (Figure 5d). These results suggested a novel role for UNC-10 in the docking of DCVs.

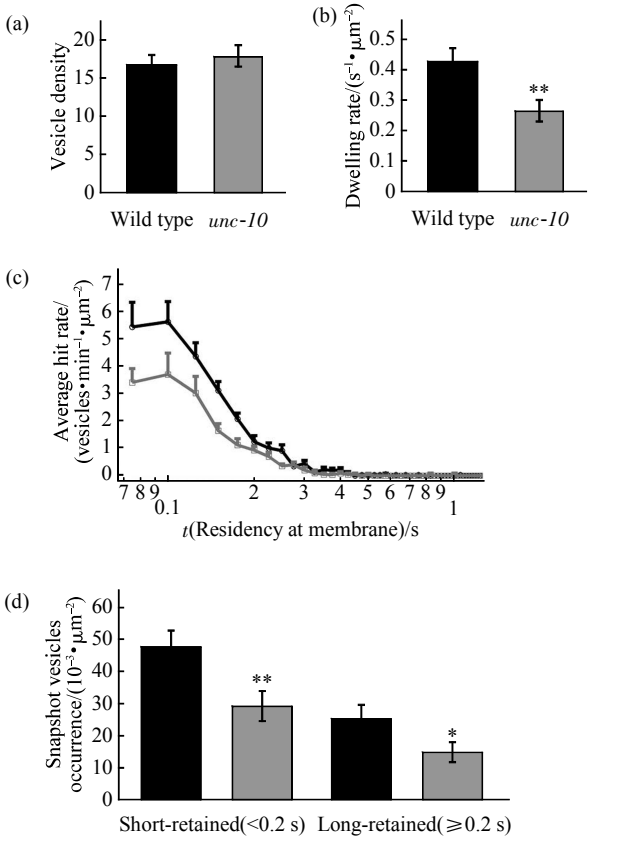

Fig. 5 Rim/UNC-10 participated in the stabilised docking of DCVs

(a) Density of total visible DCVs (per μm^2) in the evanescent field in wild type worms and *unc-10*(*md1117*) mutants. (b) The dwelling rate of DCVs (per second per unit area) in the evanescent field in wild type worms and *unc-10*(*md1117*) mutants. (c) Lifetime distribution of vesicles hitting the plasma membrane. o—o: Wild type; □—□: *unc-10*. (d) Average vesicle abundance at given times during image acquisition, grouped according to their residency times at the membrane. Error bars indicate SEM. The asterisks indicate significant differences, as determined by Student's *t* test or the Mann-Whitney test (*P < 0.05, **P < 0.01). ■: Wild type; □: *unc-10*.

3 Discussion

The *C. elegans unc-10* gene encodes a homologue of the vertebrate Rim family of active zone proteins[2]. *C. elegans* and vertebrate Rim proteins all contain highly conserved zinc finger, PDZ, C2A and C2B domains arranged in a similar topological order. Studies of Rim function in mammalian cells had indicated that Rim proteins were required for synaptic

exocytosis [1, 21], they regulated vesicle docking and activated vesicle priming at the presynaptic active zone [22–23]. Meanwhile, a recent study suggested that RIM proteins tethered Ca^{2+} Channels to presynaptic active zones *via* a direct PDZ-Domain interaction and determined Ca^{2+} channel density[23–24]. What's more, the mammalian Rim2 played a docking role in pancreatic beta cells[25]. Since there is only one Rim homologue in *C. elegans*, UNC-10 may play multiple roles as a combination of the contribution of all Rim isoforms in mammalian neurons. As a matter of fact, a previous study had demonstrated that UNC-10 played a role in SV exocytosis[2]. In this paper, our data suggested that *C. elegans* UNC-10 also regulated DCV exocytosis.

unc-10 mutant animals exhibited behavioural defects (Figure 1), though Rim mutant phenotypes were less severe than those of mutations disrupting the essential components of the *C. elegans* synaptic release machinery, such as syntaxin [26]. We then assessed the requirement for UNC-10 in DCV release *via* monitoring the uptake of ANF-GFP-labelled neuropeptides into coelomocytes *in vivo*. Applying electrophysiological recording of C_m developed by our group[20], we found a remarkable reduction in the burst amplitude (RRP) of C_m in *unc-10* mutants. The decrease in the RRP size might indicate an impairment in the late step of vesicle release. And finally, to further analyse the exact step that UNC-10 regulates, we tracked the movements of single DCVs with TIRFM. Through the combination of these assays, we found that UNC-10 was involved in DCV release through regulating the late step of DCV exocytosis.

Rim was originally identified as a putative Rab3A-effector, and a later study suggested that Rim1 might interact with several Rab proteins, including Rab3A/B/C/D, Rab10, Rab26 and Rab37 [1, 27], while Rim2 might interact with Rab3A/B/C/D and Rab8. To identify which proteins interact with UNC-10 in regulating DCV exocytosis will involve further investigation.

Acknowledgements We thank Michael Krause for the KM246 strain and the (*p*)*ida-1*::*IDA-1*::*GFP* plasmid; Thomas F. J. Martin for the EG3680 strain and the (*p*)*aex-3*::*ANF*::*GFP* plasmid; and *Caenorhabditis* Genetic Center for the other strains.

References

[1] Wang Y, Okamoto M, Schmitz F, *et al*. Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature, 1997, **388**(6642): 593−598

[2] Koushika S P, Richmond J E, Hadwiger G, *et al*. A post-docking role for active zone protein Rim. Nat Neurosci, 2001, **4**(10): 997−1005

[3] Castillo P E, Schoch S, Schmitz F, *et al*. RIM1alpha is required for presynaptic long-term potentiation. Nature, 2002, **415**(6869): 327−330

[4] Schoch S, Castillo P E, Jo T, *et al*. RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. Nature, 2002, **415**(6869): 321−326

[5] Ozaki N, Shibasaki T, Kashima Y, *et al*. cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol, 2000, **2**(11): 805−811

[6] Wang Y, Sugita S, Sudhof T C. The RIM/NIM family of neuronal C2 domain proteins. Interactions with Rab3 and a new class of Src homology 3 domain proteins. J Biol Chem, 2000, **275**(26): 20033−20044

[7] Betz A, Thakur P, Junge H J, *et al*. Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. Neuron, 2001, **30**(1): 183−196

[8] Wang X, Hu B, Zimmermann B, *et al*. Rim1 and rabphilin-3 bind Rab3-GTP by composite determinants partially related through N-terminal alpha -helix motifs. J Biol Chem, 2001, **276**(35):32480−32488

[9] Ohtsuka T, Takao-Rikitsu E, Inoue E, *et al*. Cast: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and munc13-1. J Cell Biol, 2002, **158**(3): 577−590

[10] Wang Y, Liu X, Biederer T, *et al*. A family of RIM-binding proteins regulated by alternative splicing: Implications for the genesis of synaptic active zones. Proc Natl Acad Sci USA, 2002, **99** (22): 14464−14469

[11] Brenner S. The genetics of *Caenorhabditis elegans*. Genetics, 1974, **77**(1): 71−94

[12] Stiernagle T. Maintenance of *C. elegans*. WormBook, 2006: 1−11

[13] Cai T, Fukushige T, Notkins A L, *et al*. Insulinoma-associated protein IA-2, a vesicle transmembrane protein, genetically interacts with UNC-31/CAPS and affects neurosecretion in *Caenorhabditis elegans*. J Neurosci, 2004, **24**(12): 3115−3124

[14] Speese S, Petrie M, Schuske K, *et al*. UNC-31 (CAPS) is required for dense-core vesicle but not synaptic vesicle exocytosis in *Caenorhabditis elegans*. J Neurosci, 2007, **27**(23): 6150−6162

[15] Branicky R, Hekimi S. What keeps *C. elegans* regular: the genetics of defecation. Trends Genet, 2006, **22**(10): 571−579

[16] Christensen M, Estevez A, Yin X, *et al*. A primary culture system for functional analysis of *C. elegans* neurons and muscle cells. Neuron, 2002, **33**(4): 503−514

[17] Strange K, Christensen M, Morrison R. Primary culture of *Caenorhabditis elegans* developing embryo cells for electrophysiological, cell biological and molecular studies. Nat Protoc, 2007, **2**(4): 1003−1012

[18] Olivo-Marin J C. Extraction of spots in biological images using multiscale products. Pattern Recogn, 2002, **35**(9): 1989−1996

[19] Jaqaman K, Loerke D, Mettlen M, *et al*. Robust single-particle tracking in live-cell time-lapse sequences. Nat Methods, 2008, **5**(8): 695−702

[20] Zhou K M, Dong Y M, Ge Q, *et al*. PKA activation bypasses the requirement for UNC-31 in the docking of dense core vesicles from *C. elegans* neurons. Neuron, 2007, **56**(4): 657−669

[21] Schoch S, Mittelstaedt T, Kaeser P S, *et al*. Redundant functions of RIM1alpha and RIM2alpha in Ca(2+)-triggered neurotransmitter release. EMBO J, 2006, **25**(24): 5852−5863

[22] Deng L, Kaeser P S, Xu W, *et al*. RIM proteins activate vesicle priming by reversing autoinhibitory homodimerization of Munc13. Neuron, 2011, **69**(2): 317−331

[23] Han Y, Kaeser P S, Sudhof T C, *et al*. RIM determines Ca(2)+ channel density and vesicle docking at the presynaptic active zone. Neuron, 2011, **69**(2): 304−316

[24] Kaeser P S, Deng L, Wang Y, *et al*. RIM proteins tether Ca^{2+} channels to presynaptic active zones *via* a direct PDZ-domain interaction. Cell, 2011, **144**(2): 282−295

[25] Yasuda T, Shibasaki T, Minami K, *et al*. Rim2alpha determines docking and priming states in insulin granule exocytosis. Cell Metab, 2010, **12**(2): 117−129

[26] Ogawa H, Harada S, Sassa T, *et al*. Functional properties of the unc-64 gene encoding a *Caenorhabditis elegans* syntaxin. J Biol Chem, 1998, **273**(4): 2192−2198

[27] Fukuda M. Distinct Rab binding specificity of Rim1, Rim2, rabphilin, and Noc2. Identification of a critical determinant of Rab3A/Rab27A recognition by Rim2. J Biol Chem, 2003, **278**(17): 15373−15380

UNC-10 在致密核心囊泡分泌过程中的作用 *

冯婉娟[1)] 周 围[1)] 章永登[1)] 吴政星[1)] 徐 涛[1, 2)**]

([1)] 华中科技大学生命科学与技术学院，武汉 430074；[2)] 中国科学院生物物理研究所生物大分子国家重点实验室，北京 100101)

摘要 Rim 是囊泡分泌活性区中的重要组成蛋白，它与细胞分泌和突触可塑性相关．在秀丽隐感线虫中只存在一种编码 Rim 的基因即 *unc-10*．我们的研究发现，在线虫中 Rim 的基因突变 *unc-10*(*md1117*)会导致致密核心囊泡的分泌缺陷．在活体中，*unc-10* 突变虫系的神经多肽分泌显著下降．此外，在主要分泌致密核心囊泡的 ALA 神经元内，钙光解释放促发的快相分泌也比野生型减少．运用全内反射荧光显微成像技术，我们观察在 *unc-10* 缺失的情况下 ALA 神经元中致密核心囊泡的锚定过程，结果显示在细胞膜附近停留的囊泡数目减少，表明囊泡锚定受到阻碍．上述试验结果表明，UNC-10 能够影响致密核心囊泡的分泌过程，其机制可能是影响了囊泡的锚定过程．

关键词 秀丽隐杆线虫，致密核心囊泡，UNC-10，细胞分泌

学科分类号 Q7

DOI: 10.3724/SP.J.1206.2011.00374

* 国家重点基础研究发展计划(973)(2010CB833701), 国家自然科学基金(30870564, 90913022)和中国科学院创新方向(KSCX2-SW-224)资助项目.

** 通讯联系人.

Tel: 010-64888469, E-mail: xutao@ibp.ac.cn

收稿日期：2011-08-15，接受日期：2011-09-19