Duration of fusion pore opening and the amount of hormone released are regulated by myosin II during kiss-and-run exocytosis

Ryo AOKI†1, Tetsuya KITAGUCHI‡1, Manami OYA*, Yu YANAGIHARA*, Mai SATO*, Atsushi MIYAWAKI†‡ and Takashi TSUBOI*1,2

*Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba,Meguro, Tokyo 153-8902, Japan, †Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan, ‡Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan

INTRODUCTION

Hormone secretion mediates intercellular communication to control various activities in multicellular organisms and requires a sophisticated release mechanism that is highly regulated both temporally and quantitatively. Regulated hormone secretion, exocytosis, was originally thought to require the fusion of a dense-core vesicle with the plasma membrane (i.e. formation of an exocytotic fusion pore) that eventually collapses via an ‘omega’ figure to permit the release of the dense-core vesicle cargoes (i.e. full fusion exocytosis) [1,2]. Other studies have suggested the existence of ‘kiss-and-run’ exocytosis, which allows the partial release of certain protein cargoes from the dense-core vesicles during transient fusion with the plasma membrane [3–6]. However, the molecular mechanism(s) that regulates and maintains the transient fusion pore is still unclear.

The exocytosis process of dense-core vesicles in neuroendocrine cells consists of four steps: (i) the transported dense-core vesicle morphologically attaches to the plasma membrane (transport and tethering step); (ii) the tethered vesicle forms a tight-core SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) complex at the target plasma membrane (docking step); (iii) preparation of exocytosis-competent vesicles (priming step); and (iv) finally, fusion of the vesicle with the plasma membrane (fusion step). Recent studies have suggested that the myosin family of proteins are responsible for vesicle dynamics [7]. For example, myosin Va participates in dense-core vesicle transport as a molecular motor [8,9] and in the docking process [9]. Meanwhile, myosin VI is the only known motor protein that transports cargoes to the minus ends of the actin filament [10], and is involved in endocytosis by associating with clathrin-coated vesicles [11]. Several research groups have also shown that myosin II plays important roles in shifting the exocytotic mode from kiss-and-run exocytosis to full fusion exocytosis and speeds up the release of catecholamine in full fusion exocytosis [12–14]. However, relatively little is known about the role of myosin II in kiss-and-run exocytosis.

In the present study, to clarify the role of myosin II in kiss-and-run exocytosis, we used TIRF (total internal reflection fluorescence) microscopy to analyse single exocytotic events at a high spatiotemporal resolution [15]. We found that myosin II increases the duration of tPA (tissue plasminogen activator) and BDNF (brain-derived neurotrophic factor) release by slowing fusion pore closure, suggesting that myosin II activity controls the amount of hormone released from single vesicles in neuroendocrine cells.

EXPERIMENTAL

Plasmid construction

An EGFP (enhanced green fluorescent protein) fragment was amplified by PCR using the primers BamHI-EGFP-F (5′-CCGGATCCCATGTTAGCAAGGGCCAGG-3′; BamHI site underlined) and EcoRI-EGFP-R (5′-GGAATTCTTACT-TGTACAGCTCGTCC-3′; EcoRI site underlined). The amplified

Abbreviations used: BDNF, brain-derived neurotrophic factor; DN, dominant-negative; EGFP, enhanced green fluorescent protein; F-actin, filamentous actin; FI, fluorescence index; FP, fluorescent protein; mRFP, monomeric red fluorescent protein; NA, numerical aperture; NPY, neuropeptide Y; RBC, Ringer buffer; RLC, myosin II regulatory light chain; spH, synaptopHluorin; TIRF, total internal reflection fluorescence; TPA, tissue plasminogen activator; VAMP2, vesicle-associated membrane protein 2; WT, wild-type.

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email takatsuboi@bio.c.u-tokyo.ac.jp).
EGFP fragment was digested with BamHI/EcoRI and inserted into the BamHI/EcoRI site of pcDNA3 (Invitrogen) as pcDNA3 EGFP. The cDNA clone of the mouse RLC (myosin II regulatory light chain) was obtained from the FANTOM RIKEN full-length cDNA clone collection (FANTOM clone ID I73003N21) [16]. The deduced amino acid sequence of RLC was the same as that of mouse myl12b (NCBI accession number NM_023402). The RLC fragment was amplified by PCR using the primers HindIII-RLC-F (5′-CCCAAGCTTTGTCTTTCGCGC-3′; HindIII site underlined) and BamHI-RLC-R (5′-CCGGATCCCGTATCTTTGTCTTTCGCGC-3′; BamHI site underlined). The amplified RLC fragment was digested with HindIII/BamHI and inserted into the HindIII/BamHI site of pcDNA3-EGFP as pcDNA3-RLC-EGFP. An unphosphorylatable DN (dominant-negative) form of RLC (RLC T18A/S19A) was generated by sequential PCR. Venus-tagged BDNF [17], mRFP (monomeric red fluorescent protein)-tagged human insulin and mRFP-tagged VAMP2 (vesicle-associated membrane protein 2) were generously provided by Dr Kinji Inoue (Division of Life Science, Graduate School of Science and Engineering, Saitama University, Saitama, Japan). The sequences of all plasmids were verified by automated sequencing. Other expression constructs were prepared as described previously [5,18–21].

Cell culture

PC12 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) (Invitrogen) supplemented with 10% fetal bovine serum, 10% horse serum, 100 units/ml penicillin G and 100 μg/ml streptomycin, at 37°C under 5% CO2. For live-cell imaging, the PC12 cells were plated on to poly-L-lysine-coated coverslips, and the cells were co-transfected with 3 μg of each FP (fluorescent protein)-tagged hormone plasmid or VAMP2-tagged pH-sensitive green fluorescent protein [spH (synapto-pHluorin)] plasmid, and either 3 μg of WT (wild-type) RLC–EGFP or DN RLC–EGFP plasmid using LipofectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions.

TIRF microscopy

TIRF imaging was performed in modified RB (Ringer buffer: 130 mM NaCl, 3 mM KCl, 5 mM CaCl2, 1.5 mM MgCl2, 10 mM glucose and 10 mM Hepes, pH 7.4) at 37°C. High-KCl stimulation was achieved by perfusion with RB containing 70 mM KCl (the NaCl concentration was reduced to maintain the osmolarity). We mounted a high NA (numerical aperture) objective lens (Olympus Plan Apochromatic, 100×, NA=1.45, infinity-corrected) on an inverted microscope (Olympus IX71) and introduced an incident light for total internal reflection illumination through the high NA objective lens via a single-mode optical fibre and two illumination lenses (Olympus IX2-RFAEVA-2). To excite EGFP and mRFP simultaneously, we used a diode-pumped solid-state 488-nm (HPU50100, 20 mW, Furukawa Electronic) and a 561-nm (85YCA020, 20 mW, Melles Griot) laser respectively. For simultaneous imaging of green and red fluorescence, an image splitter (Dual View; Optical Insights) divided the green and red components of the images with a 565-nm dichroic mirror (Chroma) and passed the green component through a 500–540nm bandpass filter (Chroma) and the red component through a 580-nm long pass filter (Chroma). Images were acquired every 300 ms.

Image analysis

To analyse the TIRF imaging data, single exocytotic events were selected manually, and the average fluorescence intensity of an individual vesicle was calculated as described previously [22]. To distinguish between fusion events and vesicle movement (i.e. vesicles that pause at the plasma membrane and then move back inside the cell without fusing), we focused on fluorescence changes just before the decrease in fluorescent signals. It is known that a rapid transient increase in fluorescence intensity occurs during a fusion event, whereas, during vesicle movement, the fluorescence intensity gradually decreases to the background level [19,23]. For the analysis of spH fluorescence, the average spH fluorescence intensity for each cell in the first frame was subtracted (see Figure 3B). The number of fusion events during a 5-min period was counted manually on the basis of the above criteria (see Figure 6). The same squares were used to select the corresponding point in the other colour image (see Figure 5) using split-view analysis software (MetaMorph 7.5, Molecular Devices).

Measurement of stimulus-induced release of NPY (neuropeptide Y)- and tPA–mRFP

PC12 cells were plated at a density of 3 × 10^5 cells per six-well dish, and the cells were co-transfected with 3 μg of the tPA–mRFP or NPY–mRFP plasmid, and 3 μg of either WT RLC–EGFP or DN RLC–EGFP plasmid using LipofectamineTM 2000. At 2 days after transfection, the culture medium was removed and replaced with RB. The cells were then incubated for 60 min in high-KCl RB to induce depolarization. The amount of tPA– or NPY–mRFP released into the buffer was measured by detecting fluorescence using a spectrophotofluorimeter from 550 to 650 nm (Hitachi F-7000).

Immunocytochemistry

For immunocytochemical analysis, PC12 cells were transfected with tPA–mRFP and fixed with 2% (w/v) parafomaldehyde (Wako Pure Chemicals) in PBS on ice for 5 min. The cells were then incubated with anti-RLC antibody (sc-28329, 1:100 dilution, Santa Cruz Biotechnology) at room temperature (25°C) for 1 h. The cells were then immunostained with Alexa Fluor® 488-labelled secondary IgG (1:1000 dilution, Molecular Probes) and imaged under the TIRF microscope.

Data analysis

Results are presented as means ± S.E.M. The statistical significance of differences between means was assessed by ANOVA followed by a Newman–Keuls test for multiple comparisons, or by an unpaired Student’s t test when only two groups were compared. All analyses were conducted using GraphPad Prism software.

RESULTS

Hormone-release kinetics are modulated by myosin II

To visualize the dense-core vesicles in PC12 cells, we used FP-tagged peptide hormones (i.e. tPA–mRFP, BDNF–Venus, NPY–mRFP and insulin–mRFP). As reported previously by ourselves and by other groups, overexpression of these FP-tagged hormones in PC12 cells produced a highly punctate pattern of fluorescence under the TIRF microscope (Figures 1 and 2, and see Supplementary Figure S1 at http://www.BiochemJ.org/bj/429/bj4290497add.htm) [4,6,18]. To elucidate the effect of myosin II on the release kinetics of dense-core vesicle exocytosis, we monitored the dynamics of single exocytotic events of these vesicle cargoes displaying distinct release kinetics (i.e. slower release for tPA–mRFP and BDNF–Venus; faster release for...
Myosin II modulates fusion pore dynamics

NPY–mRFP and insulin–mRFP) in PC12 cells overexpressing either WT RLC or a DN form of RLC (RLC T18A/S19A, mutated at its two phosphorylation sites [24]). After stimulation with high-KCl (70 mM), some tPA–mRFP fluorescent spots brightened and subsequently dimmed (Figure 1A, top row, 6.0 s panel), implying that the dense-core vesicles cargoes were released into the extracellular space. When we analysed the time course of fluorescence intensity in the centre of the tPA–mRFP vesicles, the vesicles did not completely release their contents (Figure 1B), consistent with previous reports [4,18]. Overexpression of either WT or DN RLC did not affect the peak amplitude of normalized fluorescence intensity of tPA–mRFP release (Figure 1B). However, overexpression of WT RLC significantly slowed the kinetics of tPA–mRFP release (n = 132 and 153 vesicles from control and WT RLC respectively; P < 0.05, ANOVA), whereas overexpression of DN RLC significantly shortened the duration of its release (n = 131 vesicles; P < 0.05, ANOVA). We also analysed the release kinetics of another cargo, BDNF–Venus, which also shows slow kinetics and partial release (Figures 1C and 1D) [25]. Consistent with tPA–mRFP release kinetics, WT RLC prolonged the duration of BDNF–Venus release (n = 73 and 85 vesicles from control and WT RLC respectively; P < 0.05, ANOVA), whereas overexpression of DN RLC shortened the release duration (Figures 1C and 1D) (n = 103 vesicles; P < 0.05, ANOVA).

We next investigated whether myosin II modulates the release kinetics of the rapidly released cargoes NPY–mRFP and insulin–mRFP. As described previously [18], high-KCl stimulation caused NPY–mRFP- or insulin–mRFP-containing spots to brighten and disappear suddenly (Figures 2A and 2C), reflecting the faster release kinetics for these cargoes. Overexpression of WT or DN RLC did not affect the release kinetics of NPY–mRFP (Figure 2B; n = 78, 91 and 82 vesicles from control, WT and DN RLC respectively) or insulin–mRFP (Figure 2D; n = 83, 98 and 85 vesicles from control, WT and DN RLC respectively). These results suggest that myosin II specifically regulates the release kinetics of slowly released cargoes.

Fusion pore closure is slowed by myosin II

To confirm whether the modulatory effects of myosin II on the release kinetics of tPA–mRFP and BDNF–Venus are due to an altered duration of fusion pore opening, we observed the time course of the fusion pore opening using spH. spH is a pH-sensitive green fluorescent protein-tagged VAMP2 and is retained inside the vesicle during kiss-and-run exocytosis [26]. By exploiting this property, it is possible to monitor the connection between the vesicle lumen and the extracellular space during exocytosis by sensing pH changes [27]. The punctate structure of spH or VAMP2–mRFP was co-localized with tPA–mRFP, BDNF–Venus, NPY–mRFP or insulin–mRFP (Supplementary Figure S1), suggesting that the distinct release kinetics of each cargo are derived from the properties of the cargo rather than the vesicle itself. Therefore the measurement of spH kinetics enabled us to analyse the duration of fusion pore opening of the dense-core vesicles in Figures 1 and 2, regardless of the distinct release kinetics of the cargoes. After stimulating cells with high-KCl, the fluorescence brightened immediately and subsequently
Figure 2  Myosin II has no effect on the kinetics of NPY–mRFP or insulin–mRFP release

(A and C) Typical sequential images of a single NPY–mRFP- (A) and insulin–mRFP- (C) containing vesicle observed after high-KCl stimulation of cells overexpressing WT RLC or DN RLC, or without exogenous RLC (Control) observed under a TIRF microscope. Scale bars, 1 μm. (B and D) Time course of changes in fluorescence intensity measured in the centre of NPY–mRFP- (B) and insulin–mRFP- (D) containing vesicles in the control (thick lines), WT RLC- (broken lines) and DN RLC- (thin lines) overexpressing cells. The mean fluorescence intensity before exocytosis was normalized to 100 %. The normalized intensity at 5.0, 5.4 or 6.0 s did not differ significantly between the three types of cells (P > 0.05, ANOVA). a.u., arbitrary units.

dimmed (Figure 3), reflecting the vesicular neutralization and reacidification processes, as reported previously by ourselves [5]. When we analysed the time course of changes in fluorescence intensity in the centre of the spH vesicles, the duration of spH brightening was increased by overexpression of WT RLC and decreased by overexpression of DN RLC (n = 53, 58 and 61 vesicles from control, WT and DN RLC respectively; P < 0.05, ANOVA) (Figure 3). These results indicate that the slower release kinetics of tPA–mRFP and BDNF–Venus by myosin II can be attributed to prolonged fusion pore opening.

Myosin II increases tPA–mRFP release, but not that of NPY–mRFP

To examine whether the changes in duration of fusion pore opening regulate the amount of hormone released, we stimulated cells for 60 min and collected the culture medium, and measured the fluorescence intensity of FP-tagged hormones released into the medium using a spectrophotofluorimeter. The FI (fluorescence index) was calculated by integrating the fluorescence intensity of the medium from 550 to 650 nm to assess the amount of tPA–mRFP or NPY–mRFP. When WT RLC was overexpressed in PC12 cells, the FI for tPA–mRFP was significantly increased (Figure 4A) compared with the FI in control cells (n = 8 trials; P < 0.01, ANOVA). In contrast, the FI for NPY–mRFP, the kinetics of which was not modulated by myosin II activity (Figure 2A), was unchanged (Figure 4B; n = 8 trials; P > 0.05, ANOVA). Interestingly, the FI values for tPA–mRFP and NPY–mRFP in cells overexpressing DN RLC were comparable with their respective values in control cells. These results suggest that myosin II regulates the amount of hormones released into the medium, although the amount of released hormone is not proportional to the duration of the fusion pore opening.

RLC is co-localized with tPA–mRFP-containing vesicles

To clarify whether myosin II directly associates with the dense-core vesicles to modulate the duration of fusion pore opening, we simultaneously observed the dynamics of vesicle cargo and RLC during exocytosis. Under the TIRF microscope, immunostained endogenous RLC showed a punctate and filamensous structure beneath the plasma membrane [28]. In addition, with the resolution of TIRF microscopy (>200 nm), a substantial proportion of the tPA–mRFP-containing vesicles was co-localized with endogenous RLC (Figure 5A). Consistently, overexpressed WT and DN RLC–EGFP also showed punctate and filamentous distribution (Figures 5B and 5C). Furthermore, some spots for tPA–mRFP were co-localized with them in living cells (Figures 5B and 5C). These results suggest that myosin II is involved in the dense-core vesicle dynamics at the site of exocytosis, and that the ATPase activity of myosin II is not necessary for co-localization with the vesicles. After stimulating the cells with high-KCl, the WT RLC–EGFP spots that were co-localized with tPA–mRFP quickly disappeared from the subplasmalemmal region (dispersal; 76.3 ± 6.5 %, n = 13 cells) (Figure 5B). In contrast, almost half of the DN RLC–EGFP spots were immobile (immobile; 52.5 ± 8.3 %, c⃝ The Authors Journal compilation c⃝ 2010 Biochemical Society.
Myosin II modulates fusion pore dynamics

Myosin II modulates fusion pore dynamics

Figure 3 Myosin II slows the fluorescence kinetics of spH

(A) Typical sequential images of a single spH-containing vesicle observed after high-KCl stimulation of cells overexpressing WT RLC or DN RLC, or without exogenous RLC (Control) observed under a TIRF microscope. Scale bar, 1 μm. (B) Time course of changes in fluorescence intensity measured in the centre of spH-containing vesicles in the control (thick line), WT RLC- (broken line) and DN RLC- (thin line) overexpressing cells. The mean fluorescence intensity before exocytosis was normalized to 100%. The normalized intensity at 9.0 s differed significantly between the three types of cells (*P < 0.05).

Figure 4 Myosin II enhances the release of tPA–mRFP, but not NPY–mRFP, into the medium

PC12 cells co-transfected with WT RLC or DN RLC, or without exogenous RLC (Control) were stimulated and the FI values for tPA–mRFP (A) and NPY–mRFP (B) were measured using a spectrophotofluorimeter. The FI value of control cells without stimulation (Resting) was normalized to 1 (eight dishes per experiment). **P < 0.01; n.s., not significant.

Figure 5 Dynamics of WT or DN RLC at the site of exocytosis

(A) Co-localization of endogenous RLC with tPA–mRFP-containing vesicles. Scale bar, 10 μm. (B and C) The left-hand panels show the distribution of tPA–mRFP and WT RLC–EGFP (B) or DN RLC–EGFP (C) fluorescence within the cells under a TIRF microscope. The right-hand panels show typical three sequential dual-colour TIRF images representing the behaviour of a single WT RLC–EGFP (B) or DN RLC–EGFP (C) with tPA–mRFP fluorescent spots during exocytosis of a single vesicle after stimulation with high-KCl. The circle indicates the vesicle position before exocytosis. Scale bars, 10 μm (left-hand panels) and 2 μm (right-hand panels).

n = 15 cells, P < 0.01, Student’s t test) (Figure 5C). Interestingly, the disappearance of WT RLC–EGFP from the plasma membrane occurred before the decay in the tPA–mRFP signal, indicating that myosin II leaves the vesicles before fusion pore closure (Figure 5B; 10.0 s), even though myosin II regulates the duration of fusion pore opening (Figure 1B).

Myosin II is not involved in the vesicle transport, docking or priming steps of exocytosis

So far, we have shown that myosin II regulates the duration of fusion pore opening at the site of exocytosis in an ATPase-dependent manner. However, it is still possible that myosin II modulates the duration of fusion pore opening by regulating the earlier exocytotic steps. To investigate this possibility, we examined whether myosin II regulates the transport, docking or priming steps of exocytosis. Overexpression of WT or DN RLC did not affect the density of plasma-membrane-docked NPY– or

© The Authors Journal compilation © 2010 Biochemical Society
Figure 6  Effects of myosin II on vesicle transport, docking and priming

(A) Typical TIRF microscopy images of plasma-membrane-docked NPY– or tPA–mRFP-containing vesicles in NPY– or tPA–mRFP only (Control), and WT RLC- or DN RLC-overexpressing cells. Scale bar, 5 µm. (B) The density of the plasma-membrane-docked NPY– or tPA–mRFP-containing vesicles was measured by counting the vesicles in each image (n = 8 cells per group; P > 0.05 compared with the control, ANOVA). (C) Determination of the number of NPY– and tPA–mRFP release events (R) during the 5-min stimulation period to the number of plasma-membrane-docked vesicles (D) before stimulation in WT RLC- or DN RLC-overexpressing cells obtained by TIRF microscopy (n = 5 cells per group; P > 0.05 compared with the control, ANOVA).

tPA–mRFP vesicles (Figures 6A and 6B; P > 0.05, ANOVA). Since the density of the plasma-membrane-docked vesicles is altered by interfering with the vesicle transport [8] or docking steps [29], these results suggest that myosin II does not contribute to the vesicle transport or docking steps of exocytosis.

We next analysed the ratio of the number of NPY– or tPA–mRFP release events during the 5-min stimulation period (R) to the plasma membrane-docked vesicles before stimulation (D). The ratio of R to D was approx. 20 %, and no significant differences were observed among WT or DN RLC-expressing cells and control cells (Figure 6C; P > 0.05, ANOVA), indicating that myosin II does not regulate the priming step of exocytosis. Taken together, our results indicate that myosin II regulates hormone release by modulating the fusion pore, rather than modulating the earlier exocytotic steps.

DISCUSSION

There are two mechanisms for exocytosis in neuroendocrine cells, namely full fusion exocytosis and kiss-and-run exocytosis. Although myosin II is implicated in the acceleration of fusion pore dilation in full fusion exocytosis, the function of myosin II in kiss-and-run exocytosis is largely unknown. Therefore we used TIRF microscopy to analyse the fusion pore dynamics in kiss-and-run exocytosis by combining several FP-tagged hormones showing different kinetics. In the present study, we found that the fluorescence intensity of tPA–mRFP and BDNF–Venus after peak induced by stimulation with high-KCl decayed more slowly with myosin II activation without changes in peak amplitude (Figure 1). This suggests that myosin II slows fusion pore closure, but does not affect the size of the fusion pore because the expansion of the fusion pore size in kiss-and-run exocytosis is accompanied by an increase in the peak amplitude of fluorescence intensity [30]. Therefore myosin II is likely to behave differently in terms of fusion pore regulation between kiss-and-run and full fusion exocytosis. However, when we hypothesize that myosin II prevents the closure of the fusion pore in both types of exocytosis, the different effects by myosin II are explained by the same function.

Figure 5 shows the dynamics of RLC at the site of exocytosis. Interestingly, the disappearance of WT RLC from the site of exocytosis occurred before the decay of the tPA–mRFP signal, even though myosin II regulates the duration of fusion pore opening. Thus we speculate that other molecules that interact with myosin II play a crucial role in fusion pore regulation. One candidate molecular target for myosin II is cortical F-actin (filamentous actin). The subplasmalemmal F-actin network runs between the plasma membrane and secretory vesicles [31,32] and acts as a barrier between the secretory vesicles and the plasma membrane [33,34]. Thus myosin II might regulate the fusion pore dynamics by modifying the subplasmalemmal actin cortex.

The release kinetics of tPA–mRFP and BDNF–Venus were prolonged by myosin II, but we found no changes in the release kinetics of NPY–mRFP or insulin–mRFP (Figures 1 and 2). This difference could be due to differences in the molecular properties of each hormone, such as molecular mass. Since larger hormones tend to display slower release kinetics than smaller hormones [4,18], it is likely that the release of tPA–mRFP and
BDNF–Venus is slow enough to be controlled by modulating the duration of fusion pore opening, whereas the release of NPY–mRFP and insulin–mRFP is completed before fusion pore closure, even when the duration of fusion pore opening is shortened by DN RLC overexpression. In addition, mature BDNF dimerizes [25] and certain vesicle cargoes interact with other luminal components [35–37], which could slow the release kinetics of the vesicle cargoes. Therefore the differences in release kinetics caused by the molecular mass and other properties of the cargoes contained in each vesicle could allow cells to selectively control the amount of cargo released through myosin II activation. Interestingly, the release kinetics for BDNF–Venus kinetics, as shown in Figure 1, are slower than those for spH, a marker for the fusion pore opening, as shown in Figure 3. This can be explained by the fact that some BDNF is stably deposited at the site of exocytosis after release [36].

Both depletion of the cargo from vesicles by release and vesicle internalization are responsible for the decrease in fluorescence intensity observed under the TIRF microscope. In addition, some members of the myosin family are responsible for vesicle transportation [8]. Therefore myosin II appears to be involved in the modulation of internalization kinetics rather than in the release kinetics. However, this interpretation is apparently inconsistent with the fact that the amount of released iPA–mRFP was increased by the overexpression of WT RLC (Figure 4A), because vesicle internalization should not affect the amount of hormone to be released. Therefore we conclude that the slower decrease of fluorescence intensity with WT RLC shown in Figure 1 is due to slowing of fusion pore closure, rather than the vesicle internalization. Interestingly, we also found that the amount of iPA–mRFP released is not decreased by DN RLC overexpression. Solubilizing the peptide released from the vesicle core protein by vesicle neutralization is critical to accelerate peptide release [38,39]. This mechanism may provide a non-linear relationship between the duration of fusion pore opening and the amount of hormone released.

Since overexpression of WT or DN RLC did not affect the number of plasma-membrane-docked vesicles or the exocytotic response, we conclude that activated myosin II does not contribute to the earlier exocytotic steps, including transport, docking or priming (Figure 6). However, we cannot completely exclude the possibility that myosin II contributes to earlier exocytotic steps such as vesicle transport in a motor-activity-independent manner. In fact, we found that the co-localization of myosin II with plasma-membrane-docked vesicles was retained without its ATPase activity (Figure 5). Myosin II contains two functional domains. One is the head domain, which has ATPase activity and is required for motor activity. The other is the long rod domain, which is required for the assembly of myosin II monomers into bipolar filaments. Myosin II lacking ATPase activity can still bind actin filaments to bundle them [40,41], and the rod domains can interact with the lipid membrane [42,43]. Therefore myosin II could participate in the transport step of exocytosis via its adaptor function.

In summary, we have demonstrated using live-cell TIRF imaging that myosin II controls the duration of fusion pore opening to regulate the amount of hormone released from a single vesicle.

**AUTHOR CONTRIBUTION**

Ryo Aoki, Tetsuya Kitaguchi, Manami Oya, Yu Yanagihara, Mai Sato and Takashi Tsuboi conceived the experiments. Ryo Aoki, Tetsuya Kitaguchi and Takashi Tsuboi co-wrote the paper. Atsushi Miyawaki provided scientific guidance and financial support. All authors discussed the results and commented on the paper.

Received 2 December 2009/5 May 2010; accepted 8 June 2010
Published as BJ Immediate Publication 8 June 2010, doi:10.1042/BJ20091839

© The Authors. Journal compilation © 2010 Biochemical Society
Duration of fusion pore opening and the amount of hormone released are regulated by myosin II during kiss-and-run exocytosis

Ryo AOKI*, Tetsuya KITAGUCHI†, Manami OYA*, Yu YANAGIHARA*, Mai SATO*, Atsushi MIYAWAKI†‡ and Takashi TSUBOI*†

*Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro, Tokyo 153-8902, Japan, †Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan, and ‡Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan

Figure S1 Co-localization of spH/VAMP2–mRFP and FP-tagged hormones

TIRF images of paraformaldehyde-fixed PC12 cells showing the distribution of spH or VAMP2–mRFP with tPA–mRFP, BDNF–Venus, NPY–mRFP and insulin–mRFP. Scale bars, 10 μm.

Received 2 December 2009/5 May 2010; accepted 8 June 2010
Published as BJ Immediate Publication 8 June 2010, doi:10.1042/BJ20091839

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email takatsuboi@bio.c.u-tokyo.ac.jp).