Effects of phorbol ester on vesicle dynamics as revealed by total internal reflection fluorescence microscopy

Zhang, Enming; Xue, Renhao; Soo, Jianchow; Chen, Peng

2008

Zhang, E., Xue, R., Soo, J. & Chen, P. (2008). Effects of phorbol ester on vesicle dynamics as revealed by total internal reflection fluorescence microscopy. *Pflügers Archiv European Journal of Physiology*, 457, 211-222.

<https://hdl.handle.net/10356/94319>

<https://doi.org/10.1007/s00424-008-0485-6>

© 2008 Springer-Verlag. This is the author created version of a work that has been peer reviewed and accepted for publication by *Pflügers Archiv European Journal of Physiology*, Springer-Verlag. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [DOI: <http://dx.doi.org/10.1007/s00424-008-0485-6>]

Downloaded on 26 Apr 2023 17:53:17 SGT

**Effects of phorbol ester on vesicle dynamics as revealed by total
internal reflection fluorescence microscopy**

Enming Zhang, Renhao Xue, Jianchow Soo, Peng Chen^{*}

Division of Bioengineering, Nanyang Technological University, Singapore 637457

* to whom correspondence should be addressed. Tel.: +65-65141086; Fax:

+65-67911761; E-mail: chenpeng@ntu.edu.sg

Abstract

Exocytosis of neurotransmitter or hormone-filled vesicles is a highly dynamic process regulated by various proteins and lipids. As mainly revealed indirectly by the electrophysiological methods, exocytosis is believed to involve multiple kinetic steps in which vesicles transit from one state to another. Using total internal reflection fluorescence microscopy (TIRFM) which enables direct visualization of individual vesicles, we developed an analytical framework to track and analyze vesicle dynamics. We demonstrated that all subplasmalemmal vesicles generally undergo constant and caged Brownian motion. And they can be classified into three populations that differ in their motion characteristics and fusion competence. Furthermore, we showed that these vesicle pools are differentially modulated by phorbol-12-myristate-13-acetate (PMA), a phorbol ester analog to endogenous diacylglycerol, through both protein kinase C dependent and independent pathways.

Keywords exocytosis · TIRFM · vesicle dynamics · protein kinase C · diacylglycerol

Introduction

Exocytosis, a process by which secretory vesicles are directed to outer cell membrane and subsequently discharge their contents to extracellular environment upon triggering, is highly regulated through the orchestrated actions of various proteins and lipids [1-4]. It is predominantly believed that exocytosis process proceeds with multiple sequential events including vesicle transport, tethering and docking onto plasma membrane, priming (or maturation), and final Ca^{2+} -dependent vesicle fusion [5, 6]. Diacylglycerol (DAG), a secondary messenger lipid generated by phospholipase C dependent cleavage of phosphatidylinositol bisphosphate in response to a variety of biological signals, modulates multiple stages along exocytotic cascade through various mechanisms [2, 7, 8].

It has long been recognized that DAG potentiates the release of neurotransmitters at neuronal synapses and hormone release from endocrine cells through activation of protein kinase C (PKC) [9-12]. A number of proteins which involve in different aspects of exocytosis have been identified as PKC substrates. For example, PKC exerts its influences by phosphorylating SNAP-25 [13], a member of the core exocytotic machinery (SNARE protein complex) which drives membrane fusion in the final stage of exocytosis. Phosphorylation of SNAP-25 potentiates vesicle recruitment [14] and sensitize vesicle release [15]. Munc18, a secretory protein which plays an important role in vesicle docking, is another target of PKC [16]. Its phosphorylation enhances vesicle replenishment likely *via* inhibiting the association of Munc18 with syntaxin and consequently increasing free syntaxin for SNARE

complex formation [17]. Furthermore, activation of PKC causes disassembly of peripheral F-actin cytoskeleton and thus facilitates vesicle access to the plasma membrane [18], likely via phosphorylation of MARCK [19] or other actin filament associated proteins [20]. More recently, it has been revealed that, in parallel to its classic action on PKC, DAG also binds with Munc13 and consequently induces augmentation of exocytosis [21]. Munc13 is believed to be an essential priming protein which renders vesicle fusion competence by assisting in the assembly of the functional SNARE complex [22, 23]. PKC dependent and independent pathways converge on exocytosis machinery in a collaborative and mutually dependent manner [24].

The studies of DAG effects on the dynamics of exocytosis have mainly relied on electrophysiological methods such as membrane capacitance measurements and carbon fiber amperometry. These methods, however, can only provide indirect information about how secretory vesicles transit from one molecular state to another. Direct observation of vesicle dynamics become possible with the emergence of total internal reflection fluorescence microscopy (TIRFM), which enables real-time visualization and tracking of individual fluorescent-tagged secretory vesicles [25-27]. And such observation may provide insightful information on vesicle's molecular state. Because of the selective evanescent illumination of the thin section (~200 nm) just above the interface between the glass coverslip and the adhered cell, TIRFM is instrumental to gain insights into dynamic events occurring at, or close to, the plasma membrane of living cells with outstanding optical contrast and resolution.

Phorbol-12-myristate-13-acetate (PMA), a synthetic phorbol ester, is commonly used as the stable analog of the endogenous lipid DAG to examine DAG pathways. In the present study, we investigated the effects of PMA on vesicle dynamics in rat pheochromocytoma (PC12) cells using TIRFM. We found that secretory vesicles in the subplasmalemmal membrane region can be differentiated into three distinct populations based on their characteristic dynamics. And PMA differentially modulates these vesicle pools in PKC dependent and independent manners.

Materials and Methods

Cell culture and solutions

PC12 cells (American Type Culture Collection, Rockville, Md., USA) were cultured in RPMI 1640 medium (Gibco, Rockville, Md., USA) supplemented with 10% (vol/vol) heat-inactivated bovine calf serum (Gibco), 5% heat-inactivated horse serum (Gibco), and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂/95% air.

All chemicals were purchased from Sigma (St. Louis, MO, USA). The bath solution contains (in mM): 150 NaCl, 5.4 KCl, 2 MgCl₂, 1.8 CaCl₂, 5 mM glucose, and 10 HEPES (titrated to pH 7.4). High K⁺ solution used to induce exocytosis in PC12 cells contains (in mM): 37 NaCl, 105 KCl, 5 CaCl₂, 2 MgCl₂, and 10 HEPES (titrated to pH 7.4). For PMA (phorbol-12-myristate-13-acetate) treatment, cells were incubated in

the bath solution with addition of 0.1 μ M PMA for 10 minutes. In some experiments, 2.5 μ M BIS (bisindoylmaleimidine) was applied to the bath solution for 30 minutes before the introduction of PMA.

TIRFM imaging

Neuropeptide Y (NPY)–enhanced green fluorescence protein (EGFP) plasmid was a kind gift from Dr. Wolf Almers (Vollum Institute, Oregon Health Sciences University). Cells were plated onto poly-L-lysine (Sigma)-coated glass coverslips and transfected with 1 μ g of NPY-EGFP plasmid using FuGENE6 Transfection Reagent (Roche, Indianapolis, USA), 1-2 days before the imaging experiments.

Total internal-reflection fluorescence microscopy (TIRFM) was carried out using a Zeiss Axiovert 200 inverted microscope system (Carl Zeiss, Inc., Germany). EGFP was excited by 488 nm laser and the emission wavelength was collected at 520 nm. Time-lapse digital images were acquired by a CCD camera with exposure time of 20 milliseconds and 2 Hz sampling frequency. Image stacks were collected using a 100 $\times$ 1.45 NA TIRF objective and MetaMorph 6.3 program (Molecular Devices, Downingtown, Pa., USA). The imaging chamber was maintained constantly at 25 °C throughout the experiment. Cells, which appear unhealthy or are apparently differentiated or are clustered or have less than 10 fluorescently labeled vesicles, are considered not typical and thus not chosen for experiments.

Analysis of vesicle motion

Vesicles were individually tracked using either MetaMorph or Image J (an open-source Java-based image processing program developed at National Institute of Health, <http://rsb.info.nih.gov/ij/>). The vesicle velocity, dwell time and coverage diameters were obtained by a program based on Igor (WaveMetrics, Lake Oswego, Or., USA). Statistic analyses, namely, SEM and *t*-test were performed to assess cell-to-cell variation. The simulations of vesicle random walk were also realized using an Igor routine. In the simulations, each step of the Brownian random walk was assumed constant while its direction was random in two dimensions. To simulate the caging effect, a reflecting round boundary with various sizes was assumed.

Amperometry measurement

Amperometry recording were performed at room temperature using 5 μm carbon fiber electrodes (ALA Scientific Instruments, Westbury, Ny., USA). The carbon fiber with a freshly cut tip was biased at 700 mV and gently positioned against the cell. The amperometry signals were filtered at 1 kHz and sampled at 4 kHz using an EPC-10 double patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The data was analyzed using a Igor program, Amperometric Spike Analysis 8.15, developed by Dr. Eugene Mosharov (Department of Neurology, Columbia University) [28]. Only

spikes with amplitudes larger than 2 pA (5 times of the background noise) were considered as amperometric events.

Results

Large dense core vesicles (LDCV) in rat PC12 cells were specifically labeled by overexpressing neuropeptide Y fused to enhanced green fluorescence protein (NPY-EGFP). While staying within ~200 nm thin layer of cytosol immediately adjacent to the plasmalemma, they can be clearly resolved under evanescent-wave excitation using a total internal reflection fluorescence microscope. Time-elased images were taken over two minutes at 0.5 seconds time interval to reveal vesicle dynamics. Agreeing with previously reported [27, 29], none of the vesicles in the evanescent field adopted stable docking onto the plasma membrane, which might be intuitively assumed as a prelude to fusion. Instead, they generally manifested constant, but apparently confined, lateral motion, as well as vertical trafficking (transition between the inner cytosol and subplasmalemmal region). Vesicles in a cell were individually tracked and analyzed to obtain information about their trajectories, velocities, and dwell time.

Subplasmalemmal vesicles can be classified into three populations according to their motion dynamics

We define vesicles that appeared at the beginning of the time-resolved imaging as pre-docked vesicles; and vesicles that arrived from the inner cytosol during the imaging as visiting vesicles. Comparing to previously docked vesicles, visitors moved much rapidly and returned back into the cytosol after a short period of dwell time. Pre-docked vesicles can be further distinguished by two types: 1) sedentary pre-docked vesicles, which stayed up to the end of the recording (2 minutes) and were most inactive in motion; 2) mobile pre-docked vesicles, which were lost into the cytosol during the imaging period and moved in moderate speeds. Analyses of vesicle dynamics indicate that the three types of vesicles are distinct in their motion characteristics, release competence, and dependence on phorbol ester modulations. Therefore, they may represent distinct functional pools of vesicles characterized by different molecular states. Nevertheless, it should be pointed out that our visual classifications on these vesicles types are by no means clear-cut criteria. Hence, such distinction between vesicle groups can be somewhat blurred.

Fig. 1a illustrates the scatter plot of dwell time versus average velocity, in which each dot represents one vesicle (the total of 495 vesicles from 10 cells). Average velocity is defined as the total distance of vesicle motion divided by vesicle dwell time. It is evident that vesicles were clustered into three groups. The visiting vesicles (blue) were short lived with an average dwell time of 7.9 ± 0.77 seconds (SEM - standard error of the mean) and moved rapidly with an average velocity of 0.74 ± 0.024 $\mu\text{m/s}$. The mobile pre-docked vesicles (red) lingered in the near plasma region 5 times longer than the visitors (43.3 ± 3.09 seconds) and with a much slower velocity

($0.39 \pm 0.021 \mu\text{m/s}$). The sedentary pre-docked vesicles (green) were most stably leashed onto the subplasmalemmal membrane region with the slowest mobility ($0.16 \pm 0.014 \mu\text{m/s}$).

In agreement with the previous studies [30-32], vesicle motion was found to be highly restricted as if vesicles were confined in cages. An example trajectory of a mobile pre-docked vesicle is depicted in Fig. 1b. We define the longest distance between any two vesicle footprints as the long coverage diameter (LCD), as indicated by the arrowed-and-dashed red line. The sum of the longest perpendicular distances to the LCD axis from two footprints at each side is defined as the short coverage diameter (SCD), as indicated by two arrowed-and-dashed black lines. All the vesicle footprints fall into the rectangular area specified by the long and short coverage diameters.

In Fig. 1c, each trace demonstrates how LCD of a vesicle evolves with time (from its appearance to disappearance) in a typical cell. Visiting vesicles, mobile pre-docked vesicles and sedentary pre-docked vesicles, which are represented by different colors (blue, red and green, respectively), showed distinct kinetics. If a vesicle undergoes unbounded Brownian random walk, its motion coverage would diverge over time, i.e., $LCD \propto \sqrt{t}$. On the contrary, vesicle LCD approaches a limit in a time scale of ten seconds, suggesting that vesicle motion is confined within small cages delimited by certain physical barriers or due to some tethering forces. The traces from different types of vesicles are well separated, indicating characteristic differences in their motion.

The average behaviors of the three vesicle pools are obtained from 10 cells (Fig. 1d). Due to varying dwell time, we average the LCD curves of the visiting vesicles over the initial 10 seconds, only for those which survived up to 10 seconds or more (blue circles; the average from 50 vesicles). Mobile pre-docked vesicles are averaged over 20 second time frame, while those whose dwell time were less than 20 seconds are not used (red circles; 74 vesicles). Sedentary pre-docked vesicles are also averaged over 20 seconds (green circles; 77 vesicles). The 10 second and 20 second time frames are selected for averaging to balance two considerations: first, to assure that not too many vesicles are left out for analysis; on the other hand, only vesicles staying long enough to reflect the motion trend should be chosen for analysis. Clearly from their averaged LCD curves, the three types of vesicles differ in their motion kinetics.

In contrast to the case of unlimited Brownian random walk in which LCD curve would diverge with $\sqrt{t}$, the averaged vesicle LCD curves can be well fitted by a double-exponential. And they converge to a limit rapidly with a fast time constant of ~ 0.5 s and a slower time constant of ~ 10 s (Fig. 1d). The approaching limit predicts the ultimate boundary (cage size) of vesicle motion regardless of how long the vesicle remains in the subplasmalemmal membrane region before losing into the cytosol and how fast the vesicle moves. The time constants, on the other hand, reflect the mobility of the vesicle. Specifically, the averaged LCD curve of the visiting vesicles approaches (as $t \rightarrow \infty$) the cage size of $1.60 \mu\text{m}$ with an initial fast kinetics (time constant $\tau_1 = 0.45$ s) followed by a slower kinetic component (time constant $\tau_2 = 7.74$

s). Comparing to the visiting vesicles, the sedentary pre-docked vesicles showed a much smaller cage size (0.69 μm) and slower kinetics ($\tau_1 = 0.77$ s; $\tau_2 = 15.6$ s), whereas the mobile pre-docked vesicles showed a moderate cage size (0.98 μm) and moderate kinetics ($\tau_1 = 0.38$ s; $\tau_2 = 13.76$ s).

Vesicle lateral motion is modulated by PMA

When the cells ($n = 9$) were treated with PMA, interestingly, the motion of the visiting vesicles became confined to a much greater extent with a reduced cage size of 1.0 μm (blue squares in Fig. 1d; averaged from 104 vesicles). To test the roles of PKC isoforms in this effect, bisindolylmaleimide (BIS) which is a specific PKC inhibitor through competitive inhibition of ATP binding within the PKC catalytic domain was applied in addition to PMA treatment (7 cells). BIS should not affect non-kinase phorbol ester / DAG-binding proteins such as Munc13. As shown in Fig. 1d, BIS (7 cells) was not able to relief the PMA induced further motion restriction (blue triangles; 35 vesicles). Hence it is likely that PMA exaggerates the confinement of the visiting vesicle's lateral motion through PKC independent mechanisms. By contrast, PMA treatment only slightly reduced the cage size of the sedentary pre-docked vesicles to 0.55 μm (green squares in Fig. 1d; 70 vesicles); and did not affect the motion of the mobile pre-docked vesicles appreciably (red squares in Fig. 1d; 48 vesicles). Whereas, additional application of BIS significantly reduced the cage sizes of both the sedentary and mobile pre-docked vesicles to 0.45 μm (green triangles in Fig. 1d; 84

vesicles) and 0.53 μm (red triangles in Fig. 1d; 43 vesicles), respectively. Intriguingly, these results imply that although boosting PKC activation by PMA does not impact the movement of pre-docked vesicles significantly, inhibition of PKCs leads to severe motion confinement.

Comparing the velocities and the cage sizes of the three types of vesicles, it is noted that less mobile vesicles tend to be imprisoned within a smaller area. In addition, vesicle velocities of the three types of vesicles responded to the application of PMA and BIS similarly as the induced changes in their cage sizes, as demonstrated in Fig. 2. For example, PMA reduces both the cage size and the velocity of the visiting vesicles. It is conceivable that vesicle mobility and how far the vesicle can travel may be determined by the same tethering or confining mechanisms.

PMA reduces the number of unreleasable sedentary pre-docked vesicles

Sedentary vesicles made up ~35% of all pre-docked vesicles (Fig. 3a, 10 cells). PMA treatment (9 cells) significantly lowered this percentage to ~14%. This reduction was reversed by BIS (7 cells), giving rise to ~51% and hence implying the involvement of PKCs. The sedentary pre-docked vesicles were generally brighter than other vesicles. Under total internal reflection illumination, the fluorescence brightness is exponentially inverse-proportional to the distance between the fluorescent vesicle and the membrane-coverslip interface. Therefore, sedentary vesicles are more closely attached onto the plasma membrane. But somewhat surprisingly, these vesicles are

often reluctant to be released upon triggering. The left panel in Fig. 3b is the last TIRFM image of a two-minute recording on a control cell. The sedentary pre-docked vesicles which stayed on throughout the two-minute recording are identified and indicated by arrows. The right panel in Fig. 3b shows the TIRFM image of the same cell 48 seconds after the introduction of high K^+ solution. High K^+ solution is commonly used to depolarize the cell membrane and subsequently induce exocytosis. All the sedentary pre-docked vesicles remained after high K^+ stimulation, whereas disappearance of the other vesicles was observed.

Subject to high K^+ stimulation, the averaged release time courses of the pre-docked vesicles in three conditions (14 control cells, 13 cells with PMA treatment, and 7 cells treated with PMA plus BIS) are shown in Fig. 3c. The percentages of the remaining (unreleased) vesicles are consistent with the percentages of the sedentary vesicles shown in Fig. 3a. It could be speculated that the sedentary vesicles are in a docked but unprimed state. And PKC activation by PMA primes these vesicles to the readily releasable state, *via* phosphorylation of secretory proteins such as Munc18 [16] and SNAP-25 [15]. In comparison to sedentary vesicles, mobile pre-docked vesicles are essentially readily releasable upon stimulation.

PMA enhances exocytosis mainly by increasing the delivery of visiting vesicles

Phorbol ester is known to enhance exocytosis in various cell types. The mechanisms underlying, however, are not fully understood yet. In some studies, it has been

attributed to the increase in the number of vesicles close to the plasma membrane as a result of PKC activation based on electron microscopy observations [18, 33]. From TIRFM imaging on live PC12 cells, we however found that, PMA did not increase the total number of the visible vesicles in subplasmalemmal membrane region at any given snapshot in time (average from 6 cells, open squares in Fig. 4a). On the other hand, the arrival rate of visiting vesicles was dramatically increased by PMA (6 cells, filled squares in Fig. 4a). PKC activation is likely responsible for this increased vesicle transport from the cytosol, because it can be completely blocked by the application of BIS (7 cells, filled triangles in Fig. 4a).

PMA enhancement in exocytosis was confirmed by carbon fiber amperometry (Fig. 4b). Carbon fiber electrode, with a diameter of 5 μm and positioned against the cell membrane, likely can only detect vesicle release from the membrane area just underneath, which only accounts for a small fraction (~5 - 10 %) of the total cell membrane area. Considering that the total number of pre-docked vesicles is typically less than 40, most of the exocytotic events recorded by carbon fiber must be produced by newly arrived visiting vesicles. Therefore, it can be concluded that the PMA enhancement reported by amperometry recording is achieved by increasing delivery of releasable visiting vesicles to the plasma membrane. Large portion of the PMA recruited visiting vesicles, however, are not fusion competent, since the PMA induced increase (33%) in the amperometric signal is much less than the increase (230%) in the arrival rate of visiting vesicles as observed under TIRFM.

Vesicle dwell time is reduced by PMA

Increased delivery of visiting vesicles does not cause accumulation of vesicles in the subplasmalemmal membrane region (Fig. 4a) because PMA also reduces the dwell time of both pre-docked vesicles and visiting vesicles, as if the interactions to keep the vesicles attaching to the membrane are weakened (Fig. 5). In other words, PMA does not alter the equilibrium between the vesicle recruitment and vesicle retrieval. PMA shortens vesicle dwell time probably through PKC dependent pathways as BIS treatment fully abolishes this effect.

Vesicle motion coverage is regulated by PMA

As discussed above, PMA modulates boundary, velocity, and dwell time of vesicle motion. Consequently, the total coverage area of vesicle lateral motion, which can be estimated by LCD multiplies SCD, are subject to PMA regulation. As seen in Fig. 6a, PMA largely shrunk the motion coverage of visiting vesicles and mobile pre-docked vesicles, but not that of sedentary pre-docked vesicles. BIS failed to antagonize PMA effect. Instead, it further reduced the motion coverage of mobile pre-docked vesicles and also caused decrease of the coverage of sedentary pre-docked vesicles. Demonstrated in Fig. 6b, both PMA and BIS, did not cause appreciable changes in the aspect ratios of motion coverage which is defined as SCD divided by LCD.

Discussion

Using TIRFM, we visualized individual secretory vesicles that are immediately adjacent to the plasma membrane in PC12 cells and analyzed their movement. We classify these vesicles into three groups: visiting vesicles, mobile pre-docked vesicles, and sedentary pre-docked vesicles. These populations of vesicles differ in their motion characteristics, fusion competence, and dependence to PMA modulation, suggesting that they constitute molecularly heterogeneous vesicles.

All vesicles are constantly moving. This observation contradicts the once widely recognized hypothesis that a stable docking state at the plasma membrane is a prerequisite for vesicle fusion. An interesting question to ask is that, as the vesicle moves constantly, whether the associated tethering, docking and priming proteins (e.g., SNAREs) move along as a whole like a drifting exocytotic raft? Or vesicles leap between the relatively stationary and abundant exocytotic sites defined by clustered secretory proteins?

Interestingly and perhaps counter-intuitively, sedentary pre-docked vesicles, which are most strongly attached onto the subplasmalemmal membrane region indicated by their lowest mobility, longest dwell time and high fluorescence brightness, are most fusion incompetent. It is plausible that tethering and docking interactions between vesicles and plasma membrane are actually fusion-inhibitory to prevent them from being constitutively released. We nevertheless could not completely rule out the possibility that the sedentary pre-docked vesicles undergo

kiss-and-run fusion under high K^+ stimulation which only leads to release of a small amount of NPY-EGFP and thus undistinguishable reduction in vesicle brightness. In comparison, the mobile pre-docked vesicles are more loosely associated with plasma membrane. But they are fusion competent and may be responsible for the initial phase of release in response to intracellular calcium elevation because of their proximity to the exocytotic site. Visiting vesicles arrived from the inner cytosol can only be transiently captured in the subplasmalemmal membrane region. Despite the high lateral mobility, they are releasable, and in fact, account for the majority of the fusion events recorded by carbon fiber microelectrode. It has been demonstrated that these recruited vesicles can be immediately released once they reach the plasma membrane and they are responsible for the late and sustained phase of exocytosis [34].

Here we describe a new method to characterize vesicle motion which allows us to distinguish the motion characteristics of different vesicle populations and monitor kinetics of vesicle movement over time. Two parameters, termed as long coverage diameter (LCD) and short coverage diameter (SCD), are introduced to simply quantify the coverage of vesicle lateral movement. The evolvement of LCD over time reflects the nature of vesicle motion. If vesicles undergo unbounded Brownian random walk, LCD would increase in proportional to square root of time. Specifically, $LCD = \sqrt{4Dt}$, where D is the diffusion coefficient of the vesicle [35]. To illustrate this, we performed Monte Carlo simulations. In simulations, the time interval (τ) between random walk steps is 0.5 seconds, the same as the TIRFM imaging interval we used. The step size of random walk (δ) is a constant, which is determined based on

an assumed vesicle velocity ($v = \delta/\tau$). The step size relates to the diffusion coefficient by equation: $D = \delta^2/(2\tau)$ [35]. An example of stimulated trajectory in 120 seconds duration is depicted in Fig. 7a, assuming the mean velocity of the mobile pre-docked vesicles ($v = 0.39 \mu\text{m/s}$). Fig. 7b exhibits the averaged LCD curves, each obtained from 100 simulations, by assuming the vesicle velocity of $0.16 \mu\text{m}$ (triangles), $0.39 \mu\text{m}$ (squares), and $0.74 \mu\text{m/s}$ (circles), respectively corresponding to the mean velocities of the sedentary pre-docked, the mobile pre-docked and the visiting vesicles. These curves agree well with theoretical predictions, i.e., $LCD = \sqrt{4Dt} = \delta\sqrt{2t/\tau} = v\sqrt{2\pi}$ (fitted solid curves). They diverge towards infinite as time increases. The actual vesicle motions are apparently discrepant from such unbounded Brownian random walk.

In Fig. 7c, a random walk trajectory is obtained by repeating simulation procedure as in Fig. 7a while imposing a confining round boundary (a hypothetical cage). In this example, we assumed a diameter of $0.98 \mu\text{m}$ for the confining cage, identical to the cage size of the mobile pre-docked vesicles. In Fig. 7d, three averaged LCD curves were obtained, each from 100 simulations, by assuming the mean velocities and the cage sizes of the three types of vesicles, respectively. These curves can be well fitted by a double-exponential (solid curves). And they agree very well with the actual experimental data shown in Fig. 1d (re-plotted in Fig. 7d as thick solid curves). These simulation results unambiguously demonstrate that the motion of subplasmalemmal vesicle is caged Brownian random walk. Nevertheless, some vesicles exhibited directed motion (travel directionally for a relatively long distance),

deviating from their typical behavior. These events, however, are rare, particularly within the short time frames (10s or 20s) that we used for analysis.

The cortical actin meshwork probably is the dominant factor that hinders vesicle motion [18]. This view was supported by the experiments using latrunculin A which disrupts actin cortex by preventing actin polymerization. Vesicle motion in latrunculin A treated cells is analogous to unbounded Brownian random walk (data not shown). Interestingly, a recent study reported the existence of cytoskeletal polygonal cages in bovine chromaffin cells that physically confine the movement of the vesicle inside [36]. The estimated sizes of the cortical cages and the interior cages are about 0.48 μm and 0.64 μm in diameter, comparable to the predicted cage sizes of the pre-docked vesicles in our study. It appears that the cage size is inversely proportional to the vesicle velocity. It is possible that the same tethering or confining interaction may slow down the vesicle velocity and set the limits for vesicle motion as well.

Further supporting the notion that the three types of vesicles are molecularly heterogeneous, we show that PMA modulates these morphologically defined vesicle pools differentially. PMA has long been recognized as a potent exocytosis promoter. Phorbol ester enhancement in exocytosis has been attributed to the induced PKC activation and the resulting increase in the size of the readily releasable vesicle pool (RRP) [9, 11]. Mainly derived from the membrane capacitance measurement of exocytosis, the release of RRP is defined as the initial rapid phase in the exocytosis kinetics responding to a step-like increase in intracellular calcium concentration by calcium uncaging. Vesicles in RRP are considered to be appropriately docked and

fully primed and thus ready to be released immediately upon triggering [37]. As a prerequisite to be readily releasable, these vesicles are presumably in close proximity with the plasma membrane (< 25 nm away from the membrane) to assure the critical SNARE protein pairing, i.e., complexing between vesicle SNARE (VAMP) and target SNAREs (syntaxin and SNAP25) [38]. Therefore, vesicles in RRP must be closely attached to the plasma membrane and visible in evanescent field. In this study on PC12 cells, we, however, found that phorbol ester does not increase the number of vesicles within subplasmalemmal membrane region.

Although fail to increase the number of docked vesicles, PMA facilitates priming and subsequent release of sedentary pre-docked vesicles likely through PKC phosphorylation directly on fusion proteins. In PC12 cells, because the number of docked vesicles is small (typically, 20 – 40 vesicles), such facilitation in exocytosis is not significant. But for chromaffin and neuron cells which has hundreds of morphologically docked vesicles, such PMA facilitation is prominent and may underlie the observed increase in RRP. As demonstrated by our experiments, in PC12 cells, PMA enhances exocytosis largely by increasing the rate of vesicle delivery to the plasma membrane, also in a PKC dependent manner. Kinetic analyses of individual amperometric spikes (data not shown) indicate that PMA also accelerated the fusion rate of the exocytotic events leading to amperometric signals of larger amplitude (Fig. 4b). PMA enhanced fusion rate has also been reported in chromaffin cells [39]. And Munc18 phosphorylation by PKC was suggested to account for this phenomenon because expression of Munc18 phosphomimetic for PKC

phosphorylation produced similar effects [17]. In contrast to our observation, the peak amplitude of amperometric spikes was not apparently increased in these studies on chromaffin cells because PMA, at the same time, decreased the quantal size of vesicle release as well because of increase in kiss-and-run type of fusion.

Neuroendocrine cells possess a dense actin meshwork underneath the plasma membrane. This cortical F-actin network acts as a physical barrier separating a large pool of the reserved vesicles in the cytosol and the docked vesicles. On the other hand, once docked on the plasma membrane, F-actin serves as an active regulator to keep the vesicles in place and drive the final membrane fusion [40]. Several lines of evidences have been documented that PKC activation (e.g., by PMA) causes disassembly and re-organization of dense actin cortex clearing the way for myosin dependent transport of vesicles from the reserve pool to the plasma membrane along the remaining fine actin filament [41]. PKC activation disrupts and re-organizes actin networks probably through multiple pathways, including recruitment of actin severing protein scinderin [40] and phosphorylation of MARCKS [19, 41] or phosphorylation of other actin filament associated proteins [20]. It explains our observation that PMA largely increased the traffic between the reserved vesicle pool and docked vesicle pool. Specifically, both the delivery rate of visiting vesicles and the retrieval of cortical vesicles back into the cytosol were increased after PMA application. PKC specific inhibitor, BIS, completely abolished this increased vesicle traffic. Nonetheless, such an actin centric view is perhaps too simple to picture the likely complicated mechanisms underlying. Increased trafficking may be resulted from the combined

actions of multiple PKC effector proteins that participate in vesicle tethering and docking [5, 6, 42]. For instance, PKC phosphorylation on Munc18 could be responsible for increased vesicle trafficking by PMA, in line with a recent demonstration that phosphomimetic mutant of Munc18 increased the rate of vesicle pool replenishment [17].

Seemingly contradictory to its disrupting action on actin meshwork, PMA causes restriction on vesicle motion, particularly on visiting vesicles. Unlike other actin disassembly drugs such as latrunculin A, PMA only reduces the density of actin meshwork instead of dissolving all the actin filaments. Therefore, PMA induced re-organization of actin cortex does not necessarily decrease the sizes of actin cages that confine vesicle motion. PMA restrains vesicle motion by decreasing its caging boundary and vesicle velocity, possibly through phorbol ester / DAG receptor proteins, other than PKCs such as Munc13, that involve in vesicle tethering, docking and priming. The overall consequence of reduced vesicle motion and dwell time by PMA is the significant reduction in the coverage area of vesicle motion. This is consistent with the finding from a recent paper [32] which showed that both PMA treatment and overexpression of Munc13 largely eliminated long-range vesicle motion. But the authors in that study did not report PMA induced decreases in vesicle velocity and dwell time as we did, maybe because they used a different cell model – bovine chromaffin cells.

In addition to PKC, there are alternative DAG/phorbol ester receptors involving in various cellular processes, including Munc13, chimaerins, protein kinase D,

RasGRPs, and DAG kinase γ [43]. BIS is commonly used to assess PKC dependence of phorbol ester effects. However, it has been previously reported that BIS causes irreversible rundown in neuronal exocytosis *via* nonspecific actions because phorbol esters can still similarly potentiates exocytosis in BIS treated cells [23]. We showed that BIS completely reversed the PMA induced increase in vesicle delivery (Fig. 4a) and PMA induced decrease in vesicle dwell time (Fig. 5) and concluded that these PMA effects are likely PKC dependent. These conclusions are further supported by the observations that PMA failed to potentiate vesicle delivery and did not cause significant reduction in vesicle dwell time in BIS pre-treated cells, suggesting that BIS nonspecific actions likely do not play an important role. Specifically, Cells with only BIS treatment ($n = 6$) showed vesicle delivery rate of 14.5 ± 1.1 vesicles in 300s, comparable to that after additional PMA application (13.8 ± 1.5 vesicles in 300s; 6 cells). And cells with only BIS treatment ($n = 7$) showed a dwell time of 12.6 ± 1.9 seconds for visiting vesicles and a dwell time of 86.3 ± 3.8 seconds for pre-docked vesicles, comparable to the values obtained with additional PMA treatment (8.6 ± 1.6 seconds and 81.3 ± 3.7 seconds, respectively; 7 cells). For the cases where PMA had no effect, yet BIS was inhibitory (Fig. 1d and Fig. 2), it is possible that PMA was not able to further boost PKC activities while BIS specifically blocked basal PKC activities. Alternatively, it could be nonspecific actions of BIS. To avoid the caveats of pharmacological assessment of PKC function, other more specific and informative methodologies can be applied, for examples, overexpression of wide-type and

dominant interfering PKC mutants, antisense knockdown of PKC expression, and inducing or interfering PKC phosphorylation on the targeting proteins [43].

In summary, this study presents an approach to analyze the heterogeneous dynamics of secretory vesicles using total internal reflection fluorescence microscopy, and provides further evidence that the PMA regulation in exocytosis is multifaceted and complex. This study demonstrates an analytical framework which, together with genetic manipulations, may allow quantitative assessment of the roles of specific secretory proteins in regulating various aspects of vesicle dynamics.

Acknowledgement

This study was supported by grants from the Ministry of Education (Singapore) to PC (AcRF tire 2 -T206B3220 and AcRF tire 1 - RG 41/05).

References

1. Burgoyne RD, Morgan A (2003) Secretory granule exocytosis. *Physiological Reviews* 83:581-632
2. Wenk MR, De Camilli P (2004) Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: Insights from vesicle recycling in nerve terminals.

Proceedings of the National Academy of Sciences of the United States of America
101:8262-8269

3. Barclay JW, Morgan A, Burgoyne RD (2005) Calcium-dependent regulation of exocytosis. *Cell Calcium* 38:343-353
4. Rohrbough J, Broadie K (2005) Lipid regulation of the synaptic vesicle cycle. *Nature Reviews Neuroscience* 6:139-150
5. Becherer U, Rettig J (2006) Vesicle pools, docking, priming, and release. *Cell and Tissue Research* 326:393-407
6. Izumi T, Kasai K, Gomi H (2007) Secretory vesicle docking to the plasma membrane: molecular mechanism and functional significance. *Diabetes Obesity & Metabolism* 9:109-117
7. Roldan ERS, Harrison RAP (1992) The Role of Diacylglycerol in the Exocytosis of the Sperm Acrosome - Studies Using Diacylglycerol Lipase and Diacylglycerol Kinase Inhibitors and Exogenous Diacylglycerols. *Biochemical Journal* 281:767-773
8. Davletov B, Connell E, Darios F (2007) Regulation of SNARE fusion machinery by fatty acids. *Cellular and Molecular Life Sciences* 64:1597-1608
9. Gillis KD, Mossner R, Neher E (1996) Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. *Neuron* 16:1209-1220
10. Yang Y, Udayasankar S, Dunning J, Chen P, Gillis KD (2002) A highly Ca^{2+} -sensitive pool of vesicles is regulated by protein kinase C in adrenal chromaffin

cells. Proceedings of the National Academy of Sciences of the United States of America 99:17060-17065

11. Stevens CF, Sullivan JM (1998) Regulation of the readily releasable vesicle pool by protein kinase C. Neuron 21:885-893

12. Morgan A, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciufo LF, Evans GJO, Graham ME (2005) Regulation of exocytosis by protein kinase C. Biochemical Society Transactions 33:1341-1344

13. Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M (1996) Phosphorylation of 25-kDa synaptosome-associated protein - Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. Journal of Biological Chemistry 271:14548-14553

14. Nagy G, Matti U, Nehring RB, Binz T, Rettig J, Neher E, Sorensen JB (2002) Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser(187) potentiates vesicle recruitment. Journal of Neuroscience 22:9278-9286

15. Yang Y, Craig TJ, Chen XH, Ciufo LF, Takahashi M, Morgan A, Gillis KD (2007) Phosphomimetic mutation of Ser-187 of SNAP-25 increases both syntaxin binding and highly Ca²⁺-sensitive exocytosis. Journal of General Physiology 129:233-244

16. Fujita Y, Sasaki T, Fukui K, Kotani H, Kimura T, Hata Y, Sudhof TC, Scheller RH, Takai Y (1996) Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C - Its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. Journal of Biological Chemistry 271:7265-7268

17. Nili U, De Wit H, Gulyas-Kovacs A, Toonen RF, Sorensen JB, Verhage M, Ashery U (2006) Munc18-1 phosphorylation by protein kinase C potentiates vesicle pool replenishment in bovine chromaffin cells. *Neuroscience* 143:487-500
18. Gil A, Rueda J, Viniegra S, Gutierrez LM (2000) The F-actin cytoskeleton modulates slow secretory components rather than readily releasable vesicle pools in bovine chromaffin cells. *Neuroscience* 98:605-614
19. Park YS, Hur EM, Choi BH, Kwak E, Jun DJ, Park SJ, Kim KT (2006) Involvement of protein kinase C-epsilon in activity-dependent potentiation of large dense-core vesicle exocytosis in chromaffin cells. *Journal of Neuroscience* 26:8999-9005
20. Larsson C (2006) Protein kinase C and the regulation of the actin cytoskeleton. *Cellular Signalling* 18:276-284
21. Basu J, Betz A, Brose N, Rosenmund C (2007) Munc13-1 C1 domain activation lowers the energy barrier for synaptic vesicle fusion. *Journal of Neuroscience* 27:1200-1210
22. Kwan EP, Gaisano HY (2007) New insights into the molecular mechanisms of priming of insulin exocytosis. *Diabetes Obesity & Metabolism* 9:99-108
23. Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Sudhof TC, Takahashi M, Rosenmund C, Brose N (2002) beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. *Cell* 108:121-133

24. Wierda KDB, Toonen RFG, de Wit H, Brussaard AB, Verhage M (2007) Interdependence of PKC-dependent and PKC-independent pathways for presynaptic plasticity. *Neuron* 54:275-290
25. Holz RW (2006) Analysis of the late steps of exocytosis: Biochemical and total internal reflection fluorescence microscopy (TIRFM) studies. *Cellular and Molecular Neurobiology* 26:439-447
26. Allersma MW, Wang L, Axelrod D, Holz RW (2004) Visualization of regulated exocytosis with a granule membrane probe using total internal reflection microscopy. *Molecular Biology of the Cell* 15:4658-4668
27. Steyer JA, Horstmann H, Almers W (1997) Transport, docking and exocytosis of single secretory granules in live chromaffin cells. *Nature* 388:474-478
28. Mosharov EV, Sulzer D (2005) Analysis of exocytotic events recorded by amperometry. *Nature Methods* 2:651-658
29. Degtyar VE, Allersma MW, Axelrod D, Holz RW (2007) Increased motion and travel, rather than stable docking, characterize the last moments before secretory granule fusion. *Proceedings of the National Academy of Sciences of the United States of America* 104:15929-15934
30. Johns LM, Levitan ES, Shelden EA, Holz RW, Axelrod D (2001) Restriction of secretory granule motion near the plasma membrane of chromaffin cells. *Journal of Cell Biology* 153:177-190
31. Oheim M, Stuhmer W (2000) Tracking chromaffin granules on their way through the actin cortex. *European Biophysics Journal with Biophysics Letters* 29:67-89

32. Nofal S, Becherer U, Hof D, Matti U, Rettig J (2007) Primed vesicles can be distinguished from docked vesicles by analyzing their mobility. *Journal of Neuroscience* 27:1386-1395
33. Danks K, Wade JA, Batten TFC, Walker JH, Ball SG, Vaughan PFT (1999) Redistribution of F-actin and large dense-cored vesicles in the human neuroblastoma SH-SY5Y in response to secretagogues and protein kinase C alpha activation. *Molecular Brain Research* 64:236-245
34. Mica OI, Nishiwaki C, Kikuta T, Nagai S, Nakamichi Y, Nagamatsu S (2004) TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic beta-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat beta-cells. *Biochemical Journal* 381:13-18
35. Berg HC (1983) *Random walks in biology*, Princeton University Press
36. Giner D, Lopez I, Villanueva J, Torres V, Viniegra S, Gutierrez LM (2007) Vesicle movements are governed by the size and dynamics of F-actin cytoskeletal structures in bovine chromaffin cells. *Neuroscience* 146:659-669
37. Sorensen JB (2004) Formation, stabilisation and fusion of the readily releasable pool of secretory vesicles. *Pflugers Archiv-European Journal of Physiology* 448:347-362
38. Jahn R, Scheller RH (2006) SNAREs - engines for membrane fusion. *Nature Reviews Molecular Cell Biology* 7:631-643

39. Graham ME, Fisher RJ, Burgoyne RD (2000) Measurement of exocytosis by amperometry in adrenal chromaffin cells: Effects of clostridial neurotoxins and activation of protein kinase C on fusion pore kinetics. *Biochimie* 82:469-479
40. Eitzen G (2003) Actin remodeling to facilitate membrane fusion. *Biochimica Et Biophysica Acta-Molecular Cell Research* 1641:175-181
41. Trifaró JM, Lejen T, Rose SD, Pene TD, Barkar ND, Seward EP (2002) Pathways that control cortical F-actin dynamics during secretion. *Neurochemical Research* 27:1371-1385
42. Barclay JW, Craig TJ, Fisher RJ, Ciufo LF, Evans GJO, Morgan A, Burgoyne RD (2003) Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *Journal of Biological Chemistry* 278:10538-10545
43. Brose N, Rosenmund C (2002) Move over protein kinase C, you've got company: Alternative cellular effectors of diacylglycerol and phorbol esters. *Journal of Cell Science* 115:4399-4411

Fig. 1 Subplasmalemmal vesicles can be classified into three populations: visiting vesicles (blue), mobile pre-docked vesicles (red) and sedentary pre-docked vesicles (green). And their motion kinetics was differential modulated by PMA and BIS. **a** The scatter plot of vesicle dwell time versus average velocity. Each dot represents one vesicle. There are total of 495 vesicles from 10 cells. **b** An example trajectory of a mobile pre-docked vesicle is depicted. Color scales with time in a duration of 46.5

seconds. The motion coverage can be described by two parameters: long coverage diameter (LCD) and short coverage diameter (SCD). LCD is defined as the longest distance between any two vesicle footprints (red arrowed-and-dashed line). The sum of the longest perpendicular distances to the LCD axis from two footprints at each side is defined as SCD (the total length of the two black arrowed-and-dashed lines). **c** Each trace represents how a vesicle LCD evolves with time. All the 40 vesicles are from the same cell. **d** LCD curves are averaged from: 50 visiting vesicles in 10 control cells (blue circles); 104 visiting vesicles in 9 PMA treated cells (blue squares); 25 visiting vesicles in 7 PMA and BIS treated cells (blue triangles); 74 mobile pre-docked vesicles in 10 control cells (red circles); 70 mobile pre-docked vesicles in 9 PMA treated cells (red squares); 43 mobile pre-docked vesicles in 7 PMA and BIS treated cells (red triangles); 77 sedentary pre-docked vesicles in 10 control cells (green circles); 48 sedentary pre-docked vesicles in 9 PMA treated cells (green squares); 84 sedentary pre-docked vesicles in 7 PMA and BIS treated cells (green triangles). Each curve is fitted by a double-exponential whose approaching limit predicts the cage size of the vesicle motion

Fig. 2 Vesicle velocity was modulated by PMA and BIS. 10 control cells, 9 PMA treated cells, 7 PMA and BIS treated cells are used for the statistics. The error bars indicate SEMs. Asterisk, $p < 0.01$ versus control, Student's t test

Fig. 3 PMA reduced the number of unreleasable sedentary pre-docked vesicles. **a** The percentage of sedentary pre-docked vesicles in all pre-docked vesicles was significantly reduced by PMA (13 cells) comparing to the control (14 cells). And BIS (7 cells) reversed the PMA effect. **b** Sedentary vesicles were reluctant to be released. The left panel shows the last TIRFM image after a 2-minute recording. Sedentary pre-docked vesicles are identified by arrows. The right panel shows the TIRFM image 48 seconds after the introduction of high K^+ stimulation. All the sedentary pre-docked vesicles (indicated by arrows) remained while disappearing of other vesicles was evident (highlighted by circles). The squares indicate two recently arrived vesicles.

The scale bar corresponds to 5 μm . **c** The percentage of the remaining vesicles in presence of high K^+ stimulation was largely decreased by PMA treatment (squares, 13 cells) comparing to the control (circles, 14 cells). On the other hand, BIS reversed such PMA effect (triangles, 7 cells). *Asterisk, $p < 0.01$ versus control, Student's t test*

Fig. 4 PMA enhanced exocytosis by increasing the delivery of visiting vesicles in PKC dependent pathways. **a** PMA application dramatically increased the delivery of visiting vesicles (filled squares averaged from 6 cells versus filled circles for 6 control cells) without appreciably affect the total number of the subplasmalemmal vesicles (open squares versus open circle for the control cells). BIS treatment (filled triangles, 7 cells) completely abolished the PMA enhancement in visiting vesicle delivery. **b** Cumulative fusion events detected by carbon fiber amperometry demonstrates that

PMA caused more exocytosis (black, 11 cells) than the control cells (light gray, 22 cells) in response to high K^+ stimulation, whereas BIS (dark gray, 11 cells) completely eliminated the PMA enhancement. The right panel illustrates example amperometry traces from 3 representative cells. The black bars underneath each trace indicate 60-second stimulation of high K^+ solution

Fig. 5 PMA decreased the dwell time of the visiting and pre-docked vesicles, and BIS effectively antagonized the PMA effect. The statistics are obtained from 10 control cells, 9 PMA treated cells, 7 PMA and BIS treated cells. *Asterisk, $p < 0.01$ versus control, Student's t test*

Fig. 6 PMA and BIS changed the coverage area of vesicle motion but not aspect ratio.

The statistics are obtained from 10 control cells, 9 PMA treated cells, 7 PMA and BIS treated cells. **a** PMA largely reduced the motion area of the visiting and mobile pre-docked vesicles, whereas BIS reduced the motion area of the mobile and sedentary pre-docked vesicles. **b** Neither PMA nor BIS treatments altered the aspect ratio of the motion coverage (SCD / LCD). Asterisk, $p < 0.01$ versus control, Student's t test

Fig. 7 Monte Carlo simulations of vesicle lateral motion at 0.5 seconds time interval.

a A stimulation of unbounded Brownian random walk in a duration of 120 seconds.

The size of each random walk step is $0.195 \mu\text{m}$ corresponding to an instantaneous velocity of $0.39 \mu\text{m/s}$. Scale bars indicate $1 \mu\text{m}$ in length. **b** Averaged LCD curves.

Each is obtained from 100 simulations of 20-second unbounded random walk by assuming a constant instantaneous velocity of $0.16 \mu\text{m/s}$ (triangles) or $0.39 \mu\text{m/s}$ (squares) or $0.74 \mu\text{m/s}$ (circles). Each LCD curve can be well fitted (solid curves) by the theoretical calculation (solid curves, see explanations in text). **c** An stimulated

caged Brownian random walk with the same duration and step size as in **a**. But a confining round boundary with a diameter of $0.98 \mu\text{m}$ is assumed. The scale bars

correspond to 0.3 μm . **d** Averaged LCD curves were obtained similarly as in **b**, except that a confine cage with a diameter of 0.69 μm (triangles) or 0.98 μm (squares) or 1.60 μm (circles) is assumed. The averaged vesicle LCDs in 10 control cells from Fig. 1d are re-plotted (dark curves) for comparison