LIPID REGULATION OF EXOCYTOSIS

ZHANG JING

School of Chemical and Biomedical Engineering

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LIST OF ABBREVIATIONS

\[ [\text{Ca}^{2+}]_i \]  
intracellular free $\text{Ca}^{2+}$ concentration

24-OHC  
24-hydroxycholesterol

7-KC  
7-ketocholesterol

7\(\beta\)-OHC  
7\(\beta\)-hydroxycholesterol

APTES  
3-aminopropyltriethoxysilane

BODIPY  
boron-dipyrrromethene

BSA  
bovine serum albumin

\text{C-5a,6a-epoxide}  
cholesterol-5\(\alpha\),6\(\alpha\)-epoxide

\text{C-5\(\beta\),6\(\beta\)-epoxide}  
cholesterol-5\(\beta\),6\(\beta\)-epoxide

CFE  
carbon fiber microelectrode

CTA  
subunit A of cholera toxin

CTB  
subunit B of cholera toxin

CTB-488  
Alexa Fluor 488 conjugated CTB

CTB-594  
Alexa Fluor 594 conjugated CTB

DAG  
diacylglycerol

DI water  
deionized water

DMSO  
dimethyl sulfoxide

DRMs  
detergent resistant membranes

EDTA  
ethylenediamine-tetraacetic acid

EMCCD  
electron multiplying charge coupled device

ER  
endoplasmic reticulum

FBS  
fetal bovine serum
GA  glutaraldehyde
GM1  monosialotetrahexosylganglioside
GSL  glycosphingolipids
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP3  inositol 1,4,5-trisphosphate
K-S test  Kolmogorov-Smirnov test
Ld  liquid-disordered phase
LDCV  large dense core vesicle
Lo  liquid-ordered phase
LPA  lysophosphatidic acid
LPC  lysophosphatidylcholine
LPDS  lipoprotein deficient serum
LPI  lysophosphatidylinositol
LPLs  lysophospholipids
LPS  lysophosphatidylserine
MβCD  methyl-β-cyclodextrin
NGF  never growth factor
neuropeptide Y-enhanced green fluorescent protein
NPY-EGFP  N-ethylmaleimide-sensitive factor
OC  oseltamivir carboxylate
PA  phosphatidic acid
PBS  phosphate buffered saline
PC  phosphatidylcholine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PSF</td>
<td>pre-spike feature</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SM proteins</td>
<td>Sec1/Munc18 proteins</td>
</tr>
<tr>
<td>SNAP</td>
<td>soluble NSF attachment protein</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>synaptosomal-associated protein of 25 kDa</td>
</tr>
<tr>
<td>SNAREs</td>
<td>soluble NSF attachment protein receptors</td>
</tr>
<tr>
<td>SPANs</td>
<td>snake PLA2 neurotoxins</td>
</tr>
<tr>
<td></td>
<td>total internal reflection fluorescence</td>
</tr>
<tr>
<td>TIRFM</td>
<td>microscopy</td>
</tr>
<tr>
<td>VAMP</td>
<td>vesicle-associated membrane protein</td>
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SUMMARY

Exocytosis is a finely tuned program in which the secretory vesicles are directed to fuse with plasma membrane and discharge neurotransmitter or hormone content to the extracellular space. It is responsible for fundamental cellular activities, such as neuron communications and endocrine. Exocytosis is precisely regulated by numerous protein factors, among which SNAREs are well recognized as the core machinery. However, increasing evidence shows that lipids also participate in the exocytotic process as essential regulators.

Lipids may modulate exocytosis via interplays with exocytotic proteins, or via their influence on the physicochemical and structural properties of the plasma membrane. In the current work, we explored the multiple roles played by lipids in the regulation of exocytosis. We mainly focused on (1) sterols and sphingolipids which are enriched in membrane functional microdomains (lipid rafts) for spatial regulation of exocytosis; and (2) lysophospholipids which are implicated in Ca\textsuperscript{2+} signaling. Integrative single-cell study platform was employed to study the lipid regulations, involving total internal reflection fluorescence microscopy, confocal microscopy, amperometry measurement, electrophysiological recording, surface functionalization, and molecular biology.

It was found that cholesterol deprivation impairs the extent of triggered exocytosis, the kinetics of quantal release, vesicular trafficking, and vesicle docking. This study highlights the versatile roles of cholesterol in facilitating exocytosis and the importance of cholesterol enriched lipid rafts in exocytosis. Oxysterols, the important mediators for cholesterol homeostasis, were observed
to enhance exocytosis. It suggests the implication of oxysterols in neuropathology. Lysophospholipids, which involve in various signaling transductions including Ca\(^{2+}\) signaling, were also found to trigger exocytosis. It is postulated that lysophospholipids may be active participants in phospholipase A2-induced exocytosis in central nervous system. Finally, we investigated the roles of ganglioside GM1 using surface immobilized and patterned GM1-binding probes (cholera toxin B). It was discovered that vesicular trafficking, docking and exocytosis were facilitated as GM1 molecules were recruited by CTB probes and thus enriched in the entire cell membrane or defined regions. This study demonstrates a novel strategy to stably examine the functions of membrane proteins and corroborates the notion that GM1, enriched in lipid rafts, play multiple roles in exocytosis, particularly in spatial regulation of exocytosis.

In summary, lipids regulate multiple aspects of exocytosis. Understanding the regulatory roles of lipids in the dynamic exocytotic cascade would assist to fully reveal this fundamental cell function and to deal with diseases related to exocytosis.
CHAPTER 1 INTRODUCTION

1.1 Regulated cell secretion or exocytosis is a programmed event in cells

Regulated exocytosis or non-constitutive secretion ("exocytosis" for short in this thesis), is a highly dynamic process in which the secretory vesicles are transported to and fuse with the cell plasma membrane to discharge their inner contents (e.g. neurotransmitter, hormones, signaling peptides) to the extracellular space upon triggering [1-4]. It is a fundamental and ubiquitous process underlying neuron communications, signal transduction, immune responses, hormone secretion, etc. [5-6]. Tremendous attentions were given to the orchestrated route of exocytosis in the past decades, and many regulatory factors have been recognized to function at different stages in dynamic exocytotic process. Nevertheless, there are still many questions to be answered in this delicate program.

It has been identified by various studies, carried in mast cells, neurons, chromaffin cells and adrenal phaeochromocytoma (PC12) cells, that secretory vesicles go through four steps to release their inner cargo, namely trafficking, docking, priming, and fusion [7-10]. A schematic illustration is shown in Figure 1.1.

Firstly, the loaded secretory vesicles are transported by motor proteins from trans-Golgi complex to the vicinity of plasma membrane along microtubules and actin filaments [11-13]. The arrived vesicles are brought closer to the plasma membrane (termed "docking") via the weak interactions between VAMP (vesicle-associated membrane protein) and plasma membrane proteins, i.e.
SNAP-25 (synaptosomal-associated protein of 25 kDa) and syntaxin [14]. These proteins are named SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) since they share a conserved SNARE motif consisting of approximately 60-70 amino acids [15-16]. In the presence of Ca\(^{2+}\), VAMP, syntaxin and SNAP-25 assemble a tight bundle of four helices (termed SNARE complex) that repels the aqueous phase between vesicle and plasma membrane, bridging the contact of two opposing membranes. After priming (maturation), vesicles gain the competency for fusion. Immediately after triggering by the influx of Ca\(^{2+}\), the contact site expands, leading to the formation of a hemifusing diaphragm and the subsequent opening of fusion pore. Finally vesicle membrane merges with the plasma membrane and discharges its inner content [17].

![Figure 1.1](image.png)

**Figure 1.1** The process of exocytosis. The figure is taken from reference [17].
SNARE proteins are widely accepted as the core machinery mediating exocytosis, many other proteins exert their regulatory roles by interplay with SNAREs. For example, synaptotagmin, which binds to Ca$^{2+}$ via its C2 domain, interacts with SNAP-25 for vesicle docking; it triggers SNARE complex assembly for fusion upon receiving Ca$^{2+}$ influx signal [18-19]. NSF (N-ethylmaleimide-sensitive factor) and SNAP (soluble NSF attachment protein) serve SNAREs as molecular chaperons that they prepare SNARE proteins configuring for assembly and disassembly [20]. SM (Sec1/Munc18) proteins show high affinity for syntaxin and are involved in vesicle docking [21]. Complexin may stabilize SNARE assembly by forming a fifth helix to the core complex [22].

Although the central role of protein regulations is well recognized, there is growing evidence that membrane lipids also play a critical role in regulating exocytosis. Lipids, which take more than 30% of the dry weight of cell plasma membrane, are the primary components that determine the structure of membrane bilayer [23]. Their importance is more than merely composing the membrane scaffold for protein embedding, rather they directly participate in various biological activities including exocytosis.

**1.2 Roles of cholesterol and cholesterol-enriched lipid rafts in exocytosis**

Cholesterol is the main sterol contained in plasma membrane. It determines multiple physical properties of biological membrane, such as membrane thickness, ordering, fluidity, and permeability [24]. Cholesterol is also implicated in actin polymerization [25], vesicle budding [26], trafficking [27], and fusion pore formation and expansion [28].
1.2.1 Lipid rafts are cholesterol-enriched microdomains on cell membrane

Cholesterol fills the spaces between phospholipids on both leaflets of the bilayers, orienting in parallel to the non-polar tails of phospholipids. Nonetheless, it is not evenly distributed, but concentrated in particular regions on the plasma membrane which are distinguished as lipid rafts [29]. The term “lipid rafts” are referred to “small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes”, which was officially defined in the Keystone Symposium on Lipid Rafts and Cell Function of 2006 [30]. Lipid rafts have been found to participate in various cell activities, like signal transduction [31], polarized cell sorting [32], virus infections [33], endocytosis [34] and exocytosis [35-37].

Lipid rafts are well known for their insolubility in cold detergent Triton X-100 [38-39], which allows biochemists to analyze the composition of theses detergent resistant membranes (DRMs). Up to date, over one hundred proteins, especially those involved in vesicle cycling, have been identified in DRMs [40]. Synaptic proteins that directly regulate vesicle docking and fusion, e.g. synaptobrevin 2, SNAP-25, synaptotagmin 1, and Munc 18-1, were found to associate with DRMs. Therefore, it is not surprising that lipid rafts are believed to be the platforms for exocytosis [35-36, 41]. In addition, lipid rafts are also critically involved in viral infection. It is notable that infection by HIV-1 virions without lipid rafts does not proceed to AIDS [42]. Influenza virus preferentially buds out from cell membrane at the location where it is enriched in raft lipids and incorporates these lipids in its own envelope [43].

Since cholesterol is the essential lipid on raft, people developed different
strategies to alter membrane cholesterol content in order to disrupt lipid rafts and investigate its function. Presently, a family of cyclodextrins becomes quite popular as cholesterol extractor due to their non-toxicity and easy manipulation. Cyclodextrins are water soluble oligosaccharides with a ring structure which can incorporate cholesterol molecules. The size of the hydrophobic pocket inside the ring is dependent on polymerization degree of cyclodextrins. The commonly used cyclodextrins are $\beta$-cyclodextrins, characterized by the highest affinity for cholesterol. Modified with additional methyl group, methyl-$\beta$-cyclodextrin (M$\beta$CD) is mostly used for extracting cholesterol from cells [44]. Established protocol by mild M$\beta$CD treatment (5~10 mM, 0.5~1 hour incubation) efficiently removes 30~60% of membrane cholesterol without doing harm to cells [45-47].

More and more evidence shows that exocytosis is impaired after cholesterol removal by M$\beta$CD treatment. Cholesterol depletion inhibits synaptosomal neurotransmitter release [48], and it blocks excitatory transmission in hippocampal and neuromuscular cultures [49-50]. Dispersion of lipid rafts by cholesterol extraction may induce alteration of membrane organization, which, in turn, affects exocytosis. Although cholesterol depletion does not eliminate the synaptic proteins and SNAREs from DRMs, it severely blocks glutamate secretion in synaptosomes, suggesting that the integrity and function of lipid rafts is impaired [51].

1.2.2 Lo/Ld phase separation in model membranes

The evidence of cholesterol mediated membrane organization has been provided by the experiments with model membranes. In artificial vesicles
composing of lipid mixtures, phase separation can be distinguished according to the difference of lipids packing and their lateral diffusion [52]. The liquid-ordered (Lo) phase that contains saturated lipids such as cholesterol, is featured by more ordered organization and constrained lipid diffusion; whilst liquid-disordered (Ld) phase consists of unsaturated lipids with less condensed molecular arrangement and higher lipid mobility [53]. This self-driven formation and coexistence of Lo/Ld phases in model systems suggests the heterogeneity of cell membranes [54-55]. Cholesterol is a crucial molecule that facilitates the assembly of raft-like or Lo domains [56], and influences the lateral diffusion of phospholipids [57].

1.2.3 Oxysterols and cholesterol homeostasis

The imbalance (either excess or deficiency) of cholesterol would cause changes in cellular functions. The homeostasis of cholesterol is achieved between cholesterol import, metabolism and export [58]. The excess cholesterol can be brought down by different pathways: (1) esterification, producing cholesteryl esters; (2) oxidation, generating oxysterols at different positions; (3) metabolism and secreted within lipoprotein complexes. Oxidation pathway is a major regulatory mechanism which produces a variety of derivatives. Non-enzymatic oxidations occur at the 5,6-double bond, producing \( \alpha \)- or \( \beta \)-epoxy cholesterol; oxidation happening on carbon 7 position generates 7-ketocholesterol, 7\( \alpha \)- or \( \beta \)-hydroxycholesterol [59]; and 24-, 25- and 27-hydroxycholesterol are the products where oxidation happens in the side chains [60].

Oxysterols can insert to bilayer membrane. But insertion of polar oxygen
moiety into the hydrophobic portion of the lipid bilayer is thermodynamically unstable [61]. Oxysterols can be tilted along the orientation of cholesterol in the membrane bilayer, so that both its oxygen moieties are at the lipid-water interface [62]. This could disrupt ordered lipid domains on the cell membrane [63]. Oxysterols formed by oxidative mechanisms are cytotoxic and are implicated in the pathophysiology of atherosclerosis [58, 64]. It was reported that oxysterols inhibit cell growth and induce apoptosis by stimulating DNA fragmentation in many cell types [65]. It is postulated that oxysterols play key roles in various brain diseases. 24-hydroxycholesterol is found in the brains of patients suffering Alzheimer’s disease [66-67]; increased levels of cholesterol, 24-hydroxycholesterol and 7-ketocholesterol are detected in the rat hippocampus after neuronal injury induced by the excitotoxin kainate [68-69].

### 1.3 Functions of sphingolipids in exocytosis

Sphingolipids are large aliphatic molecules containing a sphingosine base and a fatty acid base. According to their different head groups, sphingolipids are classified into ceramide, sphingomyelins, and glycosphingolipids. Ceramide can be derived from sphingomyelin hydrolysis by sphingomyelinase. It was reported that this ceramide/sphingomyelin pathway is involved in dopamine release from PC12 cells [70]. Monosialotetrahexosylganglioside (GM1) is one of the important glycosphingolipids in cell membrane. It was well known for its neurotrophic effects in nervous systems and neuritogenetic facilitation of neurons [71-72]. Recent years, studies on GM1 have shown that GM1 is enriched within lipid raft to stabilize its structure and mediate its functions [73-74]. Furthermore, GM1 is able to interact with Ca²⁺ ion channel to modulate
Ca$^{2+}$ signaling [75].

1.4 Phospholipids and their regulation in exocytosis

Phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinerine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylinositol 4,5-bisphosphate (PIP2), are amphipathic molecules that build membrane lipid bilayers. They do not randomly distribute on the plasma membrane, but adopt preference for different leaflet. For instance, PC and PIP2 mainly reside in the exoplasmic leaflet whereas PE and PS dominate in the cytosolic leaflet of the plasma membrane [76]. Maintenance of their asymmetric distribution is important for normal cell function [77]. Exposure of PS from cytosolic leaflet to exoplasmic leaflet was suggested to serve as a signal for recognition of apoptotic cells [78].

Phospholipids have been implicated in multiple aspects of exocytosis. For instances, insertion of PC, PE and PS affect kinetics of exocytosis differently. PC decelerates the release of neurotransmitter whereas PE accelerates it. And PS boosts exocytosis by promoting vesicle recruitment to the readily releasable pool [79]. The presence of PIP2 on the cytosolic leaflet of target membrane provides the landmarks for synaptotagmin to recognize the binding site [80]. PIP2 can be hydrolyzed by membrane phospholipase C (PLC) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG is well known as the activator of protein kinase C (PKC) which potently promotes exocytosis [81]. And IP3 causes Ca$^{2+}$ release from endoplasmic reticulum into cytosol and subsequently triggers exocytosis [82].
1.5 Membrane fusion regulated by lipid geometry

SNARE-driven vesicle-plasma membrane fusion has been widely explored. However, increasing evidence revealed that the geometry of lipids is also critical in determining the kinetics of vesicle fusion [83-84]. Lipids with smaller heads and larger hydrophobic tails, like PA, PE, and cholesterol, have a cone shape that contribute negative curvature and induce the local membrane bending towards intracellular space (Figure 1.2). On the contrary, lipids with bulky headgroups, such as lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), glycosphingolipids (GSL) and PIP2, have an inverted cone shape. They generate positive curvature and induce the membrane bending towards extracellular space. And cylindrical lipids such as PC and sphingomyelin induce zero curvature [85-89]. The lipid curvature is critically involved in the pathway of membrane fusion as it determines the formation of hemifusion intermediate and the fusion pore.

During initial contact, inner leaflet of plasma membrane and the outer leaflet of vesicular membrane bend towards each other where positive curvature is formed in the proximal leaflets and negative curvature is formed in the remaining or distal leaflets (Figure 1.3). Subsequently, a small connection (stalk) is established between two proximal leaflets. The stalk structure is dominated by negative curvature. Hence its formation is inhibited by lipids inducing positive curvature and promoted by lipids of negative curvature [90]. With the extension of stalk, local connection expands and develops to an intermediate termed “hemifusion diaphragm”, in which halves of the opposing membrane leaflets merge and the distal leaflets become closer while keeping separated from each other. Finally, a fusion pore is formed in the middle of the
hemifusion structure [83, 88, 91-93]. The separated membrane bilayers enclosing fusion pore mainly adopt positive curvature. Therefore, lipids generating positive curvature, e.g. LPC, facilitate the formation of fusion pore [90].

Figure 1.2 Curvatures of monolayer formed by different lipids. The figure is modified accordingly from reference [94].

Figure 1.3 Membrane fusion regulated by lipid geometry. The figure is modified from reference [93].
1.6 Membrane lipids reporters

It is instrumental to specifically label the interested membrane lipid in order to study its distribution and organization using fluorescent microscopy. The fluorescent probes, based on their incorporation pathways, can be categorized into two classes: lipids binding molecules and fluorescent lipid analogues that can directly insert into the bilayers as membrane native lipids [95].

Filipin, a UV excited polyene antibiotic, has selective affinity to cholesterol molecules on membrane [96]. Filipin staining is inappropriate to perform on living cells because of its cytotoxicity and fast photobleaching [95]. For cholesterol labeling in living cells, a protease-nicked and biotinylated derivative of perfringolysin O (BC0) and BODIPY-cholesterol is usually used in place of filipin. BC0 reserves the same affinity towards cholesterol without cytotoxicity [97]. BODIPY-cholesterol is a non-cytotoxic fluorescent cholesterol analogue that can mimic trafficking and incorporation as membrane native cholesterol [98].

Non-toxic B subunit of cholera toxin (CTB) is a homopentamer with molecular weight of 58 kDa [99]. It binds to membrane lipid ganglioside, especially GM1, with high affinity [100]. Theoretically, each copy of the homopentameric subunit binds to one GM1 molecule, so the pentamer can grab five GM1. Fluorescent CTB is commonly utilized to visualize the distribution of lipid rafts [101-102]. However, concerns arise when using CTB as membrane probe. One major problem is the rapid internalization of CTB through endocytotic pathways [103]. Consequently, approach of CTB labeling is limited by low temperature condition (4°C) or cell fixation to prevent CTB uptake.
Alternatively, membrane GM1 can be labeled by GM1 analogue with additional BODIPY moiety [105].

1.7 Measurement of exocytosis from single cell

1.7.1 TIRFM imaging

Total internal reflection fluorescence microscopy (TIRFM) is a new technique to study vesicle exocytosis. Comparing to the conventional epi-fluorescence microscopy, TIRFM selectively illuminates the thin membrane region < 200 nm thick. Hence, the unwanted background signal from the cell body is largely reduced [106-108]. Concept of TIRFM is derived from total internal reflection. It is known that refraction occurs when laser beam (with wavelength of $\lambda$) passes from glass substrate (refractive index $n_1$) at angle $\theta_1$ into the cell (refractive index $n_2$) with refractive angle $\theta_2$. According to the law of refraction,

**Equation 1.1**

$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$

As $n_1 > n_2$, $\theta_2$ will be greater than $\theta_1$. $\theta_2$ keeps increasing with increment of $\theta_1$ till $\theta_2$ reaches 90°. $\theta_1$ at this moment is the critical angle $\theta_c$, which means all the light beam exceeding $\theta_c$ will be reflected back to glass. The critical angle $\theta_c$ is defined by:

**Equation 1.2**

$$\theta_c = \arcsin(n_2 / n_1)$$

Under this condition, an electromagnetic field, or “evanescent wave”, is generated above the glass coverslip and cell membrane interface (Figure 1.4).
The penetration depth $d$ can be calculated by:

**Equation 1.3**

$$d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta - n_2^2}}$$

When $n_1$ is about 1.5, $n_2$ is around 1.33~1.37 and $d$ will be ~200 nm. Using TIRFM, vesicle trafficking and fusion can be resolved [109-111].

Energy intensity distribution ($I_z$ at position $z$) of the electromagnetic field decays exponentially with increasing $d$, and can be defined as:

**Equation 1.4**

$$I_z = I_0 \exp(-z/d)$$

This property enhances signal-to-noise ratio and minimizes the effect of photobleaching.

---

**Figure 1.4** Principle of TIRFM. The picture is taken from Nikon website (http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html).

TIRFM is a powerful imaging tool to capture the dynamic events happening at subplasmalemma, such as vesicular exocytosis and lateral motion of large dense core vesicle (LDCV), with high contrast as compared to conventional fluorescence imaging and high temporal resolution as compared to confocal
imaging [112-116].

1.7.2 Amperometry measurement using carbon fiber electrode

Exocytosis of secretory vesicles containing neurotransmitters i.e. catecholamines (including dopamine, epinephrine, norepinephrine) can be detected by amperometry using a carbon fiber microelectrode (CFE) (~5 µm in diameter) positioned on the cell surface. Biasing CFE at ~700 mV, the electroactive molecules (e.g., catecholamines) released from a vesicle will be oxidized and produce a current spike reflecting the kinetics of quantal vesicle release [117].

![Figure 1.5](image.png)

**Figure 1.5** Demonstration of amperometric measurement using a carbon fiber electrode. The electrode is mounted on a micromanipulator and freshly-exposed tip is gently positioned on the cell surface. A glass pipette is used for local delivery of stimulation solution. This figure is taken from reference [118].
Amperometry provides a profound insight for understanding fusion kinetics and the underlying regulatory mechanisms. It has been widely applied to monitor exocytosis, with single-vesicle-release sensitivity, either from cells that can secrete oxidizable neurotransmitters/hormones [117, 120-121], or from cells that are loaded with oxidizable compounds (e.g., dopamine) [122]. Each current spike corresponds to single vesicular release. It reveals the kinetics of quantal fusion with millisecond resolution. As illustrated in Figure 1.6, a pre-spike feature (PSF) or foot signal, which arises before the burst of main current spike, is resulted from the small amount of catecholamine leakage through the initial opening of a fusion pore [123-124]. The following steep climbing phase can be assessed by rise time ($t_r$) and rise slope, which manifests the time-course and rate for the current rising from 35% to 90% of the amperometric peak ($I_{max}$). They indicate how fast the fusion pore expands. Half-width of the peak ($t_{1/2}$), which is defined by the width of the peak at half of its maximum amplitude, reflects how long it takes a vesicle to discharge its content [119, 125].

**Figure 1.6** Kinetic parameters obtained from a typical current spike. The schematic picture is modified from reference [119].
integral area under the current spike, termed as quantal size \((Q)\), is directly proportional to the quantity of released catecholamine molecules \((N)\). The relation is defined as [126]:

**Equation 1.5** \[Q = 2FN\]

Where \(F\) is the Faraday constant \(= 96,485\) C/mol. However, only those events occurring near the CFE can be detected (~5%) due to the small size of the CFE. Therefore, amperometry is unable to measure the exocytosis from the whole cell.

*1.7.3 Exocytosis measured by membrane capacitance based on whole-cell patch clamp*

During exocytosis, vesicle membrane is incorporated with plasma membrane which leads to the increase of cell surface area. Employing whole-cell mode patch clamp technique, exocytosis can be monitored by changes of membrane capacitance which is directly proportional to the cell surface area [127-128].

Patch clamp employs a fine-polished glass pipette with tip size of 3~5 µm in diameter. An electrode is inserted in the glass pipette containing suitable solution that mimics the cell interior environment. The glass pipette is pressed against the cell membrane and suction is applied to establish resistance of “gigaohm seal” which ensures current recording with low noise (< 1 pA). The membrane capacitance measurement is established on the circuit model composing of three elements, namely, access resistance \(R_a\), membrane resistance \(R_m\), and membrane capacitance \(C_m\). \(\Delta C\), indicating exocytosis, is
acquired by:

**Equation 1.6** \[ \Delta C = C_{ves} \left[ 1 + (\omega C_{ves} R_p)^2 \right] \]

Where \( C_{ves} \) is the capacitance of a single vesicle, \( \omega \) is the frequency of applied sine wave, \( R_p \) is the resistance of fusion pore [129].

Temporal resolution of membrane capacitance measurement can achieve millisecond scale, providing detailed information on the time course of exocytosis from individual cell. However, the membrane capacitance measurement reflects the overall changes of cell surface area with possible interference of endocytosis [130].

1.8 Research motivation and objectives

Exocytosis is the fundamental process underlying neuron communication, immune response, and hormone secretion. This orchestrated program is precisely tuned by hundreds of factors, including proteins and lipids. Historically, research attentions were given to the regulatory roles played by SNARE proteins and other synaptic proteins [20]. However, increasing evidence has demonstrated that membrane lipids and membrane lipid organizations also participate in controlling exocytosis. Dysfunction of lipid regulation leads to critical diseases [131-132]. One such example is Alzheimer’s disease. Presently, the number of patients suffering from Alzheimer’s disease is 26.6 million in worldwide and it keeps increasing. Lipids and lipid rafts are believed to implicate in the development of Alzheimer’s disease [42, 133-134]. Studying the lipid regulations would help to reveal the complete picture of the exocytotic process, which is not only important for fundamental cell biology
but also for fighting with related diseases. In this thesis, lipid regulations on trafficking and exocytotic fusion of secretory vesicles were studied using electrophysiology, bioimaging, cellular and molecular biology approaches.

Traditional studies of exocytosis were based on large population of cells employing biochemical assays that are incapable of resolving the dynamic exocytosis with sufficient temporal and spatial resolution [48, 135]. Our goal is to establish an integrative platform to study the effect of different lipids on exocytosis at single-cell level and with millisecond time resolution.

To investigate the vertical trafficking, docking and lateral diffusion of secretory vesicles, LDCVs in PC12 are selectively labeled by transfection of EGFP conjugated neuropeptide Y (NPY-EGFP), which is a peptide neurotransmitter secreted in neurons and neuroendocrine cells [110]. Lateral motion of single LDCV at subplasmalemma is individually tracked and analyzed by computer program to assess vesicle trafficking, e.g. velocity, dwelling time, motion area, diffusion constant, mean square displacement, and the rate of vesicle arrival. A computer program will be used to analyze individual amperometric spikes to obtain information about rise slope, quantal size, foot signals, which reflect the quantal fusion kinetics such as fusion pore formation, dilation, quantal release, etc. As exocytosis is triggered by the elevation of intracellular free Ca$^{2+}$ ([Ca$^{2+}]_{i}$), we employ photometry to reveal the dynamics of [Ca$^{2+}]_{i}$.

Using the integrative experimental platform, this thesis aims to examine the regulatory roles of membrane lipids on multiple aspects of exocytosis, namely, extent of exocytosis, vesicle lateral and vertical trafficking, vesicle docking, and quantal fusion kinetics. In Chapter 3, we investigate the roles of cholesterol and
cholesterol enriched lipid rafts while membrane cholesterol is manipulated in different ways. The effects of several oxidation products of cholesterol will be examined in Chapter 4. In Chapter 5, the effects of lysophospholipids (phospholipid derivatives) are studied. Conventionally, the studies of lipids rely on the lipid probes applied in the bulk solution, which suffer from several problems such as internalization via endocytotic routes. In Chapter 6, a novel strategy by surface immobilization of the functional lipid probes is developed and used to investigate the effects of surface immobilized cholera toxin B (which binds to a rafts marker, GM1).
CHAPTER 2 MATERIALS AND METHODS

2.1 PC12 cell culture

PC12 is a cell line derived from rat pheochromocytoma which is known to secrete excessive catecholamines, therefore PC12 is widely adopted as a model to study neurotransmitter secretion [136]. PC12 cell line used in the experiments was purchased from ATCC (American Type Culture Collection, VA). Cells are cultured in growth medium consisting of Advanced RPMI 1640 medium, 10% fetal bovine serum (FBS), 5% horse serum, 1% penicillin-streptomycin, and maintained in 37°C incubator (NuAire, Plymouth, MN) with 95% air-5% CO₂. Growth medium is renewed every 2 days, and cells are subcultured weekly.

For long term preservation, cells are detached and resuspended in freezing medium containing 95% growth medium supplemented with 5% dimethyl sulfoxide (DMSO). Cells suspension is transferred to a freezing vial and stored in liquid N₂. To recover the cells, preservation is transferred from liquid N₂ to a 37°C water bath immediately, and add two volume of growth medium after it is thawed. Suspension is subjected for centrifuge at 1200 rpm (MIKRO 22R, Hettich Zentrifugen, MA) for 5 min, followed by removal of supernatant and addition of growth medium to resuspend cell pellet. Afterwards, cells are maintained in culture condition as described.

Medium and supplements are from GIBCO (Grand Island, NY).
2.2 Chemicals and solutions

Bath solution contains (in mM): 150 NaCl, 2.4 KCl, 2 MgCl₂, 2 CaCl₂, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); titrated to pH 7.2.

0 Ca²⁺ bath solution (in mM): 150 NaCl, 2.4 KCl, 2 MgCl₂, 10 ethylenediamine-tetraacetic acid (EDTA); titrated to pH 7.2.

High K⁺ stimulation solution: 37 NaCl, 105 KCl, 5 CaCl₂, 2 MgCl₂, 10 HEPES; titrated to pH 7.2.

Dopamine loading buffer contains (in mM): 68 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.2). 1.5 mM ascorbate and 70 mM dopamine was added shortly before cell loading.

Cholesterol stimulant: Cholesterol was dissolved in 100% ethanol as stock. When used, cholesterol stock was diluted in bath solution to final concentration of 2 µM.

Oxysterol stimulants: Oxysterol stock was prepared in 100% ethanol, and diluted in bath solution to final concentration of 2 µM when used.

Lysophospholipid stimulants: Lysophospholipid stock was prepared in 100% ethanol, and diluted in bath solution to working concentration of 2 µM when used.

All chemicals are from Sigma-Aldrich.
2.3 Modulation and measurement of cholesterol content

2.3.1 Cholesterol depletion

Cholesterol depletion using MβCD: To deplete cholesterol, cells were incubated in bath solution with additional 5 mM MβCD (Sigma) for 30 min at room temperature, and followed by extensive rinsing.

Cholesterol deprivation by lipoprotein deficient serum (LPDS): Cells were cultured with Advanced RPMI 1640 medium supplemented with 10% LPDS (from fetal calf, Sigma) and 1% penicillin-streptomycin 4~6 days before experiment.

Cholesterol segregation by filipin: Filipin (Sigma) stock solution was prepared by dissolving the powder in DMSO to 25 mg/ml. When used, cells were incubated with 4 µg/ml filipin (diluted in bath solution) for 15 min in darkness at room temperature and washed thoroughly afterwards with bath solution.

2.3.2 Cholesterol loading

For exogenous cholesterol loading, 5% MβCD aqueous solution was heated to 80°C, and dissolved cholesterol (in chloroform:methanol = 1:2, v/v) was added drop-wise till the solution was clear. The molar ratio of MβCD:cholesterol is 9.78:1. The complex film was prepared by evaporating water in a vacuum oven for 1 hour, and was finally dissolved in water as MβCD-cholesterol stock. When used, cells were incubated with 5 mM of MβCD-cholesterol complex (diluted with bath solution) for 1 hour at 37°C.
2.3.3 Measurement of cholesterol content

To harvest cell pellet, cells were washed twice and suspended with bath solution, then subjected to centrifuge at 2000 rpm 4°C (Hettich Zentrifugen) for 10 min, and followed by 4°C lysis with complete RIPA cell lysis buffer, which contains 1 RIPA lysis buffer (Santa Cruz, CA), 1% phenylmethylsulfonyl fluoride (PMSF) solution, 1% sodium orthovanadate solution and 1% protease inhibitor cocktail provided in the same package. The cell lysate was centrifuged at 4000 rpm 4°C for 30 min, and the supernatant was subjected to ultracentrifuge at 32,000 rpm 4°C (Optima Max Ultracentrigue, Beckman Coulter, CA) for 30 min. The resulting membrane pellet was then dissolved in 50 mM Tris buffer (pH 7.4) to final concentration of 0.1 mg/µl. And cholesterol content was determined using Amplex Red Cholesterol Assay Kit (Invitrogen, CA) and an Flx 800 fluorometer (BioTek, VT) following the manufacturer instructions.

2.4 Assessment of cell viability

The viability of cells (cultured in 60 mm dish) after different treatments was determined by trypan blue assay. Treated cells were washed extensively, and then were suspended with 500 µl of bath solution by pipetting. 250 µl of cell suspension was stained with 50 µl 0.4% trypan blue (Sigma) for 5 minutes and cells were counted using a hemocytometer (Heinz Herenz Medizinalbedarf, Hamburg, Germany).
2.5 Surface modifications

2.5.1 Coverglass functionalization

Glass coverslips were cleaned in piranha solution, and repetitively washed with ethanol and deionized (DI) water followed by blowing drying with nitrogen. The cleaned coverglass were silanized by soaking in 5% 3-aminopropyltriethoxysilane (APTES, Sigma) in ethanol for 1 hour with agitation. The silanized coverslips were vigorously washed with ethanol and heated at 120°C for 1 hour. Subsequently, the coverslips were treated with 5% glutaraldehyde (GA) in water for 1 hour followed by repetitive washing with DI water. Cholera toxin subunit B (CTB, Sigma) were dissolved (100 µg/ml) in phosphate buffered saline (PBS) titrated to pH 9.0. The coverslips were covered with the CTB solution for 6 hours. After crosslinking of CTB, the coverslips was extensively washed with DI water to remove CTB residues. The whole process was conducted in a sterilized environment.

2.5.2 Micro-contact printing

Silicon masters with line trenches (with height, width, and spacing distance of 2 µm) were fabricated by photolithography and silanized with octadecyltrichlorosilane (OTS) to facilitate the releasing of polydimethylsiloxane (PDMS) stamps. PDMS stamps were made by casting Sylgard 184 (Dow Corning) on the silicon masters followed by curing at 70°C overnight. The elastomeric stamps bearing negative pattern of the masters were peeling off from the masters, washed with ethanol, and dried under nitrogen.

Sterilized coverslips were incubated in 0.01% poly-L-lysine (PLL) aqueous
solution for 1 hour, followed by rinsing with DI water and drying with nitrogen. One drop of CTB or Alexa Fluor 594 conjugated CTB (CTB-594, Invitrogen) (100 µg/ml in PBS) was pipetted onto PDMS stamp (pre-sterilized with 70% ethanol) to ensure full coverage of the stamp. After inking for 30 min, the stamp was washed with DI water and blown with a mild stream of nitrogen. It was then brought into contact with PLL coated coverslip for 1 min. The coverslip with contact-printed proteins was finally rinsed with DI water and dried with nitrogen.

2.6 TIRFM imaging

Cells were subcultured at concentration of 2×10^5 on sterilized 25 mm (diameter) high refractive-indexed coverglass (No.1, Paul Marienfeld GmbH, Germany) precoated with 0.01% PLL. Vesicles were visualized by expression of plasmid neuropeptide Y tagged with enhanced green fluorescent protein (NPY-EGFP), which was a kind gift from Dr. Wolfhard Almers (Vollum Institute, Oregon Health & Science University). Plasmid transfection was carried out using FuGENE 6 Transfection Reagent (Roche Diagnostics GmbH) 1–2 days before experiments to achieve the highest expression of fluorescent protein. On the day of experiment, coverglass was mounted on a stainless chamber (Bioscience tools, San Diego, CA), and cells were visualized through an inverted total internal reflection fluorescence microscope (Axiovert 200, Carl Zeiss, Germany) equipped with an oil-immersed 100× objective (1.45 numerical aperture, Carl Zeiss) and 488 nm laser excitation. Digital images were collected at 2 Hz by an electron multiplying charge coupled device (EMCCD) camera, both of which were linked and controlled by MetaMorph.
6.3 (Universal Imaging, Downingtown, PA) with pixel size of 0.248 µm. Motions of individual vesicles were tracked using Image J (National Institute of Health, Bethesda, MD) and analyzed by Igor (WaveMetrics, Lake Oswego, OR) routines. Statistics were showed in mean ± standard error (SE), and p values were calculated by Student’s t-test.

For actin examination using TIRFM, cells were firstly fixed in 3.7% (v/v, diluted in PBS) formaldehyde (Sigma), then permeablized by 0.1% (v/v) Triton X-100 (Sigma) in PBS, and blocked with 1.5% (w/v, in PBS) bovine serum albumin (BSA, Sigma). Each step was followed by extensive wash with PBS. Actin filament was stained by Alexa Fluor 488 phalloidin (1:500, Invitrogen) for 40 min.

2.7 Confocal imaging

PC12 cells were fixed in 3.7% formaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. Non-specific binding sites were blocked with 1.5% BSA for 1 hour. Focal adhesion complexes were stained with FITC conjugated mouse monoclonal anti-vinculin antibodies (40 µg/ml, Sigma) in PBS for 40 min and subsequently imaged by a LSM510 Meta confocal laser scanning microscope equipped with 63× oil-immersion objective (1.4 numerical aperture, Carl Zeiss).

2.8 Amperometry recording

Exocytosis of dopamine-loaded PC12 cells was induced by local delivery of stimulation solution (high K⁺ or cholesterol) through a fine glass micropipette
(tip size of 2~3 µm) positioning ~10 µm away. Amperometric current was detected by a 5 µm carbon fiber electrode (ALA Scientific Instruments, Westbury, NY) that was gently placed on the cell membrane. The tip of electrode was cut to expose a fresh surface before each recording, and the voltage of carbon fiber electrode was held constantly at 700 mV to ensure consistent and high sensitivity. Amperometric signals were sampled at 4 kHz and filtered at 1 kHz using an EPC-10 double patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany). Recording spikes were analyzed with an Igor program, Amperometric Spike Analysis 8.15, developed by Dr. Eugene Mosharov at Columbia University. Only spikes with amplitude over 2 pA (10 times of background noise) were considered as true signals and used for analysis. For foot analysis, spikes with foot amplitude which was greater than 0.4 pA (two-fold of the noise) were eligible. Statistics were presented in mean ± SE, and $p$ values were calculated by Kolmogorov-Smirnov test (K-S test).

### 2.9 Capacitance measurement

Membrane capacitance measurement was carried out on PC12 cells under whole-cell voltage clamp condition, using glass capillaries with resistance of 3~7 MΩ. The series resistance ranged from 8 to 16 MΩ. An EPC-10 double patch-clamp amplifier was used together with the Pulse software package (HEKA Electronik). Capacitance measurements were performed on the cells while applying stimulation (oxysterols) externally, using the Lindau-Neher “sine + dc” technique [137] implemented in Pulse. This allowed long duration capacitance measurements in a single sweep. A 1000 Hz, 50 mV peak-to-peak sinusoid voltage stimulus was superimposed onto a DC holding potential of -70
mV.

Only cells that were stably voltage-clamped were analyzed. These showed fairly stable series resistance throughout the recording (10~12 MΩ) with no sudden changes of more than 10%. Capacitance values were recorded from each cell during the first 5 seconds after addition of the stimuli (oxysterols), and divided by the value immediately before addition of oxysterol to yield a normalized value. This was to eliminate the slight differences of initial capacitances between cells of different sizes. Seven to twelve cells were recorded in each group. Spearman’s test was used to examine the distinction between two groups, difference was considered statistically significant when $p < 0.05$.

2.10 Photometry measurement

A photometry system (TILL Photonics, GmbH) was used to measure the intracellular Ca$^{2+}$ concentration, plasma membrane content GM1 and cholesterol. The fluorescent probes were excited at desired wavelength using a monochromator and the emission fluorescence was collected by a photodiode.

2.10.1 Intracellular Ca$^{2+}$ imaging

For Ca$^{2+}$ imaging, PC12 cells were first incubated with 5 µM membrane permeable Ca$^{2+}$ dye, Fura-2 acetoxymethyl ester (Molecular Probes), in serum-free medium for 45 min. The cells were incubated in fresh medium > 30 min to allow hydrolysis of the ester and retaining of the dye before imaging in the bath solution. The fluorescence intensity ratio at 340 nm and 380 nm excitation
(F340/F380) was measured to indicate the intracellular Ca\(^{2+}\) concentration and its change upon stimulation by high K\(^+\) solution.

2.10.2 Membrane GM1, cholesterol quantification

To assess membrane GM1, PC12 cells were fixed and stained with Alexa Fluor 488 conjugated CTB (CTB-488, Invitrogen) (1 µg/ml in PBS) followed by extensive wash with PBS. The fluorescent CTB was excited at 488 nm. Measuring membrane cholesterol, PC12 cells were fixed and stained with filipin (0.05 mg/ml in PBS) and washed with PBS. Filipin was excited at 340 nm. The emitted fluorescence intensity was subtracted by background intensity to indicate the amount of GM1 and cholesterol on cell membrane.
CHAPTER 3 ROLES OF CHOLESTEROL IN VESICLE FUSION AND MOTION

3.1 Introduction

Intensive research in the past four decades has revealed that the regulated secretion, or exocytosis, of neurotransmitters and hormones is achieved through the temporally and spatially coordinated actions of numerous secretory proteins [138]. Most notably, complexing between SNARE proteins, involving VAMP on the vesicle and plasma membrane-associated syntaxin and SNAP-25, is believed to be essential for enabling membrane fusion [139]. Although this classic protein-centric view is well established, there is an increasing appreciation of the importance of the chemophysical properties and organization of membrane lipids to various aspects of the exocytotic program [80, 140-142]. In particular, growing evidence suggests that cholesterol, an abundant and essential constituent of both plasma and vesicular membranes, is a critical regulator of exocytosis through collaboration with protein factors [27, 50, 131, 135, 143-146].

It has been demonstrated in several cell types that cholesterol acts as a prefusion organizer by stabilizing lipid membrane microdomains or rafts, where secretory proteins, particularly SNAREs, assemble to define vesicle docking and fusion sites [35-36, 135, 147]. Cholesterol may promote membrane anchoring of the secretory proteins to fusion sites via cholesterol-recognition elements on proteins [148]. Cholesterol deprivation disrupts cholesterol-enriched functional microdomains and inhibits exocytosis in these cells.
Cholesterol is also an important factor in determining membrane properties such as fluidity, rigidity, and curvature [146, 149-150]. Modulation of cholesterol content in the plasma membrane changes the energy barrier for coalescence between two lipid bilayers and consequently alters the vesicle fusion kinetics [151]. Furthermore, cholesterol may exert its influence on exocytosis indirectly by modulating the actin cytoskeleton [152-153].

Based on amperometric recordings by carbon fiber microelectrodes, we show that deprivation of membrane cholesterol by methyl-β-cyclodextrin (MβCD) not only inhibited the extent of membrane depolarization-induced exocytosis, it also adversely affected the kinetics and quantal size of vesicle fusion in neuroendocrine PC12 cells. In addition, total internal fluorescence microscopy (TIRFM) studies revealed that cholesterol depletion impaired vesicle docking and trafficking, which are believed to correlate with the dynamics of exocytosis [154]. Furthermore, we found that free cholesterol is able to directly trigger vesicle fusion, albeit with less potency and slower kinetics as compared to membrane depolarization stimulation. These results underscore the versatile roles played by cholesterol in facilitating exocytosis.

3.2 MβCD treatment removes cholesterol without affecting cell morphology or cell viability

MβCD is a commonly used pharmacological agent to deplete membrane cholesterol through different concentration and various incubation time [45-46, 155-159]. MβCD is a water-soluble molecule with cavity to encapsulate cholesterol inside its hydrophobic pocket, therefore it is recognized as potential vehicle to remove cholesterol in the studies of cell biology. As shown in Figure
3.1 A and B, mild treatment with MβCD (5 mM, 30 min) did not cause changes in cell morphology (Figure 3.1 A) or affect cell viability in treated cell cultures (Figure 3.1 B, 82.10% ± 1.31% vs. 82.19 ± 1.21%, respectively), but this treatment was efficient enough to remove 32.3% ± 2.84% of membrane cholesterol (Figure 3.1 C, gray column), which is consistent with previous study [44, 144]. In other circumstance when it is preloaded with cholesterol to saturation, MβCD can be used as cholesterol donor to delivery cholesterol onto cell membrane [50]. After cholesterol was supplemented to depleted cells as described in Chapter 2, cholesterol level was elevated to 167.6% ± 25.4%. Cholesterol content was measured by fluorometric method using Amlex Red Cholesterol Assay Kit.
Figure 3.1 Treatment using 5 mM MβCD depletes cell membrane cholesterol effectively without affecting cell morphology or viability. (A) Bright field images of cell cultures before (left) and after 5 mM MβCD treatment. (B) Cell viability measurement by trypan blue assay of control (blank column) and 5 mM MβCD-treated (gray column) cells. (C) Cholesterol level of 5 mM MβCD-treated (gray column) and cholesterol-replenished (dark column) cells of the same passage. Data were averaged among 3 independent experiments.
3.3 The frequency of exocytotic events is reduced by cholesterol depletion

MβCD sequesters cholesterol in its hydrophobic pocket [156] and has been widely used to remove cholesterol from the plasma membrane. To examine the effects of cholesterol depletion on exocytosis, we compared high K⁺-triggered exocytosis from the same cell before and after MβCD treatment based on amperometric measurements. Amperometry, in contrast to electrophysiological membrane capacitance measurements [160-161], directly detects exocytosis with single-vesicle resolution without interference by endocytosis. It is especially useful to reveal, with millisecond resolution, the details of the vesicle fusion kinetics catalyzed by fusion proteins [162].

As shown in Figure 3.2 A (top), superfusion of the cell with high K⁺ solution for 2 min by means of an application pipette, which depolarizes the cell membrane, gave rise to many amperometric current spikes as detected by the carbon fiber microelectrode. Each current spike corresponds to the release of catecholamine molecules from a single secretory vesicle upon its fusion with the plasma membrane. In comparison, the same cell responded to high K⁺ stimulation with a much-reduced number of amperometric signals after incubation with 5 mM MβCD for 30 min (Figure 3.2 A, middle). The MβCD treatment removed 32.3% ± 2.8% (three cell cultures; Figure 3.1) of membrane cholesterol, which is comparable to previous findings [144]. The average time courses of the exocytotic responses (14 cells) to the first and second stimulations were normalized to the average total number of spikes resulting from the first high K⁺ stimulation before 5 mM MβCD treatment (Figure 3.2 A, bottom). Exocytosis elicited by high K⁺ stimulation after cholesterol deprivation was reduced to 39.9% ± 16.0% of the normal response. This is
consistent with the results of a previous study, in which a statistical comparison was performed between groups of control and MβCD-treated cells [36]. Cholesterol depletion also affected the kinetics of exocytosis. The accumulative number of amperometric spikes increased exponentially with a significantly slower time constant (> 140 s) in MβCD-treated cells compared to controls (~40 s; Figure 3.2 A, bottom).

Since the second stimulation was applied 30 min after the first, however, it can be argued that the observed decrease in the frequency of exocytotic events may be simply due to deterioration of cells. This possibility was ruled out by control experiments (11 cells), which showed that the paired high K⁺ stimulations, 30 min apart and without MβCD application, elicited similar exocytotic responses (Figure 3.2 B). When MβCD is preloaded with cholesterol to saturation, it can be used as a cholesterol donor to replenish cholesterol in the cell membrane [50]. Using this strategy, cholesterol molecules carried by a 5 mM MβCD-cholesterol complex were reloaded back into the cells after initial MβCD depletion. After reloading was completed, the level of membrane cholesterol was elevated to 167.6% ± 25.4% of the normal level without MβCD depletion (three cell cultures; Figure 3.1), similar to previously reported values [44]. Despite the full recovery of the membrane cholesterol level, however, the rescue of MβCD-induced inhibition by cholesterol reloading was insignificant (dashed curve in Figure 3.2 A). This raises a concern about the specificity of the MβCD treatment.
Figure 3.2 Cholesterol depletion inhibits exocytosis. The amperometric responses from two PC12 cells to high K\(^+\) stimulation are shown in the top row in A and B. The amperometric responses to the second high K\(^+\) stimulation from the same cells, with (A) or without (B) treatment with 5 mM M\(\beta\)CD for 30 min, are displayed in the middle row. The average time courses of the accumulative number of elicited amperometric spikes to the first (gray) and second (dark) stimulations are shown in pairs for the two protocols and are normalized to the average total spike number resulting from the first stimulation (bottom row). These traces are averaged from (A) 14 cells in which the average spike numbers in response to the first and second stimulations after 30 min incubation of 5 mM M\(\beta\)CD are 49.9 ± 7.5 and 19.9 ± 4.4, respectively (\(p\) < 0.001); (B) 11 cells in which the average spike numbers in response to the first and second stimulations after 30 min and without application of M\(\beta\)CD are 45.3 ± 3.8 and 45.5 ± 11.8, respectively. The dashed curve in A is the response (26.0 ± 5.1 events) from the cells (n = 17) whose membrane cholesterol was first depleted by 5 mM M\(\beta\)CD and then reloaded by the application of 5 mM M\(\beta\)CD-cholesterol complex; it is normalized to the average response to high K\(^+\) stimulation without any treatments. Replenishment of cholesterol provided only insignificant exocytotic recovery (\(p\) > 0.05, dashed versus dark curve). The dotted curve in A is the average response from 15 cells cultured in LPDS medium (28.1 ± 7.9 events) normalized by the average response from 15 cells cultured in FBS medium (56.1 ± 6.5 events; \(p\) < 0.001 between the two cell cultures). All the statistic analyses were based on at least 3 independent experiments.
As an alternative strategy, cellular cholesterol was metabolically deprived [163]. Specifically, cells were cultured in a medium supplemented with lipoprotein deficient serum (LPDS) in place of fetal bovine serum (FBS) 4–6 days before the experiments to block cholesterol synthesis and deplete cholesterol stores [164]. In the LPDS-cultured cells, exocytotic responses triggered by high K\(^+\) were significantly reduced to ~50% of those in the FBS-cultured cells (Figure 3.2 A, bottom), similar to the consequence caused by MβCD. This experiment corroborates the notion that cholesterol is crucial in supporting exocytosis.

### 3.4 The rate and quantal size of vesicular release, and prespike foot are reduced by cholesterol depletion

It is also evident from Figure 3.2 that the amplitudes of the amperometric events became generally smaller after depletion of membrane cholesterol. This suggests that cholesterol is involved in the process of quantal vesicle fusion. To investigate this phenomenon, individual amperometric signals in the same experiments presented in Figure 3.2 were extracted and analyzed. The normalized averaged amperometric spikes from the first and second stimulations are displayed in pairs for the two different experimental protocols in Figure 3.3 A. The average amplitude of the amperometric spikes after cholesterol depletion was decreased to 62.6% ± 4.6%. In line with this, the rate of release, or the rate of fusion pore expansion, which is quantified by the linear slope as the amperometric spike rises from 35% to 90% of the peak amplitude, is reduced to 56.0% ± 6.5% (Figure 3.3 C). The quantal size of vesicle release (Q), which is the total charge (integration) of the amperometric current spike,
was also reduced to 61.9% ± 3.9% by cholesterol depletion (Figure 3.3 D). Again, the control experiments confirmed that these observations were not due to deterioration of cells. The average amplitude, rise slope, and quantal size resulting from the second high K\(^+\) stimulation in the control cells were even slightly enhanced, possibly because of activity-dependent potentiation. Replenishment of cholesterol in the cholesterol-depleted cells completely rescued the reductions in amperometric amplitude, fusion rate, and quantal size (Figure 3.3, B–D), suggesting that our observations were due to specific effects of MβCD on cholesterol depletion. In addition, when cholesterol was depleted by LPDS culturing, the rise slope and quantal size also decreased to 29.4% ± 2.1% and 71.7% ± 3.1%, respectively (Figure 3.3, B–D).
Figure 3.3 Cholesterol depletion impairs vesicle fusion. Amperometric spikes obtained in the experiments presented in Figure 3.2 were individually extracted and analyzed. (A) The average spikes of the amperometric responses to the first (gray) and second (dark) stimulations are shown in pairs for the two protocols. The amplitudes for the two paired spikes are normalized based on the average amperometric amplitude responding to the first stimulation, which is 17.31 ± 0.69 pA (left, 5 mM MβCD protocol) or 21.69 ± 0.96 pA (right, control). (B) The average spike from the cells replenished with cholesterol after initial depletion is shown as the dashed trace on the left. Its amplitude (20.03 ± 1.14 pA, 17 cells) is normalized to the amplitude of the gray trace shown in A (left). Average spikes from cells cultured in FBS medium (gray, 16.40 ± 0.66 pA) or LPDS medium (dark, 5.40 ± 0.21 pA) are shown in pairs on the right. (C) The statistics of the rise slope of vesicle fusion defined by the linear slope between 35% and 90% of the peak amperometric amplitude. In the 5 mM MβCD protocol (the first paired columns on the left, normalized to the response from the first stimulation), the rise slopes are 10.10 ± 0.62 pA/ms (blank) and 5.66 ± 0.66 pA/ms (gray), from the first and second stimulations, respectively. In the control experiments (the second paired columns, normalized to the response from the first stimulation), the rise slopes are 14.01 ± 0.89 pA/ms (blank) and 16.25 ± 1.00 pA/ms (gray), from the first and second stimulations, respectively. The rise slope after reloading of cholesterol (dark, normalized to the first response in the MβCD protocol) is 9.63 ± 0.80 pA/ms. Rise slopes from FBS-medium cultured cells (dotted gray, 10.54 ± 0.61 pA/ms) and LPDS-medium cultured cells (dotted dark, 3.10 ± 0.22 pA/ms) are normalized and displayed in
pairs on the right. (D) The statistics of the quantal size defined by the total charge integration of the amperometric current spike. The data are normalized as described in C. In the 5 mM MβCD protocol (the first paired columns), the quantal sizes are 64.22 ± 2.01 fC (blank) and 39.76 ± 2.51 fC (gray), from the first and second stimulations, respectively. In the control experiments (the second paired columns), the quantal sizes are 60.34 ± 2.28 fC (blank) and 74.88 ± 2.19 fC (gray), from the first and second stimulations, respectively. The quantal size after reloading of cholesterol (dark) is 74.75 ± 2.78 fC. Quantal sizes from FBS-medium cultured cells (dotted gray, 74.14 ± 2.20 fC) and LPDS-medium cultured cells (dotted dark, 53.13 ± 2.28 fC) are normalized and displayed in pairs on the right. ** p < 0.01, *** p < 0.001.

A substantial energy barrier needs to be overcome to enable fusion between the vesicular and plasma membranes. When the driving force provided by fusion proteins barely overcomes such an energy barrier, a small foot preceding the amperometric spike occurs as a result of slow expansion of the fusion pore [165]. As shown in Figure 3.4, cholesterol depletion by MβCD or metabolic depletion reduced the percentage of footed events and the foot size.
Figure 3.4 Cholesterol depletion reduces the pre-spike foot signal. Analyses are based on the same data set presented in Figure 3.2 and Figure 3.3. (A) An example amperometric signal with prespike foot. The duration of the foot segment \( t_{\text{foot}} \) starts from the time point when the current signal exceeds the baseline noise and ends at the time point where the linear extrapolation of the main-spike rising phase intercepts with the baseline (dark dashed line). \( Q_{\text{foot}} \) is the charge integral of the foot segment. \( I_{\text{foot}} \) is the approaching plateau value determined by exponential fitting of the foot signal. (B), (C) and (D) Normalized percentage of footed-event, foot amplitude, and foot charge. In 5 mM MβCD protocol, the foot percentage decreased from 30.2% to 15.8%; \( I_{\text{foot}} \) decreased from 1.56 ± 0.11 pA to 1.32 ± 0.18 pA; \( Q_{\text{foot}} \) decreased from 6.45 ± 0.62 fC to 4.65 ± 0.74 fC (the first paired columns; normalized to the first exocytotic response; 211 footed-events in the first response and 44 footed-events in the second response from 14 cells). In the control protocol, the foot percentage increased from 24.1% to 36.1%; \( I_{\text{foot}} \) increased from 1.43 ± 0.12 pA to 1.65 ± 0.11 pA; \( Q_{\text{foot}} \) increased from 6.73 ± 0.88 fC to 7.36 ± 0.93 fC (the second paired columns; normalized to the first exocytotic response; 120 footed-events in the first response and 158 footed-events in the second response from 11 cells). When cholesterol was supplemented to the depleted cells foot percentage, \( I_{\text{foot}} \) and \( Q_{\text{foot}} \) were recovered to normal (146 events from 17 cells: 33.0%, 1.83 ± 0.12 pA, and 10.56 ± 1.38 fC). In comparison between cells cultured in FBS (232 events from 15 cells) and LPDS (38 events from 15 cells), the reduction in the percentage and the size of foot signal by metabolic
depletion of cholesterol was more prominent: (27.6% to 9.0%, 1.42 ± 0.089 pA to 0.70 ± 0.056 pA, 6.66 ± 0.74 fC to 4.14 ± 0.73 fC). ** p < 0.01, *** p < 0.001.

3.5 Vesicle fusion time is increased after cholesterol depletion

In a previous study of rat chromaffin cells [166], it was suggested that small and large vesicles may differ in terms of the molecular machinery (i.e., the number and/or composition of proteins and/or lipids) required to drive fusion pore expansion. For small vesicles, the half-width time of the amperometric spike ($t_{1/2}$), which indicates the duration of vesicle fusion, linearly scales with the cubic root of the quantal size ($Q^{1/3}$), which reflects the vesicle size. For large vesicles, $t_{1/2}$ levels off at a plateau, meaning that large vesicles dilate relatively more rapidly. It has been speculated that additional fusion machines are accommodated in the fusion complex of large vesicles and account for their expedited fusion [166]. As shown in Figure 3.5 (open circles), a similar trend was also seen in PC12 cells. Specifically, for vesicles $> 3.5$ fC$^{1/3}$, $t_{1/2}$ no longer increases linearly with $Q^{1/3}$, but instead remains at ~3.2 ms. Of interest, the capping of $t_{1/2}$ was relieved by MβCD, i.e., a linear $t_{1/2}$ and $Q^{1/3}$ relation resulted (Figure 3.5, gray circles). These data suggest that cholesterol is critical for ensuring the quick release of large vesicles. Moreover, for a given vesicle size ($Q^{1/3}$), the cholesterol-depleted cells took a longer time to discharge their contents, and this effect was more prominent for large vesicles. The MβCD-induced slowdown in vesicle fusion is caused by depletion of membrane cholesterol rather than nonspecific actions of MβCD, because $t_{1/2}$ and its saturation at large $Q^{1/3}$ were recovered after the cholesterol-depleted cells were reloaded with cholesterol (Figure 3.5, solid dark circles). Metabolic depletion of
cholesterol by LPDS medium similarly impaired the fast fusion of large vesicles, and increased the fusion time to an even greater extent as compared to MβCD.

**Figure 3.5** Relation between the half-width time of the amperometric spike ($t_{1/2}$) and the cubic root of quantal size ($Q^{1/3}$) is shown. These analyses are based on the amperometric signals obtained from the same experiments presented in Figure 3.2 and Figure 3.3. Each open circle represents the average of 80 vesicle fusion events in response to high K$^+$ stimulation without MβCD treatment. These data points can be well fitted by an exponential (dashed curve). Each solid gray circle represents the average of 20-vesicle fusion in response to high K$^+$ stimulation after a 30 min treatment with 5 mM MβCD. To indicate the trend, the gray circles are fitted by a line (gray line). Each dark circle represents the average of 40-vesicle fusion from the cells whose membrane cholesterol was reloaded after depletion. The data is fitted by an exponential (dark curve).

### 3.6 Exogenous cholesterol as secretagogue

Cholesterol not only makes exocytosis and vesicle fusion more efficient, it can also directly stimulate exocytosis after insertion into the exoplasmic leaflet of the plasma membrane. As demonstrated in Figure 3.6 A, amperometric
signals appeared after local superfusion with a cholesterol-containing bath solution (2 µM diluted from 2 mM cholesterol-ethanol stock) to cells via an application pipette. The vehicle control (0.1% v/v ethanol in bath) was not able to elicit any response (Figure 3.7). The average increases in the accumulative spike number over time, in response to 2 min of cholesterol stimulation (dark curve; 22 cells) and high K$^+$ stimulation in parallel experiments (gray curve; 10 cells), are depicted in Figure 3.6 B. Notably, cholesterol stimulation is much less potent than high K$^+$ stimulation.

The average amperometric signals from 272 cholesterol-induced events and 474 high K$^+$-induced events are plotted in Figure 3.6 C. Compared to that induced by high K$^+$, cholesterol-induced vesicle fusion manifested much slower kinetics. Specifically, the rise slope of cholesterol-induced fusion (3.47 ± 0.59 pA/ms) was much smaller than that of high K$^+$-induced fusion (10.90 ± 0.77 pA/ms), indicating a slower rate of fusion pore expansion (Figure 3.6 D). The quantal size of cholesterol-induced fusion, however, is larger than that of the high K$^+$-induced fusion events (81.82 ± 6.76 vs. 68.91 ± 2.67 fC; Figure 3.6 D). In the case of cholesterol stimulation, the fusion pore opening ($t_{1/2}$) is linearly proportional to the vesicle size ($Q^{1/3}$; Figure 3.6 E), and for the same vesicle size, cholesterol-induced vesicle fusion took a much longer time ($t_{1/2}$) to release as compared to high K$^+$-stimulated fusion. Insertion of exogenous cholesterol by micropipette delivery of cholesterol-loaded MβCD also induced similar amperometric responses with slow kinetics (Figure 3.8).
Figure 3.6 Free cholesterol triggers exocytosis. (A) Local application of 2 µM cholesterol to the cell for 2 min elicited amperometric responses (left). A high K⁺ triggered amperometric recording in a parallel experiment on a different cell is shown (right) for comparison. (B) The average increase of accumulative spike number over time, in response to cholesterol stimulation (dark curve; 22 cells) or high K⁺ stimulation (gray curve; 10 cells). (C) The average amperometric spikes from cholesterol stimulation (dark; 272 events) and high K⁺ (gray; 474 events). (D) The rise slope of cholesterol-induced vesicle fusion (dark column on the left; 3.46 ± 0.59 pA/ms) is smaller than that of high K⁺-induced fusion (gray column on the left; 10.90 ± 0.77 pA/ms), whereas the quantal size of cholesterol-induced fusion (dark column on the right; 81.82 ± 6.76 fC) is larger than that of high K⁺-induced fusion (gray column on the right; 68.91 ± 2.67 fC). (E) For cholesterol-induced vesicle fusion, the half-width time of the amperometric spike (t₁/₂) is linearly proportional to the cubic root of the quantal size (Q₁/₃; gray circles fitted by a line; each circle represents the average from 40 vesicles). For a given Q₁/₃ (vesicle size), cholesterol-triggered fusion takes longer to release compared to high K⁺-induced fusion (open circles fitted by an exponential; same data set as shown in Figure 3.5). * p < 0.05, *** p < 0.001.
Figure 3.7 Carbon fiber recordings while superfusing bath solution (top) or bath solution containing ethanol as vehicle control (bottom) onto PC12 cells.

Figure 3.8 Amperometric response induced by cholesterol loaded MβCD. (A) A typical amperometric recording in response to MβCD-cholesterol stimulation. (B) The average amperometric spike averaged from the all the responses from 186 spikes from 17 MβCD-cholesterol stimulated cells (left). The evoked response is much less compared to high K⁺ stimulation (10.9 ± 2.7 spikes/cell, p < 0.001 vs. high K⁺ stimulation). The rise slope and quantal size of MβCD-cholesterol stimulated amperometric spikes are 5.81 ± 0.65 pA/ms and 96.87 ± 5.18 fC, respectively (right). As compared to the high K⁺ stimulation, MβCD-cholesterol stimulation, similar to free cholesterol stimulation, resulted in slower rise slope (p < 0.001) and larger quantal size (p < 0.001).
The ability of cholesterol to elicit vesicle fusion was confirmed by total internal reflection fluorescence microscopy (TIRFM). With selective evanescent illumination of the thin section (~200 nm) just above the interface between the glass coverslip and the adhered cell, TIRFM can provide insights into dynamic events occurring at or close to the plasma membrane of living cells, with outstanding optical contrast and resolution. Subplasmalemmal secretory vesicles in PC12 cells, specifically labeled by neuropeptide Y conjugated with enhanced green fluorescent protein (NPY-EGFP), were individually visualized and tracked at 0.5 s intervals with the use of TIRFM.

Figure 3.9 A presents a pair of TIRFM images of a PC12 cell immediately before (top) and 100 s after (bottom) the application of 2 µM cholesterol to the bath solution. Vesicles in the subplasmalemmal region can be clearly resolved. In this experiment, the total number of the original subplasmalemmal vesicles was reduced from 19 to 10 in < 2 min. In the resting state, the total number of vesicles in the subplasmalemmal region stayed constant (open circles in Figure 3.9 B; averaged from 5 cells). In contrast, after the application of cholesterol, the total vesicle number decreased over time (solid circles in Figure 3.9 B; average from 6 cells). The delivery rate of newly arrived vesicles (solid triangles in Figure 3.9 B) was enhanced in the presence of free cholesterol, presumably to compensate for vesicle release.
Figure 3.9 Cholesterol-stimulated vesicle release revealed by TIRFM. (A) NPY-EGFP-labeled subplasmalemmal vesicles are visualized by TIRFM before (top) and 100 s after (bottom) application of 2 µM cholesterol to the bath solution. The vesicles are highlighted by circles or squares. The squares indicate vesicles newly arrived from the cytosol. (B) The total number of visible subplasmalemmal vesicles decreased over time in the presence of cholesterol (solid circles; average from six cells), whereas it remained stable without cholesterol (open circles; average from five cells). The accumulative number of newly arrived vesicles was enhanced in the presence of cholesterol (solid triangles) compared to that without cholesterol (open triangles).
3.7 Cholesterol depletion impairs vesicle trafficking and docking

Secretory vesicles undertake constant lateral movements in the subplasmalemmal membrane region, where they explore, interact with various proteins, and eventually are released at exocytotic sites upon triggering. How vesicles move in the near membrane region directly relates to the dynamics of exocytosis [154]. The lateral motion of individual vesicles was tracked, at 0.5 s intervals for 2 min, from their appearance at the beginning of imaging (for “predocked” vesicles) or from their arrival to the subplasmalemmal region (for newly arrived vesicles) until their retrieval back to the inner cytosol. Figure 3.10 A presents the trajectories of vesicle lateral motion in a typical control (left) and an MβCD-treated PC12 cell (right). An example trajectory from the control cell is illustrated with an expanded scale (middle). From this representative trace, and as demonstrated in our previous study [167] and by others [106, 168], it is clear that random vesicle lateral movement is not free Brownian motion; rather, it is confined as if the vesicle were “caged” by certain physical barriers or tethering interactions. Of interest, vesicle lateral motion was more severely restricted in the cholesterol-depleted cells as compared to the control cells.
Figure 3.10 Vesicle trafficking and docking is cholesterol-dependent. (A) The motion trajectories of the subplasmalemmal vesicles in a control cell (left) and a cell depleted of cholesterol by 5 mM MβCD (right). The cell contours are outlined by dashed lines. An example trajectory from the control cell is illustrated with an expanded scale (the horizontal and vertical scale bars indicate 200 nm). The smallest rectangle that just encases all the vesicle footprints (gray and dashed rectangle) gives the first-order estimation of the coverage area of vesicle motion. (B) and (C) The statistics of the vesicle motion area and the number of subplasmalemmal vesicles in control and differently treated cells. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
The area of the smallest rectangle that just encases all the vesicle footprints gives a first-order estimation of vesicle motion coverage, as indicated in Figure 3.10 A (middle). In the cholesterol-depleted cells, the average area of motion coverage was $0.18 \pm 0.01 \mu m^2$ (466 vesicles in 16 cells), i.e., only ~31% of that in control cells ($0.58 \pm 0.03 \mu m^2$, averaged from 509 vesicles in 10 cells; Figure 3.10 B). The analyses included all predocked and newly arrived vesicles. Vesicles also transit vertically between the inner cytosol and the subplasmalemmal region, such that the vesicles near the membrane are recycled and replenished. However, the total vesicle number in the subplasmalemmal region remains stable (Figure 3.9 B, open circles) due to the balance between vesicle arrival and retrieval. Also as revealed by TIRFM, the number of subplasmalemmal vesicles was significantly smaller in the MβCD-treated cells than that in the control cells ($23.6 \pm 2.7$ vs. $11.9 \pm 2.6$; Figure 3.10 C) whereas the dwell time of newly-delivered vesicles was greater in treated cells ($14.82 \pm 1.27$ s vs. $7.58 \pm 0.67$ s), indicating that the removal of cholesterol impaired vesicle tethering and docking. Adding cholesterol back into the cholesterol-
depleted cells restored the number of subplasmalemmal vesicles (22.8 ± 3.0, 16 cholesterol-reloaded cells; Figure 3.10 C), implying the specificity of the cholesterol effect. On the other hand, replenishment of membrane cholesterol was unable to rectify the MβCD effect on vesicle motion (Figure 3.10 B). The motion coverage of the cholesterol-replenished cells was 0.17 ± 0.01 µm² (523 vesicles in 16 cells), similar to that in the MβCD treated cells. The reason for this is unknown, but it could be due to damage to the cholesterol-protein assemblies, after cholesterol depletion, that cannot be reversed by replacement of cholesterol.

As an alternative to MβCD treatment, the availability of membrane cholesterol was reduced by sequestration of cholesterol with filipin [169], which binds specifically to membrane cholesterol. The filipin treatment also led to a significant reduction in the motion area of the vesicles (0.12 ± 0.01 µm², 328 vesicles in 17 cells) and the number of subplasmalemmal vesicles (15.4 ± 1.8). Furthermore, when cholesterol was depleted metabolically (by LPDS medium), the cells also manifested severely restricted vesicle motion (0.12 ± 0.01 µm², 286 vesicles in 24 cells) and a reduced number of subplasmalemmal vesicles (9.5 ± 1.3), similar to the MβCD-treated and filipin-treated cells. The reduced vesicle motion was not correlated to the change in the vesicle dwell time. The average vesicle dwell times in the subplasmalemmal region for the untreated cells, the MβCD-treated cells, the cholesterol-reloaded cells, the filipin-treated cells, and the cells after metabolic deprivation of cholesterol were 36.8 ± 2.0 s, 25.5 ± 1.8 s, 44.3 ± 1.9 s, 62.6 ± 2.4 s, and 59.9 ± 2.6 s, respectively. Therefore, the reduced motion coverage is due to a reinforced confinement of vesicle movement imposed by certain cholesterol-dependent
mechanisms.

The similar observations from three different protocols to decrease the availability of membrane cholesterol corroborate the notion that membrane cholesterol is critical for vesicle docking and trafficking. This could also provide an explanation for the observed effects of cholesterol depletion on the frequency and fusion kinetics of the exocytotic events.

3.8 Conclusion and discussion

As a key constituent of the cell membranes and a precursor to steroid hormones, cholesterol is implicated in numerous structural and functional capacities. Therefore, cholesterol levels are highly regulated at both body and cellular levels. Abnormalities in its homeostasis can lead to various diseases, such as developmental abnormalities, neurodegenerative disease, or hormone disorders [42, 131]. It is becoming increasingly clear that cholesterol is critically involved in membrane fusion and exocytosis [36, 144, 151]. In this study, we carefully examined and demonstrated the importance of cholesterol for various aspects of vesicular exocytosis in PC12 cells by (1) comparing exocytotic responses from the same cell before and after MβCD-induced cholesterol depletion to avoid the typically large cell-to-cell variation; (2) addressing concerns about the specificity of MβCD by replenishing cholesterol in the cell membrane and using alternative strategies for cholesterol deprivation; (3) analyzing in detail the effects of cholesterol on quantal vesicle fusion; (4) performing TIRFM imaging of vesicle docking and trafficking, which is directly related to the exocytotic competence and dynamics [154]; and (5) demonstrating the ability of exogenous cholesterol to induce vesicle fusion.
Several lines of evidence have suggested the existence of membrane microdomains enriched with cholesterol and sphingolipids [29, 86, 170-171]. These microdomains are usually called “lipid rafts” because they exist in a less fluid and more ordered state than glycerophospholipid-rich domains of the membrane. Small cholesterol molecules are thought to intercalate between hydrocarbon chains of sphingolipids, acting as a dynamic glue to stabilize the raft assembly. The ability of lipid rafts to concentrate specific proteins and exclude others makes them ideal functional platforms for spatially organizing cellular events with high efficiency on the plasma membrane. In view of the raft theory [86], it is plausible that a reduction of membrane cholesterol can disrupt raft structures and change the biological functions mediated by raft proteins. Cholesterol-rich microdomains have been proposed to be the sites of vesicle docking and fusion because proteins such as syntaxin and SNAP-25, which are critically implicated in exocytosis, have been demonstrated to associate with these domains [35, 37], and cholesterol depletion dissociates SNAREs from rafts, leading to severe inhibition of exocytosis [36, 135]. Our observations that deprivation of membrane cholesterol by different means significantly reduced the number of membrane-tethered or -docked vesicles (Figure 3.10 C) and the extent of Ca\(^{2+}\)-triggered exocytosis (Figure 3.2) lend further support to this hypothesis.

Depletion of membrane cholesterol led to markedly reduced lateral motion of the subplasmalemmal vesicles. This may partly account for the inhibition in exocytosis caused by cholesterol depletion, because the constant movement of the subplasmalemmal vesicles directly relates to the dynamics of exocytosis [154, 167]. Studies have demonstrated that depletion of membrane cholesterol
by MβCD causes the formation of solid-like membrane domains and consequently impedes the diffusion of membrane proteins and lipids [172-173]. The hindered protein diffusion may be related to the reduction in vesicle lateral motion, since subplasmalemmal vesicles are presumably tethered to the plasma membrane through a set of membrane-associated proteins (e.g., SNAREs) and move together with these protein complexes as a drifting raft. The ability of cholesterol to facilitate vesicle motion may help vesicles to explore sufficiently near the plasma membrane and become fully assembled to fusion competence. Since vesicle motion depends on the cortical actin network [174], cholesterol depletion could exert its effect by modulating actin polymerization [152]. However, fluorescence staining and imaging of the cortical actin network using TIRFM and confocal imaging did not show any obvious differences between MβCD-treated and untreated cells (Figure 3.11). Nonetheless, it is noteworthy that any possible alterations in the nanostructured actin meshwork with a size below the diffraction limit (~150 nm) cannot be resolved by optical imaging.

It has been hypothesized that, before fusion, the outer leaflet of the vesicle membrane and the inner leaflet of the plasma membrane merge first while the distal membrane leaflets remain separated [93]. This intermediate hemifusion structure is delicately stabilized by the proteins and particular lipid species until the final thrust, probably provided by twisting of the SNARE complex upon triggering, causes the formation of a small aqueous fusion pore (~1.5 nm). Soon after formation, the fusion pore dilates rapidly to release the vesicular contents. The reduction in exocytosis by cholesterol depletion may be partly attributable to an inhibition of hemifusion formation, and thus decrease in the number of releasable vesicles.
In a previous study on PC12 cells [36], it was reported that cholesterol deprivation by MβCD reduced the extent of exocytosis but did not affect the quantal release. Here, we carefully revisited that issue by comparing the exocytotic events before and after MβCD treatment in the same cell. We found that depletion of cholesterol caused slower fusion kinetics and a smaller quantal size (Figure 3.3). This is in accordance with the notion that negatively curved cholesterol [175] facilitates the formation and expansion of the fusion pore. The percentage and size of the foot signal were reduced by cholesterol depletion, indicating that the initial opening of the fusion pore was hindered. It is consistent with the view that depletion of cholesterol impairs the efficiency of the Ca\(^{2+}\)-dependent fusion process [144]. In addition, vesicles took a longer time to discharge their contents after cholesterol depletion (Figure 3.5). In particular, the molecular machine that has been postulated to facilitate the fusion of large vesicles was disabled by cholesterol depletion. The roles of cholesterol in fusion therefore appear to be threefold, as it facilitates the formation of hemifusion structure, fusion pore expansion, and the assembly of the “additional” fusion machines in large vesicles.

Because the large dense core vesicles in neuroendocrine cells, and synaptic vesicles in neurons are small (< 100 nm) and their vesicular fusion occurs rapidly (on the millisecond scale) with involvement of various protein and lipid factors, it is difficult to identify their hemifusion structure and determine how it transits to full fusion upon triggering. Other model systems have been utilized to study membrane fusion and the regulatory roles of cholesterol. Hemifused intermediate was identified in artificial liposomes by means of microspectrofluorometry [176]. Garcia et al. [177] found that cholesterol
stabilizes hemifused phospholipid bilayer vesicles. The same study, however, suggested that cholesterol inhibits transition from hemifusion to full fusion, contradicting our observation that removal of cholesterol slowed the fusion pore expansion. Homotypic fusion between cortical vesicles, which possess protein machinery that is critical for exocytosis and are much larger than large dense core vesicles and synaptic vesicles, has been used as a reduced model system for studying vesicular fusion [93, 178]. Churchward et al. [144, 179] demonstrated that cholesterol, by virtue of its intrinsic negative curvature, promotes the formation of a highly curved fusion intermediate and Ca\(^{2+}\)-triggered fusion pore formation and expansion. Fusion between cell membranes catalyzed by minimal fusion proteins (viral protein hemagglutinin) is instrumental in investigating the roles of various lipids, such as cholesterol [180]. A recent study [151] based on this technique corroborated the hypothesis that cholesterol acts during two stages in membrane fusion: before fusion pore opening and during fusion pore expansion.

It has been theoretically demonstrated that membrane tension created by adding cholesterol molecules to the exoplasmic leaflet of the plasma membrane may provide a direct driving force to cause full expansion of the hemifusion diaphragm [181]. It explains our observation that the addition of free cholesterol to the bath solution triggered vesicle fusion, albeit with less potency compared to high K\(^+\) stimulation (Figure 3.6 B). Although cholesterol-induced fusion is distinctly different from native Ca\(^{2+}\)-triggered vesicular fusion, the results suggest that the semistable hemifusion structure is in a delicate energetic balance, and the addition of negatively curved cholesterol favors the transition from hemifusion to “spontaneous” full fusion. Not unexpectedly, cholesterol-
induced quantal release, without the driven force provided by secretory proteins, manifested a much slower expansion of the fusion pore and longer fusion time for a given vesicle size (Figure 3.6, C–E). When subjected to the same uniform driving force (membrane stress introduced by the addition of cholesterol), $t_{1/2}$ scales linearly with $Q^{1/3}$ for both small and large vesicles, i.e., larger vesicles take a longer time to release their contents. The comparison between lipid-driven fusion and protein-driven fusion may allow us to decipher the energetic actions provided by the fusion proteins at different stages of fusion.

Functional studies of membrane cholesterol have relied mostly on pharmacological agents ($\beta$-cyclodextrins, particularly MβCD) as cholesterol acceptors to remove membrane cholesterol, or cholesterol donors to load cell membranes with cholesterol. Although this is regarded as the most popular and effective method to manipulate membrane cholesterol content, nonspecific effects of cyclodextrin have also been reported, and hence one should use caution when interpreting the data [44]. We used different methods to deplete cholesterol to confirm the specificity of cholesterol. In addition, we showed that cholesterol depletion did not affect cell viability, morphology, or adhesion (Figure 3.1). Furthermore, the replenishment of cholesterol was able to completely rescue the fusion kinetics (i.e., the amplitude, rise slope, quantal size, and fusion time) and vesicle docking. On the other hand, MβCD-induced inhibition of exocytosis and confinement of vesicle motion could not be reversed by replenishment of membrane cholesterol. However, it is unlikely that the observed phenomena resulted from nonspecific MβCD actions, because additional application of cholesterol-loaded MβCD did not cause further
suppression (Figure 3.2 and Figure 3.10). Instead, it is conceivable that disruptions (e.g., on cholesterol-protein assemblies or membrane microdomains) caused by cholesterol depletion that are relevant to vesicle competence and movement cannot be reversed by simply adding cholesterol back into the plasma membrane.

In conclusion, the data presented in this study establish that, in addition to its known effect in maintaining the extent of exocytosis, cholesterol plays multiple roles in facilitating exocytosis. The efficiency of exocytosis depends on the availability of membrane cholesterol, which is important for maintaining vesicle fusion/docking structures and facilitating vesicle trafficking. Cholesterol also ensures the efficiency of quantal vesicle fusion by promoting the formation of negatively curved fusion structures and by stabilizing fusion machines.

3.9 Acknowledgements

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CHAPTER 4 EFFECTS OF DIFFERENT OXYSTEROLS ON EXOCYTOSIS

4.1 Introduction

In Chapter 3, we have demonstrated that cholesterol is important to ensure the efficiency of exocytosis in PC12 cells by facilitating vesicles docking, trafficking and fusion. Depletion of cholesterol causes disruption of membrane microdomains. It is reported that low cholesterol content in cell plasma membrane (< 40 mol%) would result in failure of microdomain formation [182], which in turn impairs vesicles docking and fusion. On the other hand, excess of cholesterol also induces problems. High level of cholesterol in plasma membrane increases membrane rigidity that inhibits activities of membrane associated proteins, such as β-amyloid precursor protein involved in development of Alzheimer’s disease [183]. Excess cholesterol accumulation in blood leads to atherosclerosis which may cause heart diseases and stroke [184]. Therefore, it is obvious that cholesterol homeostasis is important at both cellular level and body level.

Cholesterol homeostasis is delicately regulated throughout its metabolic pathways which involve esterification and oxidation. Oxidation generates additional carbonyl, hydroxyl, or epoxide moieties at 7 position, 5, 6-double bond positions, 24, 25, or 27 position [60, 63]. Oxysterols have been implicated for multiple cytotoxicities and pathological effects. For example, cholesterol-5,6-epoxide, 7-hydroxycholesterol, and 7-ketocholesterol induce apoptosis through different pathways [185-186]; 7-ketocholesterol and 25-
hydroxycholesterol inhibit cell migration by altering cellular cytoskeleton organization [153]; 24-hydroxycholesterol is the primary form of brain cholesterol metabolin that can pass the blood-brain barrier, and accumulation of 24-hydroxycholesterol has been detected in patients suffering Alzheimer’s disease [66].

The addition of oxygen moieties modulates the physicochemical properties of oxysterols. They have been shown to be less potent than cholesterol for Lo phase formation [63]. The additional oxygen moiety is responsible for the greater hydrophilicity of oxysterol comparing to cholesterol. Positions of oxygen modification determine the orientation of oxysterols with respect to the membrane that may lead to the disturbance of lipids packing and increase of membrane permeability [187].

Thus far, little is understood about the roles of oxysterols in exocytosis. Herein, we examined their effects on exocytosis in this chapter. Employing approaches of TIRFM imaging and membrane capacitance measurement, we found that external oxysterols, specifically, cholesterol-5α,6α-epoxide (C-5α,6α-epoxide), cholesterol-5β,6β-epoxide (C-5β,6β-epoxide), 7β-hydroxycholesterol (7β-OHC), 7-ketocholesterol (7-KC) and 24-hydroxycholesterol (24-OHC) are able to trigger exocytosis. Among them, 7-KC is the most potent secretagogue. The exocytotic efficiency of 7-KC is attenuated by thapsigargin (Thaps) treatment that depletes intracellular Ca^{2+}. It is also blocked by MβCD treatment that disrupts lipid rafts. These data implied that 7-KC may trigger exocytosis by increasing Ca^{2+}, and exocytotic effect of 7-KC is dependent on the integrity of membrane microdomains.
4.2 Oxysterols trigger exocytosis

Since oxysterols can insert into plasma membrane and modify membrane physicochemical properties [188], we postulated that oxysterols may be able to trigger or influence exocytosis. Indeed, as revealed by TIRFM, the LDCVs in PC12 cells labeled by NYP-EGFP were depleted by oxysterol stimulation in 2 min. Representative footprint images of secretory vesicles at subplasmalemmal region in response to external 7-KC challenge at different time points are shown in Figure 4.1. Predocked vesicles on subplasmalemmal membrane (highlighted by circles) were released shortly after 7-KC stimulation (Figure 4.1 A middle and right). The total number of subplasmalemmal vesicles remained low, presumably because the newly arrived vesicles were also quickly released in the presence of the oxysterol.

Similar as 7-KC, 24-OHC, C-5α,6α-epoxide, C-5β,6β-epoxide, 7β-OHC were able to induce vesicles release as well, but with less prominent efficiency (Figure 4.1 B). The cumulative release of predocked vesicles was normalized to the initial predocked vesicle number. The plotting curves were fitted by exponential approximation with time constants of 35 s (7-KC), 42 s (24-OHC), 60 s (C-5β,6β-epoxide), 77 s (C-5α,6α-epoxide), 108 s (7β-OHC), respectively. It is obvious that 7-KC and 24-OHC had the most potent effects, whereas 7β-OHC is least competent. By investigating the number of new vesicles coming from cytosol, we found that delivery rate of new vesicles is significantly boosted in the presence of oxysterols (Figure 4.1 C), likely due to the effort to compensate for ongoing vesicle exocytosis.
Figure 4.1 Oxysterols stimulate exocytosis in PC12 cells. (A) Release of subplasmalemmal vesicles at 0 s, 50 s and 100 s after external 2 µM 7-KC stimulation. Scale bar = 5 µm. (B) and (C) The exocytosis kinetics of predocked and newly arrived vesicles in response to the challenge of different oxysterol. Data were acquired from 7~12 cells for each treatment.
Figure 4.2 Membrane capacitance measurement of oxysterols stimulated exocytosis. (A) Representative recording of membrane capacitance increase after 2 µM 7-KC application. The arrow indicates when 7-KC was applied. (B) Normalized membrane capacitance measurement 35 s after oxysterols addition. Data were acquired and averaged from 10 cells for each treatment. * $p < 0.05$. 
To confirm our observations with TIRFM, we also monitored exocytosis using membrane capacitance measurement based on whole-cell patch clamping. Significant increment of membrane capacitance was observed in the presence of exogenous oxysterols. A sample of the typical recording is presented in Figure 4.2 A. Membrane capacitance measurement confirmed our previous observation under TIRFM. 7-KC and 24-OHC were able to elicit significant exocytosis whereas 7β-OHC hardly triggered any (Figure 4.2 B).

4.3 7-KC triggered exocytosis is lipid raft- and Ca\(^{2+}\)-dependent

We found that 7-KC stimulation elicits the burst of [Ca\(^{2+}\)]\(_e\), elevation using Fura-2 imaging, and this effect is abolished by MβCD treatment and Thaps (Figure 4.3). At the same time, exocytosis induced by 7-KC is also inhibited by the same treatment (Figure 4.4), suggesting that elevation of [Ca\(^{2+}\)]\(_i\) is responsible for 7-KC triggered exocytosis, and the induced exocytosis is dependent on intracellular Ca\(^{2+}\) and the integrity of lipid rafts.
Figure 4.3 Fura-2 imaging of [Ca\(^{2+}\)]\(_i\) in response to 7-KC stimulation. Plots are averaged from 10–12 cells. Arrow indicates when 7-KC was applied.

Figure 4.4 Membrane capacitance of cells pretreated with MβCD or Thaps in response to 7-KC stimulation.
4.4 Conclusion and discussion

In this chapter, we studied the effects of oxysterols on exocytosis in neuroendocrine PC12 cells. It was found that 7-KC, 24-OHC and C-5β,6β-epoxide can trigger exocytosis in Ca\(^{2+}\)- and rafts-dependent ways and they also enhance the vertical trafficking of vesicles.

The different potency of these oxysterols may be attributed to the difference in their properties and their orientation in the cell membrane. 7-KC locates atilt in Ld phase [62], whereas 24-OHC aligns horizontally to membrane plane [187]. The insertion of oxysterols alters the membrane organization and properties, which in turn changes the functions of membrane-associated exocytotic proteins (e.g. SNAREs).

Our findings that oxysterols are competent to stimulate exocytosis may provide some insights to neuropathology. Increased level of 7-KC, 24-OHC and C-5β,6β-epoxide is found in rat hippocampal neurons after neuronal injury [68]. Accumulation of oxysterols in brains can lead to various diseases, such as Alzheimer’s disease [189] and Niemann-Pick type C disease [190]. Our results suggest the excitotoxicity introduced by oxysterols in neuropathological conditions.

4.5 Acknowledgements:

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CHAPTER 5 LYSOPHOSPHOLIPIDS TRIGGERED EXOCYTOSIS IN PC12 CELLS

5.1 Introduction

Phospholipase A2 (PLA2) is a super family of enzymes that can specifically hydrolyze membrane phospholipids at sn-2 site to produce arachidonic acid and lysophospholipids (LPLs). PLA2 may disrupt plasma membrane and induce the release of neurotransmitter amino acids from cerebral cortex [191]. One of its subfamily, secretory PLA2 (sPLA2), has been found in various locations in the central nervous system [192]. It is reported that sPLA2 stimulates neurotransmitter release from neuronal cells [193-194].

LPLs, the products of PLA2, are implicated in many biological functions. For example, lysophosphatidic acid (LPA) boosts Ca$^{2+}$ concentration in bovine chromaffin cells [195]; lysophosphatidylserine (LPS) induces histamine release in mast cells [196]; lysophosphatidylcholine (LPC) promotes insulin secretion from pancreatic β cells [197]. It is noteworthy in the study of PLA2 by O’Regan that LPC and arachidonic acid are able to regulate amino acids release from cerebral cortex as PLA2 [198]. A recent study showed that the mixture of LPC and fatty acid gives similar biological effect as snake PLA2 neurotoxins (SPANs) [199]. These findings suggest that LPLs may be participants of PLA2-mediated activities. In addition, LPLs are able to regulate the function of ion channels either by direct interaction with ion channel proteins [200], or by binding with their receptors [201]. Hence, it is not surprising that LPLs are critically involved in Ca$^{2+}$ homeostasis and signal transduction [202].
Little is known about the contributions of LPLs to exocytosis. In this Chapter, we aim to (1) characterize the exocytotic effect of LPLs, particularly LPC, LPS and LPI (lysophosphatidylinositol), at single cell level; and (2) find out the possible regulatory factors involved in LPLs-induced vesicle fusion. Using TIRFM, amperometric measurement and Fura-2 imaging, we showed that external application of LPLs stimulates exocytosis from PC12 cells, among which LPI is the most potent secretagogue to trigger exocytosis by depleting the predocked vesicles and increasing delivery of vesicles from cytosol for replenishment. The exocytotic effect of LPI was confirmed and characterized by amperometric measurement. Moreover, treatments of thapsigargin (Thaps), pertussis toxin (PTX) and U73122 were employed to elucidate the involvement of phospholipase C (PLC) and G proteins in LPI-evoked exocytosis.

5.2 Lysophospholipids evoke exocytosis in PC12 cells

External perfusion of lysophospholipids triggers exocytosis in PC12 cells as observed by TIRFM. Representative images of a single cell subjected to LPI stimulation are shown in Figure 5.1 A. It is obvious that LDCVs docking at subplasmalemma (highlighted by circles) were released upon LPI stimulation. Figure 5.1 B illustrates the process of a vesicle fusing with plasma membrane. LPI significantly depletes predocked vesicles by 58.3%, whereas LPC and LPS does not show pronounced exocytotic effect comparing to vehicle control (Figure 5.1 C). Moreover, LPI also accelerates the delivery of new vesicles to the plasma membrane. 53.9 ± 4.4 vesicles arrived from inner cytosol in response to LPI stimulation in 2 min, while 18.6 ± 5.2 and 13.0 ± 1.7 vesicles arrived in response to LPC and LPS stimulation (Figure 5.1 D).
**Figure 5.1** LPLs stimulate vesicle fusion revealed by TIRFM imaging. (A) NPY-EGFP-labeled LDCVs are depleted by LPI stimulation. Predocked LDCVs are highlighted by circles, and newly arrived LDCVs are highlighted by squares. (B) Successive frames show the fusion process of a single LDCV. (C) LPLs trigger exocytosis. (D) LPLs induce exocytosis by promoting vesicle delivery to the plasma membrane. * $p < 0.05$ in Student’s $t$-test.
5.3 Fusion kinetics of LPI-evoked exocytosis

It was revealed by amperometry studies that fusion kinetics of LPI triggered exocytosis is different from that elicited by membrane depolarization (by high K⁺ stimulation). First of all, LPI evokes exocytosis from PC12 cells, but with a much less extent as compared with high K⁺ triggered events (Figure 5.2 A). LPI stimulation elicits 17.6 ± 5.1 amperometric spikes (averaged from 8 cells), only 42% of high K⁺ evoked events, which is 41.8 ± 1.6 (averaged from 8 cells). Secondly, average current spike of LPI-induced fusion is distinct from high K⁺ induced fusion (Figure 5.2 B, dark vs. gray). The rise slope of LPI stimulated vesicle fusion is comparable with that of high K⁺ elicited fusion, i.e. 9.98 ± 1.13 pA/ms vs. 9.68 ± 0.83 pA/ms (Figure 5.2 C). These data suggested that LPI is able to initiate the opening of fusion pore as fast as high K⁺. The half-width time (t₁/₂) of LPI evoked amperometric spike, however, is characterized by greater duration (4.60 ± 0.22 ms vs. 3.32 ± 0.12 ms), indicating that LPI is able to stabilize the fusion pore and allows discharge of more vesicular content (Figure 5.2 D).
Figure 5.2 Comparisons of high K\textsuperscript{+}- and LPI-stimulated exocytosis. (A) Number of high K\textsuperscript{+}- and LPI-triggered fusion events in 2 min. (B) Average current spikes extracting from high K\textsuperscript{+}- and LPI-evoked exocytosis. (C) and (D) Statistics of rise slope and $t_{1/2}$ of current spikes. *** $p < 0.001$ in K-S test.
5.4 LPI-evoked exocytosis is Ca^{2+}-, G protein- and phospholipase C-dependent

The possible mechanism and regulatory factors underlying LPI-induced exocytosis were explored. As presented in Figure 5.3 A, LPI stimulation elicits an immediate and long lasting elevation of [Ca^{2+}]_{i}, indicating that exocytosis is triggered by [Ca^{2+}]_{i} elevation. The elevation of [Ca^{2+}]_{i} is completely abolished by treatment of Thaps, which depletes intracellular Ca^{2+} stores [203]. These data suggested that intracellular Ca^{2+} stores are responsible for LPI-elicited [Ca^{2+}]_{i} elevation.

Considering the fact that both inositol 1,4,5-trisphosphate (IP3) and G proteins are account for Ca^{2+} release from intracellular stores [204-205], we further investigated their roles in LPI-raised [Ca^{2+}]_{i} using U73122 and PTX (Fig. 5.3). IP3 is generated from PLC-catalyzed reaction. It binds to IP3 receptor located on endoplasmic reticulum (ER) membrane to open the Ca^{2+} channel and cause release of Ca^{2+} ions. U73122 blocks the activity of PLC, hence suppresses Ca^{2+} release from ER stores [195]. Treatment of U73122 impairs LPI-induced [Ca^{2+}]_{i} elevation, implying PLC-IP3 pathway is involved in triggering Ca^{2+} flux from intracellular stores. Similar inhibition was also observed in cells pretreated with PTX (G protein inhibitor), suggesting G protein is also required for LPI-elicited [Ca^{2+}]_{i} increment. Taken together, our data convince that [Ca^{2+}]_{i} elevation is dependent on G protein-sensitive factor and PLC activation.

Subsequently, roles of PLC-IP3 and G proteins in LDCV release and replenishment were examined via TIRFM. As shown in Figure 5.3 B and C,
LPI-induced depletion of predocked vesicles is inhibited by 65%, and delivery rate of new vesicles is significantly reduced from 53.9 ± 4.4 to 16.3 ± 4.5 in 2 min after treatment of U73122. In comparison, treatment of PTX and Thaps hardly affects the predocked vesicles, but severely decreases the number of new vesicles to 21.0 ± 2.4 and 7.9 ± 1.8, respectively. In summary, exocytotic effect of LPI is dependent on both PLC and G protein.

Figure 5.3 LPI-stimulated exocytosis and elevation of intracellular Ca$^{2+}$ concentration are attenuated by treatments of pertussis toxin, thapsigargin and U73122. (A) Elevation of intracellular Ca$^{2+}$ concentration by LPI is blocked in cells that are pre-incubated by PTX, Thaps and U73122. Arrow indicates when LPI was applied. (B) U73122 significantly reduces LPI-evoked exocytosis whereas PTX or Thaps have no such inhibitory effects. (C) LPI-increased delivery of new vesicles is abolished after treatments of PTX, Thaps and U73122. * $p < 0.05$, ** $p < 0.01$ in Student’s $t$-test.
5.5 Conclusion and discussion

We demonstrated that LPI, comparing to LPS and LPC, have potent effect in triggering exocytosis from PC12 cells. As revealed by TIRFM imaging and amperometric measurement, LPI depletes the predocked vesicles, facilitates the delivery of new vesicles, and alters the quantal fusion kinetics by stabilizing the fusion pore, by causing internal Ca\(^{2+}\) release in PLC and G protein dependent ways.

As LPI is the product of PLA2 activities. Our study postulated that PLA2 may involve in pathological conditions of central nervous system [206]. Studies have demonstrated that PLA2 regulates neuroinflammatory responses [207], and enhanced activity of PLA2 is detected in patients suffering Alzheimer’s disease [208].

5.6 Acknowledgements

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CHAPTER 6 SURFACE IMMOBILIZED CHOLERA TOXIN B

SUBUNIT FACILITATES VESICLE DOCKING, TRAFFICKING

AND EXOCYTOSIS

6.1 Introduction

The subunit B of cholera toxin (CTB) specifically binds to gangliosides on the cell membrane, particularly to monosialotetrahexosylganglioside (GM1) with high affinity [209]. GM1 is predominately clustered in lipid rafts which are believed to be relay points for various cellular signaling events by selectively recruiting certain proteins or lipids while excluding others [29, 209]. Upon binding with GM1, CTB is quickly endocytosed and enters a retrograde trafficking pathway through endosomes to the Golgi apparatus [210]. In addition to triggering endocytosis [211], crosslinking between GM1 and CTB can elicit other cellular responses, e.g., enrichment of β1 integrins in lipid rafts [212], increase or inhibition of cell proliferation [213-215], increase of cytoplasmic free Ca$^{2+}$ [216-217], inhibition of T cells activation [214], phosphorylation of various signaling molecules [218]. As endocytosis and exocytosis are intimately linked [185] and GM1 enriched lipid rafts are critically involved in docking and exocytosis of secretory vesicles [86], we conceive that CTB binding on the cell surface may modulate the exocytotic pathways.

Although CTB has been widely used as an instrumental membrane probe with multiple-engagement of cell functions, elucidation of the molecular transductions induced by crosslinking between GM1 and CTB is inevitably
complicated by several problems. Firstly, CTB molecules are rapidly internalized after binding due to triggered endocytosis. Thus, the effects induced by stable interaction of CTB with the cell membrane cannot be examined. In addition, trace contamination of the subunit A of cholera toxin (CTA) in the commercially prepared CTB samples will be uptaken into the cells and potently trigger cAMP pathways by activating adenylate cyclase. Some of the reported CTB effects can be attributed to the influences of CTA [219]. Furthermore, when membrane probes are applied uniformly in the bulk aqueous solution, it is not possible to investigate the effects due to their localized interaction with heterogeneous membrane domains. To avoid these problems, we studied the effects of surface immobilized CTB on vesicle docking, trafficking and exocytosis of neuroendocrine PC12 cells using integrative approaches including surface functionalization, micro-contact printing, TIRFM, amperometric detection of exocytosis using carbon fiber microelectrodes, and single cell photometry.

6.2 Surface functionalization and micro-contact printing

As illustrated in Figure 6.1 A, CTB molecules were immobilized onto a glass coverslip through a series of functionalization steps [220]. The glass coverslip was first silanized with 3-aminopropyltriethoxysilane (APTES) which reacts with hydroxyl groups on the surface. The silanized coverslip was then covalently modified with glutaraldehyde (GA) which, in turn, reacts with the primary amine group on CTB to form the covalent linkage. The success and uniformity of CTB conjugation were confirmed by the binding of green fluorescent BODIPY-FL-C5-GM1 (BODIPY-GM1) as shown in Figure 6.1 B.
The CTB coated surface which interfaces with the entire membrane of the adherent cell ensures the stable and homogeneous transmembrane signaling triggered by the crosslinking of CTB with GM1. To elucidate the spatially heterogeneous signaling, micro-patterns of CTB molecules were non-covalently created on the surface by micro-contact printing as schematically shown in Figure 6.2 A. Red fluorescent Alexa Fluor 594 conjugated CTB (CTB-594) was printed on a PLL coated glass coverslip using a polydimethylsiloxane (PDMS) stamp to produce line patterns with the nominal width and spacing distance of 2 μm. As shown in Figure 6.2 B, BODIPY-GM1 selectively binds to the CTB patterns but not to the PLL patterns between the CTB-594 micro-lines, indicating the effectiveness of the micro-patterning and that CTB-594 is able to interact normally with GM1.
Figure 6.1 Surface functionalization. (A) Illustration of covalent conjugation of CTB molecules on glass surface. (B) BODIPY-GM1 binds to the CTB coating (left) but not the glutaraldehyde coating (right), indicating the effectiveness of CTB functionalization and preserved functionalities of immobilized CTB. The scale bars = 10 µm.
Figure 6.2 Surface micro-contact printing. (A) Schematic illustration of micro-contact printing of CTB patterns on PLL coated glass coverslip, using an elastomer stamp made of PDMS. (B) Printed micro-line-patterns of CTB-594 (top) and binding of BODIPY-GM1 with CTB-594 patterns (bottom), indicating the effectiveness of micro-contact printing and the preserved functionality of immobilized CTB-594. The scale bars = 5 µm.
6.3 Surface immobilized CTB does not affect cell morphology, proliferation and adhesion

The chemical properties of the cell growth substrate could cause alterations in morphology, proliferation and adhesion of the cells, which, in turn, may profoundly affect cell functions in intricate ways [221]. To assess the CTB effects on these phenotypes of PC12 cells, we have compared the surface coating of CTB with the coating of PLL which is a cell adhesion promoting poly-peptide commonly used for cell culturing. As shown in Figure 6.3 A (left), cells can grow well on the coverslips which were covalently and uniformly decorated by CTB molecules. The morphology of PC12 cells grown on the CTB substrates appeared normal and was indistinguishable to that of the cells cultured in parallel on the PLL coated substrates. In addition, the rate of cell proliferation was nearly identical on both types of substrates (Figure 6.3 B). When cells grew on the substrates with alternating CTB and PLL micro-patterns, the cells did not exhibit any preference to either pattern. In other words, cells spread across the two kinds of patterns, and orientation or polarization of the cell body correlated with the orientation of the patterns was not observed (Figure 6.3 C). Vinculin, a protein which links the actin cytoskeleton to the transmembrane adhesion molecule integrin in focal adhesion plaques, was fluorescently stained to indicate the focal adhesion points of the cells. As seen in Figure 6.3 C, the focal points were not preferentially colocalized with either pattern, indicating that CTB and PLL similarly facilitate cell adhesion. Consistently, the distributions of focal adhesion points of the cells on the substrates with homogeneous CTB or PLL coatings were similar.
Figure 6.3 Effects of immobilized CTB on cell morphology, proliferation and adhesion. (A) Optical images of PC12 cells grown on CTB-coated (left) and PLL-coated (right) substrates. The scale bars = 50 µm. (B) Growth curves of cells grown on CTB- or PLL-coated surfaces. Each data is normalized to the initial seeding density (~180 cells/mm$^2$) at day 0, and averaged from 30 areas (~1.1 mm$^2$) in 3 different samples. (C) Confocal image showing a PC12 cell grown on a surface with dual patterns of CTB-594 (red) and PLL (dark). The green spots indicate the cell focal adhesion points stained by FITC conjugated antibodies against vinculin. The scale bar = 5 µm.
6.4 Immobilized CTB facilitates vesicle docking and trafficking in a cholesterol dependent manner

Exocytosis of hormones, neurotransmitters or other signaling molecules is a dynamic process highly regulated by the combined actions of various proteins and lipids [222]. The exocytotic process involves sequential steps of secretory vesicles, including their arrival to the subplasmalemmal region, docking and priming to become readily releasable, and final fusion with plasma membrane upon triggering. TIRFM (Figure 6.4 A, left top) is instrumental in revealing the vesicle dynamics near the cell membrane [154].

NPY-EGFP labeled secretory vesicles in the subplasmalemmal region can be individually resolved under TIRFM. Figure 6.4 A presents a typical TIRFM image of a PC12 cell grown on the CTB functionalized coverslip. As revealed by TIRFM studies [154, 167, 223], secretory vesicles undertake constant lateral movement (parallel to the cell membrane) and vertical movement (transition between inner cytosol and subplasmalemmal region). The trafficking dynamics of the secretory vesicles are highly relevant to the vesicle fusion competence and exocytotic kinetics [167, 224-225]. Vesicles in PC12 cells were individually tracked at 2 Hz for 2 min to obtain the trajectory of their lateral movement, diffusion constant, motion area, and the vertical transport. Figure 6.4 A (left bottom) depicts a typical trajectory of a vesicle’s lateral movement, whose coverage area can be approximated by the rectangular region that just encases all the vesicle footprints.
Figure 6.4 Surface coated CTB facilitates vesicle docking and trafficking in a cholesterol dependent manner. (A) Total internal reflection fluorescence microscopy (TIRFM) to reveal vesicle trafficking. Left top: the schematics of evanescent illumination of the thin subplasmalemmal region (< 200 nm). Right: A typical TIRFM image of the footprints of secretory vesicles in PC12 cells. The vesicles (bright dots, highlighted in circles) are enlightened by overexpression of NPY-EGFP. The cell contour is outlined by the dashed line. The scale bar = 5 μm. Left bottom: A typical trajectory of vesicle lateral
movement. The rectangle (dotted line) that just encases all the footprints of the vesicle gives the first estimation of the coverage area of vesicle lateral motion. The scale bars = 100 nm. (B)-(E) The statistics of the average vesicle number in the subplasmalemmal region (B), the vesicles arrived from the cytosol (C), the diffusion constant (D), and the motion coverage area (E) for cells grown on PLL- (open columns) or CTB-coated (dark columns) coverslips without (w.o. MβCD) or with MβCD treatment (w. MβCD). Each data is analyzed from the time-lapse images with a duration of 2 min and an interval of 0.5 s, from 11 cells with 618 vesicles (PLL, w.o. MβCD), 11 cells with 960 vesicles (CTB, w.o. MβCD), 10 cells with 329 vesicles (PLL, w. MβCD) and 10 cells with 300 vesicles (CTB, w. MβCD). *** p < 0.001 in Student’s t-test.

Vesicles constantly arrive from the inner cytosol to the subplasmalemmal region and retreat back after some dwell time. However, the total number of the subplasmalemmal vesicles stays nearly constant as the arrival and retrieval rates are balanced. It was found that the mean total number of the subplasmalemmal vesicles in the cells grown on CTB coated coverslips was significantly greater than that in the cells on PLL coated coverslips, implying that CTB interaction facilitates vesicle docking (Figure 6.4 B). In addition, CTB coating enhanced the vertical trafficking as evidenced by the increase in vesicle arrival from the inner cytosol (Figure 6.4 C) and enhanced the lateral motion as evidenced by the increased average diffusion constant (Figure 6.4 D) and motion coverage area (Figure 6.4 E).

GM1 is largely clustered in cholesterol rich membrane domains (lipid rafts). It has been found that methyl-β-cyclodextrin (MβCD), a cholesterol extracting agent which disrupts lipid rafts, diminishes the clustering of GM1 on the cell membrane [226] and impairs CTB binding to the lipid rafts [227]. Here, we found that MβCD treatment (incubation with 5 mM MβCD for 30 min at 37°C) completely abolished the CTB effects on vesicle docking and trafficking (Figure 6.4), indicating that the observed CTB effects are cholesterol
dependent. Membrane cholesterol was removed by 37.04 ± 2.57% (3 independent experiments) after MβCD treatment.

The facilitating roles of CTB on vesicle docking and trafficking were corroborated by the TIRFM experiments on cells grown on dual micropatterns of CTB and PLL. As shown in Figure 6.5 A and B, more vesicles were localized on the CTB patterns rather than on the PLL patterns. Consistent with the results demonstrated in Figure 6.4, vesicles on the CTB patterns exhibited enhanced vertical and lateral trafficking, and cholesterol depletion by MβCD completely eliminated the facilitating effects of the CTB patterns (Figure 6.5). Deprivation of membrane cholesterol by culturing the cells in the lipoprotein deficient serum (LPDS) for 5 days gave similar results as MβCD treatment (Figure 6.6).

In contrast to effects induced by immobilized CTB, applying CTB molecules in the solution did not cause enhanced vesicle docking or trafficking (Figure 6.7).
Figure 6.5 Micropatterned CTB facilitates vesicle docking and trafficking in a cholesterol dependent manner. (A) Distribution of secretory vesicles in a PC12 cell grown on the CTB-594 (highlighted by circles) and PLL (indicated with arrows) dual patterns without (left) or with (right) MβCD treatment. The scale bars = 5 µm. (B)-(E). The statistics of the average vesicle number in the subplasmalemmal region (B), the vesicles arrived from the cytosol (C), the diffusion constant (D), and the motion coverage area (E) for cells localized on PLL- (open columns) or CTB-patterns (dark columns) without (w.o. MβCD) or with MβCD treatment (w. MβCD). Each data is analyzed from the time-lapse
images with a 2 min duration and a 0.5 s interval, from 22 cells (w.o. MβCD) with 514 vesicles on PLL patterns and 770 vesicles on CTB patterns, 22 cells (w. MβCD) with 457 vesicles on PLL patterns and 424 vesicles on CTB patterns. ** $p < 0.01$, *** $p < 0.001$ in Student’s $t$-test.

Figure 6.6 Similar to MβCD treatment, deprivation of membrane cholesterol by culturing the cells in the lipoprotein deficient serum (LPDS) for 5 days eliminated effects of CTB patterns on vesicle docking and trafficking. (A) A typical TIRFM image showing that the secretory vesicles in PC12 cells did not show preferential colocalization with CTB patterns (highlighted by circles). Vesicles that occupied in PLL area were indicated by arrow. Scale bar = 5 µm. (B) The statistics of average vesicle number in the subplasmalemmal region; (C) vesicles arrived from cytosol, (D) diffusion constant; and (E) motion coverage area for cells colocalized with PLL-(open columns) or CTB-patterns.
(dark columns) after culturing the cells in LPDS medium, in comparison to the results from MβCD treatment as presented in Figure 6.5. Data for LPDS medium culturing is analyzed from 10 cells with 203 vesicles on PLL patterns and 207 vesicles on CTB patterns.

**Figure 6.7** Vesicle trafficking of PC12 cells revealed by TIRFM after 1 day incubation with (w., dark column) or without (w.o, open column) 10 µg/ml CTB in culture medium. Statistical analysis includes average vesicle number in the subplasmalemmal region (A), the number of vesicles arrived from the cytosol (B), diffusion constant (C), and motion coverage area (D). Each data is analyzed from 2 min time-lapse images with 0.5 s interval from 10 cells with 459 vesicles (w.o CTB) and 10 cells with 472 vesicles (w. CTB).
6.5 Immobilized CTBs enhance vesicular exocytosis in a cholesterol dependent manner

As the interaction between the cell membrane and the surface immobilized CTB molecules promotes vesicle docking and trafficking, it is thus likely that it would promote vesicular exocytosis. Single-cell amperometric measurement based on a carbon fiber microelectrode (Figure 6.8 A), which is able to electrochemically detect single vesicle release of catecholamine molecules from PC12 cells, was employed to test this hypothesis. Figure 6.8 presents amperometric recordings from a cell on a PLL coated surface and from a cell on a CTB coated surface in response to administration of high K\(^+\) solution by an application pipette. High K\(^+\) solution induces Ca\(^{2+}\) influx which, in turn, triggers vesicular exocytosis. Each amperometric current spike corresponds to single vesicle release of catecholamines.

As demonstrated in Figure 6.8 A and B, the extent of exocytosis was significantly augmented when the cells grew on CTB substrates as compared to those grown on PLL substrates. Such increase in the frequency of exocytotic response is likely attributable, at least in part, to the enhanced vesicle docking and trafficking (Figure 6.4 and 6.5). The total charge (quantal size) of each amperometric spike, which reflects the total number of released molecules, was also significantly increased by CTB coating (Figure 6.8 C and D). The CTB-enhanced quantal vesicle release was completely abolished by deprivation of membrane cholesterol using M\(\beta\)CD. Interestingly, although crosslinking of CTB occurs only at the adherent cell membrane, the enhanced vesicular exocytosis was detected by the carbon fiber electrode on the upper cell membrane. It implies that local crosslinking induces global effects. In contrast
to effects induced by immobilized CTB, applying CTB molecules in the solution did not increase vesicle exocytosis (Figure 6.9).

Figure 6.8 Immobilized CTBs enhance vesicular exocytosis in a cholesterol dependent manner. (A) Left: Illustration of single-cell amperometric measurement using a carbon fiber microelectrode (CFE). Right: two typical amperometry recordings from a PC12 cell grown on PLL- or CTB-coated coverslip, in response to a 2 min stimulation of high K\(^+\) solution delivered by an application pipette. Each spike corresponds to single vesicle release of catecholamines. (B) The average total number of amperometric spikes from cells grown on PLL coating (open columns) or CTB coating (dark columns) without (w.o. M\(\beta\)CD) or with M\(\beta\)CD treatment (w. M\(\beta\)CD). ** \(p < 0.01\) in Student’s t-test. The statistics were obtained from 18 cells (CTB, w.o. M\(\beta\)CD), 19 cells (PLL, w.o. M\(\beta\)CD), 16 cells (CTB, w. M\(\beta\)CD) and 16 cells (PLL, w. M\(\beta\)CD), respectively. (C) Averaged amperometric spikes based on the experiments shown in B from 18 cells on CTB coating without M\(\beta\)CD treatment (2165 spikes, dark curve on the left), 19 cells on PLL coating without M\(\beta\)CD treatment (1041 spikes, gray curve on the left), 16 cells on CTB coating with M\(\beta\)CD treatment (357 spikes, dark curve on the right), 16 cells on PLL coating with M\(\beta\)CD treatment (394 spikes, gray curve on the right). (D) The average quantal size (total charge per amperometric spike) under the four conditions. *** \(p < 0.001\) in K-S test.
Figure 6.9 Amperometric measurement of secretion of PC12 cells induced by high K⁺ stimulation after 1 day incubation with (w., dark traces, dark columns) or without (w.o, gray traces, open columns) 10 µg/ml CTB in culture medium. (A) Typical recordings with 2 min high K⁺ stimulation from cells incubated without or with CTB. (B) The average number of amperometric spikes from 15 cells in each case. The averaged amperometric spike (C) and quantal size (D) from 687 events (w.o CTB) and 831 events (w. CTB).
6.6 CTB effects may be attributable to increase of GM1, cholesterol and Ca\textsuperscript{2+} signaling

Figure 6.10 A demonstrates that the stable interaction between the surface immobilized CTB molecules and the adherent cell membrane resulted in a drastic increase of overall membrane GM1 which was reported by the increased fluorescence staining of membrane GM1 by Alexa Fluor 488 conjugated CTB molecules (CTB-488). GM1 molecules are segregated in the cholesterol enriched membrane domains that are believed to serve as the functional domains for vesicular exocytosis [86]. Interestingly, increase of GM1 induced by CTB was accompanied by increase of membrane cholesterol which was assayed using the increased fluorescence staining with filipin. Therefore, it is conceivable that CTB binding to GM1 on the adherent membrane leads to overall increase of GM1-cholesterol enriched functional domains, and thus enhances vesicle docking, trafficking and exocytosis.

We measured the basal intracellular Ca\textsuperscript{2+} concentration and its increase upon high K\textsuperscript{+} stimulation which excites the cell membrane and induces Ca\textsuperscript{2+} influxes through voltage gated Ca\textsuperscript{2+} channels, using single cell Ca\textsuperscript{2+} ratiometric photometry. As demonstrated in Figure 6.10 C, the basal Ca\textsuperscript{2+} level was higher in the cells grown on CTB substrates as compared to that in the cells grown on PLL substrates. In addition, the elicited Ca\textsuperscript{2+} signal by high K\textsuperscript{+} solution was stronger in the cells on the CTB substrates. It has been shown that application of CTB in the aqueous phase leads to Ca\textsuperscript{2+} influx [217] through voltage gated Ca\textsuperscript{2+} channels which are likely intrinsically modulated by GM1 [228-229]. Our observation is consistent with the previous findings and suggests that the enhanced Ca\textsuperscript{2+} signaling is achieved through transmembrane signaling of CTB.
binding instead of through formation of CTB channels or through intracellular signaling triggered by internalized CTB molecules. The CTB facilitated Ca\(^{2+}\) signal likely in part accounts for the enhanced Ca\(^{2+}\) dependent vesicular exocytosis in PC12 cells.

![Figure 6.10](image)

**Figure 6.10** Photometric measurement of GM1, cholesterol and Ca\(^{2+}\) signaling. (A) Intensity of fluorescent staining of membrane GM1 using CTB-488 from cells grown on PLL coating (n = 200) or CTB coating (n = 200). (B) Intensity of fluorescent staining of membrane cholesterol using fluorescent filipin from cells grown on PLL coating (n = 200) or CTB coating (n = 200). (C) Intracellular free Ca\(^{2+}\) reported by emission ratio of Ca\(^{2+}\) indicator Fura-2 excited at 340 and 380 nm, from 16 cells on PLL coating and 16 cells on CTB coating. *** \(p < 0.001\) in Student’s t-test.
6.7 Conclusion and discussion

Membrane signaling is conventionally studied by applying membrane probes to bulk solution. Stable, long-term, and specific effects induced by the binding probes, however, are often difficult to be examined as the probes may be quickly internalized into the cells due to triggered endocytosis or constant constitutive membrane turnover. Once internalized, the probes may trigger additional intracellular reactions. In addition, some membrane probes require organic solvent, e.g. DMSO, as a vehicle which may affect cell behaviors. Furthermore, homogenous aqueous application of membrane probes does not allow investigation of the spatial regulation of membrane signaling. Here, we demonstrated that surface immobilized membrane probes, with possibility of engineered micro/nano patterns, provide a novel alternative to study membrane signaling. Using this strategy and the integrative functional study platform at single cell level, we discovered that membrane binding of CTB, which is known to trigger endocytosis, modulates vesicular exocytosis which is intimately linked with endocytosis to ensure balanced membrane turnover [185].

Gangliosides, particularly GM1 with which CTB binds with high affinity, are critical membrane lipid species involved in micro-organization of cell membrane and various cellular processes via interactions with other membrane lipids and membrane proteins such as ion channels, receptors, and membrane associating kinases [230-232]. Crosslinking of CTB with GM1 is known to elicit multiple cellular signaling [75, 218]. In this study, it was found that CTB binding facilitates vesicle docking, trafficking and exocytosis in a cholesterol dependent manner. This study implies the important involvement of GM1 in
exocytosis, and lends further support to the widely recognized notion that GM1-cholesterol enriched membrane microdomains (lipid rafts) play critical roles in spatially defined vesicle docking and trafficking, and final vesicle fusion [86, 223].

GM1 and cholesterol enriched lipid rafts recruit secretory proteins such as SNARE proteins and are believed to serve as vesicle docking and fusion sites. Disruption of lipid rafts by depleting membrane cholesterol impairs vesicle docking, trafficking and exocytosis [35, 223]. As membrane molecules and structures are highly mobile within the lipid bilayer, the CTB patterns underlying the cell membrane presumably recruit GM1 and GM1 associated membrane structures, creating GM1 patterns on the cell membrane. Consistent with the raft theory, we show that secretory vesicles preferentially colocalize with CTB patterns (thus GM1 patterns on the cell membrane) with enhanced trafficking in a cholesterol dependent way, indicating the preferential interactions between the vesicles and the GM1-cholesterol enriched functional domains.

The exact molecular mechanisms underlying the CTB enhanced exocytosis require further investigations. It is conceivable that the interaction between the adherent membrane and immobilized CTB molecules increases the number of GM1-cholesterol enriched functional domains on the entire cell membrane based on the observations that both membrane GM1 and cholesterol were increased and that increased exocytosis was detected on the non-adherent upper membrane. And as vesicle docking, the kinetics and extent of exocytosis, quantal vesicle fusion, and replenishment of releasable vesicles are Ca\(^{2+}\) dependent [233], the CTB induced Ca\(^{2+}\) signal may be partly responsible for the
observed facilitation of vesicle docking, trafficking and exocytosis. Furthermore, gangliosides are known to interact and inhibit exocytosis promoting enzymes such as protein kinase C [234] and phospholipase C [235]. Binding of CTB may relieve such inhibitions and consequently facilitates exocytosis.

Using immobilized CTB molecules as the probes can avoid effects that are not directly caused by membrane binding of CTB with GM1, and make it possible to reveal phenomena that are exclusive when membrane binding of CTB is only transient.

We showed that immobilized CTB did not affect the morphology, proliferation, and focal adhesion of PC12 cells. Such phenotypes may directly or indirectly influence exocytosis. For example, it was reported that cell adhesion is directly involved in spatial regulation of vesicle exocytosis [236]. Therefore, it is likely that the observed CTB effects are directly and specifically related to membrane binding of CTB. In contrast to effects induced by immobilized CTB, applying CTB molecules in the solution did not cause enhanced vesicle docking or trafficking (Figure 6.7) or increase vesicle exocytosis (Figure 6.9). Also in contrast to our observations, it has been shown that applying CTB molecules in the solution promotes cell proliferation of 3T3 fibroblasts [217] and neuritogenesis of PC12 cells [219]. The discrepancy between the effects induced by immobilized and dissolved CTB molecules may be attributable to the transient binding of CTB due to internalization, or intracellular reactions caused by internalized CTB molecules or CTA contaminants. Clearly, immobilizing CTB is a novel or better alternative to interrogate the functions of GM1.
Surface functionalization and patterning have been utilized to study how cell anchoring affects cell proliferation, migration, polarization and differentiation [237-240]. Our study postulates that surface immobilized and patterned probes can be used to study membrane signaling mediated by membrane functional domains. Using patterning techniques such as dip-pen lithography, chemically functionalized nanopatterns with various geometries (e.g., dots, meshes) can be readily created. With appropriate chemistry, membrane probes targeting on other membrane lipid species or membrane proteins can be attached to the surface. This strategy promises many applications that would be instrumental to fundamental biology.

6.8 Acknowledgements

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CHAPTER 7 GENERAL CONCLUSION AND PERSPECTIVES

7.1 Lipids affect multi-aspect of exocytosis

It has been well established during the past four decades or so that exocytosis can be finely tuned by proteins, especially SNARE proteins and synaptic proteins. In recent years, however, lipids are receiving increasing attention on their regulatory roles in exocytotic process. Our work focuses on the regulation of lipids (i.e. cholesterol, oxysterols, lysosphospholipids, GM1) in the aspects of vesicular trafficking, docking, and quantal release. Using our integrative platform based on single-cell functional studies (bioimaging, electrophysiology, and electrochemical techniques), molecular biology and surface functionalization, we have studied the lipid regulations on the dynamic exocytotic process with high temporal resolution and exquisite details.

We have found that cholesterol deprivation inhibits the extent of triggered exocytosis, affects the kinetics of quantal release, restricts vesicular trafficking, and impairs vesicle docking. These observations support the notion that cholesterol-enriched lipid rafts are critically involved in multiple steps of exocytosis [36-37]. The study of the effects of surface-immobilized GM1-binding CTB molecules further support the important of lipid rafts in spatially regulated vesicle docking and fusion. Moreover, we have demonstrated that exogenous free cholesterol, oxysterols and lysosphospholipids are able to induce exocytosis. This highlights that the balance of lipid composition in the cell membrane is critical and imbalance of these lipid species may lead to profound neuropathological implications. The regulations of different lipids are briefly
summarized in Table 7.1.

7.2 Future perspectives

In a recent study, we investigated the effect of oseltamivir carboxylate (OC) on exocytosis in neuroendocrine PC12 cells [241]. OC is an inhibitor of neuraminidase (or called sialidase) which cleaves sialic acid residue of polysialylated ganglioside to produce monosialogangliosides, such as, GM1 enriched in lipid rafts [242].

It was found that OC treatment did not affect cell morphology, actin polymerization, or never growth factor (NGF)-induced differentiation (Figure 7.1). But, OC impaired vesicular trafficking by reducing the average velocity, confining motion area, increasing dwell time and suppressing vesicle replenishment (Table 7.2 and Figure 7.2). We also found that OC treatment inhibited the extent of high K⁺-elicited exocytosis, decreased rise slope, reduced the quantal size, and elongated t₁/₂ (Table 7.3 and Figure 7.3). Taken together, our findings showed that OC significantly impaired both fusion competence and quantal release efficiency.

It has been shown that oral administration of Tamiflu caused GM1 reduction in mice T cells [243]. And our studies in Chapter 6 have demonstrated that GM1 regulates exocytosis. Hence, the inhibitory effect of OC may due to its inhibition on Neu3 (the enzyme that mediates production of GM1) and subsequent alteration in the lipid raft compositions and thus functions. Further investigations on the mechanisms underlying OC-induced inhibition on exocytosis would help to elucidate the regulatory roles of GM1. This study
stresses the importance of lipid enzymes in exocytosis.

**Table 7.1** Different effects of lipids regulation on exocytosis.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol extraction vs. Intact</th>
<th>Cholesterol stimulation vs. High K⁺ stimulation</th>
<th>Oxysterols stimulation vs. Vehicle control</th>
<th>LPI stimulation vs. High K⁺ stimulation</th>
<th>CTB coating vs. PLL coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total exocytosis</td>
<td>- - -</td>
<td>- - -</td>
<td>+</td>
<td>- - -</td>
<td>+ +</td>
</tr>
<tr>
<td>Rise slope</td>
<td>- - -</td>
<td>- - -</td>
<td>N.A.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Quantal size</td>
<td>- - -</td>
<td>+</td>
<td>N.A.</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>N.S.</td>
<td>+ + +</td>
<td>N.A.</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Delivery of new vesicles</td>
<td>- - -</td>
<td>N.A.</td>
<td>N.A.</td>
<td>+ +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Motion area</td>
<td>- - -</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

+ positive effect; - negative effect. (+, -) p < 0.05, (+ +, - -) p < 0.01, (+ + +, - - -) p < 0.001 by either Student’s t-test or K-S test.

N.S., not significant; N.A., not available.

**Table 7.2** OC impaired vesicle lateral motion and vertical trafficking.

<table>
<thead>
<tr>
<th></th>
<th>OC treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velocity (nm/s)</td>
<td>55.1 ± 1.7</td>
<td>183.1 ± 5.5</td>
</tr>
<tr>
<td>Motion area (µm²)</td>
<td>0.27 ± 0.02</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>Dwell time (s)</td>
<td>63.8 ± 1.8</td>
<td>41.2 ± 1.5</td>
</tr>
<tr>
<td>Arrived vesicle</td>
<td>23.3 ± 3.1</td>
<td>52.1 ± 3.6</td>
</tr>
</tbody>
</table>

**Table 7.3** OC inhibited quantal fusion kinetics.

<table>
<thead>
<tr>
<th></th>
<th>OC treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion events per cell</td>
<td>22.8 ± 3.8</td>
<td>45.4 ± 4.1</td>
</tr>
<tr>
<td>Rise slope (pA/ms)</td>
<td>5.11 ± 0.46</td>
<td>10.58 ± 0.46</td>
</tr>
<tr>
<td>t₁/₂ (ms)</td>
<td>6.15 ± 0.19</td>
<td>5.13 ± 0.09</td>
</tr>
<tr>
<td>Quantal size (fC)</td>
<td>87.87 ± 2.03</td>
<td>69.69 ± 3.19</td>
</tr>
</tbody>
</table>
Figure 7.1 Comparisons between OC treated and non-treated PC12 in cell morphology, actin polymerization, and differentiation. (A) Brightfield cell morphology images of treated (left) and untreated cells (right). (B) Actin filaments staining of OC treated (left) and intact cells (right) after fixation. (C) NGF-induced differentiation was similar in cells treated with (left) or without OC (right).
Figure 7.2 OC impaired vesicle trafficking. (A) TIRFM imaging of NPY-EGFP labeled vesicles at subplasmalemma of control (upper panel) and OC-treated cells (lower panel). (B) Representative trajectories of a travelling vesicle from control (upper panel) and OC-treated cell (lower panel). Scale bar = 200 nm. (C)-(E) Statistics of motion parameters describing the lateral mobility of vesicles. (F) Vertical trafficking of vesicles in 2 min. *** $p < 0.001$. 
Figure 7.3 OC inhibited exocytosis in PC12 cells. (A) A typical amperometric recording of high K⁺-evoked neurotransmitter release. (B) Total number of exocytotic events of control (gray column) and OC-treated cells (dark column) in response to 2 min of high K⁺ stimulation. (C) Averaged fusion spike acquired from control (gray) and OC-treated cells (dark curve). (D) Comparisons of fusion kinetics using rise slope, t₁/₂ and quantal size. The data were normalized to control. *** p < 0.001.
Another important lipid species, which is known to critically regulate exocytosis, is phosphatidylinositol 4,5-bisphosphate (PIP2). It functions as a key regulator in actin organization that mediates vesicular trafficking [244]. It serves as landmark for synaptotagmin to recognize binding site for vesicle docking [17]. PIP2 can be hydrolyzed by phospholipase C (PLC) to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), both of which are important second messengers to facilitate exocytosis. IP3 binds to IP3 receptor on ER membrane and release Ca\(^{2+}\) from ER stores. DAG together with Ca\(^{2+}\) activate protein kinase C (PKC), which is implicated to trigger a cascade of cellular responses including exocytosis [81]. In our preliminary study (unpublished), we examined the functions of PIP2 by functionalizing neomycin on a surface (the same strategy presented in Chapter 6). Neomycin can specifically bind to PIP2 with high affinity [245]. We discovered that immobilized neomycin impaired exocytosis by decreasing fusion kinetics whereas did not affect the extent of exocytosis (Figure 7.4).

Sphingomyelin is another major lipid residing in the lipid rafts. However, the function of sphingomyelin in regulating exocytosis has not been well established. Sphingomyelin can be hydrolyzed by sphingomyelinase to produce ceramide, the lipophilic second messenger in signal transduction [246]. Studies showed that ceramide modulates the formation and organization of lipid rafts [247]. Therefore, it is not surprising that sphingomyelin/ceramide pathway is critically involved in exocytosis. Again, we used surface immobilized sphingomyelin-binding probes lysenin [248-249] to examine the functions of sphingomyelin. Our data showed that immobilized lysenin inhibited exocytosis by reducing high K\(^{+}\)-elicited spike number, decreasing rise slope and elongating
$t_{1/2}$ (Figure 7.5). More experiments will be carried out in the near future.

**Figure 7.4** Immobilized neomycin impaired quantal fusion kinetics but did not affect vesicular later motion. (A) High $K^+$-elicited current spikes from cells seeded on PLL-coated surface (5 cells) or neomycin-coated surface (11 cells). (B) Averaged amperometric spikes based on the experiments shown in (B). (C) Comparison of quantal fusion kinetics. (D) Comparisons of vesicular lateral motion. ** $p < 0.01$, *** $p < 0.001$. 
Figure 7.5 Surface immobilized lysenin impaired quantal fusion. (A) Number of high K$^+$-elicited current spikes from cells seeded on PLL-coated surface (14 cells) or lysenin-coated surface (15 cells). (B) Averaged amperometric spikes based on the experiments shown in (B). (C) Comparisons of quantal fusion kinetics. *** $p < 0.001$.

This thesis only focuses on several lipid species, leaving a large portion of lipids unexplored. Hence, much effort is still needed to elucidate the specific roles of other lipids, especially those enriched in lipid rafts. And enzymes or other factors involved in lipid homeostasis are largely unexplored and demand more investigations.
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